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Review

Genetic Transformation of Common Beans (*Phaseolus vulgaris*): Achievements and Challenges

Matheus da Costa Moura [1,2], Patricia Valle Pinheiro [2,*], Rosana Pereira Vianello [2], Natália Lima de Sousa [3], Josias Correa de Faria [2] and Francisco José Lima Aragão [3,*]

[1] Department of Molecular Biology, University of Brasilia, Brasilia 70910-900, Brazil; matheus.d.c.moura@gmail.com
[2] Embrapa Arroz e Feijão, Santo Antônio de Goiás 75375-000, Brazil; rosana.vianello@embrapa.br (R.P.V.); josias.faria@embrapa.br (J.C.d.F.)
[3] Embrapa Recursos Genéticos e Biotecnologia, Brasilia 70770-917, Brazil; natlimasousa@gmail.com
* Correspondence: patricia.pinheiro@embrapa.br (P.V.P.); francisco.aragao@embrapa.br (F.J.L.A.)

Abstract: Genetic transformation is a valuable tool for the development of plant varieties with desirable traits that are present in the species germplasm with low genetic variability, i.e., resistance to pests and diseases and nutritional improvements. Although transgenic and edited crops have been successfully obtained for many plant species, it remains difficult for common beans (*Phaseolus vulgaris*), due to their recalcitrance to in vitro regeneration. This review discusses various methods employed, such as *Agrobacterium*-mediated transformation, biolistic (particle bombardment), and hairy root systems, noting their respective efficiencies and limitations. While there has been progress, including the development of the first transgenic common bean cultivar approved for commercialization (Embrapa 5.1), the article emphasizes the need for improved protocols and techniques for more efficient genetic transformation. It also touches upon the potential of gene editing technologies like CRISPR/Cas9 in overcoming existing challenges and facilitating the development of resilient bean varieties.

Keywords: plant breeding; genetically modified organism; transgenic; in vitro regeneration; Leguminosae; *Agrobacterium tumefaciens*; gene editing; biolistic; hairy root; recalcitrant species

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1. Introduction

Common bean (*Phaseolus vulgaris* L.) is one of the most important protein sources for the human diet in developing countries, feeding over 300 million people daily worldwide [1–3]. The development of agricultural practices and techniques has contributed to a significant increase in common bean yield and production. Among these, plant breeding is one of the most successful tools for the development of not only more productive cultivars, but also cultivars with several other desirable traits, such as the preferred market class, reduced cooking time, increased levels of minerals (zinc and iron), short cycle, and upright architecture [4,5]. Nevertheless, for some of the important farmers' demands, the desirable traits are not available in the common bean germplasm for conventional genetic breeding. In part, this is due to the narrowing of the genetic diversity, which is a common phenomenon in crops that have been subjected to an intensive process of genetic breeding and recurrent selection. However, for some traits, we can assume that the genetic variability has not been identified, such as resistance to most pests and diseases. In this case, genetic engineering might help, by introducing foreign genes (transgenes) from different organisms or even synthetic genes, and, more recently, by editing the target genes in the genome, a novel and promising breeding technique.

In recent decades, the development of transgenic plants has been successfully achieved for several plant species, such as soybean, maize, cotton, sugar beet, potato, and even

common beans. Despite advances in gene delivery and plant tissue culture, genetic transformation of common beans remains difficult. Several methods for transferring genetic material into plant cells have been developed, such as *Agrobacterium*-mediated transformation and particle bombardment. However, effective gene transfer technologies require efficient plant regeneration systems through tissue culture, which come with a range of challenges, limiting the effectiveness and repeatability of biotechnology methods [6–8].

Even though genetic transformation of common beans is a challenging, laborious, and inefficient process (i.e., with a low transformation rate), one transgenic line has been developed and commercially released (Figure 1) [9,10] and another one is in the process of breeding with elite cultivars [11]. In this review, we will discuss the available techniques for plant genetic transformation, the advances obtained in the transformation of common beans, challenges, and future perspectives.

Figure 1. The first transgenic line of common bean (*Phaseolus vulgaris*) developed in the world and released for commercial use in Brazil. (**a**) A commercial field of transgenic bean resistant to the bean golden mosaic virus (cv. BRS FC401 RMD) in the State of Goias, Brazil. (**b**) Transgenic labeled packages of common beans for sale in a Brazilian groceries store.

2. *Agrobacterium* Method

Early in the 1980s, plant genetic engineering was still in its early days, or maybe it was only vague ideas. Local newspapers with headlines such as "scientists playing God" were common. However, it was realized that delivering genes into plant cells or organs would greatly accelerate the knowledge of gene function and, eventually, bring about themes such as genetic evolution, and even the opportunity to introduce foreign genes that could be useful to cultivated crops. At this point, *Agrobacterium tumefaciens* was the only known way to introduce foreign nucleic acid into a plant species. Isolated cells such as protoplasts could be transformed, but were difficult to regenerate. The drawback of these techniques was their limitation to the host range of the agrobacteria or plant species recalcitrant to cell or tissue culture.

Nonetheless, genetic transformation mediated by *A. tumefaciens* has revolutionized plant biotechnology, enabling horizontal gene transfer in plants via the Ti plasmid. This process involves modifying native plasmids, removing tumor-inducing genes, and co-cultivating the plant explant with the bacteria, using wounds to promote T-DNA insertion. Efficiency depends on several factors such as explant type, wounding method, and the use of infection elicitor compounds [12]. This method generally results in the insertion of few transgene copies, and it is crucial that the host plant be susceptible to the bacteria [13,14]. In addition, it is a low-cost and simple method that does not require any specific complex equipment [15].

