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REVIEW ARTICLES

Advancements and challenges in CRISPR/cas genome editing for legume crops

Tatiana Faria Maia[a,b], Joaquin Felipe Roca Paixão[c], Marcia Soares Vidal[b], Krzysztof Szczyglowski[d,e], Jean Luiz Simões de Araújo[b]*

[a]*Postgraduate Program in Plant Biotechnology and Bioprocesses, University of Rio de Janeiro, CEP 21941-853, Rio de Janeiro, RJ, Brazil*
[b]*Embrapa Agroibiology - Highway BR-465, Km 7, Ecologia, Zip Code 23891-000, Seropédica - RJ, Brazil*
[c]*Laboratory of Plant Molecular Biology, University of Rio de Janeiro, Zip code CEP 21941-853, Rio de Janeiro, RJ, Brazil*
[d]*Agriculture and Agri-Food Canada, London Research and Development Centre, 1391 Sandford Street, London, ON N5V 4T3, Canada*
[e]*Department of Biology, University of Western Ontario, London, ON N6A 3K7 Canada*

Highlights

- The CRISPR/Cas has significant potential to increase legume production without requiring an increase in planted area.
- Major obstacle in implementing genome editing in legumes is the difficulty of transforming and regenerating plants.
- As a simpler and faster alternative method, hair root transformation mediated by *Agrobacterium rhizogenes* has gained widespread use in studying the biochemical and molecular functions of legume root genes through CRISPR/Cas editing.

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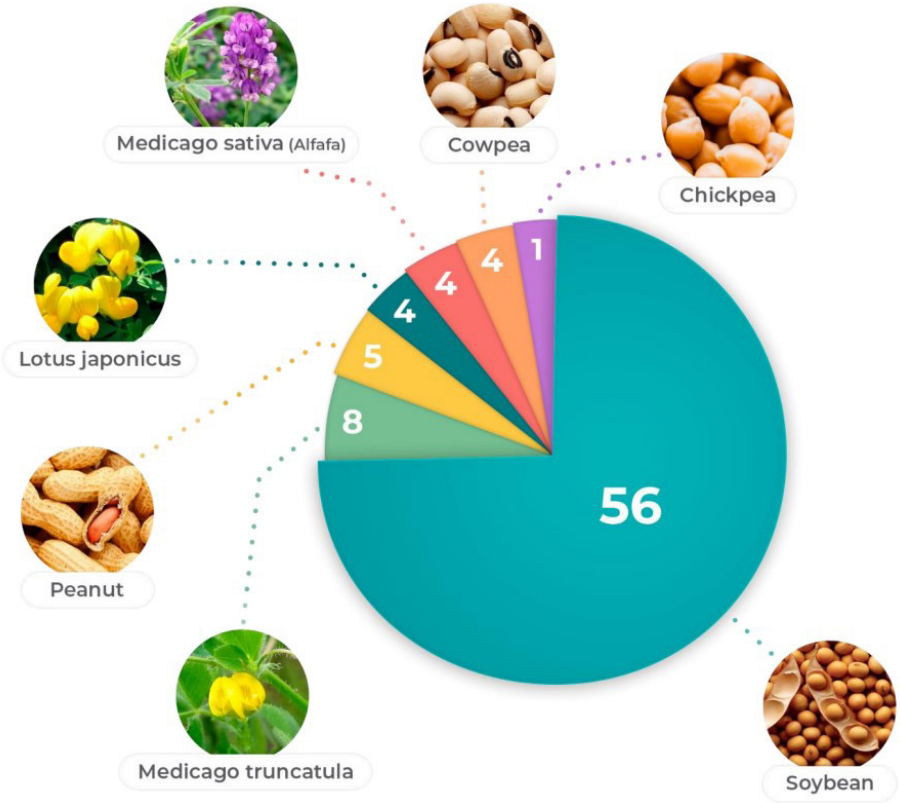
Abstract: Agricultural systems are facing significant pressure due to population growth, water scarcity, and the impacts of climate change, which collectively pose a threat to global food security. In this scenario, leguminous plants play a crucial role as they are an excellent source of protein for human nutrition. Additionally, they can symbiotically fix nitrogen with soil bacteria, thereby reducing soil contamination and emissions of nitrous oxide and ammonia. However, the yield of legumes is threatened by biotic stresses, such as diseases and pests, as well as abiotic environmental factors, which ultimately affect nutritional quality and productivity. Advancements in genetic engineering offer new approaches for improving crops. The CRISPR/Cas system, an innovative biotechnology tool, allows for precise and efficient modifications of the genome, enabling the creation of new traits in legume plants. In this review, we explore the relevant applications, challenges, and potential of the CRISPR/Cas system in enhancing the yield and adaptability of leguminous crops.

*Corresponding Author:
E-mail: jean.araujo@embrapa.br (J. L. S. Araújo)

Graphical Abstract



Number of legume crop edited using the CRISPR/Cas9 system from 2012 to 2022. The modifications include improved yield and quality, tolerance to abiotic and biotic stress.

Introduction

Legumes (Fabaceae or Leguminosae) are the third largest family among angiosperms, comprising approximately 751 genera and more than 19,500 species (The Legume Phylogeny Working Group, 2017). Legumes encompass a wide range of plant types, including trees, shrubs, and herbs. They are easily identifiable by their characteristic pod-shaped fruits (Lewis et al., 2005) and are a vital source of proteins, fibers, vitamins, and antioxidants. Moreover, legumes play a crucial role in promoting human health, as they provide protection against certain types of cancer (World Cancer Research Fund, 2014), diabetes, aging, obesity (Mudryj et al., 2014), and cardiovascular diseases (Arnoldi et al., 2015). Their high protein content, ranging from 23% to 40% (Zander et al., 2016), makes them an essential staple food in many regions of the world, particularly in developing countries. Legumes not only help combat malnutrition but also serve as valuable forage for animal feeding (Tharanathan & Mahadevamma, 2003).

One notable characteristic of legumes is their capacity to form root nodules and engage in biological nitrogen fixation (BNF) through a symbiotic relationship with rhizobia bacteria (Stacey, 2007). BNF enables legumes to obtain nitrogen from the atmosphere, reducing the reliance on nitrogen fertilizers. The benefits of leguminous crops extend beyond their nutritional value and impact the environment and socioeconomic aspects of agriculture. They also contribute to the reduction of greenhouse gas emissions; legumes release five to seven times less gases per unit area compared to other crops (Stagnari et al., 2017).

According to the United Nations (2022), the global population is projected to surpass 9.7 billion by 2050. To meet the increasing world demand, the total global food demand is expected to rise by 35% to 56% between 2010 and 2050 (van Dijk et al., 2021). Meeting this challenge requires the agricultural sector to develop crop varieties with higher yields and improved adaptability to climate change, ensuring food and nutritional security for future generations. Additionally, the need for more sustainable and low-input agricultural systems is evident. In recent decades, significant progress has been made in developing new varieties within legume communities (Pandey et al., 2016). Furthermore, advances in molecular marker technologies, quantitative trait loci (QTL) mapping, and next-generation sequencing have contributed to enhancing legume breeding (Varshney et al., 2015; Varshney, 2016; Valdisser et al., 2017). These advancements in genetic research and technology have unlocked new possibilities for improving legume crops and addressing the challenges faced by agriculture. Nevertheless, there is a need for new tools to accelerate the development of improved high-yield varieties that are stress-tolerant, along with genetic improvement programs that can generate superior varieties in a more predictable, rapid, cost-effective, and sustainable manner. While first-

generation genome editing tools, such as meganucleases, Zinc Finger Nucleases (ZFNs), and Transcription Activator-Type Effector Nucleases (TALENs), have allowed for precise editing of genome sequences by altering gene expression patterns in specific regions (Razzaq et al., 2019), they have certain drawbacks that limit their applicability in improving plant traits. Disadvantages of these platforms include their lengthy development time and high costs, difficulties in designing ZFNs and TALENs due to highly repetitive sequences, the complex nature of ZFN-DNA interactions (Bortesi & Fischer, 2015), mutagenic effects, toxicity at the target site, and off-target effects. These limitations have prompted the search for more advanced and efficient genome editing technologies.

The development of genome editing technology based on Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) (Doudna & Charpentier, 2014) has revolutionized the field of genome editing, including its application in plant genomes (Li et al., 2013; Nekrasov et al., 2013; Shan et al., 2013; Ma et al., 2015). CRISPR/Cas-mediated genome editing has gained significant attention in plant research due to its effectiveness, simplicity, affordability, and precision. This technology has been successfully used for gene manipulation in various crop species, including grain legumes like soybean (Jacobs et al., 2015; Wang et al., 2016; Čermák et al., 2017; Gao et al., 2018; Ji et al., 2019; Cai et al., 2020a). However, there is a limited amount of CRISPR research available for a wide range of other legume species, such as beans, peas, chickpeas, and lentils. Several challenges still need to be addressed when applying CRISPR technology to these and other legume species to develop desirable traits. In this review, we emphasize the applications of CRISPR/Cas systems in the genome editing of legumes and discuss the future challenges associated with its implementation in this diverse plant group.

Discovery of CRISPR and CRISPR/Cas

The discovery of CRISPR can be traced back over 30 years. It began with the identification of an unusual repetitive DNA sequence in the *Escherichia coli* genome during studies of the inhibitor of apoptosis (IAP) gene responsible for converting the alkaline phosphatase isoenzyme (Ishino et al., 1987). Subsequently, in 1993 and 1995, multiple copies of a nearly perfect repetitive sequence separated by spacers were first described in Archaea, specifically in *Haloferax mediterranei* (Mojica et al., 1993; Mojica et al., 1995). Fifteen years later, the CRISPR array was recognized as a locus consisting of repeated sequences and interspersed spacer sequences in prokaryotes (Mojica et al., 2000). In 2002, Jansen et al. from the University of Utrecht, Netherlands, observed this sequence structure in the genomes of other bacteria, including *Salmonella typhimurium* and *Streptococcus pyogenes*. They coined the term CRISPR, which stands for "Clustered Regularly Interspaced Short Palindromic Repeats". While the researchers were not yet aware of the functionality of CRISPR, they noted that CRISPR sequences were consistently followed by a collection of nearby genes encoding DNA repair proteins. These genes were later designated as CRISPR-associated genes (Cas) (Jansen et al., 2002; Makarova et al., 2002).

The origin of spacer sequences within CRISPR arrays remained a mystery until their similarity to viral and plasmid genomes were discovered. It was found that these spacer sequences served as a record of previous viral and plasmid infections (Bolotin et al., 2005; Mojica et al., 2005; Pourcel et al., 2005). A year later, a detailed analysis of Cas protein sequences encoded by Cas genes suggested that the CRISPR/Cas system functions as a prokaryotic RNA interference-based immune system (Makarova et al., 2006). In 2007, a study on the bacterium *S. thermophilus* provided evidence for the function of the CRISPR/Cas system as an adaptive immune system in prokaryotes (Barrangou et al., 2007). Subsequent studies further confirmed this function and characterized other genetic elements involved in adaptive immunity (Brouns et al. 2008; Deveau et al., 2008; Marraffini & Sontheimer 2008; Horvath et al., 2009).

In the following years, the structural and functional characteristics of proteins encoded by Cas genes were described, leading to the classification of the CRISPR/Cas system, and providing the basis for its application in genome editing (Garneau et al., 2010; Deltcheva et al., 2011; Makarova et al., 2015).

Mechanism of editing of the CRISPR/Cas9 system

The transition of the CRISPR/Cas system to a genome editing tool began with groundbreaking work in 2012 by Martin Jinek, Jennifer Doudna, and Emmanuelle Charpentier. They published a paper describing the use of CRISPR/Cas9 to induce a double-strand break (DSB) in target DNA, marking a significant advancement in the field. The study showed that the Cas9 enzyme requires two distinct RNA molecules, CRISPR RNA (crRNA) and trans-activating CRISPR RNA (tracrRNA), which together form a single guide RNA (gRNA) to facilitate the cutting of specific DNA sequences (Jinek et al., 2012). In early 2013, five papers were published demonstrating successful genome editing in humans, mice, and zebrafish using the CRISPR/Cas9 system, showcasing its functionality in eukaryotes (Cho et al., 2013; Cong et al., 2013; Hwang et al., 2013; Jinek et al., 2013; Mali et al., 2013). These findings collectively highlighted the significance of CRISPR/Cas9 in the development of a new generation of genome editing technologies (Ishino et al., 2018). Among the CRISPR/Cas9 systems, the type II system derived from *S. pyogenes*, known as SpCas9, has been the most widely used. It has gained significant popularity in plant science applications and has been successfully employed in various crops such as barley, brassica, cucumber, maize, potato, and tomato (Lawrenson et al., 2015; Chandrasekaran et al., 2016; Shi et al., 2017; Nakayasu et al., 2018; Ortigosa et al., 2019). Given its extensive usage, SpCas9 is the primary focus of this review, particularly in the context of editing legumes.

Cas9, which was formerly termed Cas5, Csn1, or Csx12, is a well-characterized effector protein in the CRISPR/Cas9 system. It functions as an endonuclease and consists of two domains: RuvC and HNH. These domains are essential for inducing a double-strand break (DSB) in the target DNA. The HNH domain cleaves the DNA strand that is complementary

to the spacer region of the guide RNA (gRNA), while the RuvC domain breaks the non-complementary DNA strand. The Cas9 nuclease binds to a single guide RNA (sgRNA), which is formed by combining the crRNA and tracrRNA. The sgRNA contains a 20-nucleotide sequence that binds to the specific target DNA sequence (spacer) and a complementary repeat sequence. Together, these components enable the formation of a DSB that is recognized by the Cas9 enzyme. Once the Cas9/gRNA complex is formed, it scans the DNA to locate the protospacer adjacent motif (PAM) sequence. Upon recognizing a PAM, the complex separates the double-stranded DNA (dsDNA) and checks if the spacer in the crRNA is complementary to the target DNA sequence near the PAM site. After the formation of a heteroduplex between the RNA and DNA, the Cas9 protein domains (RuvC and HNH) are activated and induce DSBs in the target DNA, typically three to four nucleotides upstream of the PAM site (Gupta & Musunuru, 2014; Mahfouz et al., 2014). The resulting DSBs can be repaired through two DNA repair mechanisms: non-homologous end-joining pathway (NHEJ) and homology-directed repair (HDR). NHEJ joins the broken ends of DNA without specific sequence requirements, often resulting in insertions or deletions (indels) of short nucleotide stretches at the repair site (Voytas & Gao, 2014). These mutations can lead to loss of function, allowing for gene knockout and determining genetic functions or eliminating undesirable traits. On the other hand, HDR is a more precise repair mechanism that requires a DNA template with homology to the sequences flanking the DSB. By providing a donor DNA template, this new fragment can be incorporated into the original DNA sequence through homologous recombination, resulting in gene replacement and the production of a modified protein. These mechanisms of DNA repair play a crucial role in genome editing using the CRISPR/Cas9 system. NHEJ is commonly utilized for gene knockout, while HDR allows for precise modifications by introducing specific DNA sequences through homologous recombination. Indeed, the efficiency of homology-directed repair (HDR) in plants is often lower compared to non-homologous end-joining (NHEJ), limiting its application in plant genome editing. This is due to various factors, including the limited delivery of donor repair templates (DRTs) to plant cells caused by the presence of a cell wall. Additionally, HDR-mediated editing is often restricted to dividing cell types, further limiting its utility in plants.

To overcome these limitations, researchers have developed alternative genome editing tools such as DNA base editing (BE) and prime editing (PE). These methods offer precise nucleotide substitutions without the need for a donor template, eliminating the requirement for double-strand breaks (DSBs) and enhancing efficiency.

Base editing involves fusing a Cas9 nickase with a DNA deaminase enzyme. The deaminase enzyme catalyzes the conversion of specific base pairs, such as C·G to T·A or A·T to G·C, without inducing DSBs. This technique enables targeted point mutations in the genome with high precision (Komor et al., 2018).

Prime editing is a more recent genome editing tool that combines an RNA-programmable nickase, reverse transcriptase, and a prime editing guide RNA (pegRNA). The nickase creates a nick in the DNA strand, and the reverse transcriptase uses the pegRNA as a template to synthesize a new DNA strand that is inserted into the nicked site (Anzalone et al., 2019). Prime editing offers advantages such as the ability to perform precise sequence deletions, additions, and substitutions (Anzalone et al., 2020; Kantor et al., 2020). While base editing and prime editing have been tested in various organisms, including plants, their application specifically in legumes is an area that requires further investigation. The potential of these techniques in legume species, including beans, peas, chickpeas, and lentils, is yet to be fully explored.

CRISPR/Cas application for Legume Crop Improvement

The green revolution of the past 50 years has significantly increased food production, leading to profound social, economic, and environmental impacts worldwide. However, the focus of crop research during this period was primarily on cereal crops, given their economic and caloric significance, while other important crops like legumes received less attention. Legumes have a significant presence in cultivated lands globally, with large-scale legume farms of over 200 hectares found in regions such as Australia, New Zealand, South America, and North America. In other regions like Southeast Asia, Sub-Saharan Africa, and West and North Africa, legumes are predominantly cultivated on smaller farms ranging from 2 to 20 hectares in size. Soybean is an example of a legume crop that has experienced substantial expansion, with its global production reaching 382 million tons in 2021 (Guo et al., 2022), more than 13 times higher than its production in the early 1960s. However, there is still significant potential to further increase legume yields to meet the nutritional demands of the growing global population, without requiring an increase in the planted area. This aspect is crucial for conserving natural areas and promoting sustainability in agriculture.

The advent of new gene editing technologies holds promise for the second green revolution, which will focus on nourishing the expanding population while promoting sustainable agricultural practices. By using gene editing techniques, researchers can develop high-yield legume varieties that are resilient to stress and possess desirable traits. This approach can contribute to global food security while minimizing the environmental impact of agriculture. After sequencing the genome of the model plant *Arabidopsis thaliana* (Arabidopsis Genome Initiative, 2000), modern sequencing technologies have aided in sequencing the genomes of various crops, including legumes. Currently, there are eight fully sequenced legume species. They include the genomes of *Lotus japonicus* (472 Mb) (Sato et al., 2008), *Glycine max* (1,112 Mb) (Schmutz et al., 2010), *Medicago truncatula* (373 Mb) (Young et al., 2011), *Pigeonpea – Cajanus cajan* (833 Mb) (Varshney et al., 2011), Chickpea - *Cicer arietinum* (738 Mb) (Varshney et al., 2013), *Phaseolus vulgaris* (588 Mb) (Schmutz et al., 2014), *Vigna unguiculata* (640,6 Mb) (Lonardi et al., 2019), Groundnut - *Arachis hypogaea* (2700 Mb) (Bertioli et al., 2019; Zhuang et al., 2019) and Pea - *Pisum sativum* (4450 Mb) (Kreplak, et al., 2019).

Indeed, the advent of next-generation sequencing technologies has greatly accelerated the discovery and characterization of genes controlling important agronomic traits in legume crops. The use of these genes in genome editing has significantly influenced the realm of functional genomics and has heralded a new era in crop breeding (Sarrocco et al., 2020). The pioneering use of CRISPR/Cas9 gene editing in legumes was initially reported in a publication by Jacobs et al. in 2015. In their study, they achieved successful mutations in nine endogenous genes and a green fluorescent protein (GFP) transgene in soybean hairy roots, displaying a notable proficiency in detecting the intended genetic changes. Following this milestone, several publications in 2015 further evaluated the mutation efficiency of the CRISPR/Cas9 system in soybean genes. These studies, conducted by Cai et al. (2015); Li et al. (2015); Michno et al. (2015) and Sun et al. (2015), collectively A demonstrated the potential of CRISPR/Cas technology for improving traits in leguminous crops, specifically soybean. Furthermore, Michno et al. developed a web tool in 2015 that facilitated the identification of CRISPR/Cas9 target sites and off-target sites in legumes. This resource proved valuable for designing optimized Cas9 systems and efficiently mutating genes in soybean and *Medicago truncatula*. Overall, these early studies and developments laid the foundation for harnessing the CRISPR/Cas system as a transformative tool for trait improvement in legume crops. Subsequent research has continued to expand and refine the applications of CRISPR/Cas gene editing in legumes, enabling the development of novel varieties with improved agronomic characteristics.

Despite significant efforts, only seven legume cultivars have been successfully edited using the CRISPR/Cas9 system so far (Graphical Abstract), with soybean being the most extensively studied. However, several challenges need to be addressed to develop a more efficient genome editing system for legumes. These challenges include reducing the occurrence of off-target editing, optimizing the Cas9 enzyme and its orthologs for improved specificity, and enhancing the generation of transgenic lines. One of the major obstacles in implementing genome editing in legumes is the difficulty of transforming and regenerating legume plants. The current transformation and regeneration protocols are limited to specific tissues, genotypes, and crop varieties, and are often time-consuming and labor-intensive. Overcoming these hurdles is crucial for realizing the full potential of genome editing in legume crops.

The bottleneck of transformation and delivery of CRISPR/Cas components in legumes

The methods employed for the transformation and delivery of CRISPR reagents to plant cells pose significant challenges that need to be addressed to enable the effective application of CRISPR/Cas technology in a wide range of leguminous species. Plant transformation is a multi-step process involving the introduction of genes of interest into plant cells, followed by the selection and regeneration of transgenic or gene-edited plants (Lee & Wang, 2023). Substantial progress has been made in optimizing efficient transformation systems for legume plants (Tichá et al., 2020; Che et al., 2021; Kumar et al., 2021; Xu et al., 2022). However, the recalcitrant nature of certain legume species and the high dependence on genotype significantly impact transformation and regeneration rates, serving as major obstacles to successful transformation in legumes (Somers et al., 2003; Choudhury & Rajam, 2021; Sehaole, 2022).

