CRISPR/Cas9 gene editing in legume crops: Opportunities and challenges

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Abstract
Legumes are an excellent source of proteins and health-promoting phytochemicals. Recognizing their importance in human nutrition and sustainable agricultural production, significant efforts are currently being made to accelerate genetic gain related to yield, stress resilience, and nutritional quality. Recent increases in genomic resources for multiple legume crops have laid a solid foundation for application of transformative breeding technologies such as genomic selection and genome editing for crop improvement. In this review, we focus on the recent plant-specific advances in CRISPR/Cas9-based gene editing technology and discuss the challenges and opportunities to harnessing this innovative technology for targeted improvement of traits in legume crops. Gene-editing methods have been successfully established for soybean, cowpea, chickpea, and model legumes such as Medicago truncatula and Lotus japonicus. However, the recalcitrance of other legumes to in vitro gene transfer and regeneration has posed a serious challenge to application of gene editing. We discuss various modifications to in vitro culture methods, in terms of the choice of explant, media composition, and DNA delivery and gene-editing detection methods that can potentially improve the rate of transformation and regeneration of whole plant in legume crops. Although gene-editing technology can bring enormous benefits to legume breeding, regulatory hurdles are a cause for serious concern. We compare the regulatory environments existing in the European Union and the United States of America. A favorable regulatory framework and public acceptance are important factors in realizing CRISPR's potential benefits to global food security.

Keywords
CRISPR/Cas9, gene editing, gRNA, legumes, regeneration of plants, tissue culture, transformation
Legumes are the third largest family of angiosperms with more than 19,500 known species belonging to 751 genera (Lewis, 2005), including a diverse range of food crops that are important sources of plant-based proteins and essential amino acids. Leguminous crops also play a vital role in sustainable farming by promoting soil health through symbiotic nitrogen fixation and release of high-quality organic matter into soils. Despite the health benefits and ecological significance, legume production is affected by poor crop yields. Increasing yield potential and reliability is a complex challenge that will benefit from adoption of novel strategies such as genomics-assisted selection (marker-assisted selection or genomic selection) and precision breeding (gene editing).

In the last decade, enormous progress has been made in sequencing and structural analysis of legume genomes. Draft genome and transcriptome assemblies have been generated for more than 35 legume species (Bauchet et al., 2019). These foundational resources are facilitating discovery and deployment of molecular markers in the selection of complex traits and development of superior cultivars of grain legume crops (Varshney et al., 2019). The current status and prospects of applying advanced marker-assisted backcrossing, marker-assisted selection, and genomic selection strategies for legume improvement have been extensively reviewed elsewhere (Afzal et al., 2020; Jain et al., 2017; Mousavi-Derazmahalleh et al., 2019; Varshney et al., 2015; Varshney et al., 2019). In this review, we focus on recent advances in genome-editing technology and its utility as a precision breeding tool for the improvement of legume crops.

Gene editing is based on the use of engineered nucleases and cellular DNA repair pathways to make precise, targeted changes to the genome of an organism. The development of gene-editing methodologies began nearly three decades ago with the key discovery that specific double-stranded breaks can be introduced in chromosomes using a meganuclease I-SceI (Rouet et al., 1994). However, the application of meganucleases for gene editing was limited due to low frequency of target sites in most genes. The efforts to develop efficient gene-editing systems received a big boost with the introduction of programmable zinc finger nucleases (ZFNs; Bibikova et al., 2002) and transcription activator-like effector nucleases (TALENs; Li et al., 2011; Zhang et al., 2011). However, the real breakthrough in this revolutionary technology came with the successful adaptation of RNA-guided Cas9 nuclease harnessed from the type II prokaryotic clustered regularly interspaced short palindromic repeats (CRISPR) adaptive immune system for genome editing in eukaryotic cells (Cong et al., 2013; Mali et al., 2013).
presence of large gene families in plants and therefore high sequence similarity, off-target gRNA binding is another major concern, which can lead to unintended targeting and adverse effects. Thus, it is essential to design appropriate gRNAs that have high on-target efficiency and low off-target potential. Choosing highly active and multiple gRNAs is important for species with low transformation rates and long tissue culture timelines. For species that are readily transformable, about two gRNAs per gene would be sufficient to recover a mutant.

3.2 | gRNA delivery

3.2.1 | Transformation of legume crops

For transformation, including CRISPR/Cas9 gene editing, it is necessary to have the ability to deliver the DNA/RNA components, with the regeneration of an entire plant. Legumes are well known to be both resistant to the uptake and integration of introduced DNA (Yadav et al., 2017) and recalcitrant in terms of regeneration (Ochatt et al., 2018). This is compounded by the fact that although some legume tissues are transformable and some will regenerate, the two realities are not always in the same tissue. This is why it is important to the development of a transformation protocol where the transformation vector is incorporated from the onset rather than simply with the development of a regeneration protocol.

The majority of large-seeded legumes depend upon Agrobacterium-mediated transformation, although biolistics technologies have also been deployed (Table S1). Most successful reports involve explants from seed tissues, including developing or mature embryos in whole or in part, or the cotyledonary nodes, including de-embryonated cotyledons (Table S1). Wounding, with the addition of acetosyringone to activate virulence gene in the Agrobacterium (Lai et al., 2006), is a recurring treatment to the explants. Wounding can take place in the form of puncture, or through cutting an embryo in half, or into longitudinal slices. Additional methods to enhance uptake include sonication and/or vacuum infiltration (Chopra & Saini, 2012; Liu et al., 2005; Mahmoudian et al., 2002; Trick & Finer, 1997), as well as the addition of other compounds such as lipoic acid (Dan et al., 2009).