Transient transformation of common beans by *Agrobacterium* has been reported in several studies [16–21]. For example, *Agrobacterium*-assisted genetic transformation of

P. vulgaris cv. CIAP7247F with selective and marker genes (*bar*, *nptII*, and *uidA*) has been reported, producing chimeric regenerants through direct and indirect organogenesis using epicotyl as explants [22]. However, the established protocol did not yield stable genetic transformation [22]. An efficiency of 45% was achieved in transient genetic transformation of common bean explants using LBA4404-ElectroMAX® *A. tumefaciens* with the *gusA* gene, indicated by β-glucuronidase activity [23]. Bean regenerants were selected with kanamycin over three to five weeks, but the selection was not stringent, leading to chimeric transformants and some non-transgenic material. Stable transformation obtained for the reporter and selective gene was ca. 0.5%, which is slightly higher than that reported for this species using the biolistic process [24–26].

However, in this review, we focus on the studies that obtained common bean plants with stable genetic transformation, that is, when the exogenous DNA is integrated into the host genome and transferred to the progeny (Table 1). Even so, although there have been several reports of common bean plants with stable transformation by *Agrobacterium*, this method has not generated any commercial lines yet, to our knowledge. Indeed, *Agrobacterium*-mediated genetic transformation of common beans has been reported as a process with low efficiency and effectiveness [8,21,27,28]. The primary obstacle to generating transgenic common bean plants using this method is the lack of an established and efficient de novo regeneration protocol [8]. A de novo regeneration system has been developed, but it did not prove to be effective in generating genetically modified plants [29]. The main barrier is the recalcitrance of beans to both in vitro regeneration and genetic transformation of specific cell types [30]. This recalcitrance is related to plant adaptation and development in vitro, as well as difficulties in interacting with *Agrobacterium* and integrating exogenous DNA [31]. Plant genotype, type of explant, susceptibility to infection, in vitro morphogenetic responses, cultivation conditions, and bacterial strains also impact the outcome [6,22,23,32–39].

Susceptibility to infection of plant tissues varies significantly among genotypes, which directly affects the efficiency of transgenic plants' regeneration. The natural resistance of common beans to *Agrobacterium*, combined with genetic variability and different in vitro cultivation conditions, compromises both the integration and expression of the transgenes, exacerbating issues with reproducibility [8]. The species' recalcitrance and low efficiency in regenerating plants from callus tissue are identified as the major challenges, with suggestions that the use of specific explants, such as those derived from mature seeds germinated in vitro, could improve the process [31]. Another strategy could be the use of amenable common bean genotypes before cross breeding the transgene to other commercial varieties, as has been demonstrated in the general practice of genetic transformation of the Coker-312 cotton variety before crossing with other varieties [40]. However, an *Agrobacterium*-susceptible *P. vulgaris* genotype that would allow a high frequency of transformation of regenerable tissues has been difficult to find. In addition, the lack of reproducibility and the difficulties in applying effective protocols across different laboratories remain significant obstacles to the genetic transformation of common bean using *Agrobacterium* [8,21,27,28]. The *Agrobacterium*-mediated genetic transformation protocol generally presents higher transformation rates than the biolistics method. We calculate the transformation rate as the percentage of germ line transformed plants in relation to the total number of explants used in the experiments. However, the formula used to calculate the transformation rate varies between studies. For example, in some cases, higher transformation rates are calculated as the percentage of transformants obtained in relation to the number of explants selected on the selective medium, giving the false impression that the process is more efficient. Over the years, there have been many reports of improvements to the protocol used for *Agrobacterium*-mediated transformation of common bean to increase the efficiency of the process, with reported transformation rates varying from 0.5% to 28.6% [6,22,23,33–36,38,39]. For example, combining sonication and vacuum infiltration of germinated seeds after inoculation with *Agrobacterium* resulted in a transformation rate of 12% [35]. An average transformation rate of 15.2% was obtained when the MS medium

was supplemented with 2,4-D (4.52 µM), compared to 6.5% when the medium was added to BAP (44.4 µM) and TDZ (2.27 µM) [6]. In another study, cotyledonary nodes were used, as they are considered effective in the formation of multiple shoots. They were cultured on selective MS medium supplemented with BA (1 mg/L), NAA (0.1 mg/L), and kanamycin (50 and 100 mg/L) or phosphinothricin (1 and mg/L), with co-cultivation of explants for 3 days, resulting in a transformation rate ranging from 9% to 20% [33]. Additionally, a higher transformation rate was obtained using kanamycin (28.6%) as the selective agent, compared to glufosinate ammonium (10.2%) [34]. Different periods of pre-cultivation (2, 8, 10, 12, and 17 days) of explants were used in another study, but this did not result in a significantly higher transformation rate [23]. Higher transformation rates were obtained for pre-culture periods equal to or longer than 8 days, while a low transformation rate was observed for the explants with a short pre-culture period (2 days). Survival was higher for the explants that were pre-cultured for 12 days and co-cultured for 3 days.

More recently, the common bean cultivar Olathe Pinto has been successfully transformed using *A. tumefaciens* strain EHA105, by optimizing pre-culture and co-cultivation conditions [38]. Unlike organogenesis-based methods in common bean transformation, where plant cells differentiate directly into organs from cultured tissue, these authors developed a somatic embryogenesis protocol that induced cultured cells to form embryo-like structures, which then grew into complete plants, achieving a transformation rate of 1.5–2.5%. In this study, embryo axes were pre-cultured for 12 weeks to develop into competent cells for DNA transfer via *Agrobacterium*. The authors also mentioned that an *A. tumefaciens* culture at an optical density of 0.1 and 4 days of co-cultivation increased the survival rate of the inoculated explants, from 23 to 45%. Subsequently, another research group focused on developing *Agrobacterium*-based gene transformation techniques to develop insect-resistant bean varieties [39]. They treated embryonic axes and plumule explants from common bean cultivars Akman 98 (low protein) and Karacaşehir 90 (high protein), with *A. tumefaciens* carrying a synthetic Bt *cry1Ab* gene, finding that transformation success varied by genotype and explant type. No details are given as to the embryonic axis preparation and other aspects of the experiment. Although plumule explants could not survive kanamycin selection, two transgenic plants were successfully generated from the embryonic axes of Akman 98. These results were validated through GUS analysis, PCR, RT-PCR, bioassays, and ELISA, confirming that the plants were protected against legume seed insects (*Bruchus* spp.) [39].