The most employed methods for delivering CRISPR constructs to plant cells include (i) infection with *Agrobacterium* spp., (ii) particle bombardment, and (iii) polyethylene glycol (PEG)-treated protoplasts (Alamillo et al., 2023), with the first method being the most extensively used in legumes.

Agrobacterium tumefaciens mediated genetic transformation

Agrobacterium-mediated genetic transformation is based on the transfer of T-DNA from the bacterium into the nucleus of a host plant using the bacterial type IV secretion system (Ghogare et al., 2021). When plant cells are infected, the T-DNA carrying the CRISPR cassette integrates into the host plant genome, resulting in stable genetic transformation (Laforest & Nadakuduti, 2022). *Agrobacterium*-mediated DNA delivery is responsible for approximately 80% of mutations and editing events observed in nearly all plant species (Ran et al., 2017).

Gene editing via T-DNA delivery has been reported in various legume species, including cowpea (Che et al., 2021), chickpea (Badhan et al., 2021), soybean (Qian et al., 2022), pea (Li et al., 2023), and model legumes such as *L. japonicus* (Wang et al., 2016), *M. truncatula* (Confalonieri et al., 2021), and *M. sativa* (Miller et al., 2022). Among these studies, 60% used *Agrobacterium tumefaciens* for the delivery of CRISPR/Cas elements (Table 1S). This genetic transformation method offers several advantages, including its relatively low cost and simplicity (Hwang et al., 2017), high reproducibility, low transgene copy number in the host genome (Gelvin, 2012), and the ability to transfer larger DNA fragments (Ishizaki et al., 2008). However, the efficiency of *Agrobacterium*-mediated transformation in some legumes is hindered by limitations in *in vitro* regeneration capacity and low DNA transfer rates. Even in soybean, where transformation efficiency has been improved through the use of different genotypes and optimized protocols (Li et al., 2017b; Chen et al., 2018; Hada et al., 2018; Karthik et al., 2020), the transformation frequency remains relatively low (approximately 20%) (Pareddy et al., 2020; Tiwari et al., 2022) compared to the higher efficiency observed in rice (approximately 40%) (Mohammed et al., 2019; Xu et al., 2020). In alfalfa (*Medicago sativa*), the Regen SY genotype has shown high responsiveness for generating transgenic plants, with frequencies ranging from 80% to 100% (Tichá et al., 2020).

One limitation of this DNA delivery method in most legumes is associated with the choice of explants and the regeneration process, which often involve long protocols and low transformation recovery rates (Li et al., 2017b). Cotyledonary nodes and embryonic axes have been commonly used as suitable explants for legume transformation (Bhajan et al.,

2019; Sarker et al., 2019; Sadhu et al., 2022; Polowick & Yan, 2023). The use of cotyledonary nodes facilitates the generation of explants as they can be easily isolated from germinated seeds (Paes de Melo et al., 2020). Most successful reports of legume editing involve explants derived from seed tissues, such as developing or mature embryos, or cotyledonary nodes (Bhowmik et al., 2021). However, precise handling and skillful wounding are required, which can reduce regeneration efficiency. Furthermore, the limited host range of *Agrobacterium* strains can pose a challenge, as certain plant species or genotypes may not be efficiently transformed using available *Agrobacterium* strains (Lee & Wang, 2023). To address this issue, efforts have been made to engineer *Ochrobactrum haywardense* strain H1 (Oh H1), which has demonstrated improved transformation efficiency in soybean compared to other tested *Agrobacterium* strains such as AGL1 and LBA4404Thy- (Cho et al., 2022). Oh H1-8-mediated delivery of CRISPR/Cas9 has shown promising results, leading to high-quality transgenic events with efficient marker-free, single-copy insertions and an observed mutation rate of around 90% at the targeted soybean DD38 and DD51 sites (Kumar et al., 2022). This highlights the potential of Oh H1 strain as an effective approach for enhancing legume transformation and editing.

Although advancements have been made in improving the efficiency of *A. tumefaciens*-mediated plant transformation, there is still a need to develop legume transformation protocols that offer genotype flexibility, high transformation efficiency, and minimize or eliminate tissue cultivation steps while improving plant regeneration. These improvements would contribute to the overall efficiency and applicability of CRISPR/Cas technology in legume crop improvement.

Hair root transformation mediated by *Agrobacterium rhizogenes*

Stable genetic transformation of leguminous plants is often a time-consuming and inefficient process. However, as a simpler and faster alternative method, hair root transformation mediated by *Agrobacterium rhizogenes* has gained widespread use in studying the biochemical and molecular functions of legume root genes through CRISPR/Cas9 editing (Jacobs et al., 2015; Michno et al., 2015; Tang et al., 2016; Curtin et al., 2018; Cheng et al., 2019; Zheng et al., 2020; Niazian et al., 2022).

Hairy roots are formed through the transfer of DNA (T-DNA) from the plant pathogen *A. rhizogenes* to the host plant (Bahramnejad et al., 2019). This transformation results in the development of hairy root syndrome, where chimeric composite plants with transgenic roots and wild-type shoots are generated within a few weeks. While transgenic hairy roots lack inheritability of the introduced mutation to the progeny, they provide valuable resources for unraveling the functions of target genes before investing significant time and resources into generating transgenic plants (Cai et al., 2015; Shu et al., 2020).

One of the primary targets for legume studies utilizing *A. rhizogenes* as a delivery vector for gene editing is the investigation of genes related to nodulation and nodule development. For instance, a previous study on *V. unguiculata* transformation using *A. rhizogenes* strain K599 demonstrated the essential role of the symbiosis receptor kinase (SYMRK) in nodule organogenesis in cowpea (Ji et al., 2019). The authors achieved a 67% editing efficiency by employing various guide RNAs (gRNAs). This efficiency was notably higher than that observed by Wang et al. (2016), who disrupted the SYMRK gene using *A. rhizogenes* LBA1334 and a single gRNA for transforming *L. japonicus*, resulting in a mutation frequency of 37%. Trinh et al. (2022) further highlighted the utility of this system for validating CRISPR/Cas activities and studying gene functions. They evaluated the effectiveness of mutating different selected gRNAs using *A. rhizogenes* K599, and multiple gRNAs targeting two endogenous soybean genes (SACPD-C and SMT) achieved mutation rates of 75% and 67%, respectively, with various indels detected at the target sites. Recently, a new method for transforming hairy roots in soybean, called eR&T, has been reported. This method exhibited a transformation frequency of 63.7%, significantly higher than that of the traditional method (35.8%). Moreover, the eR&T transformation efficiency approached 100%, whereas the traditional method reached only 75%. Huang et al. (2022) investigated the efficiency of eR&T in generating CRISPR/Cas9-mediated soybean mutants targeting two nodule regulatory genes, GmNSP1a and GmNSP1b. These mutants displayed reduced nodulation when induced by *S. fredii* HH103, producing fewer nodules compared to wild-type roots.

In summary, the transformation method using *A. rhizogenes* has proven to be an excellent model system for obtaining transgenic roots in leguminous plants, significantly reducing the time between reagent delivery and mutation evaluation (Li et al., 2019; Yuan et al., 2019; Triozzi et al., 2021).

Transformation by Particle Bombardment

Particle bombardment, also known as biolistic, is an alternative method for transforming plant species that are recalcitrant to *Agrobacterium* infection (Chu & Agapito-Tenfen, 2022). It is a direct transformation approach (Altpeter et al., 2005; 2016) that involves the physical disruption of the plant cell wall and membrane. In this method, high-velocity gold or tungsten micro-projectiles are used to introduce foreign DNA into plant tissues or cells (Svitashev et al., 2015; Ozyigit & Kurtoglu, 2020). The biolistic method offers several advantages, such as the ability to deliver various cargo types including plasmid DNA, ssDNA, RNA, or RNPs (Laforest & Nadakuduti, 2022), and it is not dependent on the receptivity or genotype of the host. It can transform diverse tissue or cell types, and it does not require a binary vector (Baltes et al., 2017). This technique can be used for both transient and stable gene expression (Singh et al., 2022). The first report on delivering CRISPR/Cas9 components through biolistic transformation in legumes was published by Li et al. (2015). They co-bombarded soybean embryonic callus with plasmids expressing Cas9 and sgRNA. They achieved small insertions or deletions within the cleavage site through NHEJ, with mutation frequencies of 59% and 76% for DD20 and DD43, respectively (Li et al., 2015). However, progress in legumes using CRISPR/Cas9 via biolistic

is currently limited to soybean genotypes (Campbell et al., 2019; Adachi et al., 2021). This method presents challenges due to the high pressure applied during tissue bombardment, which can result in tissue damage (Banakar et al., 2019), thereby reducing editing efficiency (Sandhya et al., 2020), such as Cas9 gene fragmentation (Jacobs et al., 2015). Major drawbacks include random integration of the cargo at multiple genomic sites when delivered as DNA, labor-intensive explant preparation (Laforest & Nadakuduti, 2022), relatively high cost, and low accuracy (Liu et al., 2019). Therefore, the biolistic delivery method requires improvements to enhance transformation efficiency and delivery consistency.

Protoplast transformation

Protoplast transformation/transfection involves the delivery of DNA, RNA, and ribonucleoprotein (RNP) using PEG, microinjection, or electroporation (Yue et al., 2021). Protoplast transformation has some advantages over other methods, including delivery of multiple plasmids with high levels of cotransformation, use of inexpensive supplies and not having specialized equipment requirements (Rustgi et al., 2022). The introduced DNA can be expressed transiently, or it may integrate into the genome, resulting in stable transformation (Baltes et al., 2017; Jansing et al., 2019). However, for a stable transformation, regeneration of the entire plant is necessary, which is a complex, time-consuming, and expensive process.

Therefore, the transient transformation of protoplasts is an alternative strategy to rapidly unravel novel gene functions, including as transcriptional activity of promoters, subcellular localization of proteins, DNA-protein interaction, protein-protein interaction, etc. (Wu et al., 2009). This strategy has been successfully used in some legume species, including *P. vulgaris* (Nanjareddy et al., 2016), *L. japonicus*, *M. trunculata* (Jia et al., 2018), soybean (Wu & Hanzawa, 2018; Xiong et al., 2019) and chickpeas (Cheng & Nakata, 2020).

Protoplast-based transfection has been utilized in legumes to assess the functionality of the CRISPR/Cas system, including the validation of vectors for gene editing (Sun et al., 2015; Patil et al., 2022), evaluation of gene editing efficiency (Yuan et al., 2019), and primary editing optimization (Biswas et al., 2022). However, the lack of an efficient system for isolating, regenerating, and transforming legume protoplasts has limited the application of gene editing through protoplasts in many legume species, such as cowpeas (Juranić et al., 2020).

Indeed, RNP-based protoplast editing offers several advantages over other methods. The use of RNP complexes, consisting of the Cas protein and the guide RNA, allows for a simplified and direct delivery of the editing components into the living cells without the need for DNA integration. This approach bypasses the potential complications associated with DNA delivery, such as random integration and off-target effects. By delivering pre-assembled RNPs, the editing process can be more efficient and precise, leading to higher editing rates and reduced off-target effects (Huang et al., 2020).

Perspectives for legume production DNA-free edit

Although efficient, the above delivery methods such as *Agrobacterium*-mediated transformation or bombardment of particles containing CRISPR expression cassettes can cause integration of transgenes at random sites in the plant genome which can disrupt essential genes or result in variable transgene expression (Sun et al., 2016; van Kregten et al., 2016; Liu et al., 2019). Furthermore, any foreign DNA incorporated into host DNA is considered genetically modified organisms (GMOs) and requires government regulation. To overcome these issues, DNA/transgene-free genome editing technologies have emerged as a promising alternative.

An alternative solution to address the challenges associated with transgenic-based approaches is the use of pre-assembled ribonucleoproteins (RNPs) consisting of the Cas protein and chemically synthesized gRNA(s) for plant genome editing (Zhang et al., 2021). Importantly, since no recombinant DNA is involved in this process, plants edited with RNPs can be considered transgene-free. RNPs offer several advantages in terms of precision and transient presence within plant cells. They directly target the specific genomic region of interest upon delivery, minimizing off-target effects (Razzaq et al., 2019). Additionally, RNPs are transiently present in plant cells and undergo degradation by endogenous proteases and nucleases within a few hours (Metje-Sprink et al., 2019). This transient nature significantly reduces mosaicism, toxicity, and off-target effects that may arise from prolonged exposure of genomic DNA to CRISPR reagents (Kim et al., 2017a; Liang et al., 2017), thereby minimizing the chances of random DNA integration (Andersson et al., 2018; Zhang et al., 2020a).

Direct transfer of CRISPR/Cas reagents in the form of RNPs into plant cells can be achieved through various delivery methods, including particle bombardment (Liang et al., 2018), electroporation (Lee et al., 2020), lipofection (Liu et al., 2020), and the commonly used method of PEG-mediated transfection (Yu et al., 2021). However, it is important to note that the efficiency of RNP delivery can vary depending on the type of tissue being targeted. In many non-DNA approaches, protoplasts serve as the preferred recipient explant for delivering CRISPR/Cas RNPs, and this holds true for legume species as well. Protoplasts are plant cells whose cell walls have been enzymatically removed, allowing for direct access of RNPs to the cellular machinery involved in genome editing. This approach has been widely adopted due to the high delivery efficiency and the ability to target a variety of cell types within the plant (Sun et al., 2016).

By using protoplasts as the target for RNP delivery, researchers can bypass the limitations of cell walls and achieve efficient and precise genome editing. Protoplast-based RNP delivery has been successfully demonstrated in legume species such as soybean and chickpea (Badhan et al., 2021; Banakar et al., 2022). Notably, studies have reported successful editing of multiple genes simultaneously in soybean protoplasts using RNP complexes (Kim et al., 2017a). These advancements highlight the potential of protoplast-based approaches for precise genome editing in legumes and other plant species.

To date, the delivery of Cas/gRNA RNPs has primarily been demonstrated in PEG-mediated protoplast assays in

legume species such as soybean and chickpea (Badhan et al., 2021; Banakar et al., 2022). In one notable legume study by Kim et al. (2017a), CRISPR/Cas9-mediated editing using RNPs was successfully performed in soybean and tobacco protoplasts. The authors achieved simultaneous editing of two genes, FAD2-1A and FAD2-1B, based on a conserved sequence in soybean protoplasts (Kim et al., 2017a). In a more recent study, the same authors developed a screening system using soybean callus-derived protoplasts for DNA editing and observed differential indel induction in the two FAD2 genes using LbCpf1-RNPs. For the Daewon cultivar, the indel frequencies were reported as 17.5% and 10.3% for GlymaFAD2-1A and GlymaFAD2-1B, respectively, after 48 hours of incubation (Kim & Choi, 2021). Another study by Subburaj et al. (2022) used the SpCas9-RNP system to mutate the CPR5 gene, which regulates trichome growth in soybean leaf mesophyll protoplasts (*Glycine max*). The researchers achieved a higher mutation frequency of 18.1% compared to plasmid-mediated editing, demonstrating the efficiency of RNPs in this context (Subburaj et al., 2022). However, despite these advancements, the regeneration of entire plants from edited protoplasts remains challenging for many species, including legumes. To overcome this hurdle, alternative RNP transformation methods such as de novo meristem induction have been proposed (Maher et al., 2020). This approach involves the concomitant expression of developmental regulators (DRs) and gene editing cassettes, which enables the creation of gene-edited shoots through de novo meristem induction and the edited DNA can then be successfully transmitted to the next generation.

In summary, further research is urgently needed to expand, accelerate, and improve the efficiency of the transformation, selection, regeneration, and delivery methods of CRISPR reagents for different plant species, with a particular focus on legumes. Addressing these challenges will facilitate the wider application of CRISPR-based genome editing in legumes and contribute to advancements in crop improvement and agriculture.

Yield Improvement in Legumes

Crop yield is a complex, multigenic, quantitative trait. Several traits related to yield have been studied, such as root and shoot architecture, leaf and vasculature features, and flowering responses (Mathan et al., 2016, Bailey-Serres et al., 2019). These traits provide directives for the use of CRISPR strategies to improve legumes, where some advances have already been achieved. To change plant architecture, Bao et al. (2019) used CRISPR/Cas9 to edit the *GmSPL9* gene family (*GmSPL9a*, *GmSPL9b*, *GmSPL9c*, and *GmSPL9d*). Four mutants (*spl9a/spl9b-1/spl9c/spl9d*) showed a higher number of nodes. However, the SpCas9 used in this study was controlled by a 35S promoter, and the authors reported that only after the T4 generation could they observe mutations in the four genes of the family. Gao et al. (2018) also used CRISPR/Cas9-directed mutagenesis in the gene SPL9 in the legume *M. sativa* and obtained an editing frequency of 2.2%, which is relatively low compared to that obtained in other plant species. One solution to overcome the difficulties awaiting the 4th generation, or even the low-efficiency transformation in *M. sativa,* could be the use of temperature-sensitive Cas9 or Cas12 proteins. Inducing heat stress (37°C) reportedly improved the efficiency 5-fold in somatic tissues and 100-fold in germline in *Arabidopsis* with Cas9 (LeBlanc et al., 2018). Recently, another group showed that using Cas12a, the editing activity increased to more than 28°C in rice, maize, and *Arabidopsis* (Malzahn et al., 2019). Applying heat stress a few days after *Agrobacterium* inoculation could improve the efficiency or augment the number of mutations under heat treatment.

Plant height is partly determined by the phytohormone gibberellin (GA) (Hedden & Sponsel, 2015; Wang et al., 2017). Manipulation of the GA pathway has already been used to improve yields in crops and legumes, leading to an increase in leaf area and dry matter (Phillips, 2016). After biosynthesis, GA interacts with GA receptors and other phytohormones to act as hub height regulators, as well as with DELLA proteins, which in turn are GA repressors. Thus, repressing *DELLA* expression could increase GA levels and influence legume yield. CRISPR interference (CRISPRi) strategies have been established in plants and consist of mutated dead Cas9 (dCas9) proteins fused with repressor domains, such as SRDX or KRAB (Xing et al., 2014, Lowder et al., 2017). The use of CRISPRi to modulate DELLA could improve yield in legumes. However, it requires a tissue-specific promoter and one or more sgRNAs, since GA and DELLA proteins play important roles during symbiosis and nodulation in legumes (Jin et al., 2016; Dolgikh et al., 2019).

Other important traits that could be engineered to improve yield in plants include leaf morphology and anatomy, vascular bundles, and fruits, as well as control of flowering time. Leaf anatomy is directly linked to photosynthetic rate, which is related to the area exposed to sunlight (Mathan et al., 2016). Vascular bundles distribute water and nutrients and drive the mobilization of carbon (Sack & Scoffoni, 2013). Leaves and their vascular bundles are formed by a similar onset of genes that govern leaf morphogenesis. Editing these genes can increase leaf performance and impact yield. Genes that control shoot meristems, such as *WUSCHEL* and *CLAVATA*, could be good candidates. Rodríguez-Leal et al. (2017) targeted the promoter of the tomato *CLV3* gene using eight sgRNAs to produce different alleles corresponding to different expressions of the *CLV3* gene and generate a phenotypic effect on yield. Some orthologs of these genes have been found in the most studied legume species to date and could be possible candidates for editing. For instance, *GmCLV1A*, *GmCLV1B*, *GmCLV2*, *GmCLV3*, *GmNARK*, and *CLE* genes in soybean, several *WOX* and *PIN* genes, *LEAFY* orthologs, *UNIFOLIATA* (*UNI*), *SINGLE LEAFLET1* (*SGL1*), and *HEADLESS* in *M. truncatula* seem to play roles in meristem maintenance (Osipova et al., 2011, Hastwell et al., 2015; Chen, 2018; Jiao et al., 2019; Meng et al., 2019).

Several studies have shown how genes, such as *GmFT2a* and *GmFT5a*, which have been identified as flowering activators and integrators in soybean, help expand their regional adaptability (Cai et al., 2018a, 2018b). As in many plants, flowering is regulated by seasonal changes in the day, which may limit the geographic range of crops (Jung & Müller, 2009). The use of CRISPR/Cas9 to knock out these genes and modify

plant flowering time could expand the geographic range of some legumes. The *ft2aft5a* double mutants produced flowers approximately 31 days later than wild plants, and they maintained vertical growth, producing significantly increased numbers of nodes, resulting in a substantial increase in new branches and consequently more pods and seeds per plant (Cai et al., 2020a). In addition to developmental genes, modification of photosynthetic carbon assimilation (PAC) has already been accomplished (Simkin et al., 2015; Calzadilla et al., 2019). However, photosynthesis and carbon assimilation consist of a complex pathway of reactions controlled by numerous enzymes. Therefore, simple targeted modifications would not be sufficient. Some studies have highlighted how researchers can change PAC by improving some inefficient steps of photorespiration or by increasing the CO_2 concentration at the site of Rubisco by introducing CO_2 pumps (Weber & Bar Even, 2019). CRISPRi or CRISPR activation (CRISPRa). This consists of the same dCas9 as CRISPRi but fused to transcriptional activators and could be an efficient method to synthetically engineer complex pathways, such as PAC. In bacteria, CRISPRi and CRISPRa have already been used to repress or activate multiple genes (Kim et al., 2017b; Mougiakos et al., 2018; Liu et al., 2019a). In plants, engineering these strategies has already been mentioned to make more pungent tomatoes (Naves et al., 2019).

In contrast, Weiner (2019) proposed that increasing yield in plants is based on modifying characteristics by improving physiological processes that usually result in decreased plant fitness. He suggested that improving yield strategies should not focus on improving traits without considering modifying performance (fitness) under specific field conditions. His argument clarified that, for example, for genetic photosynthetic improvement, other co-factors should sometimes be added in the field as fertilizers (Weiner, 2019). Nonetheless, the advantage of CRISPR transcriptional regulation strategies broadens all and thus could hijack the lack of control already existing in classical transgenic engineering.