Typical transformation protocols consist of several distinct phases. For organogenesis in species such as pea and chickpea (Olhoft et al., 2001; Polowick et al., 2000; Polowick et al., 2004; Schroeder et al., 1993), this can include a co-cultivation period (Figure 1a), shoot initiation (Figure 1b), shoot elongation (Figure 1d), and rooting (Figure 1e), each on separate media formulations. During the development of a protocol, a reporter gene, such as gus (β-glucuronidase), is a

![Figure 1](image-url)

**Figure 1** Example of transformation and regeneration stages, as shown for lentil. (a) Slices of embryo axes after 4 days of co-cultivation with *Agrobacterium*, (b) transient gus expression in an explant at the end of co-cultivation, (c) explants at the end of a shoot induction phase, (d) explants during the shoot elongation phase, (e) rooted shoots, (f) gus expression in small shoot at the end of shoot elongation after staining with X-glucuronidase, and (g) a rooted putative transformant in soil. Scale bars: (a) 5 mm, (b) 1 mm, (c) 5 mm, (d) 3 mm, (e) 5 mm, (f) 5 mm, and (g) 4 cm. Photos courtesy of Wei Yan
convenient way to track progress (Figure 1b,f). It requires sacrificing tissue; however, it can be easier to detect than fluorescence in photosynthetic, green tissues. For both reporter methods, molecular analysis such as PCR/ddPCR is required to confirm the successful regeneration of a transgenic plant. In protocols that involve embryogenesis, the stages can include embryogenesis, proliferation of embryonic tissue for bombardment, maturation, desiccation, and germination (Simmonds & Donaldson, 2000). Media formulations, incubation conditions, and time involved vary from species to species and between individual protocols. For specific methodology, there are examples of protocols for the individual species listed in Table S1.

Browning of tissues during regeneration is a hindrance with some legume species, notably bean (Olhoft et al., 2001), faba bean (Abdelwahd et al., 2008), and lupins (Polowick et al., 2014). There are several methods to prevent the browning, including the addition of thiols (Olhoft et al., 2001; Yadav et al., 2017). It can also include physical adjustments to reduce ethylene accumulation through use of vented lids or surgical tape in replacement for Parafilm (Polowick et al., 2014). In some cases, as in the addition of activated charcoal, it has been noted that although the antibrowning treatment is successful, it may have the effect of interfering with associated plant growth regulator treatments. For this reason, it is crucial to balance any additives to avoid restricting regeneration.

Rooting in vitro has long been an issue with many large-seeded legumes. It is common to resort to grafting transgenic shoots onto seedling rootstock (Akçay et al., 2009; Bean et al., 1997; Gulati et al., 2002) or even to continued growth, including flowering, in vitro (Das et al., 2019). Success has been achieved using combinations of half-strength basal media, naphthalene acetic acid (NAA), or indole butyric acid (IBA) (Chopra & Saini, 2012; Polowick et al., 2014).

The issue of rooting can also be associated with the concentration of the chemical selection during that period of regeneration, risking either the loss of successfully transformed shoots or the prospect of nontransformed escapes. Therefore, it is essential to establish the best concentration for each step with the use of kill curves. In some cases, it is effective to use a higher concentration of selection chemical during regeneration to eliminate escapes and lower the concentration or even eliminate it for rooting (Polowick et al., 2014). There can also be an issue with chimeras, often from use of pretreatments (Gulati et al., 2002) or explants where there may have been preexisting meristems that develop quickly during the regenerative phase (Mukeshimana et al., 2013).

A survey of papers describing protocol development (Table S1) shows a frequency of success in a broad range from 0.3% (Popelka et al., 2006) to a high of 12.7% (Olhoft et al., 2001), but the majority of the protocols fall within a narrower 1–4% range. This can be influenced by genotype (Popelka et al., 2006) and Agrobacterium strain (Simmonds & Donaldson, 2000). Comparison of efficiency is complicated by the use of different denominators in the frequency calculation by different laboratories, including the number of transgenic plants per 100 explants; however, some also report on “putative” transformants, or the percentage of shoots recovered relative to the number that are confirmed to be transformed.

As the largest crop in the family, soybean represents the only legume species for which transgenic cultivars have proceeded through the regulatory process and to commercialization. A total of 41 approved cultivars have been released (https://www.isaaa.org/gmapprovaldatabase/crop/default.asp?CropID=19&Crop=Soybean), predominantly with herbicide resistance. As a result, there is more reported research in this crop, although advances in companies may also to go unreported in the published literature. Soybean is also the only legume crop for which CRISPR technology has been widely reported (Zheng et al., 2020). It is also the first for which a gene-edited product has reached market, specifically an oil profile that is high in oleic acid and reduced saturated fat (https://www.thescientist.com/news-opinion/gene-edited-soybean-oil-makes-restaurant-debut-65590), arising from the use of TALENs gene-editing technology.

Finally, within the legume producer and breeders communities in many countries, including Canada and with the exception of soybean, there has been a resistance to the possible production of GMO lines due to the lack of acceptance within their markets, which are generally smaller. However, even in the absence of an appetite for transgenic cultivars, the development of efficient transformation techniques is essential to the validation of gene function. The introduction of gene-editing tools through CRISPR technology may alleviate some of this concern and encourage more research into the field.

3.2.2 | CRISPR/Cas9 mutagenesis in doubled haploids

A functional microspore-based gRNA delivery system can offer several advantages for obtaining homozygosity quickly and to shorten the generation cycle. A large number of genetically identical and physiologically uniform microspores can be easily isolated in a relatively short time for functional validation of genes as well as genetic characterization and improvement of valuable traits. Using fluorescent reporter systems, microspores can also be used for high-throughput screening of multiple gRNAs simultaneously. One of the first experiments of microspore mutagenesis using the CRISPR/Cas9 system was carried out by Bhowmik et al. (2018) who used Neon electroporation system to deliver gRNAs into isolated wheat microspores. Bilichak et al. (2020) also demonstrated the ability of CPP–ZFN (complex of cell-penetrating peptides and ZFN) to introduce edits in haploid embryo-like structures regenerated from wheat microspores.

The microspores from legume species could be potentially used for gRNA delivery and CRISPR/Cas9-mediated gene editing. However, due to the recalcitrant nature of the legume species, the production of double haploid (DH) plants is very limited. Although regeneration of DH plants has been attempted by many groups (Bobkov et al., 2010; Gupta, 1975; Lulsdorf et al., 2011; Ochatt et al., 2009; Ribalta et al., 2012), the regeneration frequency of complete haploid plants was very low (Bobkov, 2014; Ochatt et al., 2009). Thus far, regeneration of DH plants has only been successful in chickpea using anther culture and androgenesis (Abdollahi & Rashidi, 2018).
Considering the importance of DH technology in pulse breeding, recently, several laboratories including the National Research Council Canada have started re-evaluation and improvement of strategies for the productions of DH plants.