Despite the great variation among the *Agrobacterium*-mediated transformation protocols used by different research groups, some details are common. Notably, the most frequently utilized *A. tumefaciens* strains for gene transfer were LBA4404 [6,23,35,36,39], EHA105 [6,22,38], and GV3101 [6,34]. In an attempt to develop an *A. tumefaciens*-mediated transformation protocol for common beans, various types of isolated cells and tissues have been analyzed in recent decades, including shoot apexes [41], cotyledon nodes with axillary buds [42,43], cotyledon nodes without axillary buds [16,22], embryonic axes [6,23,38,39,44,45], embryonic calluses [46], plumules [35,39], leaves [36], stems [6,36], and hypocotyl explants [34]. However, among these, embryonic axes have consistently yielded the most favorable results, particularly in protocols that successfully generated transgenic lines.

Although there are many reports of successful *Agrobacterium*-mediated transformation of common beans, a reproducible and widely adopted protocol for routine transformation has yet to be established. Additionally, most published protocols do not provide evidence of transgene inheritance in the progeny, highlighting a significant gap in the development of a reliable transformation method for this plant species.

Table 1. Common bean lines with stable * genetic transformation obtained to date (2024). * The transgene was integrated into the host genome.

Transformation Method	Genotype/Variety	Transformation Rate	Country	Reference	Genetic Engineered Trait/Expressed Foreign Genes	Regulated Traits of Exogenous Genes
Biolistic	not reported	0.9%	USA	[47]	*gus*	Production of β-glucuronidase
					nptII	Resistance to kanamycin
Biolistic and electric-discharge	Navy bean	0.03%	USA	[48]	*gus*	Production of β-glucuronidase
					bar	Tolerance to glufosinate ammonium
					BGYMV *cp*	Synthesis of BGMV coat protein
Biolistic	Olathe	0.9%	Brazil	[24]	*gus-neo* fusion	Resistance to aminoglycoside antibiotics
					gus	Production of β-glucuronidase
					2S albumin	Production of 2S albumin protein
					ACI, *AC2*, *AC3*, *BCI* genes isolate BGMV	Production of the antisense sequences of genes from BGMV
Biolistic	Olathe	16 positives	Brazil	[49]	*ACI*, *TrAP*, *REn*, and *BCI* genes isolate BGMV-BR	Production of the antisense sequences of genes from BGMV
					gus	Production of β-glucuronidase
Biolistic	Olathe and Carioca	0.5–0.6%	Brazil	[50]	*bar*	Tolerance to glufosinate ammonium
					neo	Resistance to bacterial kanamycin
Agrobacterium	Green Light	2–12%	Japan	[35]	*gus*	Production of β-glucuronidase
					ME-leaN4	Production of lea protein
Biolistic	Olathe Pinto	0.66%	Brazil	[9]	*ahas*	Tolerance to the herbicide imazapyr
					BGMV-BR *AC1*	dsRNA of the gene *AC1*
Agrobacterium	Krasnoperaya, Nezhnost, and Chudesnaya	2.8 to 17.4%	Ukraine	[36]	*ahas*	Tolerance to the herbicide imazapyr
					nptII	Resistance to kanamycin
Biolistic	Olathe Pinto, Pontal, and Jalo 308	2.7%	Brazil	[26]	*ahas*	Tolerance to the herbicide imazapyr
					bla	Resistence to ampicilin
Agrobacterium	Merlot	4.1–18.2%	USA	[6]	*nptII*	Resistance to kanamycin
					*gus*A	Production of β-glucuronidase

Table 1. *Cont.*

Transformation Method	Genotype/Variety	Transformation Rate	Country	Reference	Genetic Engineered Trait/Expressed Foreign Genes	Regulated Traits of Exogenous Genes
Biolistic	Sedona	0.1–8.4%	USA	[51]	*gus*	Production of β-glucuronidase
					HVA1	Production of protein HVA1
Agrobacterium	Fönix and Maxidor	9–20%	Egypt	[33]	*nptII*	Resistance to kanamycin
					gus	Production of β-glucuronidase
					bar	Tolerance to glufosinate ammonium
Agrobacterium	Flor de Mayo Anita and Pinto Saltillo	3.9–28.6%	Mexico	[34]	*Pdf1.2*	Production of protein defensine
					avp1	Production of proton pump pyrophosphatase
					nptII	Resistance to kanamycin
					bar	Tolerance to glufosinate ammonium
Agrobacterium	CIAP7247F	2.8%	Cuba	[22]	*gusA*	Production of β-glucuronidase
					bar	Tolerance to glufosinate ammonium
Agrobacterium	Brunca	0.5%	Costa Rica	[23]	*nptII*	Resistance to kanamycin
					TPS1	Production of enzyme trehalose-6-phosphate synthaseresistanc
					gus	Production of β-glucuronidase
Agrobacterium	Olathe Pinto	0.5–2.5%	USA	[38]	*bar*	Tolerance to glufosinate ammonium
Agrobacterium	Akman 98 and Karacaşehir 90	2%	Turkey	[39]	*cry1Ab*	Production of insecticidal crystal protein
					nptII	Resistance to kanamycin
					gus	Production of β-glucuronidase
Biolistic	Olathe Pinto	0.1%	Brazil	[11]	*ahas*	Tolerance to the herbicide imazapyr
					Bemisia tabaci vATPase	dsRNA of the gene *v-ATPase*

3. Hairy Root Transformation System in Common Beans

Another tool used for DNA transfer is the hairy root system. Mediated by *Rhizobium rhizogenes*, this tool has been widely employed as a model system for studying gene function and secondary metabolite biosynthesis in various plant species. *R. rhizogenes* is a soil-borne bacterium capable of inducing the formation of hairy roots on a wide range of plant species. The bacterium transfers its T-DNA (transfer DNA), which contains genes that promote the uncontrolled initiation and growth of roots, into plant cells. Hairy roots are characterized by their rapid growth, adventitious nature, and ability to regenerate into whole plants in some cases or in particular plant species [52].