To address the specific demands of each country, research groups worldwide study legumes that are adapted to their respective regions. A notable study by Van Loon & Van Wassenhove (2018) shed light on the challenges faced in legume farming in three East African countries. The authors emphasized that legumes such as common bean, pigeonpea, chickpea, cowpea, and groundnut have a yield potential ranging from 3 to 7 Mg/ha. However, to achieve such yields, it is crucial to develop new legume crop varieties that can thrive in various water-limited conditions. This remains a pressing concern at least until 2050, particularly for countries like Kenya and Tanzania. To address this issue, studies are conducted in numerous laboratories worldwide, with a focus extending beyond soybean, which currently receives the most research attention. By diversifying the scope of research to include a wider range of legume crops, scientists can contribute to the development of innovative solutions that meet the specific needs of different regions and enhance legume production on a global scale.

Improvement of quality and nutrition in legumes

In 2016, the Food and Agriculture Organization of the United Nations declared 2016 the international year of pulses to heighten public awareness of the nutritional benefits of legumes as part of sustainable food production for food security and nutrition (FAO, 2016). Legumes are low in fat, rich in vitamins, provide proteins and essential minerals, and have cultural and medicinal roles by their bioactive compounds (Maphosa & Jideani, 2017). Although the nutritional effects of pulses are well recognized, some regions of the world as Asia and Africa have not increased the rate of legume production to match their increasing rate of consumption, and research is almost always focused on cereal crops (Nedumaran et al., 2015). Moreover, legume consumption has important protective effects against various human diseases (Li et al., 2017a; Çakir et al., 2019). Accordingly, the biofortification of *Leguminosae* species through CRISPR technologies could contribute to the nutritional quality improvement of legume crops when they require a certain nutrient compound from a defined population.

Efforts to modify the quality of legumes have been made using CRISPR systems. Soybean oil is a source of linoleic acid (omega 6), oleic acid (omega 9) and linolenic acid (omega 3). Linoleic acid is an oxidation-prone polyunsaturated fatty acid that causes an unpleasant taste and has a short shelf life, while oleic acid is monounsaturated, has self-oxidative stability, and is beneficial to health. Therefore, changes in the fatty acid profile of soybean could be attractive for regulating the oil content of these acids. Fatty acid desaturase-2 enzyme (FAD2-1) is responsible for the conversion of oleic acid to linoleic acid in developing soybean seeds (Okuley et al., 1994; Schlueter et al., 2007). Mutations in *FAD2-2* genes using the CRISPR-Cas9 system resulted in a 65% increase in oleic acid content (Al Amin et al., 2019). The simultaneous use of two gRNA for the genes *GmFAD2-1A* and *GmFAD2-1B* resulted in dramatic increases in the oleic acid content (over 80%), whereas the linoleic acid content decreased by 1.3-1.7% (Do et al., 2019). Furthermore, 77.8% (7/9) of T0 events showed heritable mutations in either or both genes. In contrast, gene editing using the TALEN system results in only 50% transmission of both *GmFAD2-1A* and *GmFAD2-1B* mutations to T1 progenies, showing that CRISPR/Cas9 is more stable, easier to design, and achieves a high level of heritable mutations (Haun et al., 2014). In peanuts, a gene editing approach using the CRISPR/Cas9 system has also been used to improve oil quality. Yuan et al. (2019) induced mutations in *ahFAD2A* and *ahFAD2B* by transforming *Agrobacterium* protoplasts and root hair to decrease fatty acid desaturase activity and allow oleic acid accumulation during seed development.

Dietary standards are constantly changing. Vegetarianism and veganism are increasing in popularity. These dietary patterns include many legumes and soybean derivatives (Orlich et al., 2014). In legumes, methionine is considered a limiting amino acid, but they are also deficient in lysine and tryptophan (Le et al., 2016). CRISPR approaches could be used in different strategies to augment the number of missing amino acids. Introducing proteins rich in lysine, methionine, or tryptophan via HDR is one such choice.

Another CRISPR system consisting of precise genome editing by cytidine base editors (CBE) and adenine base editors (ABE) has been used and could also prove attractive for modifying the amino acid composition of proteins and increasing the total amount of a limiting amino acid. CBE fusion to Cas9 catalyzes a cytosine deamination reaction in the single-stranded non-target DNA strand, converting cytosine into uracil and resulting in C-to-T replacement. ABEs catalyze adenine deamination when Cas9 recognizes a protospacer adjacent motif (PAM) to catalyze the conversion of adenine to hypoxanthine in the PAM distal region of single-stranded non-target DNA. Hypoxanthine is recognized as G by DNA repair systems, triggering the incorporation of a C base paired with hypoxanthine (Kim, 2018; Zhang et al., 2019). With prior extensive analysis of the entire sequence, it could be possible to modulate new codons to increase the amount of certain amino acids in a certain protein that is not vital for the plant. For example, many components in legumes have been described as antinutrients and can be transformed into non-functional proteins, with increased amounts of certain amino acids (Muzquiz et al., 2012).

Antinutrients can vary from metabolites, such as vicine in faba bean, oligosaccharides, alkaloids in lupin species, and various classes of proteins, and can have toxic effects if not deactivated before consumption. Additionally, several proteins have been identified as allergens. In peanuts, *Ara h1* to *Ara h8* are considered allergens depending on the sensitivity of allergic patients. Soybean, lentil, lupins, pea, chickpea, blackgram, French bean, green gram, and red gram have already been identified as allergens, with proteins from different families, such as prolamins, cupins, profilins, defensins, and oleosins (Verma et al., 2013; Cabanillas et al., 2018). RNA interference (RNAi) has been used to silence Ara h proteins with promising results for the recognition of proteins in the sera of allergic patients (Riascos et al., 2010). However, it would be interesting to target more allergenic proteins simultaneously. Jenkins et al. (2005) showed that the universe of plant allergens is small, with few families, and that many of these so-called isoallergens contain high sequence identities. Consequently, a CRISPR strategy could be adapted to target different isoallergens with one or few sgRNAs and many allergens from different families in a single CRISPR-edited plant. Diverging from other CRISPR systems, a new Cas13 protein was found to recognize 22-28 nucleotides in RNA sequences, followed by a protospacer flanking sequence or with no specific requirement. This new technology permits simultaneous cleavage of multiple RNAs and gene expression knockdown (Zhang et al., 2019). It has already been performed in *N. benthamiana* and *Arabidopsis*, where researchers used LshCas13a to interfere with the RNA genome of the Turnip mosaic virus (Aman et al., 2018). This strategy may be interesting because it permits multiple allergen knockdowns in the same plant. In contrast to prior biotechnological plants, it would permit the creation of peanuts or soybeans with multiple targeted allergens, and thus broaden the type of allergies targeted.

Although there has been significant progress in the elucidation of transcriptomic and genomic resources in legumes, there is still a need for an exploration of whole-genome data sequences, germplasm resources, and single nucleotide polymorphisms related to metabolite production and nutrient absorption, which could help in the strategy of CRISPR biofortification (Rehman et al., 2019). CRISPR sgRNA libraries have been used to discover the functions of genes and regulatory elements. Collections of mutants with CRISPR libraries have been used for tomato and rice (Jacobs et al., 2017; Lu et al., 2017; Meng et al., 2017b). Zhang et al. (2019) proposed the delivery of sgRNA libraries into plant cells or protoplasts and linked traits of interest to selectable markets to select individual cells for analysis and regeneration. These strategies could help in adapting the improvement of a certain legume and detecting interesting traits associated with biofortification, considering a more specific strategy depending on the needs of a certain population.

Improving resistance to abiotic and biotic stresses

Abiotic stresses

The Fabaceae family comprises species growing in all global biomes. Owing to their worldwide distribution, some species have different sensitivities in their responses to abiotic challenges. The real threat to legume production in the future is the stress caused by environmental changes. Faba bean and pea are drought-sensitive, whereas lentil and chickpea are drought-resistant. Drought stress has adverse effects on the total legume biomass, pod number, seed number, weight, and quality (Latef & Ahmad, 2015). Food legumes are also relatively sensitive to salinity, which can lead to a reduction in the shoot growth of soybean, chickpea, pea, faba bean, and mung bean. Other abiotic stresses, such as waterlogging, heat stress, nutrient deficiency, and toxicity, negatively affect the adaptability and yield of legumes worldwide. More importantly, humans have taken advantage of these plants mainly because of their symbiotic relationship with BNF organisms. This association has been proven to be more vulnerable in the presence of osmotic stress (Valentine et al., 2011; Mhadhbi et al., 2015).

Curtin et al. (2018) used CRISPR/Cas9 and TALEN targeting specificity to disrupt two genes with roles as dsRNA-binding proteins in soybean (GmDrb2a and GmDrb2b), for which mutants showed an altered abundance of drought and osmotic response proteins. In addition, these mutants are more resistant to salt stress. However, *GmDrb2ab* plants were more sensitive to drought stress, even with perturbations in different microRNAs (*miR156*, *miR163*, and *miR168*). In the same year, soybean plants carrying mutations in the *GmMYB118* gene using the CRISPR/Cas9 system were reported to exhibit reduced drought and salt tolerance, indicating the importance of this transcription factor in activating genes in response to abiotic stresses (Du et al., 2018). Additionally, Li et al. (2018) constructed a dual-gRNA CRISPR vector to knock out *GmSnRK1.1* and *GmSnRK1.2* genes in soybean hairy roots, which inhibited root growth in abscisic acid (ABA) and alkaline treatments. Despite these efforts, CRISPR-edited legumes have not yet shown a real improvement. A detailed understanding of the mechanisms underlying specific stresses will result in a successful strategy. The general mechanisms in legumes remain similar to those in other plants, where

a signaling cascade is initiated after the perception of the stress factor, which involves the activation of molecular pathways that respond specifically to a certain pressure. For example, during salt stress, legumes produce metabolites that act as osmoprotectants, such as amino acids (ectoine, proline), carbohydrates (trehalose, polyols), proteins (dehydrins, chaperones), and amines (glycine betaine and dimethyl sulphonium) (Babar et al., 2015). Some studies have pointed out that the molecular approach in legumes remains comparable to that in other crops and consists of modifying the expression of regulatory genes, such as protein kinases, phosphatases, and transcription factors (Mousavi-Derazmahalleh et al., 2019; Nadeem et al., 2019). The overexpression, modification, or mutation of the encoding genes will activate stress signals and downstream "tolerance genes" that encode antioxidant enzymes that participate in reactive oxygen species detoxification, osmoprotection, and assistance in protein and ionic homeostasis (Shinozaki & Yamaguchi-Shinozaki, 2007; Lamaoui et al., 2018).

Overexpression of key genes is one of the strategies most used in plant engineering. Efforts have sought to increase the expression of genes that help at different phases of abiotic stress signaling cascades. The overexpression of transcription factors remains one of the most reliable methods because it activates a series of downstream genes that can enhance the cascade response. Transcription factors that have been studied more belong to different families (*MYB, bHLH, WRKY, bZIP,* and *NAC*). Some have been used in abiotic stress transgenic approaches (Tiwari et al., 2020; Zafar et al., 2020). Shi et al. (2017) used CRISPR HDR with the sequence of the promoter (*GOSPRO2*), which generates moderate expression, either to replace the *ARGOS8* promoter or embed into its 5' untranslated (UTR) region. To do this, the authors used a two-sgRNA system and were able to increase *ARGOS8* expression, inducing a better tolerance under drought stress in maize (Shi et al., 2017). When directed by specific gRNAs, HDR is a powerful tool for choosing the location in the genome to insert any sequence insertion. Sometimes, good transgene expression depends on the site insertion. With CRISPR HDR, it is possible to target known regions in which high gene expression is constant. Some expression quantitative trait loci (QTLs) can affect some tissues more than others (Kloosterman et al., 2012; Mizuno & Okada, 2019). Depending on the transgene, one strategy could be to target these regions for the inserted transgene to be expressed in a specific tissue with better reliability for good expression. Besides promoter replacement, other studies have attempted to increase endogen expression by modifying promoters using CRISPRi and CRISPRa strategies. When endonuclease dead Cas9 (dCas9) is fused to activation domains (VP64, EDLL, TAB), strategies in plants have shown that a promoter can be more active and increase gene expression. Fusions of dCas9 with epigenetic modulators (histone acetyltransferase) have also been shown to increase gene expression when targeted to its promoter and confer good responses to plants when subjected to H_2O deficits (Lowder et al., 2015; Piatek et al., 2015; Tang et al., 2017; Roca Paixão et al., 2019). Interestingly, the combination of modifying or replacing the structure of one or more promoters (*cis* engineering) targeted by modified and ameliorated dCas9-transcription factors (*trans* engineering) is a promising goal to procure more complex responses to abiotic stresses (Shrestha et al., 2018). Still, such strategies require a trans-sequence and thus could be contemplated differently from CRISPR gene-free strategies for agriculture. These strategies remain compelling for fundamental research.

On the other hand, genes that negatively regulate abiotic stresses are good knock-out candidates using CRISPR strategies. For example, some stress-induced genes involved in the abscisic acid (ABA) response have been used with CRISPR. Mutations in the rice gene *OsABA8ox*, which catalyzes ABA, accumulate more ABA and show increased drought tolerance (Zhang et al., 2020b). Additionally, the targeted mutation of *Semi-rolled leaf1,2* in rice resulted in higher survival rates, ABA content, superoxide dismutase (SOD) and catalase (CAT) activities, and increased drought tolerance in the mutants (Liao et al., 2019). Other potential targets for improving tolerance against abiotic stresses have been proposed and identified as "sensitivity" genes (Zafar et al., 2020). More profound research is needed to identify good candidates to ameliorate legume responses to abiotic stresses. Presently, there is a good basis for studies on other plant families.

Biotic stress

Weeds are one of the major threats to agricultural productivity, as they compete with crops for light, water, space, and nutritional resources (Délye et al., 2013; Quareshy et al., 2018). The average annual economic loss of soybean crops due to weeds in the United States alone is estimated at $16 billion (Soltani et al., 2016). The development of herbicide-resistant legume varieties by genome editing holds great promise for addressing the worsening weed problems in legume crops. Li et al. (2015) pioneered the directed editing of genes that confer resistance to soybean herbicides using Cas9-gRNA. In this study, the *ALS1* gene conferring resistance to chlorosulfuron was successfully interrupted by a sequence exchange event. Acetolactate synthase is an essential enzyme for branched-chain amino acid biosynthesis and a major target of agriculturally important herbicides. NHEJ has been used for the targeted mutagenesis (small deletions and insertions) of two DD20 and DD43 soybean target sites, with mutation frequencies of 59% and 76%, respectively, as well as replacement of HDR-directed endogenous sequences (Li et al., 2015).

Other CRISPR/Cas strategies can focus on improving plant defense against pest attacks, modifying gene expression, inserting genes that compensate for the loss of photosynthetic activity, diverting resources from growth into reproduction, or modifying phytohormone balances. Additionally, attack by pathogens or parasites generates, after the first round of constitutive response, the recognition of pathogen-associated molecular patterns (PAMPs), activating cascades that comprise the synthesis of jasmonic acid (JA), salicylic acid (SA), and ethylene, and the activation of a transcription factor that contributes to the response (Santamaria et al., 2018; Hou et al., 2019). Again, CRISPR overexpression mediated by T genes or mutation of S genes that amend the plant response to a certain attack could be an attractive possibility. Haque et al. (2018) reviewed studies that used CRISPR to mitigate pathogen attacks in plants. However, real solutions for pest management need to include other eco-

friendly practices to overcome the difficulties. Integrated pest management has already been proposed for legume production in Asia and Africa. As plant scientists, we need to emphasize these strategies (Schreinemachers et al., 2014).

Sustainability and biological nitrogen fixation

The bioavailability of soil organic nitrogen is one of the most important factors limiting plant growth and crop yield. Legumes interact with bacterial species that can fix nitrogen in the soil (Masson-Boivin & Sachs, 2018), contributing to the sustainability of agricultural systems. Rhizobium-leguminous symbiosis involves the exchange of the carbon source produced by the plant and ammonium fixed by the bacteria; this process is called biological nitrogen fixation (BNF). Is the most efficient method of N_2-fixation with amounts of 20–300 kg/ha/yr, which accounts for approximately 60% of all fixed N on earth (Soumare et al., 2020). Genetic studies have shown that effective BNF requires the coordination of multiple metabolic and transport pathways in both plants and microbes.

Over the past 23 years, surprising progress has been made in the discovery of nearly 200 genes required for BNF in legumes (Roy et al., 2020). For example, *LjSYMRK* and *MtDMI2* were the first "common symbiotic" genes required for both rhizobial and mycorrhizal symbioses (Endre et al., 2002; Stracke et al., 2002). These findings have increased our understanding of the evolution of BNF in plants and helped select breeding programs for the effectiveness of BNF in legume crops. However, the scenario has been amended since the development of the new CRISPR gene editing tool, creating the possibility of identifying genes that may contribute to the effectiveness of BNF. For example, Ji et al. (2019) developed an alternative strategy in *ex vitro V. unguiculata* plants that was successfully applied to CRISPR/Cas9 to effectively disrupt the kinase-like symbiosis receptor gene (*VuSYMRK*) in transgenic hairy roots of *A. rhizogenes*. The mutant roots did not develop nodules after inoculation with *Sinorhizobium* sp., indicating the main function of (*VuSYMRK*) in nodule formation. Yang et al. (2022) also applied CRISPR/Cas9 to edit the heat shock protein, GmHSP17.9, located in soybean nodules. The resulting mutant plants showed a remarkable alteration in nodule number, nodule fresh weight, and nitrogenase activity, which caused significant changes in the seed yield of the plants.

Lotus japonicus is one of the most widely used model legumes for BNF studies because of its relatively small genome and diploid nature. The kinase-like symbiosis receptor gene (*SYMRK)* and leghemoglobin gene (*LjLb1, LjLb2, and LjLb3*) were knocked out in the same transgenic lineage using two gRNAs (Wang et al., 2016). Leghemoglobins are essential for buffering oxygen in nodules at low levels, while transporting them rapidly to rhizobia. It was also reported that for nodule gene editing using CRISPR/Cas9, the nodulation-specific *LjLb2* promoter was more effective compared to the constitutively active CaMV35S promoter. In this study, there was a color change from pink to white nodules in addition to their reduced size, resulting in lower symbiotic nitrogen fixation efficiency in *L. japonicus* (Wang et al., 2019). Legume genomes typically encode several *Lb* genes that account for at least 10% of the total mRNA and 5% of the total protein in mature nodules (Ott et al., 2005). In this regard, novel strategies using CRISPR can be implemented to modify the elements of nitrogen incorporation, such as receptors and/or enzymes, to improve nitrogen uptake, increase species interactions, and to install mutations designed to create structural changes in sgRNA genes. All these strategies may increase the efficiency of BNF in legumes.

Advantages and limitations of the CRISPR system in legumes

The CRISPR system has made remarkable progress in recent years, continually expanding its capabilities, and improving the efficiency of genomic editing in plants. One particularly appealing feature of the CRISPR/Cas system is its ability to perform multiplex editing, enabling the simultaneous mutation of multiple genes in a single transformation event. This multiplex editing capability significantly reduces the time and cost required to generate new plant varieties (Borrelli et al., 2018). In the context of legumes, the CRISPR/Cpf1 system has emerged as an efficient multiplex genome editing tool. Studies have demonstrated its high editing efficiency, reaching up to 91.7% in soybeans (Duan et al., 2021). CRISPR/Cpf1 complements CRISPR/Cas9 and offers several advantages, including the ability to perform DNA-free genome editing and greater flexibility in targeting different genomic regions. Unlike Cas9, which requires a G-rich protospacer adjacent motif (PAM), Cpf1 use a T-rich PAM, allowing it to target a broader range of genomic sequences (Zetsche et al., 2015; Shmakov et al., 2017). However, to further enhance genome editing in legumes, it is essential to develop additional variants of nucleases that can recognize a wide array of PAM sequences. This would expand the scope of genome editing possibilities and enable more precise and efficient genetic modifications in legume crops. Overall, the advancements in CRISPR technology, particularly the development of multiplex editing systems like CRISPR/Cpf1, hold great promise for improving genomic editing in legumes. Continued research and innovation in this field will contribute to the development of new legume varieties with enhanced traits and improved adaptability to abiotic and biotic stresses, ultimately benefiting legume crop production and global food security. In addition to the CRISPR/Cas9 system, other CRISPR-based editing systems, such as adenine base editors (ABEs) and cytosine base editors (CBEs), offer precise genome editing capabilities. These base editors enable the transfer of DNA bases without breaking the template DNA, allowing for targeted nucleotide changes (Komor et al., 2016). ABE and CBE have been successfully applied in two leguminous crops, soybean, and alfalfa, demonstrating their potential for precise editing in legumes (Cai et al., 2020b; Bottero et al., 2022). However, base editing does have limitations, such as the specific nucleotide changes it can introduce (Mishra et al., 2020). To overcome this limitation, a newer addition to the CRISPR toolbox, prime editing (PE), has been developed. PE allows specific changes to be introduced at a targeted locus through a single-stranded DNA break, enabling the conversion of any base to any other base with minimized off-target effects. Although initial studies suggest lower editing efficiencies with PE, especially for generating stable transformants, it holds potential as an important tool for

genome editing in leguminous plants (Lin et al., 2020). While the CRISPR/Cas9 technique has been extensively studied for soybean modification, its use in other leguminous plants is still limited due to various challenges. Legumes often exhibit recalcitrance to *in vitro* gene transfer, which hinders efficient transformation and regeneration procedures. Additionally, leguminous crops, including soybeans, have relatively low mutation efficiency. To overcome these challenges, it is crucial to focus on developing new plant transformation and regeneration systems in combination with efficient CRISPR/Cas delivery methods. A promising advantage is the tissue culture-free genome editing system, which combines ex vitro hair root induction mediated by transient *A. rhizogenes* and the CRISPR/Cas technique. This approach offers a rapid, accurate, and cost-effective platform for functional validation of genes of interest in leguminous plants (Du et al., 2018; Alok et al., 2020; Niazian et al., 2022).