### 3.3 Detection of genetic modifications

Identifying and tracking modifications induced by genome editing are necessary at several stages, including optimization of experimental conditions and characterization, selection, and tracking of edited lines. The range of methods available for detecting modifications is wide and evolving, and there is currently no single method of choice for all genotyping needs.

General challenges to confident detection include biological aspects of gene-editing proteins such as the spectrum of possible induced mutations, and biological aspects of the target organism, including multiplicity of homologs and efficiency of creating edited lines. Due to limitations with regenerating legumes, screening large number of lines or heterogeneous tissues may be necessary, necessitating that methods be cost-effective and sensitive. Although most economically important legumes genomes are not complicated by recent polyploidization, this clade may have a greater tendency for gene duplications (Young et al., 2011). High-quality genome assemblies assist with reliable interpretation of detection experiments and are particularly important for examining off-target effects.

In this section, we touch on some of the most popular and promising methods for detecting edited sequences in legumes. For more in-depth comparisons of established methodologies, we point the reader to a relatively recent review fully devoted to the subject (Germini et al., 2018).

#### 3.3.1 Mismatch detection

One of the original and most popular methods to screen for mutations is via the detection of mismatches that are formed from denaturation and annealing heterogeneous populations of DNA. A number of endonucleases that are capable of cleaving at mismatches have been used, including Cel I (Surveyor; Qiu et al., 2004) and T7EI (Mashal et al., 1995). Although Cel I may outperform T7EI for SNP detection, T7EI is recommended for CRISPR/Cas9 experiments particularly as it appears to perform better on indels and generally generates less background signal, a limiting factor for this strategy. Although mismatch detection is straightforward to implement and requires only standard molecular biology equipment, it suffers from limited sensitivity and high rates of both false positives and negatives.

#### 3.3.2 Electrophoretic separation

Relying directly on electrophoretic mobility can allow a simpler approach to identifying mutants. Large insertions or deletions can be tracked easily by PCR followed by agarose gel electrophoresis, whereas PCR followed by non-denaturing PAGE is capable of tracking indels as small as 1 bp (Zhu et al., 2014). Although these methods can be very rapid, their ability to detect low-abundance edits is weak, and hence, they are best used in systems where editing efficiency is high or in screening later generations for homozygous lines.

Where it is possible to target a restriction enzyme recognition site, restriction enzyme-suppressed PCR (RE-PCR) can provide a more sensitive option (Xie & Yang, 2013). RE-PCR involves performing a restriction digest prior to PCR amplification such that only mutated sites are amplified.

Annealing at critical temperature PCR (ACT-PCR) is another strategy reliant on PCR failure in the presence of mutations. This strategy to detect homozygous mutant lines involves finding the highest possible annealing temperature on the wild-type template, such that annealing of a primer overlapping the target site fails on an edited template (Hua et al., 2017). It is also possible to include an additional competing flanking primer to allow for an internal positive control (Biswas et al., 2019). Although this method has minimal requirements in term of equipment, a gradient thermocycler is ideal for identifying the critical annealing temperature.

Integrating means for fluorescence-based detection can greatly improve the sensitivity of PCR-based methods. Biswas et al. (2019) demonstrated the use of fluorophore-labeled primers for generating labeled amplicons with detection by DNA capillary electrophoresis. This method improves the sensitivity to detect indels (limit of detection [LOD] ~0.1%) and can resolve populations of mutations at 1- to 2-bp resolution. This method also leverages the automation potential of capillary electrophoresis instrumentation and, although not yet demonstrated, could be used to monitor multiple targets simultaneously by using different fluorophores per amplicon in a similar fashion to that used routinely for high-throughput simple sequence repeat (SSR) analysis. The combination of low cost, high sensitivity, and scalability make this method an attractive option for screening steps in legumes, particularly where access to instrumentation will allow for quick turnaround times.

#### 3.3.3 Fluorophore binding-based detection

Several highly sensitive methods for edit detection, such as high-resolution melt analysis (Thomas et al., 2014), qPCR (Peng et al., 2018), and ddPCR (Miyaoka et al., 2018), rely on binding of fluorescent probes or dyes. Although these methods are sensitive and low cost, they require the access to the appropriate equipment.

#### 3.3.4 Sanger sequencing

Sanger sequencing is a reliable low-cost method for genotyping and can confirm the nature of the modifications. Although traditional analyses of Sanger sequence data are dependent on clonal input, a number of analytical methods such as TIDE (Tracking of Indels by
**3.3.5  |  Next-generation sequencing**

Massively parallel sequencing technologies have the capacity to simultaneously examine a large number of sites and accommodate a large number of samples. Next-generation sequencing (NGS) methods can be extremely sensitive (LOD < 1%, Kim et al., 2019) and offer the ability to characterize the spectrum of edits in a population of cells. Preparing samples for NGS involves the addition of sequencing adapters to the desired target sequences. For preparing amplicon libraries for Illumina platforms, a popular approach is to include a partial adapter sequence as a tail on the target-specific primers, followed by a second PCR that completes the adapter sequence and introduces sample barcodes. This strategy allows the use of generic indexing primers and the pooling of multiple amplicons from the same sample following the initial PCR reaction. Commercially available generic indices are now available for up to 384 dually unique index combinations and potentially exist to scale multiplexing much further. It is also possible to multiplex the initial target-specific PCR to further streamline the preparation of large number of amplicons per sample. A number of tools, such as Oligo2go (Hendling et al., 2018), are available to assist with multiplex PCR design, and approaches such as rhAmp-seq from IDT allow multiplexing of over 100 targets.

Although there are no well-established methods for using Oxford Nanopore Technologies for edit detection, the accessibility of the smaller Minion or Flongle platforms is attractive for rapid turnaround. Although the error rates for this platform are high, the technology and application strategies are evolving rapidly. One strategy to cope with higher error rates is through the incorporation of unique molecular identifiers (UMIs) into amplicons (Karst et al., 2021). Though this adds an additional step to the workflow, it can greatly improve the reliability and LOD of mutations.