Among the numerous advantages offered by the hairy root transformation system are the rapid growth, which allows for quick assessment of transgene expression and phenotypic effects; constitutive transgene expression, providing a consistent source of the gene product; and the potential to regenerate whole plants from transformed roots if the regeneration protocol is effective for the culture in question. Additionally, hairy root transformation can reduce the risk of chimerism, a condition in which different cells in the plant may have different genetic compositions, compared to other transformation methods.

Recent advancements in transgenic hairy root generation have enhanced their utility for molecular studies in common bean. For example, Brasileiro and collaborators [32] demonstrated for the first time the susceptibility of *P. vulgaris* to *A. rhyzogenes* and obtained transformed roots. A refined protocol, incorporating key steps for successful transformation and subsequent analyses, has been developed, yielding higher transformation rates, promoting activity analysis, and monitoring rhizobia infection [53,54]. These improvements significantly enhance the efficiency and reliability of using transgenic hairy roots as a tool for investigating gene function and plant–microbe interactions in common bean.

Hairy root transformation has been a valuable tool for advancing our understanding of *P. vulgaris* biology and for developing improved cultivars. In functional genomics, hairy root cultures have been used to investigate the roles of genes involved in root development, nutrient uptake, and plant–microbe interactions [55–57]. By manipulating gene expression in these cultures, hairy root transformation offers opportunities for crop improvement by introducing genes that confer resistance to diseases or enhance tolerance to abiotic stresses, which has important implications for food security and sustainable agriculture.

Despite its advantages, hairy root transformation presents several challenges and limitations that must be addressed for its effective application in *P. vulgaris*. One primary challenge is genotype specificity, as different genotypes may vary in their susceptibility to *R. rhizogenes* infection and the subsequent development of hairy roots. Additionally, transgene expression in hairy roots is typically constitutive, meaning that the transgene is expressed in all cells of the hairy root mass. This lack of tissue specificity can be a limitation in applications where targeted expression of the transgene is desired. Addressing these challenges and limitations will be crucial for further advancing the application of hairy root transformation to *P. vulgaris* and realizing its full potential for plant biotechnology [56–60].

4. Biolistic Method

Although the *Agrobacterium*-mediated genetic transformation method was the first one to be developed and is considered efficient for other plant species, the first common bean plant with stable genetic modification was obtained by another transformation method, the particle bombardment or biolistics method (Table 1). First described by Sanford and collaborators [61], genetic transformation by the biolistic method consists of a mechanism that accelerates gold or tungsten microparticles driven by gunpowder, electrical discharge, or high pressure helium. It was called a “gene gun”. These particles are coated with the plasmid DNA, which is delivered into the cells in a non-destructive manner. The main advantages of this transformation method are as follows: (1) it does not rely on biological vectors, so does not depend on the host’s susceptibility to the vector pathogen, as in the *Agrobacterium* system; (2) it does not require complex tissue culture systems, reducing it to the minimal in vitro cultivation. Altogether, these characteristics facilitate

the transformation of recalcitrant species and allow efficient insertion of multiple genes into various tissues [62,63]. The use of a "gene gun" makes the process rapid, though costly and prone to the insertion of multiple copies of transgenes [24,26,64,65]. The method has been successfully applied to delivering substances to many plant species, such as onion, tobacco, rice, and maize, and, according to the authors, the biolistic process tends to be universal regarding species and type of tissue. Indeed, not only was the biolistic method used to successfully obtain the first genetically modified (GM) and stable common bean plant (Russell et al. 1993) [48], but also, later, the first commercially available event (Figure 1) [10,66,67].

However, the biolistic method has some drawbacks. Transformation frequencies via particle acceleration are typically low, ranging from 0.02% to 0.9% [9,11,24,48,68]. Additionally, the biolistic method often results in gene chimeras due to fragmented or multiple gene copies, leading to gene silencing and instability [69], which requires extensive evaluation. Furthermore, transgenes may interact with each other, causing issues such as co-suppression, epistasis, and unstable gene expression, which can decline or disappear during plant development [70]. Despite the low transformation frequencies and unstable genetic integration, the biolistic system remains the method of choice for routine transformation of common beans [11,26,50,71].

Common bean transformation by the biolistic method was first achieved by the group of J.C. Sanford [47]. Later, Russel and collaborators reported a successful transformation of common beans using particle bombardment mediated by electric discharge, with a lower transformation rate of 0.03% [48]. Meristems from seeds of five common bean cultivars were transformed using gold particles coated with plasmids containing the *gus*, *bar*, and bean golden mosaic virus (BGMV) coat protein (*cp*) genes. The meristems were exposed by removing the seed coat, cotyledons, and primary leaves. After particle bombardment, the meristems were cultured on different media, under controlled conditions, for the development of shoots and regeneration of plants. R0 plants were acclimatized in soil and grew in a greenhouse to produce seeds. The inserted genes were transferred to the subsequent generations at an approximate rate of 75%, producing progeny with stable transgene expression.