In conclusion, exploring alternative CRISPR-based editing systems, such as base editors and prime editors, along with the development of new transformation and regeneration techniques, will contribute to overcoming the challenges associated with CRISPR/Cas genome editing in leguminous crops. These advancements hold promise for accelerating the discovery and functional validation of genes in legume plants, ultimately enhancing their agronomic traits, productivity, and adaptation to environmental stresses.

Regulation

The CRISPR/Cas system is a powerful tool for crop improvement due to its efficiency, accuracy, robustness, adaptability, and simplicity. However, the use of this method raises regulatory and biosafety concerns, particularly regarding the classification of CRISPR-edited crops as genetically modified organisms (GMOs) and the generation of transgene-free plants. Regulations for genetically modified crops vary widely across countries, with some being more stringent and others more flexible. For instance, the European Court of Justice has ruled that CRISPR/Cas-edited crops should be subjected to the same rigorous regulations as GMOs (Court of Justice of the European Union, 2018). New Zealand also considers genome-edited plants as genetically modified and regulates them under GMO legislation (Fritsche et al., 2018). In contrast, the US Department of Agriculture (USDA, 2020) has declared that CRISPR/Cas9-edited crops will not be regulated and are exempt from GMO laws (Myskja & Myhr, 2020). Several South American countries, including Brazil, Chile, Colombia, Paraguay, and Uruguay, have established clear and flexible guidelines. If the final variety does not contain foreign genes or DNA, these countries do not subject it to additional regulatory oversight (Eckerstorfer et al., 2019; Smyth, 2019). Brazil, for example, has stated that plants created using "new innovative technologies in precision improvement" (genome editing) will not be regulated by the National Technical Commission of Biosafety (CTNBio), as they are with traditional genetically modified products. However, consultation with CTNBio is necessary to confirm the use of "innovation in precision improvement" rather than genetic engineering (normative resolution No. 16/2018, Brasil, 2018). Australia, Argentina, and Canada also exempt genome-edited products from regulations if they are proven to be non-GMOs (Gao et al., 2018; Tchetvertakov, 2019). Canada is unique in that its entire GM legislation is based on the product rather than the process (Turnbull et al., 2021). Japan, on the other hand, does not regulate plants edited using SDN-1 and SDN-2 (Gupta et al., 2021). Some countries, like India and China, are still in the process of developing regulations for gene-edited crops (Wani et al., 2022). There is an urgent need for global harmonization of regulations in this field. In 2018, a drought and salt-tolerant soybean variety edited using CRISPR/Cas9 received USDA clearance as an unregulated plant (Curtin et al., 2018). However, public acceptance of genome-edited crops remains a major challenge that needs to be addressed.

Conclusion

While CRISPR genome editing technology has advanced significantly in many crops, its application in legumes is still in its early stages. The progress made in the first eight years (2015-2022) of CRISPR/Cas genome editing in legumes has been limited. One major challenge is the low rate of transformation efficiency, which affects the success of the editing process and hampers the generation of a large number of edited plants in certain legume species.

To address these challenges, efforts are needed to increase the transformation efficiency of legumes. Researchers should focus on developing specific vectors and methodologies tailored to the unique characteristics of leguminous species. These customized tools can help enhance the efficiency of the editing process, enabling the generation of a larger number of edited plants.

Overall, while CRISPR/Cas genome editing has shown great potential for legume improvement, further research and development are necessary to overcome the limitations associated with low transformation efficiency and achieve more successful editing outcomes in leguminous crops.

Conflict of interests

The authors declare that there are no conflicts of interest.

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References

Adachi, K., Hirose, A., Kanazashi, Y., Hibara, M., Hirata, T., Mikami, M., Endo, M., Hirose, S., Maruyama, N., Ishimoto, M., Abe, J., & Yamada, T. (2021). Site-directed mutagenesis by biolistic transformation efficiently generates inheritable mutations in a targeted locus in soybean somatic embryos and transgene-free descendants in the T 1 generation. *Transgenic Research*, *30*(1), 77-89. http://doi.org/10.1007/s11248-020-00229-4. PMid:33386504.

Al Amin, N., Ahmad, N., Wu, N., Pu, X., Ma, T., Du, Y., Bo, X., Wang, N., Sharif, R., & Wang, P. (2019). CRISPR-Cas9 mediated targeted disruption of FAD2-2 microsomal omega-6 desaturase in soybean (Glycine max. L). *BMC Biotechnology*, *19*(1), 9. http://doi.org/10.1186/s12896-019-0501-2. PMid:30691438.

Alamillo, J. M., López, C. M., Rivas, F. J. M., Torralbo, F., Bulut, M., & Alseekh, S. (2023). Clustered regularly interspaced short palindromic repeats/CRISPR-associated protein and hairy roots: A perfect match for gene functional analysis and crop improvement. *Current Opinion in Biotechnology*, *79*, 102876. http://doi.org/10.1016/j.copbio.2022.102876. PMid:36621223.

Alok, A., Jain, P., Kumar, J., Yajnik, K., & Bhalothia, P. (2020). Genome engineering in medicinally important plants using CRISPR/Cas9 tool. In V. Singh, & P. K. Dhar (Eds.), *Genome engineering via CRISPR-Cas9 system* (pp. 155-161). Academic Press. http://doi.org/10.1016/B978-0-12-818140-9.00014-3.

Altpeter, F., Baisakh, N., Beachy, R., Bock, R., Capell, T., Christou, P., Daniell, H., Datta, K., Datta, S., Dix, P. J., Fauquet, C., Huang, N., Kohli, A., Mooibroek, H., Nicholson, L., Nguyen, T. T., Nugent, G., Raemakers, K., Romano, A., Somers, D. A., Stoger, E., Taylor, N., & Visser, R. (2005). Particle bombardment and the genetic enhancement of crops: Myths and realities. *Molecular Breeding*, *15*(3), 305-327. http://doi.org/10.1007/s11032-004-8001-y.

Altpeter, F., Springer, N. M., Bartley, L. E., Blechl, A. E., Brutnell, T. P., Citovsky, V., Conrad, L. J., Gelvin, S. B., Jackson, D. P., Kausch, A. P., Lemaux, P. G., Medford, J. I., Orozco-Cárdenas, M. L., Tricoli, D. M., Van Eck, J., Voytas, D. F., Walbot, V., Wang, K., Zhang, Z. J., & Stewart Junior, C. N. (2016). Advancing crop transformation in the era of genome editing. *The Plant Cell*, *28*(7), 1510-1520. http://doi.org/10.1105/tpc.16.00196. PMid:27335450.

Aman, R., Ali, Z., Butt, H., Mahas, A., Aljedaani, F., & Khan, M. Z. (2018). RNA virus interference via CRISPR/Cas13a system in plants. *Genome Biology*, 1-9. https://doi.org/10.1186/s13059-017-1381-1.

Andersson, M., Turesson, H., Olsson, N., Fält, A. S., Ohlsson, P., Gonzalez, M. N., Samuelsson, M., & Hofvander, P. (2018). Genome editing in potato via CRISPR-Cas9 ribonucleoprotein delivery. *Physiologia Plantarum*, *164*(4), 378-384. http://doi.org/10.1111/ppl.12731. PMid:29572864.

Anzalone, A. V., Koblan, L. W., & Liu, D. R. (2020). Genome editing with CRISPR–Cas nucleases, base editors, transposases and prime editors. *Nature Biotechnology*, *38*(7), 824-844. http://doi.org/10.1038/s41587-020-0561-9. PMid:32572269.

Anzalone, A. V., Randolph, P. B., Davis, J. R., Sousa, A. A., Koblan, L. W., Levy, J. M., Chen, P. J., Wilson, C., Newby, G. A., Raguram, A., & Liu, D. R. (2019). Search-and-replace genome editing without double-strand breaks or donor DNA. *Nature*, *576*(7785), 149-157. http://doi.org/10.1038/s41586-019-1711-4. PMid:31634902.

Arabidopsis Genome Initiative. (2000). Analysis of the genome sequence of the flowering plant Arabidopsis thaliana. *Nature*, *408*(6814), 796-815. http://doi.org/10.1038/35048692. PMid:11130711.

Arnoldi, A., Boschin, G., Zanoni, C., & Lammi, C. (2015). The health benefits of sweet lupin seed flours and isolated proteins. *Journal of Functional Foods*, *18*, 550-563. http://doi.org/10.1016/j.jff.2015.08.012.

Babar, M. M., Zaidi, N. U. S. S., Azooz, M. M., & Kazi, A. G. Genetic and molecular responses of legumes in a changing environment. (2015). Legumes under environmental stress: yield, improvement and adaptations, 199-214. https://doi.org/10.1002/9781118917091.ch12

Badhan, S., Ball, A. S., & Mantri, N. (2021). First report of CRISPR/Cas9 mediated DNA-free editing of 4CL and RVE7 genes in chickpea protoplasts. *International Journal of Molecular Sciences*, *22*(1), 396. https://doi.org/10.3390/ijms22010396.

Bahramnejad, B., Naji, M., Bose, R., & Jha, S. (2019). A critical review on use of Agrobacterium rhizogenes and their associated binary vectors for plant transformation. *Biotechnology Advances*, *37*(7), 107405. http://doi.org/10.1016/j.biotechadv.2019.06.004. PMid:31185263.

Bailey-Serres, J., Parker, J. E., Ainsworth, E. A., Oldroyd, G. E., & Schroeder, J. I. (2019). Genetic strategies for improving crop yields. *Nature*, *575*(7781), 109-118. http://doi.org/10.1038/s41586-019-1679-0. PMid:31695205.

Baltes, N. J., Gil-Humanes, J., & Voytas, D. F. (2017). Genome engineering and agriculture: Opportunities and challenges. *Progress in Molecular Biology and Translational Science*, *149*, 1-26. http://doi.org/10.1016/bs.pmbts.2017.03.011. PMid:28712492.

Banakar, R., Eggenberger, A. L., Lee, K., Wright, D. A., Murugan, K., Zarecor, S., Lawrence-Dill, C. J., Sashital, D. G., & Wang, K. (2019). High-frequency random DNA insertions upon co-delivery of CRISPR-Cas9 ribonucleoprotein and selectable marker plasmid in rice. *Scientific Reports*, *9*(1), 19902. http://doi.org/10.1038/s41598-019-55681-y. PMid:31882637.

Banakar, R., Schubert, M., Kurgan, G., Rai, K. M., Beaudoin, S. F., Collingwood, M. A., Vakulskas, C. A., Wang, K., & Zhang, F. (2022). Efficiency, specificity and temperature sensitivity of Cas9 and Cas12a RNPs for DNA-free genome editing in plants. *Frontiers in Genome Editing*, *3*, 41. http://doi.org/10.3389/fgeed.2021.760820. PMid:35098208.

Bao, A., Chen, H., Chen, L., Chen, S., Hao, Q., Guo, W., Qiu, D., Shan, Z., Yang, Z., Yuan, S., Zhang, C., Zhang, X., Liu, B., Kong, F., Li, X., Zhou, X., Tran, L. P., & Cao, D. (2019). CRISPR/Cas9-mediated targeted mutagenesis of GmSPL9 genes alters plant architecture in soybean. *BMC Plant Biology*, *19*(1), 1-12. http://doi.org/10.1186/s12870-019-1746-6. PMid:30961525.

Barrangou, R., Fremaux, C., Deveau, H., Richards, M., Boyaval, P., Moineau, S., Romero, D. A., & Horvath, P. (2007). CRISPR provides acquired resistance against viruses in prokaryotes. *Science*, *315*(5819), 1709-1712. http://doi.org/10.1126/science.1138140. PMid:17379808.

Bertioli, D. J., Jenkins, J., Clevenger, J., Dudchenko, O., Gao, D., Seijo, G., Leal-Bertioli, S. C. M., Ren, L., Farmer, A. D., Pandey, M. K., Samoluk, S. S., Abernathy, B., Agarwal, G., Ballén-Taborda, C., Cameron, C., Campbell, J., Chavarro, C., Chitikineni, A., Chu, Y., Dash, S., El Baidouri, M., Guo, B., Huang, W., Kim, K. D., Korani, W., Lanciano, S., Lui, C. G., Mirouze, M., Moretzsohn, M. C., Pham, M., Shin, J. H., Shirasawa, K., Sinharoy, S., Sreedasyam, A., Weeks, N. T., Zhang, X., Zheng, Z., Sun, Z., Froenicke, L., Aiden, E. L., Michelmore, R., Varshney, R. K., Holbrook, C. C., Cannon, E. K. S., Scheffler, B. E., Grimwood, J., Ozias-Akins, P., Cannon, S. B., Jackson S. A., & Schmutz J. (2019). The genome sequence of segmental allotetraploid peanut *Arachis hypogaea*. *Nature Genetics*, *51*(5), 877-884. http://doi.org/10.1038/s41588-019-0405-z. PMid:31043755.

Bhajan, S. K., Begum, S., Islam, M. N., Hoque, M. I., & Sarker, R. H. (2019). In vitro regeneration and Agrobacterium-mediated genetic transformation of local varieties of mungbean (*Vigna radiata* (L). Wilczek). *Plant Tissue Culture & Biotechnology*, *29*(1), 81-97. http://doi.org/10.3329/ptcb.v29i1.41981.

Bhowmik, P., Konkin, D., Polowick, P., Hodgins, C. L., Subedi, M., Xiang, D., Yu, B., Patterson, N., Rajagopalan, N., Babic, V., Ro, D.-K., Tar'an, B., Bandara, M., Smyth, S. J., Cui, Y., & Kagale, S. (2021). CRISPR/Cas9 gene editing in legume crops: Opportunities and challenges. *Legume Science*, *3*(3), e96. **https://doi.org/10.1002/leg3.96**.

Biswas, S., Bridgeland, A., Irum, S., Thomson, M. J., & Septiningsih, E. M. (2022). Optimization of prime editing in rice, peanut, chickpea, and cowpea protoplasts by restoration of GFP activity. *International Journal of Molecular Sciences*, *23*(17), 9809. http://doi.org/10.3390/ijms23179809. PMid:36077206.

Bolotin, A., Quinquis, B., Sorokin, A., & Ehrlich, S. D. (2005). Clustered regularly interspaced short palindrome repeats (CRISPRs) have spacers of extrachromosomal origin. *Microbiology*, *151*(8), 2551-2561. http://doi.org/10.1099/mic.0.28048-0. PMid:16079334.

Borrelli, V. M., Brambilla, V., Rogowsky, P., Marocco, A., & Lanubile, A. (2018). The enhancement of plant disease resistance using CRISPR/Cas9 technology. *Frontiers in Plant Science*, *9*, 1245. http://doi.org/10.3389/fpls.2018.01245. PMid:30197654.

Bortesi, L., & Fischer, R. (2015). The CRISPR/Cas9 system for plant genome editing and beyond. *Biotechnology Advances*, *33*(1), 41-52. http://doi.org/10.1016/j.biotechadv.2014.12.006. PMid:25536441.

Bottero, E., Gómez, C., Stritzler, M., Tajima, H., Frare, R., Pascuan, C., Blumwald, E., Ayub, N., & Soto, G. (2022). Generation of a multi-herbicide-tolerant alfalfa by using base editing. *Plant Cell Reports*, *41*(2), 493-495. http://doi.org/10.1007/s00299-021-02827-w. PMid:34994854.

Brasil. Comissão Técnica Nacional de Biossegurança. (2018). Estabelece os requisitos técnicos para apresentação de consulta à CTNBio sobre as Técnicas Inovadoras de Melhoramento de Precisão *(Resolução Normativa Nº 16, de 15 de janeiro de 2018)*.

Brouns, S. J. J., Jore, M. M., Lundgren, M., Westra, E. R., Slijkhuis, R. J. H., Snijders, A. P. L., Dickman, M. J., Makarova, K. S., Koonin, E. V., & Van Der Oost, J. (2008). Small CRISPR RNAs guide antiviral defense in prokaryotes. *Science*, *321*(5891), 960-964. http://doi.org/10.1126/science.1159689. PMid:18703739.

Cabanillas, B., Jappe, U., & Novak, N. (2018). Allergy to peanut, soybean, and other legumes: Recent advances in allergen characterization, stability to processing and IgE cross-reactivity. *Molecular Nutrition & Food Research*, *62*(1), 1700446. http://doi.org/10.1002/mnfr.201700446. PMid:28944625.

Cai, Y., Chen, L., Liu, X., Sun, S., Wu, C., Jiang, B., Han, T., & Hou, W. (2015). CRISPR/Cas9-mediated genome editing in soybean hairy roots. *PLoS One*, *10*(8), 0136064. https://doi.org/10.1371/journal.pone.0136064.

Cai, Y., Chen, L., Liu, X., Guo, C., Sun, S., Wu, C., Jiang, B., Han, T., & Hou, W. (2018a). CRISPR/Cas9-mediated targeted mutagenesis of GmFT2a delays flowering time in soya bean. *Plant Biotechnology Journal*, *16*(1), 176-185. http://doi.org/10.1111/pbi.12758. PMid:28509421.

Cai, Y., Chen, L., Sun, S., Wu, C., Yao, W., Jiang, B., Han, T., & Hou, W. (2018b). CRISPR/Cas9-mediated deletion of large genomic fragments in soybean. *International Journal of Molecular Sciences*, *19*(12), 3835. http://doi.org/10.3390/ijms19123835. PMid:30513774.

Cai, Y., Wang, L., Chen, L., Wu, T., Liu, L., Sun, S., Wu, C., Yao, W., Jiang, B., Yuan, S., Han, T., & Hou, W. (2020a). Mutagenesis of GmFT2a and GmFT5a mediated by CRISPR/Cas9 contributes for expanding the regional adaptability of soybean. *Plant Biotechnology Journal*, *18*(1), 298-309. http://doi.org/10.1111/pbi.13199. PMid:31240772.

Cai, Y., Chen, L., Zhang, Y., Yuan, S., Su, Q., Sun, S., Wu, C., Yao, W., Han, T., & Hou, W. (2020b). Target base editing in soybean using a modified CRISPR/Cas9 system. *Plant Biotechnology Journal*, *18*(10), 1996-1998. http://doi.org/10.1111/pbi.13386. PMid:32311214.

Çakir, Ö., Uçarli, C., Tarhan, Ç., Pekmez, M., & Turgut-Kara, N. (2019). Nutritional and health benefits of legumes and their distinctive genomic properties. *Food Science and Technology (Campinas)*, *39*(1), 1-12. http://doi.org/10.1590/fst.42117.

Calzadilla, P. I., Vilas, J. M., Escaray, F. J., Unrein, F., Carrasco, P., & Ruiz, O. A. (2019). The increase of photosynthetic carbon assimilation as a mechanism of adaptation to low temperature in *Lotus japonicus*, 1–15. https://doi.org/10.1038/s41598-018-37165-7.

Campbell, B. W., Hoyle, J. W., Bucciarelli, B., Stec, A. O., Samac, D. A., Parrott, W. A., & Stupar, R. M. (2019). Functional analysis and development of a CRISPR/Cas9 allelic series for a CPR5 ortholog necessary for proper growth of soybean trichomes. *Scientific Reports*, *9*(1), 1-11. http://doi.org/10.1038/s41598-019-51240-7. PMid:31611562.

Čermák, T., Curtin, S. J., Gil-Humanes, J., Čegan, R., Kono, T. J., Konečná, E., Belanto, J. J., Starker, C. G., Mathre, J. W., Greenstein, R. L., & Voytas, D. F. (2017). A multipurpose toolkit to enable advanced genome engineering in plants. *The Plant Cell*, *29*(6), 1196-1217. http://doi.org/10.1105/tpc.16.00922. PMid:28522548.

Chandrasekaran, J., Brumin, M., Wolf, D., Leibman, D., Klap, C., Pearlsman, M., Sherman, A., Arazi, T., & Gal-On, A. (2016). Development of broad virus resistance in non-transgenic cucumber using CRISPR/Cas9 technology. *Molecular Plant Pathology*, *17*(7), 1140-1153. http://doi.org/10.1111/mpp.12375. PMid:26808139.

Che, P., Chang, S., Simon, M. K., Zhang, Z., Shaharyar, A., Ourada, J., O'Neill, D., Torres-Mendoza, M., Guo, Y., Marasigan, K. M., Vielle-Calzada, J.-P., Ozias-Akins, P., Albertsen, M. C., & Jones, T. J. (2021). Developing a rapid and highly efficient cowpea regeneration, transformation and genome editing system using embryonic axis explants. *The Plant Journal*, *106*(3), 817-830. http://doi.org/10.1111/tpj.15202. PMid:33595147.

Chen, L., Cai, Y., Liu, X., Yao, W., Guo, C., Sun, S., Wu, C., Jiang, B., Han, T., & Hou, W. (2018). Improvement of soybean Agrobacterium-mediated transformation efficiency by adding glutamine and asparagine into the culture media. *International Journal of Molecular Sciences*, *19*(10), 3039. http://doi.org/10.3390/ijms19103039. PMid:30301169.

Chen, R. (2018). Functional genomics and genetic control of compound leaf development in Medicago truncatula: An overview. Functional Genomics in Medicago truncatula. *Methods and Protocols*, *1822*, 197-203. http://doi.org/10.1007/978-1-4939-8633-0_14. PMid:30043306.

Cheng, N., & Nakata, P. A. (2020). Development of a rapid and efficient protoplast isolation and transfection method for chickpea (Cicer arietinum). *MethodsX*, *7*, 101025. http://doi.org/10.1016/j.mex.2020.101025. PMid:32874941.

Cheng, Q., Dong, L., Su, T., Li, T., Gan, Z., Nan, H., Lu, S., Fang, C., Kong, L., Li, H., Hou, Z., Kou, K., Tang, Y., Lin, X., Zhao, X., Chen, L., Liu, B., & Kong, F. (2019). CRISPR/Cas9-mediated targeted mutagenesis of GmLHY genes alters plant height and internode length in soybean. *BMC Plant Biology*, *19*(1), 1-11. http://doi.org/10.1186/s12870-019-2145-8. PMid:31852439.