Though per experiment costs remain high relative to the other strategies presented here, the massive data content and multiplexing scalability of NGS methods have the potential to translate into lower costs per sample than any other method presented above. NGS of genomic libraries is also the only current method that can provide comprehensive genome coverage to exhaustively examine genomic alterations (e.g., Tang et al., 2018). Though powerful, NGS approaches can have long turnaround times and require batching to fully exploit their benefits. NGS methods provide information for individual library fragments, thus providing some tolerance for off-target amplification, but the presence of off-target sequences, and differences between the line used and the reference genome are primary drivers for false positives. Therefore, including negative controls is critical for clear interpretation of variant calling data. We anticipate that the popularity of NGS screening methods will continue to grow as editing experiments move toward higher multiplexity and efficiency of NGS experiments continues to increase.

### TABLE 1  |  Examples of CRISPR/Cas9-mediated gene editing in legumes

| Species                     | Target genes                                           | References |
|-----------------------------|--------------------------------------------------------|------------|
| **Medicago truncatula**     | Hua enhancer1, phytoene desaturase, and symbiosis receptor-like kinase | (Curtin et al., 2018; Meng et al., 2017; Michno et al., 2015; Wolabu et al., 2020) |
| **Lotus japonicus**         | Legemoglobin 1/2/3, symbiosis receptor-like kinase (LjSYMRK), and Lotus histidine kinase 1 (LHK1)-interacting protein (LjCFZ1-2) | (Cai, Yin, et al., 2018; Wang et al., 2016; Wang et al., 2019) |
| **Glycine max**             | Phytoene desaturase, glutamine synthase, chalcone-flavanone isomerase, glucosyltransferase, DDM1 orthologs, MET1 orthologs, dicer-like3a, double-stranded RNA-binding2a/b, fatty acid desaturase 2, and Squamosa promoter-binding protein-like | (Al Amin et al., 2019; Bao et al., 2019; Bao et al., 2020; Curtin et al., 2018; Du et al., 2016; Jacobs et al., 2015; Li et al., 2015; Michno et al., 2015; Sun et al., 2015) |
| **Vigna unguiculata**       | Symbiosis receptor-like kinase and VuSP011-1            | (Che et al., 2021; Ji et al., 2019; Juranić et al., 2020) |
| **Cicer arietinum**         | (protoplasts)                                          | 4-coumarate ligase (4CL) and Reveille 7 (RVE7) |

**4  |  CRISPR/Cas9 FOR CROP IMPROVEMENT IN LEGUMES**

CRISPR/Cas9-mediated gene editing has been established in some legume crops (Table 1), including the model legumes Medicago truncatula and Lotus japonicus, and in soybean, cowpea, and chickpea for which transformation protocols are available. Below, we discuss the current status and prospects of using CRISPR/Cas9 for improvement of various traits in legume crops.
4.1 Model legume plants (M. truncatula Gaertn. and L. japonicus (Regel) K. Larsen)

The ease of transformation, diploidy, self-fertility, small genome, and short life cycle of M. truncatula and L. japonicus has benefited the genetic and physiological studies of nitrogen fixation in root nodules and other aspects of legume-specific physiology in these two model species (Sato & Tabata, 2006; Young & Udvardi, 2009). In addition, whole-genome sequences of M. truncatula (500 Mb) and L. japonicus (472 Mb) have further facilitated various molecular studies of these species (Sato et al., 2008; Young et al., 2011). Although they share many of the same characteristics, M. truncatula and L. japonicus form different types of nodules (Hirsch, 1992). M. truncatula forms indeterminate nodules, which are also found in pea (Pisum sativum L.), chickpea (Cicer arietinum L.), lentil (Lens culinaris Medik.), and faba bean (Vicia faba L.), whereas L. japonicus organizes determinate nodules, a feature it shares with soybean (Glycine max (L.) Merr.), mung bean (Vigna radiate (L.) Wilczek), and cowpea (Vigna unguiculata L.). Hence, M. truncatula and L. japonicus have unique advantages in studying indeterminate and determinate nodulation, respectively.

The development of CRISPR/Cas9 has allowed targeted mutagenesis of nodulation-associated genes in these model legumes. Using CRISPR/Cas9 in M. truncatula, the function of candidate genes for nodulation, identified by genome-wide association studies, was examined (Curtin et al., 2017), and mutational analysis of five different nodule-specific PLAT domain (NPD1–5) and nitrate peptide family (NPD) genes has also been accomplished (Trujillo et al., 2019; Wang, Sun, et al., 2020). Similar successful cases of CRISPR/Cas9 were reported in L. japonicus. Several mutant plants for symbiosis receptor-like kinase (LjSYM1), leghemoglobin (LjLb1-3), and Lotus histidine kinase 1 (LjHK1)-interacting protein (LjCZF1-2) have been generated by CRISPR/Cas9 (Cai, Yin, et al., 2018; Wang et al., 2016; Wang et al., 2019). Besides nodulation-associated genes, the function of SUPERMAN (MtSUP) was validated as a true orthologue of its Arabidopsis counterpart ATSUP in M. truncatula (Rodas et al., 2021), and the biochemical activity encoded in CYP716A51-1 was identified to be the C28 oxidation of triterpenes in L. japonicus (Suzuki et al., 2019). Therefore, there is ample evidence for effective uses of CRISPR/Cas9 in M. truncatula and L. japonicus, which promises unprecedented opportunities to fully elucidate the nodulation process and other aspects of legume physiology.