Later, in 1996, there were two reports of genetic transformation of common beans using the biolistic method. In one of them, the authors obtained 6 transformed plants from 319 bombarded embryos (9%) [71]. The other report was the first of a series of studies that led to the development of the first commercially available transgenic common bean cultivar [9,10,24,26,49,50]. Our group successfully introduced a plasmid containing the fusion of *nptII*::*gus*, namely pBI426 [24]. Optimal parameters of helium pressure at 1200 PSI, a particle flight distance of 6 mm, and M10 tungsten particles (mean diameter of 1.07 µm) were established. Transgenic plants were then achieved with a transformation rate of 0.9%. Using the cultivar Olathe pinto, which presents an exposed apical meristem, an important trait for genetic transformation by particle bombardment, the objective of this first study was to evaluate the frequency of co-transformation and inheritance of linked and unlinked genes on transgenic plants [24]. Different plasmids containing the marker genes *neo* and *gus* and the antisense sequences of the *AC1*, *AC2*, *AC3*, and *BC1* genes from BGMV were evaluated [24]. The authors reported a transformation rate of 0.9%, obtaining 27 transgenic plants from 3079 embryos.

Then, in 1998, the authors transformed common bean plants to introduce the *Rep-TrAP-REn* and *BC1* genes from the BGMV, aiming to obtain plants resistant to this virus [49]. Four transgenic lines were generated, two of which exhibited significantly attenuated viral symptoms and delayed development after inoculation with viruliferous whiteflies. In contrast, non-transformed plants or those transformed only with the *gus-neo* gene developed typical BGMV symptoms 10 to 15 days after inoculation, demonstrating the potential of antisense RNA as a strategy to confer BGMV resistance in common beans. Next, the authors successfully introduced a transgene coding for a methionine-rich storage albumin from the Brazilian nut into the common bean genome, via the biolistic method.

Among the five transgenic lines obtained, two showed a significant increase (14–23%) in seed methionine levels [72].

In another study, a common bean line tolerant to the herbicide glufosinate ammonium was developed using the biolistic process, introducing the *bar* gene from *Streptomyces hygroscopicus* [50]. The cultivars Olathe and Carioca were transformed via particle bombardment, resulting in a transformation rate of 0.6% for Olathe and 0.5% for Carioca. Two transgenic events, PHV119 and PHV122, were obtained, with high tolerance to the herbicide in both greenhouse and field conditions, as well as growth performance comparable to non-transgenic control plants. The segregation of the transgenes followed a Mendelian pattern in subsequent generations, confirming the stable inheritance of the inserted genes.

In another attempt to introduce resistance to BGMV, Faria and collaborators transformed the cv. Olathe pinto using a construct with the BGMV *rep* gene containing a substitution of an amino acid at position 262 and the *bar* gene [25]. Of 17 T0 plants, only one passed the transgene to the progeny, and expressed very high resistance to glufosinate ammonium and some resistance to BGMV. Due to its efficient resistance to glufosinate ammonium, it was further used for different purposes, such as studies to determine gene flow frequencies in *P. vulgaris* [73].

Then, in 2007, the authors published a study in which a common bean line with a high resistance to BGMV (93% of the plants free of symptoms) was obtained by the biolistic technique, using the RNA interference (RNAi) approach to silence a region of the *AC1* viral gene [9]. A total of 2706 embryonic axes were bombarded, obtaining 18 transgenic common bean lines, which means a transformation efficiency of 0.66% (Table 1, Figure 2). This work also reported an increase in plant transformation efficiency with the use of the herbicide imazapyr as a selective agent, associated with the *Arabidopsis thaliana ahas* gene [9]. The transgenic event that was generated was named Embrapa 5.1 and was subsequently evaluated in field trials, with successful plant protection against the virus and, at the same time, no difference in agronomic traits [10]. The biosafety of this event was approved by the Brazilian Biosafety Committee in 2011 (Figure 2). The next step was the development of the first transgenic common bean cultivar, BRS FC401 RMD [66,74,75], which became available for seed commercialization later.

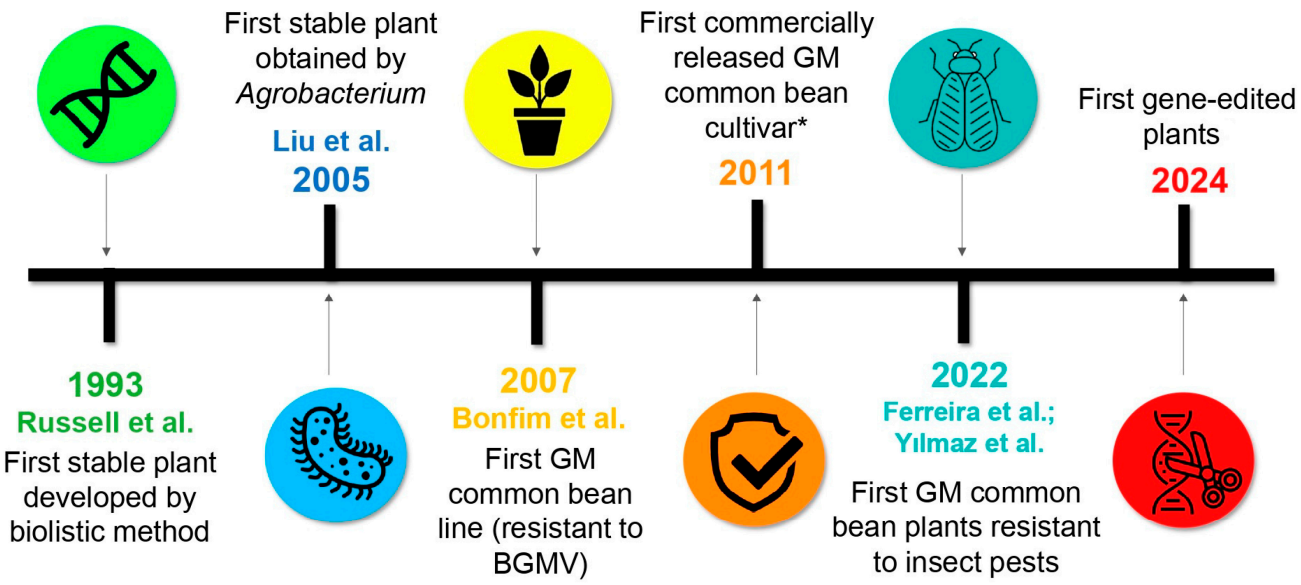

Figure 2. Milestones of the development of genetically modified common bean (*Phaseolus vulgaris*) lines around the world [9,11,35,39,48]. * Genetically modified event Embrapa 5.1 [67].