Cho, H. J., Moy, Y., Rudnick, N. A., Klein, T. M., Yin, J., Bolar, J., Hendrick, C., Beatty, M., Castañeda, L., Kinney, A. J., Jones, T. J., & Chilcoat, N. D. (2022). Development of an efficient marker-free soybean transformation method using the novel bacterium Ochrobactrum haywardense H1. *Plant Biotechnology Journal*, *20*(5), 977-990. http://doi.org/10.1111/pbi.13777. PMid:35015927.

Cho, S. W., Kim, S., Kim, J. M., & Kim, J. S. (2013). Targeted genome engineering in human cells with the Cas9 RNA-guided endonuclease. *Nature Biotechnology*, *31*(3), 230-232. http://doi.org/10.1038/nbt.2507. PMid:23360966.

Choudhury, A., & Rajam, M. V. (2021). Genetic transformation of legumes: An update. *Plant Cell Reports*, *40*(10), 1813-1830. http://doi.org/10.1007/s00299-021-02749-7. PMid:34230986.

Chu, P., & Agapito-Tenfen, S. Z. (2022). Unintended Genomic Outcomes in Current and Next Generation GM Techniques: A Systematic Review. *Plants*, *11*(21), 2997. http://doi.org/10.3390/plants11212997. PMid:36365450.

Confalonieri, M., Carelli, M., Gianoglio, S., Moglia, A., Biazzi, E., & Tava, A. (2021). CRISPR/Cas9-mediated targeted mutagenesis of CYP93E2 modulates the triterpene saponin biosynthesis in

Medicago truncatula. *Frontiers in Plant Science*, *12*, 690231. http://doi.org/10.3389/fpls.2021.690231. PMid:34381478.

Cong, L., Ran, F. A., Cox, D., Lin, S., Barretto, R., Habib, N., Hsu, P. D., Wu, X., Jiang, W., Marraffini, L. A., & Zhang, F. (2013). Multiplex genome engineering using CRISPR/Cas systems. *Science*, *339*(6121), 819-823. http://doi.org/10.1126/science.1231143. PMid:23287718.

Court of Justice of the European Union. (2018). *Organisms obtained by mutagenesis are GMOs and are, in principle, subject to the obligations laid down by the GMO directive*. Press Release.

Curtin, S. J., Xiong, Y., Michno, J. M., Campbell, B. W., Stec, A. O., Čermák, T., Starker, C., Voytas, D. F., Eamens, A. L., & Stupar, R. M. (2018). Crispr/cas9 and talen s generate heritable mutations for genes involved in small rna processing of glycine max and medicago truncatula. *Plant Biotechnology Journal*, *16*(6), 1125-1137. http://doi.org/10.1111/pbi.12857. PMid:29087011.

Deltcheva, E., Chylinski, K., Sharma, C. M., Gonzales, K., Chao, Y., Pirzada, Z. A., Eckert, M. R., Vogel, J., & Charpentier, E. (2011). CRISPR RNA maturation by trans-encoded small RNA and host factor RNase III. *Nature*, *471*(7340), 602-607. http://doi.org/10.1038/nature09886. PMid:21455174.

Délye, C., Jasieniuk, M., & Le Corre, V. (2013). Deciphering the evolution of herbicide resistance in weeds. *Trends in Genetics*, *29*(11), 649-658. http://doi.org/10.1016/j.tig.2013.06.001. PMid:23830583.

Deveau, H., Barrangou, R., Garneau, J. E., Labonté, J., Fremaux, C., Boyaval, P., Romero, D. A., Horvath, P., & Moineau, S. (2008). Phage response to CRISPR-encoded resistance in Streptococcus thermophilus. *Journal of Bacteriology*, *190*(4), 1390-1400. http://doi.org/10.1128/JB.01412-07. PMid:18065545.

Do, P. T., Nguyen, C. X., Bui, H. T., Tran, L. T., Stacey, G., Gillman, J. D., Zhang, Z. J., & Stacey, M. G. (2019). Demonstration of highly efficient dual gRNA CRISPR/Cas9 editing of the homeologous GmFAD2-1A and GmFAD2-1B genes to yield a high oleic, low linoleic and α-linolenic acid phenotype in soybean. *BMC Plant Biology*, *19*(1), 311. http://doi.org/10.1186/s12870-019-1906-8. PMid:31307375.

Dolgikh, A. V., Kirienko, A. N., Tikhonovich, I. A., Foo, E., & Dolgikh, E. A. (2019). The DELLA proteins influence the expression of cytokinin biosynthesis and response genes during nodulation. *Frontiers in Plant Science*, *10*, 432. http://doi.org/10.3389/fpls.2019.00432. PMid:31024597.

Doudna, J. A., & Charpentier, E. (2014). The new frontier of genome engineering with CRISPR-Cas9. *Science*, *346*(6213), 1258096. http://doi.org/10.1126/science.1258096. PMid:25430774.

Du, Y. T., Zhao, M. J., Wang, C. T., Gao, Y., Wang, Y. X., Liu, Y. W., Chen, M., Chen, J., Zhou, Y. B., & Ma, Y. Z. (2018). Identification and characterization of GmMYB118 responses to drought and salt stress. *BMC Plant Biology*, *18*, 320. http://doi.org/10.1186/s12870-018-1551-7. PMid:30509166.

Duan, K., Cheng, Y., Ji, J., Wang, C., Wei, Y., & Wang, Y. (2021). Large chromosomal segment deletions by CRISPR/LbCpf1-mediated multiplex gene editing in soybean. *Journal of Integrative Plant Biology*, *63*(9), 1620-1631. http://doi.org/10.1111/jipb.13158. PMid:34331750.

Eckerstorfer, M. F., Engelhard, M., Heissenberger, A., Simon, S., & Teichmann, H. (2019). Plants developed by new genetic modification techniques: Comparison of existing regulatory frameworks in the EU and non-EU countries. *Frontiers in Bioengineering and Biotechnology*, *7*, 26. http://doi.org/10.3389/fbioe.2019.00026. PMid:30838207.

Endre, G., Kereszt, A., Kevei, Z., Mihacea, S., Kaló, P., & Kiss, G. B. (2002). A receptor kinase gene regulating symbiotic nodule development. *Nature*, *417*(6892), 962-966. http://doi.org/10.1038/nature00842. PMid:12087406.

FAO - United Nations Food and Agriculture Organization. (2016). *Year of pulse crops*. http://www.fao.org/pulses-2016/en/.

Fritsche, S., Poovaiah, C., MacRae, E., & Thorlby, G. (2018). A New Zealand perspective on the application and regulation of gene editing. *Frontiers in Plant Science*, *9*, 1323. http://doi.org/10.3389/fpls.2018.01323. PMid:30258454.

Gao, R., Feyissa, B. A., Croft, M., & Hannoufa, A. (2018). Gene editing by CRISPR / Cas9 in the obligatory outcrossing *Medicago sativa*. *Planta*, *247*(4), 1043-1050. http://doi.org/10.1007/s00425-018-2866-1. PMid:29492697.

Garneau, J. E., Dupuis, M. È., Villion, M., Romero, D. A., Barrangou, R., Boyaval, P., Fremaux, C., Horvath, P., Magadán, A. H., & Moineau, S. (2010). The CRISPR/Cas bacterial immune system cleaves bacteriophage and plasmid DNA. *Nature*, *468*(7320), 67-71. http://doi.org/10.1038/nature09523. PMid:21048762.

Gelvin, S. B. (2012). Traversing the cell: Agrobacterium T-DNA's journey to the host genome. *Frontiers in Plant Science*, *3*, 52. http://doi.org/10.3389/fpls.2012.00052. PMid:22645590.

Ghogare, R., Ludwig, Y., Bueno, G. M., Slamet-Loedin, I. H., & Dhingra, A. (2021). Genome editing reagent delivery in plants. *Transgenic Research*, *30*(4), 321-335. http://doi.org/10.1007/s11248-021-00239-w. PMid:33728594.

Guo, B., Sun, L., Jiang, S., Ren, H., Sun, R., Wei, Z., Wei, Z., Hong, H., Luan, X., Wang, J., Wang, X., Xu, D., Li, W., Guo, C., & Qiu, L.-J. (2022). Soybean genetic resources contributing to sustainable protein production. *Theoretical and Applied Genetics*, *135*(11), 4095-4121. http://doi.org/10.1007/s00122-022-04222-9. PMid:36239765.

Gupta, R. M., & Musunuru, K. (2014). Expanding the genetic editing tool kit: ZFNs, TALENs, and CRISPR-Cas9. *The Journal of Clinical Investigation*, *124*(10), 4154-4161. http://doi.org/10.1172/JCI72992. PMid:25271723.

Gupta, S., Kumar, A., Patel, R., & Kumar, V. (2021). Genetically modified crop regulations: Scope and opportunity using the CRISPR-Cas9 genome editing approach. *Molecular Biology Reports*, *48*(5), 4851-4863. http://doi.org/10.1007/s11033-021-06477-9. PMid:34114124.

Hada, A., Krishnan, V., Jaabir, M., Kumari, A., Jolly, M., Praveen, S., & Sachdev, A. (2018). Improved Agrobacterium tumefaciens-mediated transformation of soybean [Glycine max (L.) Merr.] following optimization of culture conditions and mechanical techniques. *In Vitro Cellular & Developmental Biology. Plant*, *54*(6), 672-688. http://doi.org/10.1007/s11627-018-9944-8.

Haque, E., Taniguchi, H., Hassan, M. M., Bhowmik, P., Karim, M. R., Śmiech, M., Zhao, K., Rahman, M., & Islam, T. (2018). Application of CRISPR/Cas9 genome editing technology for the improvement of crops cultivated in tropical climates: Recent progress, prospects, and challenges. *Frontiers in Plant Science*, *9*, 617. http://doi.org/10.3389/fpls.2018.00617. PMid:29868073.

Hastwell, A. H., Gresshoff, P. M., & Ferguson, B. J. (2015). Genome-wide annotation and characterization of CLAVATA/ESR (CLE) peptide hormones of soybean (Glycine max) and common bean (Phaseolus vulgaris), and their orthologues of Arabidopsis thaliana. *Journal of Experimental Botany*, *66*(17), 5271-5287. http://doi.org/10.1093/jxb/erv351. PMid:26188205.

Haun, W., Coffman, A., Clasen, B. M., Demorest, Z. L., Lowy, A., Ray, E., Retterath, A., Stoddard, T., Juillerat, A., Cedrone, F., Mathis, L., Voytas, D. F., & Zhang, F. (2014). Improved soybean oil quality by targeted mutagenesis of the fatty acid desaturase 2 genes family. *Plant Biotechnology Journal*, *12*(7), 934-940. http://doi.org/10.1111/pbi.12201. PMid:24851712.

Hedden, P., & Sponsel, V. (2015). A Century of Gibberellin Research. *Journal of Plant Growth Regulation*, *34*(4), 740-760. http://doi.org/10.1007/s00344-015-9546-1. PMid:26523085.

Horvath, P., Coûté-Monvoisin, A. C., Romero, D. A., Boyaval, P., Fremaux, C., & Barrangou, R. (2009). Comparative analysis of CRISPR loci in lactic acid bacteria genomes. *International Journal of Food Microbiology*, *131*(1), 62-70. http://doi.org/10.1016/j.ijfoodmicro.2008.05.030. PMid:18635282.

Hou, S., Liu, Z., Shen, H., & Wu, D. (2019). Damage-associated molecular pattern-triggered immunity in plants. *Frontiers in Plant Science*, *10*, 646. http://doi.org/10.3389/fpls.2019.00646. PMid:31191574.

Huang, P., Lu, M., Li, X., Sun, H., Cheng, Z., Miao, Y., Fu, Y., & Zhang, X. (2022). An Efficient Agrobacterium rhizogenes-Mediated Hairy Root Transformation Method in a Soybean Root Biology Study. *International Journal of Molecular Sciences*, *23*(20), 12261. http://doi.org/10.3390/ijms232012261. PMid:36293115.

Hwang, H. H., Yu, M., & Lai, E. M. (2017). Agrobacterium-mediated plant transformation: Biology and applications. *The Arabidopsis Book / American Society of Plant Biologists*, *15*, e0186. http://doi.org/10.1199/tab.0186. PMid:31068763.

Hwang, W. Y., Fu, Y., Reyon, D., Maeder, M. L., Tsai, S. Q., Sander, J. D., Peterson, R. T., Yeh, J.-R. J., & Joung, J. K. (2013). Efficient genome editing in zebrafish using a CRISPR-Cas system. *Nature Biotechnology*, *31*(3), 227-229. http://doi.org/10.1038/nbt.2501. PMid:23360964.

Ishino, Y., Krupovic, M., & Forterre, P. (2018). History of CRISPR-Cas from encounter with a mysterious repeated sequence to genome editing technology. *Journal of Bacteriology*, *200*(7), e00580-e17. http://doi.org/10.1128/JB.00580-17. PMid:29358495.

Ishino, Y., Shinagawa, H., Makino, K., Amemura, M., & Nakata, A. (1987). Nucleotide sequence of the iap gene, responsible for alkaline phosphatase isozyme conversion in Escherichia coli, and identification of the gene product. *Journal of Bacteriology*, *169*(12), 5429-5433. http://doi.org/10.1128/jb.169.12.5429-5433.1987. PMid:3316184.

Ishizaki, K., Chiyoda, S., Yamato, K. T., & Kohchi, T. (2008). Agrobacterium-mediated transformation of the haploid liverwort Marchantia polymorpha L., an emerging model for plant biology. *Plant & Cell Physiology*, *49*(7), 1084-1091. http://doi.org/10.1093/pcp/pcn085. PMid:18535011.

Jacobs, T. B., LaFayette, P. R., Schmitz, R. J., & Parrott, W. A. (2015). Targeted genome modifications in soybean with CRISPR/Cas9. *BMC Biotechnology*, *15*, 16. http://doi.org/10.1186/s12896-015-0131-2. PMid:25879861.

Jacobs, T. B., Zhang, N., Patel, D., & Martin, G. B. (2017). Generation of a collection of mutant tomato lines using pooled CRISPR libraries. *Plant Physiology*, *174*(4), 2023-2037. http://doi.org/10.1104/pp.17.00489. PMid:28646085.

Jansen, R., Embden, J. D. V., Gaastra, W., & Schouls, L. M. (2002). Identification of genes that are associated with DNA repeats in prokaryotes. *Molecular Microbiology*, *43*(6), 1565-1575. http://doi.org/10.1046/j.1365-2958.2002.02839.x. PMid:11952905.

Jansing, J., Schiermeyer, A., Schillberg, S., Fischer, R., & Bortesi, L. (2019). Genome editing in agriculture: Technical and practical considerations. *International Journal of Molecular Sciences*, *20*(12), 2888. http://doi.org/10.3390/ijms20122888. PMid:31200517.

Jenkins, J. A., Griffiths-Jones, S., Shewry, P. R., Breiteneder, H., & Mills, E. C. (2005). Structural relatedness of plant food allergens with specific reference to cross-reactive allergens: An in silico analysis. *The Journal of Allergy and Clinical Immunology*, *115*(1), 163-170. http://doi.org/10.1016/j.jaci.2004.10.026. PMid:15637564.

Ji, J., Zhang, C., Sun, Z., Wang, L., Duanmu, D., & Fan, Q. (2019). Genome editing in cowpea Vigna unguiculata using CRISPR-Cas9. *International Journal of Molecular Sciences*, *20*(10), 2471. http://doi.org/10.3390/ijms20102471. PMid:31109137.

Jia, N., Zhu, Y., & Xie, F. (2018). An efficient protocol for model legume root protoplast isolation and transformation. *Frontiers in Plant Science*, *9*, 670. http://doi.org/10.3389/fpls.2018.00670. PMid:29915605.

Jiao, K., Li, X., Su, S., Guo, W., Guo, Y., Guan, Y., Hu, Z., Shen, Z., & Luo, D. (2019). Genetic control of compound leaf development in the mungbean (Vigna radiata L.). *Horticulture Research*, *6*(1), 23. http://doi.org/10.1038/s41438-018-0088-0. PMid:30729013.

Jin, Y., Liu, H., Luo, D., Yu, N., Dong, W., Wang, C., Zhang, X., Dai, H., Yang, J., & Wang, E. (2016). DELLA proteins are common components of symbiotic rhizobial and mycorrhizal signalling pathways. *Nature Communications*, *7*(1), 1-14. http://doi.org/10.1038/ncomms12433. PMid:27514472.

Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J. A., & Charpentier, E. (2012). A programmable dual-RNA–guided DNA endonuclease in adaptive bacterial immunity. *Science*, *337*(6096), 816-821. http://doi.org/10.1126/science.1225829. PMid:22745249.

Jinek, M., East, A., Cheng, A., Lin, S., Ma, E., & Doudna, J. (2013). RNA-programmed genome editing in human cells. *elife*, *2*, e00471. http://dx.doi.org/10.7554/eLife.00471.

Jung, C., & Müller, A. E. (2009). Flowering time control and applications in plant breeding. *Trends in Plant Science*, *14*(10), 563-573. http://doi.org/10.1016/j.tplants.2009.07.005. PMid:19716745.

Juranić, M., Nagahatenna, D. S., Salinas-Gamboa, R., Hand, M. L., Sánchez-León, N., Leong, W. H., How, T., Bazanova, N., Spriggs, A., Vielle-Calzada, J.-P., & Koltunow, A. M. G. (2020). A detached leaf assay for testing transient gene expression and gene editing in cowpea (Vigna unguiculata [L.] Walp.). *Plant Methods*, *16*(1), 1-17. http://doi.org/10.1186/s13007-020-00630-4. PMid:32549904.

Kantor, A., McClements, M. E., & MacLaren, R. E. (2020). CRISPR-Cas9 DNA base-editing and prime-editing. *International Journal of Molecular Sciences*, *21*(17), 6240. http://doi.org/10.3390/ijms21176240. PMid:32872311.

Karthik, S., Pavan, G., & Manickavasagam, M. (2020). Nitric oxide donor regulates Agrobacterium-mediated genetic transformation efficiency in soybean [Glycine max (L.) Merrill]. *Plant Cell, Tissue and Organ Culture*, *141*(3), 655-660. http://doi.org/10.1007/s11240-020-01808-3.

Kim, H., & Choi, J. (2021). A robust and practical CRISPR/crRNA screening system for soybean cultivar editing using LbCpf1 ribonucleoproteins. *Plant Cell Reports*, *40*(6), 1059-1070. http://doi.org/10.1007/s00299-020-02597-x. PMid:32945949.

Kim, J. S. (2018). Precision genome engineering through adenine and cytosine base editing. *Nature Plants*, *4*(3), 148-151. http://doi.org/10.1038/s41477-018-0115-z. PMid:29483683.

Kim, H., Kim, S. T., Ryu, J., Kang, B. C., Kim, J. S., & Kim, S. G. (2017a). CRISPR/Cpf1-mediated DNA-free plant genome editing. *Nature Communications*, *8*, 14406. http://doi.org/10.1038/ncomms14406. PMid:28205546.

Kim, S. K., Seong, W., Han, G. H., Lee, D. H., & Lee, S. G. (2017b). CRISPR interference-guided multiplex repression of endogenous competing pathway genes for redirecting metabolic flux in *Escherichia coli*. *Microbial Cell Factories*, *16*(1), 188. http://doi.org/10.1186/s12934-017-0802-x. PMid:29100516.

Kloosterman, B., Anithakumari, A. M., Chibon, P. Y., Oortwijn, M., van der Linden, G. C., Visser, R. G., & Bachem, C. W. (2012). Organ specificity and transcriptional control of metabolic routes revealed by expression QTL profiling of source-sink tissues in a segregating potato population. *BMC Plant Biology*, *12*, 17. http://doi.org/10.1186/1471-2229-12-17. PMid:22313736.

Komor, A. C., Badran, A. H., & Liu, D. R. (2018). Editing the genome without double-stranded DNA breaks. *ACS Chemical Biology*, *13*(2), 383-388. http://doi.org/10.1021/acschembio.7b00710. PMid:28957631.

Komor, A. C., Kim, Y. B., Packer, M. S., Zuris, J. A., & Liu, D. R. (2016). Programmable editing of a target base in genomic DNA without double-stranded DNA cleavage. *Nature*, *533*(7603), 420-424. http://doi.org/10.1038/nature17946. PMid:27096365.

Kreplak, J., Madoui, M. A., Cápal, P., Novák, P., Labadie, K., Aubert, G., Bayer, P. E., Gali, K. K., Syme, R. A., Main, D., Klein, A., Bérard, A., Vrbová, I., Fournier, C., d'Agata, L., Belser, C., Berrabah, W., Toegelová, H., Milec, Z., Vrána, J., Lee, H., Kougbeadjo, A., Térézol, M., Huneau, C., Turo, C. J., Mohellibi, N., Neumann, P., Falque, M., Gallardo, K., McGee, R., Tar'an, B., Bendahmane, A., Aury, J. M., Batley, J., Le Paslier, M. C., Ellis, N., Warkentin, T. D., Coyne, C. J., Salse, J., Edwards, D., Lichtenzveig, J., Macas, J., Doležel, J., Wincker, P., & Burstin, J. (2019). A reference genome for pea provides insight into legume genome evolution. *Nature Genetics*, *51*(9), 1411-1422. http://doi.org/10.1038/s41588-019-0480-1. PMid:31477930.

Kumar, A., Jaiwal, R., Sreevathsa, R., Chaudhary, D., & Jaiwal, P. K. (2021). Transgenic cowpea plants expressing Bacillus thuringiensis Cry2Aa insecticidal protein imparts resistance to Maruca vitrata legume pod borer. *Plant Cell Reports*, *40*(3), 583-594. http://doi.org/10.1007/s00299-020-02657-2. PMid:33471196.