4.2 Soybean

As an important crop for oil and protein, soybean (G. max (L.) Merr.) is among the first crop species targeted for genetic improvement by the CRISPR/Cas9 technology (for a recent review, see Bao et al., 2020). Soybean is a diploid species evolved from a palaeotetraploid. Its genome is highly duplicated and thus poses a major challenge for conventional genetic approaches for understanding gene function. Another challenge is that Agrobacterium-mediated transformation efficiency is very low in soybean, affected by tissue or cultivar. Therefore, Agrobacterium rhizogenes-mediated hairy root transformation has been widely used to quickly evaluate the efficacy of gRNAs before whole-plant transformation, as it only takes a few weeks to obtain transgenic hairy roots (Li et al., 2019). In 2015, several groups reported their successful gene-editing work using the CRISPR/Cas9 technology in soybean (Cai et al., 2015; Jacobs et al., 2015; Li et al., 2015; Sun et al., 2015). After these initial successes, continued efforts have been made by the soybean research community to improve the gene-editing technology. For example, Di et al. (2019) tested multiple GmU6 promoters (11 in total) for their suitability in driving gRNA expression in soybean hairy roots and found that the GmU6-8 and GmU6-10 promoters had higher activity in enhancing editing efficiency (20.3% and 20.6%, respectively), compared with the other nine GmU6 promoters (ranged from 2.8% to 17%). Two studies demonstrated that large genomic deletions (from around 1 kb to more than 4.5 kb) could be achieved by using dual gRNAs to cleave two adjacent loci on the same chromosome in soybean (Cai, Chen, et al., 2018; Do et al., 2019). The large deletion strategy will not only ensure complete knockout of target genes but also contribute to other fields of research, such as understanding the function of regulatory elements or noncoding genes (Li et al., 2018). Most recently, Zheng et al. (2020) tested egg cell-specific promoters and found that one of them could lead to good gene-editing efficiency (26.8%) in stable transgenic soybean lines.

More importantly, efforts have also been made to understand gene function and improve/manipulate agronomic traits, such as seed oil and storage proteins, flowering timing, and plant architecture. Kanazashi et al. (2018) used a single gRNA to edit two homologous genes GmPPD1 and GmPPD2, which encode orthologs of the Arabidopsis PEAPOD. The resulting double mutants showed severe abnormal leaf and pod phenotypes. Cai, Chen, et al. (2018) reported that homozygous mutants obtained by CRISPR/Cas9-mediated mutagenesis in FLOWERING LOCUS T2a (GmFT2a) delayed flowering in soybean. The team further mutated GmFT5a and obtained ft2aft5a double mutants by crossing with the ft2a mutant. The double mutants flowered about 31 days later than wild-type plants and produced more pods and seeds under short-day conditions (Cai et al., 2020). Bao et al. (2019) used a CRISPR/Cas9-based multiplex genome-editing tool to mutate four GmSPL9 genes, and created various soybean mutants containing different combinations of mutated loci, which showed altered node number on the main stem and branch number. Seed related traits, such as seed oil profile (Do et al., 2019), unpleasant beany flavor of soybean seed product (Wang, Kuang, et al., 2020), and isoflavone content and resistance to soybean mosaic virus (Zhang et al., 2020), have been improved by CRISPR/Cas9 technology. In addition, Li et al. (2019) tested gRNAs for generating mutants of seed storage protein genes, which will be useful for breeding food-type soybeans.

Of note, the first soybean CRISPR library targeting more than 100 candidate genes has been constructed. A collection of mutant soybean lines were also generated using improved procedures (Bai et al., 2020). Such efforts toward a collection of mutants of all soybean genes by the community will prove to be pivotal for soybean genetics and biotechnology.
4.3 | Cowpea

Cowpea (V. unguiculata (L.) Walp.) is an orphan grain legume suitable for cultivation in dry and warm climates (Cullis & Kunert, 2017). In 2019, 14.4 million tonnes of cowpeas were produced globally, an increase from 9.7 million tonnes in 2009. Cowpeas have a protein content of 25% by dry weight and have a high-lysine content, allowing them to complement cereal crop-based diets.

Cowpea is known to be recalcitrant for transformation, hindering routine practices of CRISPR/Cas9. However, recent advances have demonstrated improved transformation efficiencies of 4.5–37% across nine cowpea genotypes (Che et al., 2021). In this work, Che et al. used a CRISPR/Cas9 construct that expresses Cas9 under the soybean elongation factor (GmEF1A2) promoter and a gRNA under the VuU6 promoter to produce 35 T1 plants, of which 68.8% showed editing activity. The researchers targeted the meiosis gene VuSPO11-1 to develop asexual plants suitable for hybrid development. In another study, to overcome the difficulty of testing CRISPR/Cas9 constructs in stable cowpea transformants, Juranić et al. (2020) developed a transient method of testing CRISPR/Cas9 constructs in 48 h via Agrobacterium infiltration of detached leaflets. Using this method, the researchers were able to test a variety of constructs using the Arabidopsis 50S ribosomal protein (AtRPS5A) promoter (RPSSA) or the parsley ubiquitin4-2 (PcUBi4-2) promoter for Cas9 expression and either the VuU6 or AtU6 promoters for gRNA expression. A construct using the AtRPS5A and AtU6-26 promoters for VuSPO11-1 knockout showed a mutation frequency of 0.05–3.9% in the transient assay and was used to produce 10 T1 plants. Two of these plants displayed 50% gene editing. Only one of these plants survived, but it displayed a sterile phenotype similar to the one produced by Che et al. (2021).

4.4 | Lentils

Lentils (L. culinaris Medik.) are pulse crops consumed worldwide and are good sources of protein, carbohydrates, dietary fiber components, minerals, vitamins, and secondary metabolites that include phenolic compounds (flavan-3-ols, proanthocyanidins, and some flavonols). These secondary metabolites are associated with health benefits for human nutrition (e.g., antioxidant, antitumor, and antiheart disease properties). The seed coat color and quality usually define the market class and the value of lentils (Ganesan & Xu, 2017; Mirali et al., 2017). Lentils are ranked the sixth among the 11 primary legumes harvested as dry seeds. Canada is the world’s largest producer and exporter of lentils (Ji et al., 2019).