In 2008, the same research group published an efficient transformation protocol for common bean using the biolistic technique (transformation rate of 2.7% in the cultivars Olathe Pinto and Pontal), which has been used to this day for the genetic transformation of

common bean, soybean, and cotton in their lab [26]. The protocol involved preparing the apical meristems of the embryonic axes for bombardment, using the mutant *ahas* gene as a selective marker, and inducing multiple shoots. This approach enabled the recovery of fertile transgenic plants, offering an effective methodology for the production of transgenic common bean cultivars.

Another research group has reported the development of a transgenic common bean line using the biolistic method to introduce the marker genes *gus* and *bar*, along with the *HVA1* gene responsible for conferring drought tolerance [51]. The cultivars Condor, Matterhorn, Sedona, Olathe, and Montcalm were used in the experiment, with transformation rates varying among cultivars, achieving a maximum efficiency of 8.4% for the *gus* marker. The transgenic plants expressed resistance to the glufosinate ammonium herbicide and better drought tolerance compared to non-transgenic plants, indicating that the transformation had been successful. Later, transgenic beans biofortified with folates, transformed by the biolistic process, were reported [76].

More recently, a common bean transgenic line with tolerance to the whitefly *Bemisia tabaci* has been developed using the biolistic protocol described by Rech et al. [26]. A fragment of the *vATPAse* gene from the insect was introduced into the common bean genome for silencing via RNAi [11]. Of a total of 8764 explants subjected to particle bombardment, only nine T0 plants tested positive for the *vATPase* transgene, resulting in a low transformation rate of 0.1%. Insect bioassays showed a mortality rate of 59% in the T3 generation of the transgenic plants, which was homozygous for the transgene. This transgenic event is currently in use in crosses to transfer the transgene to commercial cultivars.

5. In Vitro Regeneration: Problems and Proposed Solutions

In vitro plant regeneration is important in the genetic improvement of plant species, leading to advances in areas such as genetic transformation, in vitro mutagenesis, and micropropagation [77]. Despite these advances, the application of this technology to crops like common bean faces several challenges. Early efforts to produce transgenic bean plants failed due to the lack of efficient DNA transfer systems and plant regeneration methods [78–81]. The tissue culture of *P. vulgaris* has evolved significantly with the development of techniques such as meristem culture [82] and direct organogenesis [42]; however, establishing an efficient and reproducible protocol for large-scale use remains a challenge.

Regeneration can mainly occur through two distinct routes: organogenesis and somatic embryogenesis, each with distinct characteristics and specific applications [77]. Organogenesis involves the formation of organs such as shoots and roots from somatic cells, either directly from the explant (direct organogenesis) or through an intermediate callus (indirect organogenesis) [83]. In direct organogenesis, organs develop without the formation of a callus, which generally results in more efficient and rapid regeneration. In contrast, in indirect organogenesis, the explant first develops a callus, which later differentiates into specific plant organs. This process is influenced by the ratio of the phytohormones cytokinin and auxin, which needs to be adjusted according to the type of explant and plant species. Organogenesis is a monopolar process, where the newly formed organs have a direct vascular connection with the explant, allowing the flow of essential nutrients and hormones for the development of the regenerated plant [83].

On the other hand, somatic embryogenesis is the process of forming embryos from somatic cells, without the need for fertilization. These embryos, known as somatic embryos, can develop into complete plants in a manner similar to embryos formed through normal fertilization, but originating from somatic cells rather than reproductive cells [41,84,85]. Somatic embryogenesis can be induced directly, when embryos form directly from the explant, or indirectly, when the explant forms a callus that subsequently produces the embryos [77]. Direct embryogenesis is generally more efficient, whereas indirect embryogenesis, although more flexible in terms of explant, may be less effective in some species. Embryogenesis is characterized by a bipolar structure, with two distinct developmental poles (apical and root), and features a closed vascular system, distinguishing it from organogenesis [81,83].

For *P. vulgaris*, tissue culture began to be explored with the use of apical meristems, followed by the proliferation of axillary shoots from apical shoot and cotyledonary nodes [82,86,87]. Explants such as leaf, petioles, and embryonic axes have also been employed in direct organogenesis regeneration protocols, with or without the presence of axillary meristems [88–90]. The induction of somatic embryogenesis and the formation of pre-embryos are possible in bean tissue culture [41,84,85], although the acquisition of regenerated plants is limited. Attempts at regeneration via indirect organogenesis have also shown low reproducibility, compromising the efficiency of genetic transformation [8,81].

The excessive production of phenolic compounds, which caused browning and cell death in the excised areas of the explants, making it difficult to develop multiple shoots, has also been reported as a challenge. To mitigate this problem, antioxidants such as activated charcoal and silver nitrate are used in the culture medium, resulting in reduced browning of shoots and increased regeneration frequency. Additionally, the combination of growth regulators, such as benzyladenine (BA), indole-3-butyric acid (IBA), and indole-3-acetic acid (IAA), results in greater efficiency and is most effective in shoot proliferation from an explant [51]. The optimal medium for multiple shoot regeneration was 2.5 mg/L BA, 0.1 mg/L IAA or IBA [51].