Kumar, S., Liu, Z. B., Sanyour-Doyel, N., Lenderts, B., Worden, A., Anand, A., Cho, H.-J., Bolar, J., Harris, C., Huang, L., Xing, A., & Richardson, A. (2022). Efficient gene targeting in soybean using Ochrobactrum haywardense-mediated delivery of a marker-free donor template. *Plant Physiology*, *189*(2), 585-594. http://doi.org/10.1093/plphys/kiac075. PMid:35191500.

Laforest, L. C., & Nadakuduti, S. S. (2022). Advances in delivery mechanisms of CRISPR gene-editing reagents in plants. *Frontiers in Genome Editing*, *4*, 830178. http://doi.org/10.3389/fgeed.2022.830178. PMid:35141701.

Lamaoui, M., Jemo, M., Datla, R., & Bekkaoui, F. (2018). Heat and drought stresses in crops and approaches for their mitigation. *Frontiers in Chemistry*, *6*, 26. http://doi.org/10.3389/fchem.2018.00026. PMid:29520357.

Latef, A. A. H. A., & Ahmad, P. (2015). Legumes and breeding under abiotic stress: an overview. In M. M. Azooz, & P. Ahmad (Eds.), *Legumes under environmental stress: Yield, improvement and adaptations* (pp. 1-20). Wiley. https://doi.org/10.1002/9781118917091.ch1.

Lawrenson, T., Shorinola, O., Stacey, N., Li, C., Østergaard, L., Patron, N., Uauy, C., & Harwood, W. (2015). Induction of targeted, heritable mutations in barley and Brassica oleracea using RNA-guided Cas9 nuclease. *Genome Biology*, *16*, 258. http://doi.org/10.1186/s13059-015-0826-7. PMid:26616834.

Le, D. T., Chu, H. D., & Le, N. Q. (2016). Improving nutritional quality of plant proteins through genetic engineering. *Current Genomics*, *17*(3), 220-229. http://doi.org/10.2174/1389202917666160202215934. PMid:27252589.

LeBlanc, C., Zhang, F., Mendez, J., Lozano, Y., Chatpar, K., Irish, V. F., & Jacob, Y. (2018). Increased efficiency of targeted mutagenesis by CRISPR/Cas9 in plants using heat stress. *The Plant Journal*, *93*(2), 377-386. http://doi.org/10.1111/tpj.13782. PMid:29161464.

Lee, K., & Wang, K. (2023). Strategies for genotype-flexible plant transformation. *Current Opinion in Biotechnology*, *79*, 102848. http://doi.org/10.1016/j.copbio.2022.102848. PMid:36463838.

Lee, M. H., Lee, J., Choi, S. A., Kim, Y. S., Koo, O., Choi, S. H., Ahn, W. S., Jie, E. Y., & Kim, S. W. (2020). Efficient genome editing using CRISPR-Cas9 RNP delivery into cabbage protoplasts via electro-transfection. *Plant Biotechnology Reports*, *14*(6), 695-702. http://doi.org/10.1007/s11816-020-00645-2.

Lewis, G. P., Schrire, B., Mackinder, B., & Lock, M. (Eds). (2005). *Legumes of the world* (pp. 127-159). Royal Botanic Gardens Kew.

Li, C., Nguyen, V., Liu, J., Fu, W., Chen, C., Yu, K., & Cui, Y. (2019). Mutagenesis of seed storage protein genes in Soybean using CRISPR/Cas9. *BMC Research Notes*, *12*(1), 176. http://doi.org/10.1186/s13104-019-4207-2. PMid:30917862.

Li, G., Liu, R., Xu, R., Varshney, R. K., Ding, H., Li, M., Yan, X., Huang, S., Li, J., Wang, D., Ji, Y., Wang, C., He, J., Luo, Y., Gao, S., Wei, P., Zong, X., & Yang, T. (2023). Development of an Agrobacterium-mediated CRISPR/Cas9 system in pea (Pisum sativum L.). *The Crop Journal*, *11*(1), 132-139. http://doi.org/10.1016/j.cj.2022.04.011.

Li, H. Q., Chen, C., Chen, R. R., Song, X. W., Li, J. N., Zhu, Y. M., & Ding, X. D. (2018). Preliminary analysis of the role of GmSnRK1.1 and GmSnRK1.2 in the ABA and alkaline stress response of the soybean using the CRISPR/Cas9-based gene double-knockout system. *Yi chuan = Hereditas / Zhongguo yi chuan xue hui bian ji*, *40*(6), 496-507. https://doi.org/10.16288/j.yczz.17-424. PMid: **29959122.**

Li, H., Li, J., Shen, Y., Wang, J., & Zhou, D. (2017a). Legume consumption and all-cause and cardiovascular disease mortality. *BioMed Research International*, *2017*, 8450618. http://doi.org/10.1155/2017/8450618. PMid:29230416.

Li, S., Cong, Y., Liu, Y., Wang, T., Shuai, Q., Chen, N., Gai, J., & Li, Y. (2017b). Optimization of Agrobacterium-mediated transformation in soybean. *Frontiers in Plant Science*, *8*, 246. http://doi.org/10.3389/fpls.2017.00246. PMid:28286512.

Li, J. F., Norville, J. E., Aach, J., McCormack, M., Zhang, D., Bush, J., Church, G. M., & Sheen, J. (2013). Multiplex and homologous recombination-mediated genome editing in Arabidopsis and Nicotiana benthamiana using guide RNA and Cas9. *Nature Biotechnology*, *31*(8), 688-691. http://doi.org/10.1038/nbt.2654. PMid:23929339.

Li, Z., Liu, Z. B., Xing, A., Moon, B. P., Koellhoffer, J. P., Huang, L., Ward, R. T., Clifton, E., Falco, S. C., & Cigan, A. M. (2015). Cas9-guide RNA directed genome editing in soybean. *Plant Physiology*, *169*(2), 960-970. http://doi.org/10.1104/pp.15.00783. PMid:26294043.

Liang, Z., Chen, K., Li, T., Zhang, Y., Wang, Y., Zhao, Q., Liu, J., Zhang, H., Liu, C., Ran, Y., & Gao, C. (2017). Efficient DNA-free genome editing of bread wheat using CRISPR/Cas9 ribonucleoprotein complexes. *Nature Communications*, *8*, 14261. http://doi.org/10.1038/ncomms14261. PMid:28098143.

Liang, Z., Chen, K., Zhang, Y., Liu, J., Yin, K., Qiu, J. L., & Gao, C. (2018). Genome editing of bread wheat using biolistic delivery of CRISPR/Cas9 in vitro transcripts or ribonucleoproteins. *Nature Protocols*, *13*(3), 413-430. http://doi.org/10.1038/nprot.2017.145. PMid:29388938.

Liao, S., Qin, X., Luo, L., Han, Y., Wang, X., Usman, B., Nawaz, G., Zhao, N., Liu, Y., & Li, R. (2019). CRISPR/Cas9-induced mutagenesis of semi-rolled leaf1, 2 confers curled leaf phenotype and drought tolerance by influencing protein expression patterns and ROS scavenging in rice (Oryza sativa L.). *Agronomy (Basel)*, *9*(11), 728. http://doi.org/10.3390/agronomy9110728.

Lin, Q., Zong, Y., Xue, C., Wang, S., Jin, S., Zhu, Z., Wang, Y., Anzalone, A. V., Raguram, A., Doman, J. L., Liu, D. R., & Gao, C. (2020). Prime genome editing in rice and wheat. *Nature Biotechnology*, *38*(5), 582-585. http://doi.org/10.1038/s41587-020-0455-x. PMid:32393904.

Liu, J., Nannas, N. J., Fu, F. F., Shi, J., Aspinwall, B., Parrott, W. A., & Dawe, R. K. (2019a). Genome-scale sequence disruption following biolistic transformation in rice and maize. *The Plant Cell*, *31*(2), 368-383. http://doi.org/10.1105/tpc.18.00613. PMid:30651345.

Liu, W., Rudis, M. R., Cheplick, M. H., Millwood, R. J., Yang, J. P., Ondzighi-Assoume, C. A., Montgomery, G. A., Burris, K. P., Mazarei, M., Chesnut, J. D., & Stewart Junior, C. N. (2020). Lipofection-mediated genome editing using DNA-free delivery of the Cas9/gRNA ribonucleoprotein into plant cells. *Plant Cell Reports*, *39*(2), 245-257. http://doi.org/10.1007/s00299-019-02488-w. PMid:31728703.

Liu, Y., Wan, X., & Wang, B. (2019). Engineered CRISPRa enables programmable eukaryote-like gene activation in bacteria. *Nature Communications*, *10*(1), 3693. http://doi.org/10.1038/s41467-019-11479-0. PMid:31451697.

Lonardi, S., Muñoz-Amatriaín, M., Liang, Q., Shu, S., Wanamaker, S. I., Lo, S., Tanskanen, J., Schulman, A. H., Zhu, T., Luo, M.-C., Alhakami, H., Ounit, R., Hasan, A. M., Verdier, J., Roberts, P. A., Santos, J. R.P., Ndeve, A., Doležel, J., Vrána, J., Hokin, S. A., Farmer, A. D., Cannon, S. B., & Close, T. J. (2019). The genome of cowpea (Vigna unguiculata [L.] Walp.). *The Plant Journal*, *98*(5), 767-782. https://onlinelibrary.wiley.com/doi/10.1111/tpj.14349.

Lowder, L. G., Paul, J. W., & Qi, Y. (2017). Multiplexed transcriptional activation or repression in plants using CRISPR-dCas9-based systems. In K. Kaufmann, & B. Mueller-Roeber (Eds.), *Plant gene regulatory networks* (pp. 167-184). Humana Press. http://doi.org/10.1007/978-1-4939-7125-1_12.

Lowder, L. G., Zhang, D., Baltes, N. J., Paul 3rd, J. W., Tang, X., Zheng, X., Voytas, D. F., Hsieh, T. F., Zhang, Y., & Qi, Y. (2015). A CRISPR/Cas9 toolbox for multiplexed plant genome editing and transcriptional regulation. *Plant Physiology*, *169*(2), 971-985. http://doi.org/10.1104/pp.15.00636. PMid:26297141.

Lu, Y., Ye, X., Guo, R., Huang, J., Wang, W., Tang, J., Tang, J., Tan, L., Zhu, J.-K., Chu, C., & Qian, Y. (2017). Genome-wide targeted mutagenesis in rice using the CRISPR/Cas9 system. *Molecular Plant*, *10*(9), 1242-1245. http://doi.org/10.1016/j.molp.2017.06.007. PMid:28645638.

Ma, X., Zhang, Q., Zhu, Q., Liu, W., Chen, Y., Qiu, R., Wang, B., Yang, Z., Li, H., Lin, Y., Xie, Y., Shen, R., Chen, S., Wang, Z., Chen, Y., Guo, J., Chen, L., Zhao, X., Dong, Z., & Liu, Y.-G. (2015). A robust CRISPR/Cas9 system for convenient, high-efficiency multiplex genome editing in monocot and dicot plants. *Molecular Plant*, *8*(8), 1274-1284. http://doi.org/10.1016/j.molp.2015.04.007. PMid:25917172.

Maher, M. F., Nasti, R. A., Vollbrecht, M., Starker, C. G., Clark, M. D., & Voytas, D. F. (2020). Plant gene editing through de novo induction of meristems. *Nature Biotechnology*, *38*(1), 84-89. http://doi.org/10.1038/s41587-019-0337-2. PMid:31844292.

Mahfouz, M. M., Piatek, A., & Stewart Junior, C. N. (2014). Genome engineering via TALENs and CRISPR/Cas9 systems: Challenges and perspectives. *Plant Biotechnology Journal*, *12*(8), 1006-1014. http://doi.org/10.1111/pbi.12256. PMid:25250853.

Makarova, K. S., Aravind, L., Grishin, N. V., Rogozin, I. B., & Koonin, E. V. (2002). A DNA repair system specific for thermophilic Archaea and bacteria predicted by genomic context analysis. *Nucleic Acids Research*, *30*(2), 482-496. http://doi.org/10.1093/nar/30.2.482. PMid:11788711.

Makarova, K. S., Grishin, N. V., Shabalina, S. A., Wolf, Y. I., & Koonin, E. V. (2006). A putative RNA-interference-based immune system in prokaryotes: Computational analysis of the predicted enzymatic machinery, functional analogies with eukaryotic RNAi, and hypothetical mechanisms of action. *Biology Direct*, *1*(1), 1-26. http://doi.org/10.1186/1745-6150-1-7. PMid:16545108.

Makarova, K. S., Wolf, Y. I., Alkhnbashi, O. S., Costa, F., Shah, S. A., Saunders, S. J., Barrangou, R., Brouns, S. J. J., Charpentier, E., Haft, D. H., Horvath, P., Moineau, S., Mojica, F. J. M., Terns, R. M., Terns, M. P., White, M. F., Yakunin, A. F., Garrett, R. A., van der Oost, J., Backofen, R., & Koonin, E. V. (2015). Backofen, R., & Koonin, E. V. (2105). An updated evolutionary classification of CRISPR–Cas systems. *Nature Reviews. Microbiology*, *13*(11), 722-736. http://doi.org/10.1038/nrmicro3569. PMid:26411297.

Mali, P., Yang, L., Esvelt, K. M., Aach, J., Guell, M., DiCarlo, J. E., Norville, J. E., & Church, G. M. (2013). RNA-guided human genome engineering via Cas9. *Science*, *339*(6121), 823-826. http://doi.org/10.1126/science.1232033. PMid:23287722.

Malzahn, A. A., Tang, X., Lee, K., Ren, Q., Sretenovic, S., Zhang, Y., Chen, H., Kang, M., Bao, Y., Zheng, X., Deng, K., Zhang, T., Salcedo, V., Wang, K., Zhang, Y., & Qi, Y. (2019). Application of CRISPR-Cas12a temperature sensitivity for improved genome editing in rice, maize, and Arabidopsis. *BMC Biology*, *17*(1), 1-14. http://doi.org/10.1186/s12915-019-0629-5. PMid:30704461.

Maphosa, Y., & Jideani, V. A. (2017). The role of legumes in human nutrition. In M. C. Hueda (Eds.), *Functional food-improve health through adequate food*. London: IntechOpen. http://doi.org/10.5772/intechopen.69127.

Marraffini, L. A., & Sontheimer, E. J. (2008). CRISPR interference limits horizontal gene transfer in staphylococci by targeting DNA. *Science*, *322*(5909), 1843-1845. http://doi.org/10.1126/science.1165771. PMid:19095942.

Masson-Boivin, C., & Sachs, J. L. (2018). Symbiotic nitrogen fixation by rhizobia-the roots of a success story. *Current Opinion in Plant Biology*, *44*, 7-15. http://doi.org/10.1016/j.pbi.2017.12.001. PMid:29289792.

Mathan, J., Bhattacharya, J., & Ranjan, A. (2016). Enhancing crop yield by optimizing plant developmental features. *Development*, *143*(18), 3283-3294. http://doi.org/10.1242/dev.134072. PMid:27624833.

Meng, X., Yu, H., Zhang, Y., Zhuang, F., Song, X., Gao, S., Gao, C., & Li, J. (2017b). Construction of a genome-wide mutant library in rice using CRISPR/Cas9. *Molecular Plant*, *10*(9), 1238-1241. http://doi.org/10.1016/j.molp.2017.06.006. PMid:28645639.

Meng, Y., Hou, Y., Wang, H., Ji, R., Liu, B., Wen, J., Niu, L., & Lin, H. (2017b). Targeted mutagenesis by CRISPR/Cas9 system in the model legume Medicago truncatula. *Plant Cell Reports*, *36*(2), 371-374. http://doi.org/10.1007/s00299-016-2069-9. PMid:27834007.

Meng, Y., Liu, H., Wang, H., Liu, Y., Zhu, B., Wang, Z., Hou, Y., Zhang, P.,Wen, J., Yang, H., Mysore, K. S., Chen, J., Tadege, M., Niu, L., Lin, H. (2019). *HEADLESS*, a *WUSCHEL* homolog, uncovers novel aspects of shoot meristem regulation and leaf blade development in *Medicago truncatula*. *Journal of Experimental Botany*, *70*(1), 149-163. https://doi.org/10.1093/jxb/ery346.

Metje-Sprink, J., Menz, J., Modrzejewski, D., & Sprink, T. (2019). DNA-free genome editing: Past, present and future. *Frontiers in Plant Science*, *1957*, 1957. http://doi.org/10.3389/fpls.2018.01957. PMid:30693009.

Mhadhbi, H., Mylona, P. V., & Polidoros, A. N. (2015). Legume-rhizobia symbiotic performance under abiotic stresses: Factors influencing tolerance behavior. In M. M. Azooz, & P. Ahmad (Eds.), *Legumes under environmental stress: Yield, improvement and adaptations* (pp. 125-131). Wiley. https://doi.org/10.1002/9781118917091.ch8.

Michno, J. M., Wang, X., Liu, J., Curtin, S. J., Kono, T. J., & Stupar, R. M. (2015). CRISPR/Cas mutagenesis of soybean and Medicago truncatula using a new web-tool and a modified Cas9 enzyme. *GM Crops and Food: Biotechnology in Agriculture and the Food Chain*, *6*(4), 243-252. http://doi.org/10.1080/21645698.2015.1106063. PMid:26479970.

Miller, S. S., Dornbusch, M. R., Farmer, A. D., Huertas, R., Gutierrez-Gonzalez, J. J., Young, N. D., Samac, D. A., & Curtin, S. J. (2022). Alfalfa (Medicago sativa L.) pho2 mutant plants hyperaccumulate phosphate. *G3*, *12*(6), jkac096. https://doi.org/10.1093/g3journal/jkac096.

Mishra, R., Joshi, R. K., & Zhao, K. (2020). Base editing in crops: Current advances, limitations and future implications. *Plant Biotechnology Journal*, *18*(1), 20-31. http://doi.org/10.1111/pbi.13225. PMid:31365173.

Mizuno, A., & Okada, Y. (2019). Biological characterization of expression quantitative trait loci (eQTLs) showing tissue-specific opposite directional effects. *European Journal of Human Genetics*, *27*(11), 1745-1756. http://doi.org/10.1038/s41431-019-0468-4. PMid:31296926.

Mohammed, S., Abd Samad, A., & Rahmat, Z. (2019). Agrobacterium-mediated transformation of rice: Constraints and possible solutions. *Rice Science*, *26*(3), 133-146. http://doi.org/10.1016/j.rsci.2019.04.001.

Mojica, F. J. M., Ferrer, C., Juez, G., & Rodríguez-Valera, F. (1995). Long stretches of short tandem repeats are present in the largest replicons of the Archaea Haloferax mediterranei and Haloferax volcanii and could be involved in replicon partitioning. *Molecular Microbiology*, *17*(1), 85-93. http://doi.org/10.1111/j.1365-2958.1995.mmi_17010085.x. PMid:7476211.

Mojica, F. J., Díez-Villaseñor, C., García-Martínez, J., & Soria, E. (2005). Intervening sequences of regularly spaced prokaryotic repeats derive from foreign genetic elements. *Journal of Molecular Evolution*, *60*(2), 174-182. http://doi.org/10.1007/s00239-004-0046-3. PMid:15791728.

Mojica, F. J., Díez-Villaseñor, C., Soria, E., & Juez, G. (2000). Biological significance of a family of regularly spaced repeats in the genomes of Archaea, Bacteria and mitochondria. *Molecular Microbiology*, *36*(1), 244-246. http://doi.org/10.1046/j.1365-2958.2000.01838.x. PMid:10760181.

Mojica, F. J., Juez, G., & Rodriguez-Valera, F. (1993). Transcription at different salinities of Haloferax mediterranei sequences adjacent to partially modified PstI sites. *Molecular Microbiology*, *9*(3), 613-621. http://doi.org/10.1111/j.1365-2958.1993.tb01721.x. PMid:8412707.

Mougiakos, I., Bosma, E. F., Ganguly, J., Van Der Oost, J., & Van Kranenburg, R. (2018). Hijacking CRISPR-Cas for high-throughput bacterial metabolic engineering: Advances and prospects. *Current*

Opinion in Biotechnology, *50*, 146-157. http://doi.org/10.1016/j.copbio.2018.01.002. PMid:29414054.

Mousavi-Derazmahalleh, M., Bayer, P. E., Hane, J. K., Valliyodan, B., Nguyen, H. T., Nelson, M. N., Erskine, W., Varshney, R. K., Papa, R., & Edwards, D. (2019). Adapting legume crops to climate change using genomic approaches. *Plant, Cell & Environment*, *42*(1), 6-19. http://doi.org/10.1111/pce.13203. PMid:29603775.

Mudryj, A. N., Yu, N., & Aukema, H. M. (2014). Nutritional and health benefits of pulses. *Applied Physiology, Nutrition, and Metabolism*, *39*(11), 1197-1204. http://doi.org/10.1139/apnm-2013-0557. PMid:25061763.

Muzquiz, M., Varela, A., Burbano, C., Cuadrado, C., Guillamón, E., & Pedrosa, M. M. (2012). Bioactive compounds in legumes: Pronutritive and antinutritive actions. Implications for nutrition and health. *Phytochemistry Reviews*, *11*(2), 227-244. http://doi.org/10.1007/s11101-012-9233-9.

Myskja, B. K., & Myhr, A. I. (2020). Non-safety assessments of genome-edited organisms: Should they be included in regulation? *Science and Engineering Ethics*, *26*(5), 2601-2627. http://doi.org/10.1007/s11948-020-00222-4. PMid:32424723.

Nadeem, M., Li, J., Yahya, M., Wang, M., Ali, A., Cheng, A., Wang, X., & Ma, C. (2019). Grain legumes and fear of salt stress: Focus on mechanisms and management strategies. *International Journal of Molecular Sciences*, *20*(4), 799. http://doi.org/10.3390/ijms20040799. PMid:30781763.