Development of improved lentil cultivars well adapted to environment and climate instability with improved meal quality is ongoing process in breeding programs and critical for long-term genetic gain. Genetic diversity plays a crucial role in the development of novel plant varieties, and genetic improvement of lentil requires the introduction of new alleles that extend beyond the existing adapted pool of germplasm. To attain further breakthroughs in enhancing yield and meal quality, and improving the stability of cultivars, new sources of genes and alleles must be identified and generated in lentil genetic resources. A reference genome of lentil (redberry) genome has been made available recently as a prerelease by Dr Kirstin Bett’s group from the University of Saskatchewan (https://knowpulse.usask.ca/lentil-genome), which will provide a foundational resource for trait improvement. Targeted genome editing using CRISPR/Cas9 genome-editing technology can provide a solution to certain constraints for lentil improvement and, combined with traditional breeding techniques, could significantly aid in improving the meal quality and yield of lentils. A number of genetic transformation methods including Agrobacterium-mediated genetic transformation have been attempted (Gulati et al., 2002; Sarker, Biswas, et al., 2003). There are a few reports on successful lentil transformation, but transformation efficiency is less than 1% (Atkins & Smith, 1997; Sarker et al., 2019). In vitro plant regeneration of explants from different lentil tissues, including shoot apices, epicotyls, nodal segments, embryo axes, cotyledonal nodes, and roots, has been attempted for genetic transformation (Akcay et al., 2009; Mahmoudian et al., 2002; Sarker, Mustafa et al., 2003; Warkentin & McHughen, 1993). Among these studies, cotyledon-attached decapitated embryo appeared to provide the best response toward in vitro regeneration following Agrobacterium-mediated genetic transformation (Sarker, Biswas, et al., 2003). As the number of shoots regenerated per explant greatly affect the transformation efficiency and success of CRISPR/Cas9-based gene editing, optimization of the protocol with an appropriate combination of mineral media and hormones is required in the near future. Random mutagenesis approaches have contributed to the understanding of the functions of genes involved in seed size (pea subtilase, SBTI.1) and tendril formation (tendril-less, tI) (D’Eruffth et al., 2012) as well as other traits, such as pod shattering and herbicide tolerance. Gene editing can provide an easier, cheaper, and more precise way of disrupting genes for lentil improvement.

4.5 | Peas

As pea (P. sativum L.) seeds are a rich source of protein with high-lysine content, the research community has had a long interest in the nutritional quality of its seeds. The sequencing of the pea genome (Kreplak et al., 2019) has provided an important resource for unlocking the nutritional and productivity potential of this crop. Pea seeds typically contain 20–25% protein and 50% starch (Gatel & Grosjean, 1990). Pea protein isolates and concentrates are key ingredients in diverse plant-based foods. Despite the excellent nutritional value of pea seeds, the uses of pea products are still not widely accepted, mainly due to the characteristic “off-flavors” often described as green, beany, hay-like, metallic, and astringent (Roland et al., 2017). These undesirable flavors are caused by a mixture of volatile organic compounds (VOCs; Figure S1A, methoxypyrazines, alcohols, ketones, aldehydes, etc.) and nonvolatile saponins (Figure S1B, triterpene glycosides). To improve the flavor quality of pea products, the genes responsible for the “off-flavors” are obvious targets for CRISPR/Cas9 gene editing. However, to date, new agro-traits
developed with CRISPR/Cas9 that remove or reduce the levels of undesirable flavors have not been reported in peas, to the best of our knowledge.

VOCs are a collection of small organic molecules enzymatically synthesized during cultivation, transportation, and storage of pea products. VOC generation in plants is initiated by lipoxygenases (LOX), which catalyze the conversion of polyunsaturated fatty acids (linoleic acid and linolenic acid) to hydroperoxide forms (HPOs) of fatty acids (Porta & Rocha-Sosa, 2002). Depending on the position of hydroperoxy conjugations, 9-HPO and 13-HPO can be formed by 9-LOX or 13-LOX, respectively. These HPOs are cleaved and/or modified by subsequent enzymes, such as hydroperoxide lyases and aldehyde dehydrogenases, resulting in the production of dozens of different VOCs. Hence, targeted mutations of LOX by CRISPR/Cas9 are expected to block the release of VOC-related “off-flavors” in peas. It should be noted that soybean mutants that have lesions in each of the three LOX isogenes (LOX1/2/3) have been reported (Davies & Nielsen, 1986; Hildebrand & Hymowitz, 1982; Kitamura et al., 1983). A null mutant of LOX2 is of particular interest because it had significantly improved flavor quality in human sensory tests, whereas null mutations of LOX1/3 did not (Davies et al., 1987). Intriguingly, the null mutation of LOX2 did not impair key agronomic traits, such as seed yield and disease resistance (Pfeiffer et al., 1992). In peas, a null mutant of LOX2, one of the two seed LOX isogenes, was identified and reported to produce decreased levels of VOCs with no compromised traits in seed weight and field yield (Forster et al., 1999). Considering the reported ecophysiological roles of VOCs (Vancanneyt et al., 2001), it is surprising to observe that the LOX mutants from both soybeans and peas maintain normal agronomic features, but modern agricultural practices may enable soybeans and peas to tolerate reduced levels of VOCs in the field. Although one pea LOX2 mutant is available, introgression of this trait into elite cultivars is a long-term goal. A group A saponin (soyasaponin β) (Reim & Rohn, 2015). These are group B saponins that possess hydroxy groups at C3/C22/C24 in the β-amyrin backbone (Figure S1B). On the other hand, soybean produces group A saponins that have an additional hydroxyl group at C21 (Yano et al., 2017). In pea flour, saponin B and DDMP saponin cause metallic, astringent, and bitter flavors (Heng et al., 2006; Price et al., 1985). Of these two compounds, DDMP saponin appears to be the key contributor to the “off-flavors” in pea flour (Heng et al., 2006). As the biosynthetic genes for these compounds have been reported in legumes (e.g., β-amyrin synthase, C22 hydroxylase, and UDP-glycosyltransferase) (Morita et al., 2000; Sundaramoorthy et al., 2019), CRISPR/Cas9 can be applied to remove or alter the levels of saponin B and DDMP saponin in pea seeds.

As pea products become more widely used, allergic reactions due to pea ingestion have also been more frequently reported in recent years. The causative proteins of the allergic reactions to peas are not fully understood, but one study using the sera from pea allergy patients suggested the pea storage proteins, vicilin and convicillin, as potential major allergens (Sanchez-Monge et al., 2004). Further studies are required to better understand pea allergens, but once we identify the responsible allergens, CRISPR/Cas9 can be used to remove the allergenic proteins.