Regeneration of non-meristematic tissues, such as stem sections and leaf explants, is a problem for common beans. Various studies have shown that regeneration of plants from these tissues did not occur across different tested culture media, highlighting the difficulty of regenerating these materials. In contrast, the use of embryonic axes as explants has proven to be more promising, with several culture media allowing the production of multiple shoots [6].

The selection of appropriate tissues for in vitro regeneration is a critical aspect for the success of the process, and, historically, tissues such as apical and axillary meristems as well as immature seeds have been widely used due to their totipotency. In the in vitro regeneration of common bean, apical meristems are typically used in direct organogenesis processes, while other explants, such as cotyledons and embryonic axes, have also been explored. However, regenerating complete plants from somatic embryos in legumes, such as beans, is often challenging [8,91,92].

Among the main issues with the in vitro regeneration of common beans are the low efficiency in forming shoots and roots and the variability in response among different genotypes to hormonal treatments. The use of growth regulators at doses of 10 mg/L of 6-benzylaminopurine (BAP) and 1 mg/L naphthaleneacetic acid (NAA) has shown promising results in shoot formation in specific cultivars, where the efficiency of shoot formation reached up to 100% in certain culture media [93,94].

In a study aiming to establish a method for direct organogenesis regeneration, the use of epicotyl and hypocotyl explants from different cultivars showed superior regenerative capacity compared to hypocotyls. Additionally, the use of activated charcoal and silver nitrate in the culture medium increased shoot formation [95]. Another study addressed the development of an efficient regeneration system for *P. vulgaris* using both direct and indirect organogenesis. By using different explants, such as cotyledonary nodes, embryonic axes, and root segments, it was identified that embryonic axes and cotyledonary nodes with two cotyledons were more effective in shoot and callus formation. The combination of BAP (7 mg/L) and NAA (0.2 mg/L) resulted in a high shoot regeneration rate, and rooting was 100% when using indolebutyric acid (IBA) (0.5 mg/L) [96].

Recently, considerable efforts have focused on refining and optimizing more efficient regeneration protocols. Successful regeneration of *P. vulgaris* from epicotyls and hypocotyls through direct organogenesis has been reported, using six common bean cultivars and two basal media [95]. The authors suggested that common bean regeneration is cultivar-specific and that the effectiveness of the basal medium depends on the genotype and additional compounds. This highlights the complexity of the regeneration process. Using artificial intelligence, Aasim and collaborators developed and optimized a protocol for common bean regeneration with machine learning models [97]. They treated mature embryos with

various concentrations (0.25, 0.50, 1.0, 1.50, 5, 10, and 20 mg/L) of BAP, identifying optimal BAP combinations for effective shoot regeneration. The multilayer perceptron model was the most accurate in predicting shoot regeneration, counts, and length.

Altogether, these studies suggest that combining different approaches, such as the use of specific explants and careful hormone modulation, can improve in vitro regeneration rates of common bean. Soares and collaborators [28] emphasize the need for further research to develop protocols that can be consistently applied in genetic improvement programs. Efficient regeneration is not only important for clonal propagation, but also for genetic transformation, allowing the introduction of desirable traits into bean genotypes.

6. Gene Editing: What Comes Next?

The CRISPR/Cas9 system has revolutionized plant genetic engineering. Since the efficiency of gene editing can be significantly influenced by the choice of single guide RNA (sgRNA) and promoter, a rapid and efficient method for validating sgRNA efficiency in common bean is crucial before proceeding with the more time-consuming stable transformation process. Hairy root transformation has potential as a valuable tool for this purpose. If a plant regeneration protocol from hairy roots has already been established, hairy root transformation can provide a convenient platform for assessing sgRNA efficiency without the need for stable plant regeneration, accelerating the development of genetically engineered common bean cultivars with desired traits [98,99]. Recently, our group developed the first gene-edited common bean plants, with modifications to genes involved in the biosynthesis of the raffinose family oligosaccharides, which can enhance the taste and digestibility of common beans (Figure 3, unpublished data). To our knowledge, there are at least two other groups that are in the process of generating gene-edited common bean lines: (1) drought-resistant plants at Universidad Autónoma de Chile [100] and (2) grains with low antinutritional factors at Istituto di Biologia e Biotecnologia Agraria, Italy (personal communication, Aragão FJL).

In addition to all the advantages conferred by gene editing technology, gene-edited plants have been considered in many countries, such as in Brazil, as non-transgenic, which provides less rigorous regulation for commercial release. In January 2024, the European Parliament voted in favor of easing standards for gene-edited crops. However, the measure is still in negotiation between the European Union (EU) member states, which remain divided over the need/obligation of patenting and labels on foods made from gene-edited plants. For now, the edited plants continue to comply with the strict legislation that regulates the commercialization of transgenic plants in the EU.