Nakayasu, M., Akiyama, R., Lee, H. J., Osakabe, K., Osakabe, Y., Watanabe, B., Sugimoto, Y., Umemoto, N., Saito, K., Muranaka, T., & Mizutani, M. (2018). Generation of α-solanine-free hairy roots of potato by CRISPR/Cas9 mediated genome editing of the St16DOX gene. *Plant Physiology and Biochemistry*, *131*, 70-77. http://doi.org/10.1016/j.plaphy.2018.04.026. PMid:29735370.

Nanjareddy, K., Arthikala, M. K., Blanco, L., Arellano, E. S., & Lara, M. (2016). Protoplast isolation, transient transformation of leaf mesophyll protoplasts and improved Agrobacterium-mediated leaf disc infiltration of Phaseolus vulgaris: Tools for rapid gene expression analysis. *BMC Biotechnology*, *16*(1), 53. http://doi.org/10.1186/s12896-016-0283-8. PMid:27342637.

Naves, E. R., de Ávila Silva, L., Sulpice, R., Araújo, W. L., Nunes-Nesi, A., Peres, L. E., & Zsögön, A. (2019). Capsaicinoids: Pungency beyond Capsicum. *Trends in Plant Science*, *24*(2), 109-120. http://doi.org/10.1016/j.tplants.2018.11.001. PMid:30630668.

Nedumaran, S., Abinaya, P., Jyosthnaa, P., Shraavya, B., Rao, P., & Bantilan, C. (2015). *Grain legumes production, consumption and trade trends in developing countries* (Working Paper Series, No. 60). CGIAR.

Nekrasov, V., Staskawicz, B., Weigel, D., Jones, J. D., & Kamoun, S. (2013). Targeted mutagenesis in the model plant Nicotiana benthamiana using Cas9 RNA-guided endonuclease. *Nature Biotechnology*, *31*(8), 691-693. http://doi.org/10.1038/nbt.2655. PMid:23929340.

Niazian, M., Belzile, F., & Torkamaneh, D. (2022). CRISPR/Cas9 in planta hairy root transformation: A powerful platform for functional analysis of root traits in soybean. *Plants*, *11*(8), 1044. http://doi.org/10.3390/plants11081044. PMid:35448772.

Okuley, J., Lightner, J., Feldmann, K., Yadav, N., Lark, E., & Browse, J. (1994). Arabidopsis FAD2 gene encodes the enzyme that is essential for polyunsaturated lipid synthesis. *The Plant Cell*, *6*(1), 147-158. http://doi.org/10.1105/tpc.6.1.147. PMid:7907506.

Orlich, M. J., Jaceldo-Siegl, K., Sabaté, J., Fan, J., Singh, P. N., & Fraser, G. E. (2014). Patterns of food consumption among vegetarians and non-vegetarians. *British Journal of Nutrition*, *112*(10), 1644-1653. http://doi.org/10.1017/S000711451400261X. PMid:25247790.

Ortigosa, A., Gimenez-Ibanez, S., Leonhardt, N., & Solano, R. (2019). Design of a bacterial speck resistant tomato by CRISPR/Cas9-mediated editing of Sl JAZ 2. *Plant Biotechnology Journal*, *17*(3), 665-673. http://doi.org/10.1111/pbi.13006. PMid:30183125.

Osipova, M. A., Dolgikh, E. A., & Lutova, L. A. (2011). Peculiarities of meristem-specific WOX5 gene expression during nodule organogenesis in legumes. *Russian Journal of Developmental Biology*, *42*(4), 226-237. http://doi.org/10.1134/S1062360411010085.

Ott, T., van Dongen, J. T., Günther, C., Krusell, L., Desbrosses, G., Vigeolas, H., Bock, V., Czechowski, T., Geigenberger, P., & Udvardi, M. K. (2005). Symbiotic leghemoglobins are crucial for nitrogen fixation in legume root nodules but not for general plant growth and development. *Current Biology*, *15*(6), 531-535. http://doi.org/10.1016/j.cub.2005.01.042. PMid:15797021.

Ozyigit, I. I., & Kurtoglu, K. Y. (2020). Particle bombardment technology and its applications in plants. *Molecular Biology Reports*, *47*(12), 9831-9847. http://doi.org/10.1007/s11033-020-06001-5. PMid:33222118.

Paes de Melo, B., Lourenço-Tessutti, I. T., Morgante, C. V., Santos, N. C., Pinheiro, L. B., de Jesus Lins, C. B., Silva, M. C. M., Macedo, L. L. P., Fontes, E. P. B., & Grossi-de-Sa, M. F. (2020). Soybean embryonic axis transformation: Combining biolistic and Agrobacterium-mediated protocols to overcome typical complications of in vitro plant regeneration. *Frontiers in Plant Science*, *11*, 1228. http://doi.org/10.3389/fpls.2020.01228. PMid:32903423.

Pandey, M. K., Roorkiwal, M., Singh, V. K., Ramalingam, A., Kudapa, H., Thudi, M., Chitikineni, A., Rathore, A., & Varshney, R. K. (2016). Emerging genomic tools for legume breeding: Current status and future prospects. *Frontiers in Plant Science*, *7*, 455. http://doi.org/10.3389/fpls.2016.00455. PMid:27199998.

Pareddy, D., Chennareddy, S., Anthony, G., Sardesai, N., Mall, T., Minnicks, T., Karpova, O., Clark, L., Griffin, D., Bishop, B., Shumway, N., Samuel, P., Smith, K., & Sarria, R. (2020). Improved soybean transformation for efficient and high throughput transgenic production. *Transgenic Research*, *29*(3), 267-281. http://doi.org/10.1007/s11248-020-00198-8. PMid:32303980.

Patil, G. B., Stupar, R. M., & Zhang, F. (2022). Protoplast isolation, transfection, and gene editing for soybean soybeans (*Glycine max*). In K. Wang, & F. Zhang (Eds.), *Protoplast technology: Methods and Protocols* (pp. 173-186). Springer. https://doi.org/10.1007/978-1-0716-2164-6_13.

Phillips, A. L. (2016). Genetic control of gibberellin metabolism and signalling in crop improvement. In P. Hedden, & S. G. Thomas (Eds.), *Annual Plant Reviews* (Vol. 49: Gibberellins, The, pp. 405-430). John Wiley & Sons, Ltd. https://doi.org/10.1002/9781119210436.ch13.

Piatek, A., Ali, Z., Baazim, H., Li, L., Abulfaraj, A., Al-Shareef, S., Aouida, M., & Mahfouz, M. M. (2015). RNA-guided transcriptional regulation in planta via synthetic dC as9-based transcription factors. *Plant Biotechnology Journal*, *13*(4), 578-589. http://doi.org/10.1111/pbi.12284. PMid:25400128.

Polowick, P. L., & Yan, W. (2023). A protocol for Agrobacterium-mediated genetic transformation of Lens culinaris Medik (lentil). *Plant Cell, Tissue and Organ Culture*, *152*(3), 605-618. http://doi.org/10.1007/s11240-022-02434-x.

Pourcel, C., Salvignol, G., & Vergnaud, G. (2005). CRISPR elements in Yersinia pestis acquire new repeats by preferential uptake of bacteriophage DNA and provide additional tools for evolutionary studies. *Microbiology*, *151*(Pt 3), 653-663. http://doi.org/10.1099/mic.0.27437-0. PMid:15758212.

Qian, L., Jin, H., Yang, Q., Zhu, L., Yu, X., Fu, X., Zhao, M., & Yuan, F. (2022). A sequence variation in GmBADH2 enhances soybean aroma and is a functional marker for improving soybean flavor. *International Journal of Molecular Sciences*, *23*(8), 4116. http://doi.org/10.3390/ijms23084116. PMid:35456933.

Quareshy, M., Prusinska, J., Li, J., & Napier, R. (2018). A cheminformatics review of auxins as herbicides. *Journal of Experimental Botany*, *69*(2), 265-275. http://doi.org/10.1093/jxb/erx258. PMid:28992122.

Ran, Y., Liang, Z., & Gao, C. (2017). Current and future editing reagent delivery systems for plant genome editing. *Science China. Life Sciences*, *60*(5), 490-505. http://doi.org/10.1007/s11427-017-9022-1. PMid:28527114.

Razzaq, A., Saleem, F., Kanwal, M., Mustafa, G., Yousaf, S., Arshad, H. M. I., Hameed, M. K., Khan, M. S., & Joyia, F. A. (2019). Modern trends in plant genome editing: An inclusive review of the CRISPR/Cas9 toolbox. *International Journal of Molecular Sciences*, *20*(16), 4045. http://doi.org/10.3390/ijms20164045. PMid:31430902.

Rehman, H. M., Cooper, J. W., Lam, H. M., & Yang, S. H. (2019). Legume biofortification is an underexploited strategy for combatting hidden hunger. *Plant, Cell & Environment*, *42*(1), 52-70. http://doi.org/10.1111/pce.13368. PMid:29920691.

Riascos, J. J., Weissinger, A. K., Weissinger, S. M., & Burks, A. W. (2010). Hypoallergenic legume crops and food allergy: Factors affecting feasibility and risk. *Journal of Agricultural and Food Chemistry*, *58*(1), 20-27. http://doi.org/10.1021/jf902526y. PMid:19921800.

Roca Paixão, J. F., Gillet, F. X., Ribeiro, T. P., Bournaud, C., Lourenço-Tessutti, I. T., Noriega, D. D., de Melo, B. P., de Almeida-Engler, J., & Grossi-de-Sa, M. F. (2019). Improved drought stress tolerance in Arabidopsis by CRISPR/dCas9 fusion with a Histone AcetylTransferase. *Scientific Reports*, *9*(1), 8080. http://doi.org/10.1038/s41598-019-44571-y. PMid:31147630.

Rodríguez-Leal, D., Lemmon, Z. H., Man, J., Bartlett, M. E., & Lippman, Z. B. (2017). Engineering quantitative trait variation for crop improvement by genome editing. *Cell*, *171*(2), 470-480. http://doi.org/10.1016/j.cell.2017.08.030. PMid:28919077.

Roy, S., Liu, W., Nandety, R. S., Crook, A., Mysore, K. S., Pislariu, C. I., Frugoli, J., Dickstein, R., & Udvardi, M. K. (2020). Celebrating 20 years of genetic discoveries in legume nodulation and symbiotic nitrogen fixation. *The Plant Cell*, *32*(1), 15-41. http://doi.org/10.1105/tpc.19.00279. PMid:31649123.

Rustgi, S., Naveed, S., Windham, J., Zhang, H., & Demirer, G. S. (2022). Plant biomacromolecule delivery methods in the 21st century. *Frontiers in Genome Editing*, *4*, 1011934. http://doi.org/10.3389/fgeed.2022.1011934. PMid:36311974.

Sack, L., & Scoffoni, C. (2013). Leaf venation: Structure, function, development, evolution, ecology and applications in the past, present and future. *The New Phytologist*, *198*(4), 983-1000. http://doi.org/10.1111/nph.12253. PMid:23600478.

Sadhu, S. K., Jogam, P., Gande, K., Banoth, R., Penna, S., & Peddaboina, V. (2022). Optimization of different factors for an Agrobacterium-mediated genetic transformation system using embryo axis explants of chickpea (Cicer arietinum L.). *Journal of Plant Biotechnology*, *49*(1), 61-73. http://doi.org/10.5010/JPB.2022.49.1.061.

Sandhya, D., Jogam, P., Allini, V. R., Abbagani, S., & Alok, A. (2020). The present and potential future methods for delivering CRISPR/Cas9 components in plants. *Journal of Genetic Engineering and Biotechnology*, *18*(1), 1-11. http://doi.org/10.1186/s43141-020-00036-8. PMid:32638190.

Santamaria, M. E., Arnaiz, A., Gonzalez-Melendi, P., Martinez, M., & Diaz, I. (2018). Plant perception and short-term responses to phytophagous insects and mites. *International Journal of Molecular Sciences*, *19*(5), 1356. http://doi.org/10.3390/ijms19051356. PMid:29751577.

Sarker, R. H., Das, S. K., Shethi, K. J., & Hoque, M. I. (2019). Genetic transformation. In M. Singh (Ed.), *Lentils* (pp. 141-202). Academic Press. http://doi.org/10.1016/B978-0-12-813522-8.00008-X

Sarrocco, S., Herrera-Estrella, A., & Collinge, D. B. (2020). Plant disease management in the post-genomic era: From functional genomics to genome editing. *Frontiers in Microbiology*, *11*, 107. http://doi.org/10.3389/fmicb.2020.00107. PMid:32117135.

Sato, S., Nakamura, Y., Kaneko, T., Asamizu, E., Kato, T., Nakao, M., Sasamoto, S., Watanabe, A., Ono, A., Kawashima, K., Fujishiro, T., Katoh, M., Kohara, M., Kishida, Y., Minami, C., Nakayama, S., Nakazaki, N., Shimizu, Y., Shinpo, S., Takahashi, C., Wada, T., Yamada, M., Ohmido, N., Hayashi, M., Fukui, K., Baba, T., Nakamichi, T., Mori, H., & Tabata, S. (2008). Genome structure of the legume, Lotus japonicus. *DNA Research*, *15*(4), 227-239. http://doi.org/10.1093/dnares/dsn008. PMid:18511435.

Schlueter, J. A., Vasylenko-Sanders, I. F., Deshpande, S., Yi, J., Siegfried, M., Roe, B. A., Schlueter, S. D., Scheffler, B. E., & Shoemaker, R. C. (2007). The FAD2 gene family of soybean: Insights into the structural and functional divergence of a paleopolyploid genome. *Crop Science*, *47*(S1), S-14. http://doi.org/10.2135/cropsci2006.06.0382tpg.

Schmutz, J., Cannon, S. B., Schlueter, J., Ma, J., Mitros, T., Nelson, W., Hyten, D. L., Song, Q., Thelen, J. J., Cheng, J., Xu, D., Hellsten, U., May, G. D., Yu, Y., Sakurai, T., Umezawa, T., Bhattacharyya, M. K., Sandhu, D., Valliyodan, B., Lindquist, E., Peto, M., Grant, D., Shu, S., Goodstein, D., Barry, K., Futrell-Griggs, M., Abernathy, B., Du, J., Tian, Z., Zhu, L., Gill, N., Joshi, T., Libault, M., Sethuraman, A., Zhang, X. C., Shinozaki, K., Nguyen, H. T., Wing, R. A., Cregan, P., Specht, J., Grimwood, J., Rokhsar, D., Stacey, G., Shoemaker, R. C., & Jackson, S. A. (2010). Genome sequence of the palaeopolyploid soybean. *Nature*, *463*(7278), 178-183. http://doi.org/10.1038/nature08670. PMid:20075913.

Schmutz, J., McClean, P. E., Mamidi, S., Wu, G. A., Cannon, S. B., Grimwood, J., Jenkins, J., Shu, S., Song, Q., Chavarro, C., Torres-Torres, M., Geffroy, V., Moghaddam, S. M., Gao, D., Abernathy, B., Barry, K., Blair, M., Brick, M. A., Chovatia, M., Gepts, P., Goodstein, D. M., Gonzales, M., Hellsten, U., Hyten, D. L., Jia, G., Kelly, J. D., Kudrna, D., Lee, R., Richard, M. M., Miklas, P. N., Osorno, J. M., Rodrigues, J., Thareau, V., Urrea, C. A., Wang, M., Yu, Y., Zhang, M., Wing, R. A., Cregan, P. B., Rokhsar, D. S., & Jackson, S. A. (2014). A reference genome for common bean and genome-wide analysis of dual domestications. *Nature Genetics*, *46*(7), 707-713. http://doi.org/10.1038/ng.3008. PMid:24908249.

Schreinemachers, P., Srinivasan, R., Wu, M. H., Bhattarai, M., Patricio, R., Yule, S., Quang, V. H., & Hop, B. T. H. (2014). Safe and sustainable management of legume pests and diseases in Thailand and Vietnam: A situational analysis. *International Journal of Tropical Insect Science*, *34*(2), 88-97. http://doi.org/10.1017/S174275841400023X.

Sehaole, E. K. M. (2022). Genetic transformation in agro-economically important legumes. In J. C. Jimenez-Lopez, & A. Clemente (Eds.), *Legumes research* (Vol. 1). London: IntechOpen. https://doi.org//10.5772/intechopen.101262.

Shan, Q., Wang, Y., Li, J., Zhang, Y., Chen, K., Liang, Z., Zhang, Z., Liu, J., Xi, J., Qiu, J. L., & Gao, C. (2013). Targeted genome modification of crop plants using a CRISPR-Cas system. *Nature Biotechnology*, *31*(8), 686-688. http://doi.org/10.1038/nbt.2650. PMid:23929338.

Shi, J., Gao, H., Wang, H., Lafitte, H. R., Archibald, R. L., Yang, M., Hakimi, S. M., Mo, H., & Habben, J. E. (2017). ARGOS 8 variants generated by CRISPR-Cas9 improve maize grain yield under field drought stress conditions. *Plant Biotechnology Journal*, *15*(2), 207-216. http://doi.org/10.1111/pbi.12603. PMid:27442592.

Shinozaki, K., & Yamaguchi-Shinozaki, K. (2007). Gene networks involved in drought stress response and tolerance. *Journal of Experimental Botany*, *58*(2), 221-227. http://doi.org/10.1093/jxb/erl164. PMid:17075077.

Shmakov, S., Smargon, A., Scott, D., Cox, D., Pyzocha, N., Yan, W., Abudayyeh, O. O., Gootenberg, J. S., Makarova, K. S., Wolf, Y. I., Severinov, K., Zhang, F., & Koonin, E. V. (2017). Diversity and evolution of class 2 CRISPR–Cas systems. *Nature Reviews. Microbiology*, *15*(3), 169-182. http://doi.org/10.1038/nrmicro.2016.184. PMid:28111461.

Shrestha, A., Khan, A., & Dey, N. (2018). cis – trans Engineering: Advances and Perspectives on Customized Transcriptional Regulation in Plants. *Molecular Plant*, *11*(7), 886-898. http://doi.org/10.1016/j.molp.2018.05.008. PMid:29859265.

Shu, H., Luo, Z., Peng, Z., & Wang, J. (2020). The application of CRISPR/Cas9 in hairy roots to explore the functions of AhNFR1 and AhNFR5 genes during peanut nodulation. *BMC Plant Biology*, *20*(1), 417. http://doi.org/10.1186/s12870-020-02614-x. PMid:32894045.

Simkin, A. J., Mcausland, L., Headland, L. R., Lawson, T., & Raines, C. A. (2015). Multigene manipulation of photosynthetic carbon

assimilation increases CO 2 fixation and biomass yield in tobacco. *Journal of Experimental Botany*, *66*(13), 4075-4090. https://doi.org/10.1093/jxb/erv204.

Singh, P., Shukla, A., Tiwari, N. N., Ansari, J., Thakur, S., Patil, P. G., Rathore, M., Verma, O. P., Singh, N. P., & Das, A. (2022). Routine and efficient in vitro regeneration system amenable to biolistic particle delivery in chickpea (Cicer arietinum L.). *Plant Cell, Tissue and Organ Culture*, *148*(3), 699-711. http://doi.org/10.1007/s11240-022-02230-7.

Smyth, S. J. (2019). Global status of the regulation of genome editing technologies. *Perspectives in Agriculture, Veterinary Science, Nutrition and Natural Resources*, *14*(21), 1-6. http://doi.org/10.1079/PAVSNNR201914021.

Soltani, N., Dille, J. A., Burke, I. C., Everman, W. J., VanGessel, M. J., Davis, V. M., & Sikkema, P. H. (2016). Potential corn yield losses from weeds in North America. *Weed Technology*, *30*(4), 979-984. http://doi.org/10.1614/WT-D-16-00046.1.

Somers, D. A., Samac, D. A., & Olhoft, P. M. (2003). Recent advances in legume transformation. *Plant Physiology*, *131*(3), 892-899. http://doi.org/10.1104/pp.102.017681. PMid:12644642.

Soumare, A., Diedhiou, A. G., Thuita, M., Hafidi, M., Ouhdouch, Y., Gopalakrishnan, S., & Kouisni, L. (2020). Exploiting biological nitrogen fixation: A route towards a sustainable agriculture. *Plants*, *9*(8), 1011. http://doi.org/10.3390/plants9081011. PMid:32796519.

Stacey, G. (2007). The Rhizobium-legume nitrogen-fixing symbiosis. In H. Bothe, S. J. Ferguson, & W. E. Newton (Eds.), *Biology of the nitrogen cycle* (pp. 147-163). Elsevier. http://doi.org/10.1016/B978-044452857-5.50011-4.

Stagnari, F., Maggio, A., Galieni, A., & Pisante, M. (2017). Multiple benefits of legumes for agriculture sustainability: An overview. *Chemical and Biological Technologies in Agriculture*, *4*(1), 1-13. http://doi.org/10.1186/s40538-016-0085-1.

Stracke, S., Kistner, C., Yoshida, S., Mulder, L., Sato, S., Kaneko, T., Tabata, S., Sandal, N., Stougaard, J., Szczyglowski, K., & Parniske, M. (2002). A plant receptor-like kinase required for both bacterial and fungal symbiosis. *Nature*, *417*(6892), 959-962. http://doi.org/10.1038/nature00841. PMid:12087405.

Subburaj, S., Zanatta, C. B., Nunn, J. A., Hoepers, A. M., Nodari, R. O., & Agapito-Tenfen, S. Z. (2022). A DNA-free editing platform for genetic screens in soybean via CRISPR/Cas9 ribonucleoprotein delivery. *Frontiers in Plant Science*, *13*, 939997. http://doi.org/10.3389/fpls.2022.939997. PMid:35903231.