### 4.6 Faba bean

Faba bean (V. faba L.), also known as broad bean, horse bean, or field bean, is one of the most important cool-season grain legumes. Owing to its nutritional significance of high-protein content and rich resource of fibers, vitamins, essential minerals, and antioxidants (Ray & Georges, 2010), faba bean has gained increasing attention in recent years with global production of 4.92 million tonnes in 2018 (http://faostat.fao.org). Although the global acreage has remained constant at about 2 million ha since 1991, the yield has tended to increase in the last few decades (Merga et al., 2019). Faba bean is a diploid (2n = 2x = 12) partial outcrossing species with a genome size of ~13 Gb, containing more than 85% of repetitive DNA (Carrillo-Perdomo et al., 2020; Sudheesh et al., 2019). A reference genome of faba bean is still lacking; however, significant progress has been made in creating genetic and genomic resources to facilitate molecular breeding. Due to the strong synteny relationships with model legume M. truncataula and other legumes (Braich et al., 2017), transcriptome analyses and comparative genomics approaches have been deployed to discover single-nucleotide polymorphisms (SNPs) for construction of high-density consensus genetic map, as well as to predict the candidate genes controlling various traits of interest.

Conventional cross-pollination remains the primary strategy to introgress favorable alleles from exotic or other adapted germplasm into elite faba bean varieties. In parallel, ethyl methanesulfonate (EMS) mutagenesis has been used to induce novel beneficial variants in breeding programs as demonstrated by the isolation of five mutants with various degrees of nodulation (Duc, 1995) and the development of herbicide tolerant faba bean varieties. Genetic modification of faba bean for trait improvement has been hindered by the lack of a robust and efficient transformation method (O’Sullivan & Angra, 2016). A few studies reported tissue culture and regeneration protocols with low transformation efficiency for faba bean (Table S1).

The absence of an annotated reference genome for the complex faba bean genome poses challenges for application of CRISPR/Cas9 gene editing, particularly with the design of specific gRNA-targeted genes of interest. To date, no CRISPR/Cas9 studies have been reported for this crop. Two major antinutritional factors in faba bean are tannins in the seed coat, and vicine and convicine (V-C) in the cotyledons (Crépon et al., 2010). Numerous efforts have been made to
identify the candidate genes involved in the V–C biosynthetic pathway (Gutiérrez et al., 2017; Khazaei et al., 2019; Ray et al., 2015). Although a number of candidate genes were suggested, the identity of the causal gene(s) still remains unknown. By deep learning of genotyping-by-sequencing (GBS) data of faba bean lines with high and low V–C content, two regulatory SNPs (rSNPs) were identified to be significantly associated with V–C content (Heinrich et al., 2020). In a recent study, mutations in Transparent Testa Globra 1 (Tv(TG1)), encoding a WD40 transcription factor, and Transparent Testa 8 (VjTT8, Vf_Mt1g072320) encoding a bHLH domain transcription factor, were reported to be responsible for zero tannin and white flower color in faba bean (Gutierrez et al., 2020; Gutierrez & Torres, 2019). However, further studies are required to elucidate the regulatory mechanism of these candidate genes and their influence on other biological processes and metabolites. The CRISPR/Cas9 technology holds great promise to fulfill this task.

CRISPR/Cas9 also provides new avenues to enhance the sulfur-containing amino acids (methionine [Met] and cysteine [Cys]) by increasing the amounts of the legumin subunit relative to vicilin. Some approaches that have been demonstrated and promising in other crops can be employed such as suppressing the genes encoding Met- and Cys-poor proteins and expressing Met- and Cys-rich seed storage proteins (Galili & Amir, 2013; Warsame et al., 2018). The technology improvements in genetic transformation and CRISPR/Cas9 system are expected to advance our understanding of gene function and to speed up the genetic improvement of traits of importance in faba bean.

### 4.7 Mung bean

Mung bean (Vigna radiata (L.) Wilczek), is a warm-season, self-pollinated, short-duration (~60 days) grain legume (Kang et al., 2014; Nair et al., 2013). Mung bean seeds are rich in carbohydrates, high-quality proteins (22–24%), fiber, antioxidants, and iron (40–70 ppm), making them an ultimate choice for balanced diets (Amaral Ana et al., 2017; Itoh et al., 2006; Vijayalakshmi et al., 2003). Modern cultivated mung bean has resulted from multiple rounds of domestication and selection and is currently distributed throughout southern and eastern Asia, Africa, and Austronesia (Lambrides et al., 2000; Vishnu-Mitre, 1974). Mung bean productivity is very low (~0.5 t ha⁻¹), mainly because of its nonsynchronous maturity, lack of improved varieties, and susceptibility to biotic and abiotic stresses (Chauhan et al., 2018; Pratap et al., 2013). In the past, crop improvement through conventional and mutation breeding approaches was successfully employed; however, molecular breeding techniques can provide fast, efficient, and cost-effective approaches to crop improvement.

Mung bean is a diploid crop (2n = 2x = 22) with a relatively small (579 Mb) genome size (Kang et al., 2014; Parida et al., 1990). Mung bean was one of the first legumes to have a genetic linkage map in the early 1990s (Fatokun et al., 1992; Fatokun et al., 1993). In the past, molecular techniques involving random amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP), SNP, and SSR markers have been applied toward improving mung bean, with a focus on yield, nutritional improvement, and disease resistance (Chankaew et al., 2011; Fatokun et al., 1993; War et al., 2017). Recently, both cultivated (V. radiata) and a wild relative (V. sublobata) of mung bean have been sequenced, along with the transcriptome sequences of 22 Vigna accessions of 18 species (Kang et al., 2014).

With the availability of whole-genome sequence, and a huge core collection of 1481 mung bean entries that have been evaluated for various important agronomic traits (Schafleitner et al., 2015), there is great scope to use CRISPR/Cas9 gene editing in mung bean breeding programs. Recently, CRISPR/Cas9-mediated gene editing was successfully used in cowpea (V. unguiculata) for the disruption of symbiotic nitrogen fixation by targeting a symbiosis receptor-like kinase gene (Ji et al., 2019). The success of CRISPR/Cas9 in a Vigna system suggests that gene editing can be applied to other species including mung bean. Disease resistance and quality traits would be some of the early targets for gene editing in mung bean. Developing mung bean varieties that can withstand changing weather patterns would help in further expansion of mung bean cultivation worldwide.