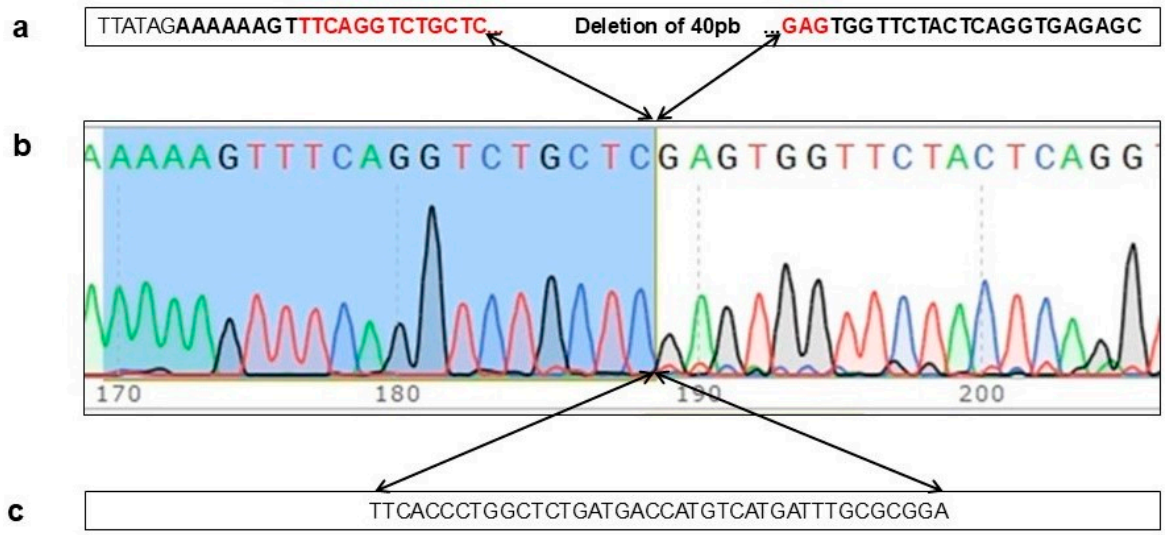

Figure 3. *Cont.*

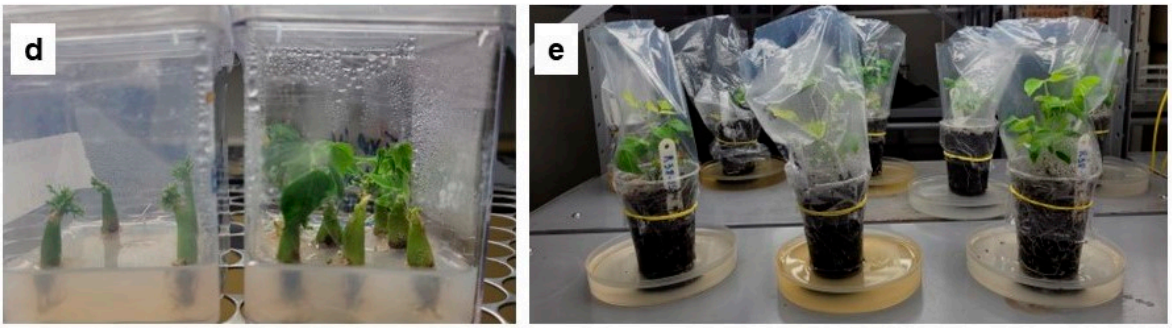

Figure 3. Illustration of the first gene-edited event developed in the common bean genome (*Phaseolus vulgaris*). The segment of the gene encoding stachyose synthase highlights the site where gene editing of 40 base pairs (bp) occurred, with a partial indication of the guide sequences used for the editing shown in red (**a**). An electropherogram displays the sequence of the gene post-editing (**b**), along with the deleted sequence fragment of the gene (**c**). Illustration of common bean tissue culture after genetic transformation: (**d**) genetically modified common bean embryos cultivated in selection medium, and (**e**) gene-edited common bean plants with modifications in genes involved in the biosynthesis of the raffinose family oligosaccharides in the process of acclimation in a potting mix substrate.

Interestingly, precisely because gene-edited plants are not obtained through the introduction of transgenes, the gene editing technique has its limitations, as it is restricted to modifications in the host's own genome. For some traits, genome editing may not be sufficient to obtain a plant with the desired phenotype, especially in the case of searching for resistance to pests and diseases, traits often not found in the plant's germplasm or at least not in monogenic inheritance.

7. Future Perspectives

Considering the economic and social importance of beans, a great effort has been made worldwide to develop systems for introducing and expressing transgenes as well as using new breeding techniques. Nevertheless, despite numerous reports on *Agrobacterium*-mediated transformation, none of the published processes have been repeated in different laboratories or appear useful for introducing stable agronomic characteristics. Using the selective agent imazapyr, the biolistic system has proven to be effective and has been used in different laboratories (Embrapa Arroz e Feijão and Embrapa Recursos Genéticos e Biotecnologia in Brazil, and Tecnologico de Monterrey in Mexico), but it cannot yet be considered routine. Although the possibility of *de novo* regeneration has been demonstrated in *P. vulgaris* [22,29], it is still too inefficient a process to be used in routine genetic transformation. More efforts are needed to develop tissue culture systems for direct or indirect de novo regeneration of cells that can be efficiently transformed by the biolistic method or *Agrobacterium*. For example, more genotypes should be tested in order to identify some that may have a high potential for regeneration via callus. Similarly, genotypes with an exposed apical meristem structure, i.e., with poorly developed leaf primodia, should be sought, while at the same time possessing a genotype with a high capacity for cytokinin-induced multiple shooting. In addition, more studies are necessary to find a better combination of *Agrobacterium*-susceptible *P. vulgaris* genotype.

Novel selective systems will be important to improve the efficiency of these processes in order to make them less laborious. Recently, a visual screening of transgenic soybean plants was developed using the *RUBY* reporter system, which consists of three genes encoding betalain biosynthetic enzymes, leading to the accumulation of purple pigment in transgenic tissue [101]. Genetically modified plants can be easily identified without the need for specialized equipment. This could be used in transformation processes aiming at gene editing in *P. vulgaris*.

The advent of CRISPR/Cas9 technology has revolutionized plant genome editing, providing unprecedented precision and efficiency in gene modification. In the context of

common bean crop improvement, CRISPR/Cas9 represents a significant breakthrough. By overcoming existing challenges and capitalizing on its potential, researchers can develop common bean varieties that are more productive, resilient, and sustainable, thus contributing to global food security and addressing future challenges. Despite its immense promise, challenges must be addressed for the successful application of CRISPR/Cas9 in legume crop improvement. Efficient protocols for the transformation and regeneration of common bean plants are crucial for effective genome editing. Additionally, identifying and targeting genes associated with desirable traits, such as disease resistance, nitrogen fixation, and abiotic stress tolerance, is essential.

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