Sun, X., Hu, Z., Chen, R., Jiang, Q., Song, G., Zhang, H., & Xi, Y. (2015). Targeted mutagenesis in soybean using the CRISPR-Cas9 system. *Scientific Reports*, *5*(1), 10342. http://doi.org/10.1038/srep10342. PMid:26022141.

Sun, Y., Li, J., & Xia, L. (2016). Precise genome modification via sequence-specific nucleases-mediated gene targeting for crop improvement. *Frontiers in Plant Science*, *7*, 1928. http://doi.org/10.3389/fpls.2016.01928. PMid:28066481.

Svitashev, S., Young, J. K., Schwartz, C., Gao, H., Falco, S. C., & Cigan, A. M. (2015). Targeted mutagenesis, precise gene editing, and site-specific gene insertion in maize using Cas9 and guide RNA. *Plant Physiology*, *169*(2), 931-945. http://doi.org/10.1104/pp.15.00793. PMid:26269544.

Tang, F., Yang, S., Liu, J., & Zhu, H. (2016). Rj4, a gene controlling nodulation specificity in soybeans, encodes a thaumatin-like protein but not the one previously reported. *Plant Physiology*, *170*(1), 26-32. http://doi.org/10.1104/pp.15.01661. PMid:26582727.

Tang, X., Lowder, L. G., Zhang, T., Malzahn, A. A., Zheng, X., Voytas, D. F., Zhong, Z., Chen, Y., Ren, Q., Li, Q., Kirkland, E. R., Zhang, Y., & Qi, Y. (2017). A CRISPR-Cpf1 system for efficient genome editing and transcriptional repression in plants. *Nature Plants*, *3*, 17018. http://doi.org/10.1038/nplants.2017.18. PMid:28211909.

Tchetvertakov, G. (2019) *Australia approves cutting-edge CRISPR gene editing technology*. https://smallcaps.com.au/australia-approves-cutting-edge-crispr-gene-editing-technology/

Tharanathan, R. N., & Mahadevamma, S. (2003). Grain legumes-a boon to human nutrition. *Trends in Food Science & Technology*, *14*(12), 507-518. http://doi.org/10.1016/j.tifs.2003.07.002.

The Legume Phylogeny Working Group. (2017) A new subfamily classification of the Leguminosae based on a taxonomically comprehensive phylogeny. *Taxon*. *66*(1), 44-77. https://doi.org/10.12705/661.3.

Tichá, M., Illésová, P., Hrbáčková, M., Basheer, J., Novák, D., Hlaváčková, K., Šamajová, O., Niehaus, K., Ovečka, M., & Šamaj, J. (2020). Tissue culture, genetic transformation, interaction with beneficial microbes, and modern bio-imaging techniques in alfalfa research. *Critical Reviews in Biotechnology*, *40*(8), 1265-1280. http://doi.org/10.1080/07388551.2020.1814689. PMid:32942912.

Tiwari, P., Indoliya, Y., Chauhan, A. S., Pande, V., & Chakrabarty, D. (2020). Over-expression of rice R1-type MYB transcription factor confers different abiotic stress tolerance in transgenic Arabidopsis. *Ecotoxicology and Environmental Safety*, *206*, 111361. http://doi.org/10.1016/j.ecoenv.2020.111361. PMid:32987264.

Tiwari, R., Singh, A. K., & Rajam, M. V. (2022). Improved and reliable plant regeneration and Agrobacterium-mediated genetic transformation in soybean (Glycine max L.). *Journal of Crop Science and Biotechnology*, *26*, 275-284. http://doi.org/10.1007/s12892-022-00179-9.

Trinh, D. D., Le, N. T., Bui, T. P., Le, T. N. T., Nguyen, C. X., Chu, H. H., & Do, P. T. (2022). A sequential transformation method for validating soybean genome editing by CRISPR/Cas9 system. *Saudi Journal of Biological Sciences*, *29*(10), 103420. http://doi.org/10.1016/j.sjbs.2022.103420. PMid:36060110.

Triozzi, P. M., Schmidt, H. W., Dervinis, C., Kirst, M., & Conde, D. (2021). Simple, efficient and open-source CRISPR/Cas9 strategy for multi-site genome editing in Populus tremula× alba. *Tree Physiology*, *41*(11), 2216-2227. http://doi.org/10.1093/treephys/tpab066. PMid:33960379.

Turnbull, C., Lillemo, M., & Hvoslef-Eide, T. A. (2021). Global regulation of genetically modified crops amid the gene edited crop boom-a review. *Frontiers in Plant Science*, *12*, 630396. http://doi.org/10.3389/fpls.2021.630396. PMid:33719302.

United Nations. (2022). *World Population Prospects 2022*. Department of Economic and Social Affairs Population Dynamics. https://population.un.org/wpp/

USDA Aphis. (2020). *7 CFR Parts 330, 340, and 372: RIN 0579-AE47*. USDA APHIS.

Valdisser, P. A., Pereira, W. J., Almeida Filho, J. E., Müller, B. S., Coelho, G. R., de Menezes, I. P., Vianna, J. P. G., Zucchi, M. I., Lanna, A. C., Coelho, A. S. G., de Oliveira, J. P., Moraes, A. C., Brondani, C., & Vianello, R. P. (2017). In-depth genome characterization of a Brazilian common bean core collection using DArTseq high-density SNP genotyping. *BMC Genomics*, *18*(1), 423. http://doi.org/10.1186/s12864-017-3805-4. PMid:28558696.

Valentine, A. J., Benedito, V. A., & Kang, Y. (2011). Legume nitrogen fixation and soil abiotic stress: From physiology to genomics and beyond. *Annual Plant Reviews*, *42*, 207-248. http://doi.org/10.1002/9781119312994.apr0456.

van Dijk, M., Morley, T., Rau, M. L., & Saghai, Y. (2021). A meta-analysis of projected global food demand and population at risk of hunger for the period 2010-2050. *Nature Food*, *2*(7), 494-501. http://doi.org/10.1038/s43016-021-00322-9. PMid:37117684.

van Kregten, M., de Pater, S., Romeijn, R., van Schendel, R., Hooykaas, P. J., & Tijsterman, M. (2016). T-DNA integration in plants results from polymerase-θ-mediated DNA repair. *Nature Plants*, *2*(11), 16164. http://doi.org/10.1038/nplants.2016.164. PMid:27797358.

van Loon, P., & Van Wassenhove, L. N. (2018). Assessing the economic and environmental impact of remanufacturing: A decision support tool for OEM suppliers. *International Journal of Production Research*, *56*(4), 1662-1674. http://doi.org/10.1080/00207543.2017.1367107.

Varshney, R. K. (2016). Emerging genomic tools for legume breeding: Current status and future prospects. *Frontiers in Plant Science*, *7*, 455. PMid:27199998.

Varshney, R. K., Chen, W., Li, Y., Bharti, A. K., Saxena, R. K., Schlueter, J. A., Donoghue, M. T., Azam, S., Fan, G, Whaley, A. M., Farmer, A. D., Sheridan, J., Iwata, A., Tuteja, R., Penmetsa, R. V., Wu, W., Upadhyaya, H. D., Yang, S. P., Shah, T., Saxena, K. B., Michael, T., McCombie, W. R., Yang, B., Zhang, G., Yang, H., Wang, J., Spillane, C., Cook, D. R., May, G. D., Xu, X., & Jackson, S. A. (2011). Draft genome sequence of pigeonpea (Cajanus cajan), an orphan legume crop of resource-poor farmers. *Nature Biotechnology*, *30*(1), 83-89. http://doi.org/10.1038/nbt.2022. PMid:22057054.

Varshney, R. K., Kudapa, H., Pazhamala, L., Chitikineni, A., Thudi, M., Bohra, A., Gaur, P. M., Janila, P., Fikre, A., Kimurto, P., & Ellis, N. (2015). Translational genomics in agriculture: Some examples in grain legumes. *Critical Reviews in Plant Sciences*, *34*(1-3), 169-194. http://doi.org/10.1080/07352689.2014.897909.

Varshney, R. K., Song, C., Saxena, R. K., Azam, S., Yu, S., Sharpe, A. G., Cannon, S., Baek, J., Rosen, B. D., Tar'an, B., Millan, T., Zhang, X., Ramsay, L D., Iwata, A., Wang, Y., Nelson, W., Farmer, A. D., Gaur, P. M., Soderlund, C., Penmetsa, R. V., Xu, C., Bharti, A. K., He, W., Winter, P., Zhao, S., Hane, J. K., Carrasquilla-Garcia, N., Condie, J. A., Upadhyaya, H. D., Luo, M. C., Thudi, M., Gowda, C. L., Singh, N. P., Lichtenzveig, J., Gali, K. K., Rubio, J., Nadarajan, N., Dolezel, J., Bansal, K. C., Xu, X., Edwards, D., Zhang, G., Kahl, G., Gil, J., Singh, K. B., Datta, S. K., Jackson, S. A., Wang, J., & Cook, D. R. (2013). Draft genome sequence of chickpea (Cicer arietinum) provides a resource for trait improvement. *Nature Biotechnology*, *31*(3), 240-246. http://doi.org/10.1038/nbt.2491. PMid:23354103.

Verma, A. K., Kumar, S., Das, M., & Dwivedi, P. D. (2013). A comprehensive review of legume allergy. *Clinical Reviews in Allergy & Immunology*, *45*(1), 30-46. http://doi.org/10.1007/s12016-012-8310-6. PMid:22555630.

Voytas, D. F., & Gao, C. (2014). Precision genome engineering and agriculture: Opportunities and regulatory challenges. *PLoS Biology*, *12*(6), e1001877. http://doi.org/10.1371/journal.pbio.1001877. PMid:24915127.

Wang, L., Rubio, M. C., Xin, X., Zhang, B., Fan, Q., Wang, Q., Ning, G., Becana, M., & Duanmu, D. (2019). CRISPR/Cas9 knockout of leghemoglobin genes in Lotus japonicus uncovers their synergistic roles in symbiotic nitrogen fixation. *The New Phytologist*, *224*(2), 818-832. http://doi.org/10.1111/nph.16077. PMid:31355948.

Wang, L., Wang, L., Tan, Q., Fan, Q., Zhu, H., Hong, Z., Zhang, Z., & Duanmu, D. (2016). Efficient inactivation of symbiotic nitrogen fixation related genes in Lotus japonicus using CRISPR-Cas9. *Frontiers in Plant Science*, *7*, 1333. http://doi.org/10.3389/fpls.2016.01333. PMid:27630657.

Wang, Y., Zhao, J., Lu, W., & Deng, D. (2017). Gibberellin in plant height control: Old player, new story. *Plant Cell Reports*, *36*(3), 391-398. http://doi.org/10.1007/s00299-017-2104-5. PMid:28160061.

Wani, A. K., Akhtar, N., & Shukla, S. (2022). CRISPR/Cas9: Regulations and challenges for law enforcement to combat its dual-use. *Forensic Science International*, *334*, 111274. http://doi.org/10.1016/j.forsciint.2022.111274. PMid:35316773.

Weber, A. P., & Bar-Even, A. (2019). Update: Improving the efficiency of photosynthetic carbon reactions. *Plant Physiology*, *179*(3), 803-812. http://doi.org/10.1104/pp.18.01521. PMid:30610109.

Weiner, J. (2019). Looking in the wrong direction for higher-yielding crop genotypes. *Trends in Plant Science*, *24*(10), 927-933. http://doi.org/10.1016/j.tplants.2019.07.001. PMid:31358472.

World Cancer Research Fund, & American Institute for Cancer Research. (2014). *Diet, nutrition, physical activity, and cancer: A global perspective* (Third Expert Report. 2014). WHO Press, World Cancer Report. http://www.aicr.org/cancer-research/dietandcancerreport/

Wu, F. H., Shen, S. C., Lee, L. Y., Lee, S. H., Chan, M. T., & Lin, C. S. (2009). Tape-Arabidopsis Sandwich-a simpler Arabidopsis protoplast isolation method. *Plant Methods*, *5*(1), 1-10. http://doi.org/10.1186/1746-4811-5-16. PMid:19930690.

Wu, F., & Hanzawa, Y. (2018). A simple method for isolation of soybean protoplasts and application to transient gene expression analyses. *JoVE (Journal of Visualized Experiments)*, (131), e57258. https://doi.org/10.3791/57258.

Xing, H.-L., Dong, L., Wang, Z.-P., Zhang, H.-Y., Han, C.-Y., Liu, B., Wang, X.-C., & Chen, Q.-J. (2014). A CRISPR/Cas9 toolkit for multiplex genome editing in plants. *BMC Plant Biology*, *14*(1), 327. http://doi.org/10.1186/s12870-014-0327-y. PMid:25432517.

Xiong, L., Li, C., Li, H., Lyu, X., Zhao, T., Liu, J., Zuo, Z., & Liu, B. (2019). A transient expression system in soybean mesophyll protoplasts reveals the formation of cytoplasmic GmCRY1 photobody-like structures. *Science China. Life Sciences*, *62*(8), 1070-1077. http://doi.org/10.1007/s11427-018-9496-5. PMid:30929191.

Xu, H., Guo, Y., Qiu, L., & Ran, Y. (2022). Progress in soybean genetic transformation over the last decade. *Frontiers in Plant Science*, *13*, 900318. http://doi.org/10.3389/fpls.2022.900318. PMid:35755694.

Yang, Z., Du, H., Xing, X., Li, W., Kong, Y., Li, X., & Zhang, C. (2022). A small heat shock protein, GmHSP17. 9, from nodule confers symbiotic nitrogen fixation and seed yield in soybean. *Plant Biotechnology Journal*, *20*(1), 103-115. http://doi.org/10.1111/pbi.13698. PMid:34487637.

Young, N. D., Debellé, F., Oldroyd, G. E., Geurts, R., Cannon, S. B., Udvardi, M. K., Benedito, V. A., Mayer, K. F., Gouzy, J., Schoof, H., Van de Peer, Y., Proost, S., Cook, D. R., Meyers, B. C., Spannagl, M., Cheung, F., De Mita, S., Krishnakumar, V., Gundlach, H., Zhou, S., Mudge, J., Bharti, A. K., Murray, J. D., Naoumkina, M. A., Rosen, B., Silverstein, K. A., Tang, H., Rombauts, S., Zhao, P. X., Zhou, P., Barbe, V., Bardou, P., Bechner, M., Bellec, A., Berger, A., Bergès, H., Bidwell, S., Bisseling, T., Choisne, N., Couloux, A., Denny, R., Deshpande, S., Dai, X., Doyle, J. J., Dudez, A. M., Farmer, A. D., Fouteau, S., Franken, C., Gibelin, C., Gish, J., Goldstein, S., González, A. J., Green, P. J., Hallab, A., Hartog, M., Hua, A., Humphray, S. J., Jeong, D. H., Jing, Y., Jöcker, A., Kenton, S. M., Kim, D. J., Klee, K., Lai, H., Lang, C., Lin, S., Macmil, S. L., Magdelenat, G., Matthews, L., McCorrison, J., Monaghan, E. L., Mun, J. H., Najar, F. Z., Nicholson, C., Noirot, C., O'Bleness, M., Paule, C. R., Poulain, J., Prion, F., Qin, B., Qu, C., Retzel, E. F., Riddle, C., Sallet, E., Samain, S., Samson, N., Sanders, I., Saurat, O., Scarpelli, C., Schiex, T., Segurens, B., Severin, A. J., Sherrier, D. J., Shi, R., Sims, S., Singer, S. R., Sinharoy, S., Sterck, L., Viollet, A., Wang, B. B., Wang, K., Wang, M., Wang, X., Warfsmann, J., Weissenbach, J., White, D. D., White, J. D., Wiley, G. B., Wincker, P., Xing, Y., Yang, L., Yao, Z., Ying, F., Zhai, J., Zhou, L., Zuber, A., Dénarié, J., Dixon, R. A., May, G. D., Schwartz, D. C., Rogers, J., Quétier, F., Town, C. D., & Roe, B. A. (2011). The Medicago genome provides insight into the evolution of rhizobial symbioses. *Nature*, *480*(7378), 520-524. http://doi.org/10.1038/nature10625. PMid: **22089132.**

Yu, J., Tu, L., Subburaj, S., Bae, S., & Lee, G. J. (2021). Simultaneous targeting of duplicated genes in Petunia protoplasts for flower color modification via CRISPR-Cas9 ribonucleoproteins. *Plant Cell Reports*, *40*(6), 1037-1045. http://doi.org/10.1007/s00299-020-02593-1. PMid:32959126.

Yuan, M., Zhu, J., Gong, L., He, L., Lee, C., Han, S., Chen, C., & He, G. (2019). Mutagenesis of FAD2 genes in peanut with CRISPR/Cas9 based gene editing. *BMC Biotechnology*, *19*(1), 24. http://doi.org/10.1186/s12896-019-0516-8. PMid:31035982.

Yue, J. J., Yuan, J. L., Wu, F. H., Yuan, Y. H., Cheng, Q. W., Hsu, C. T., & Lin, C. S. (2021). Protoplasts: From isolation to CRISPR/Cas genome editing application. *Frontiers in Genome Editing*, *3*, 717017. http://doi.org/10.3389/fgeed.2021.717017. PMid:34713263.

Zafar, S. A., Zaidi, S. S. E. A., Gaba, Y., Singla-Pareek, S. L., Dhankher, O. P., Li, X., Mansoor, S., & Pareek, A. (2020). Engineering abiotic stress tolerance via CRISPR/Cas-mediated genome editing. *Journal of Experimental Botany*, *71*(2), 470-479. http://doi.org/10.1093/jxb/erz476. PMid:31644801.

Zander, P., Amjath-Babu, T. S., Preissel, S., Reckling, M., Bues, A., Schläfke, N., Kuhlman, T., Bachinger, J., Uthes, S., Stoddard, F., Murphy-Bokern, D., & Watson, C. (2016). Grain legume decline and potential recovery in European agriculture: A review. *Agronomy for Sustainable Development*, *36*(2), 1-20. http://doi.org/10.1007/s13593-016-0365-y.

Zetsche, B., Gootenberg, J. S., Abudayyeh, O. O., Slaymaker, I. M., Makarova, K. S., Essletzbichler, P., Volz, S. E., Joung, J., van der Oost, J., Regev, A., Koonin, E. V., & Zhang, F. (2015). Cpf1 is a single RNA-guided endonuclease of a class 2 CRISPR-Cas system. *Cell*, *163*(3), 759-771. http://doi.org/10.1016/j.cell.2015.09.038. PMid:26422227.

Zhang, H., Cao, Y., Zhang, H., Xu, Y., Zhou, C., Liu, W., Zhu, R., Shang, C., Li, J., Shen, Z., Guo, S., Hu, Z., Fu, C., & Sun, D. (2020a). Efficient generation of CRISPR/Cas9-mediated homozygous/biallelic Medicago truncatula mutants using a hairy root system. *Frontiers in Plant Science*, *11*, 294. http://doi.org/10.3389/fpls.2020.00294. PMid:32265954.

Zhang, Y., Wang, X., Luo, Y., Zhang, L., Yao, Y., Han, L., Che, Z., Wang, L., & Li, Y. (2020b). OsABA8ox2, an ABA catabolic gene, suppresses root elongation of rice seedlings and contributes to drought response. *The Crop Journal*, *8*(3), 480-491. http://doi.org/10.1016/j.cj.2019.08.006.

Zhang, Y., Iaffaldano, B., & Qi, Y. (2021). CRISPR ribonucleoprotein-mediated genetic engineering in plants. *Plant Communications*, *2*(2), 100168. http://doi.org/10.1016/j.xplc.2021.100168. PMid:33898980.

Zhang, Y., Malzahn, A. A., Sretenovic, S., & Qi, Y. (2019). The emerging and uncultivated potential of CRISPR technology in plant science. *Nature Plants*, *5*(8), 778-794. http://doi.org/10.1038/s41477-019-0461-5. PMid:31308503.

Zheng, N., Li, T., Dittman, J. D., Su, J., Li, R., Gassmann, W., Peng, D., Whitham, S. A., Liu, S., & Yang, B. (2020). CRISPR/Cas9-based gene editing using egg cell-specific promoters in Arabidopsis and soybean. *Frontiers in Plant Science*, *11*, 800. http://doi.org/10.3389/fpls.2020.00800. PMid:32612620.

Zhuang, W., Chen, H., Yang, M., Wang, J., Pandey, M. K., Zhang, C., Chang, W. C., Zhang, L., Zhang, X., Tang, R., Garg, V., Wang, X., Tang, H., Chow, C. N., Wang, J., Deng, Y., Wang, D., Khan, A. W., Yang, Q., Cai, T., Bajaj, P., Wu, K., Guo, B., Zhang, X., Li, J., Liang, F., Hu, J., Liao, B., Liu, S., Chitikineni, A., Yan, H., Zheng, Y., Shan, S., Liu, Q., Xie, D., Wang, Z., Khan, S. A., Ali, N., Zhao, C., Li, X., Luo, Z., Zhang, S., Zhuang, R., Peng, Z., Wang, S., Mamadou, G., Zhuang, Y., Zhao, Z., Yu, W., Xiong, F., Quan, W., Yuan, M., Li, Y., Zou, H., Xia, H., Zha, L., Fan, J., Yu, J., Xie, W., Yuan, J., Chen, K., Zhao, S., Chu, W., Chen, Y., Sun, P., Meng, F., Zhuo, T., Zhao, Y., Li, C., He, G., Zhao, Y., Wang, C., Kavikishor, P. B., Pan, R. L., Paterson, A. H., Wang, X., Ming, R., & Varshney, R. K. (2019). The genome of cultivated peanut provides insight into legume karyotypes, polyploid evolution and crop domestication. *Nature Genetics*, *51*(5), 865-876. http://doi.org/10.1038/s41588-019-0402-2. PMid:31043757.

Supplementary Material

Supplementary material accompanies this paper.
Table 1S. Application of the CRISPR/Cas technique for legume crop improvement (2015-2022).
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