### 4.8 Chickpea

Chickpea (C. arietinum L.) is considered as one of the most important food legumes because of its nutritional value and symbiotic nitrogen fixation ability. Chickpea is the second most important food legume after common bean. It is grown in more than 50 countries with approximately 14.24 million tonnes of chickpeas produced worldwide in 2019 (http://faostat.fao.org). Although the draft sequences of the desi-type chickpea genome were published in 2013 (Jain et al., 2013; Varshney et al., 2013), the efforts of creating new variations and developing new chickpea varieties through genomics and gene-editing approaches are very limited because of the lack of efficient and reproducible plant regeneration systems. So far, chickpea has mainly been transformed to regenerate transgenic plants expressing a chimeric gene-encoding insecticidal crystal protein Cry1Aabc of Bacillus thuringiensis for pod borer (Heliothis armigera) resistance (Das et al., 2017). A robust transformation system to obtain stable transgenic lines was recently established by Das Bhowmik et al. (2019), which was successfully used generate transgenic lines expressing GUS (uidA), stress tolerance (AtBAG4 and TIBAG), and Fe biofortification (OsNAS2 and CaNAS2) genes. The availability of reference genome and transcriptome sequences as well as transformation methods provides unique opportunities for application of gene-editing technologies to characterize the functions of genes and improve agricultural traits in chickpea. The International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) is leading the efforts of developing gene-edited chickpeas to increase yield, seed size, and quality. First example of CRISPR/Cas9-mediated editing of chickpea protoplasts was recently published, in which Badhan et al. (2021) reported creation of knockouts of 4-coumarate ligase (4CL) and Reveille 7 (RVE7) genes, both associated with drought tolerance in chickpea. These recent studies have demonstrated the feasibility of gene editing in chickpea laying a technical foundation for future trait discovery and improvement.
Developing herbicide resistance and improving carotenoids content would be early targets for future gene-editing efforts in chickpea. Chickpeas are sensitive to many postemergent herbicides registered for the control of broadleaf weeds in lentils or peas. The recent discovery of the Ala251Thr substitution in psbA chloroplast gene being responsible for resistance to a photosystem II inhibitor herbicide metribuzin (McMurray et al., 2019) has opened this a new avenue for creating herbicide resistance in chickpea and other legumes using gene editing. In another study, the identification and expression analysis of candidate genes involved in carotenoid biosynthesis in chickpea (Rezaei et al., 2016) has revealed several candidate genes as early knockout targets for improving carotenoid content in chickpea.

5 | REGULATORY PERSPECTIVE ON GENOME EDITING IN LEGUME CROPS

Gene editing is viewed as a promising breeding tool to develop new crop varieties by public and private breeders alike (Gleim et al., 2020). The regulation of gene editing initially created uncertainty in many countries, which has been clarified over the past 3 years. In 2018, the global regulation of gene editing splits along the same boundaries as the regulation of genetically modified (GM) crops. Leading GM crop-producing nations, such as Argentina, Australia, Brazil, and the United States, determined that if no foreign genes were present in the final product, the product would be regulated as a conventionally bred crop variety and not as a genetically modified organism (GMO), although the European Union (EU) ruled that all crop varieties developed by gene editing would have to be regulated as equivalent to the regulations that exist for GMO products.

In March 2018, the United States Secretary of Agriculture announced that new breeding technologies, such as genome editing, that were not a plant pest or developed using plant pests, would not require additional regulatory oversight and that crop varieties developed through the use of these technologies would be viewed as conventionally bred plants (https://www.usda.gov/media/press-releases/2018/03/28/secretary-perdue-issues-usda-statement-plant-breeding-innovation). The announcement indicated that traits developed through targeted gene editing could also be developed through traditional mutagenic breeding techniques, which are not subject to additional regulatory oversight. Subsequently, other nations announced regulatory policies that aligned with that of the United States. GM crop-producing nations including Argentina, Australia, and Brazil all announced that if no foreign genes or DNA was present in the final variety, the variety would be treated as conventional and would not be subject to additional regulatory oversight (Smyth, 2019).

In July 2018, the Court of Justice of the EU ruled that gene-editing technologies would be required to be regulated as equivalent to GMO crops within the EU (https://curia.europa.eu/jcms/upload/docs/application/pdf/2018-07/cp180111en.pdf). The impact of this ruling on gene-editing research investment in the EU was immediate. Bayer and BASF announced that they would relocate all of their gene-editing research to other nations that were more receptive of innovation. By the end of 2018, smaller plant breeding firms were announcing identical investment plans to those of the large multinationals and would no longer be investing in gene-editing research within the EU (https://resource.wur.nl/en/show/Innovation-in-a-bind-European-ruling-on-CRISPR-Cas-has-major-consequences.html).

Canada regulates the novelty of new plant varieties, regardless of the breeding technology used to develop varieties. A 2018 survey of public and private plant breeders in Canada found that slightly more public breeders were using gene editing as compared with private breeders, 33% to 31% (Gleim et al., 2020). The survey additionally found that 77% of respondents believed the requirements of PNT regulations should be updated on the basis of the scientific advances of the past 25 years (Smyth et al., 2020). Again, more public breeders were supportive of revising PNT regulations as they pertain to the application of gene-editing technologies than private breeders, 46% to 31%.

6 | SUMMARY

The recent advances in CRISPR/Cas9 technology have broadened the options to precisely and efficiently modify genes through the addition or deletion of genetic material. In this article, we reviewed a wide range of tools and resources available for the design and delivery of gRNAs, as well as detection of genetic modifications. CRISPR/Cas9 technology provides novel avenues for functional genomics and improvement of various traits in grain legume crops. However, the successful application of genome editing for legume improvement will depend on the availability of efficient protocols for plant transformation and regeneration of whole plants, along with a conducive regulatory environment and evidence of public acceptance of gene-edited crops.

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CONFLICT OF INTERESTS

The authors declare that they have no competing interests.

AUTHOR CONTRIBUTIONS

P. B. and S. K. conceptualized the study. All authors contributed to the writing and critical review of the manuscript.

DATA AVAILABILITY STATEMENT

All data generated or analyzed during the study are included in the published article.

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