



# Heritability of targeted gene modifications induced by plant-optimized CRISPR systems

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**Abstract** The *Streptococcus*-derived CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)/Cas9 (CRISPR-associated protein 9) system has emerged as a very powerful tool for targeted gene modifications in many living organisms including plants. Since the first application of this system for plant gene modification in 2013, this RNA-guided DNA endonuclease system has been extensively engineered to meet the requirements of functional genomics and crop trait improvement in a number of plant species. Given its short history, the emphasis of many studies has been the optimization of the technology to improve its reliability and efficiency to generate heritable gene modifications in plants. Here we review and analyze the features of customized CRISPR/Cas9 systems developed for plant genetic studies and crop breeding. We focus on two essential aspects: the heritability of gene modifications induced by CRISPR/Cas9 and the factors affecting its efficiency, and we provide strategies for future design of systems with improved activity and heritability in plants.

**Keywords** CRISPR-Cas · sgRNA · Gene editing · Mutagenesis · Inheritance

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## Introduction

Since 1996, when the first genetically modified seeds were planted in the United States for commercial use, genetically modified crops have been grown on an accumulated area of more than 1.8 billion hectares worldwide. So far, about 80 % of the area used to grow soybeans and 70 % of cotton growing area used genetically modified cultivars [1]. Some of the benefits brought by genetic modification in agriculture to the world's growing population are increased crop yields, reduced costs for food production, reduced need for pesticides, improved food quality, resistance to biotic and abiotic stress and increased health benefits [2]. Over the years, plant genetic modification has been based on the incorporation of foreign DNA into the plant genome by plant transformation but even though transformation methods have become quite efficient, the insertion site of the transferred DNA fragments as well as the number of copies cannot be controlled [3]. With the widespread adoption of transgenics, genetically modified crops have triggered an intense debate about their potential or perceived health and environmental risks and appeals for more precise gene modification technologies are increasing [4]. In contrast to the established genetic modification methods, targeted gene modification is able to manipulate plant genes in a site-specific manner making them very appealing for functional genomics research and crop improvement [5].

The concept of targeted gene modification encompasses precise gene editing and targeted gene mutagenesis. Precise gene modification, also known as gene targeting, was first achieved in mammalian cells via homology-directed repair (HDR). The HDR process is naturally activated during the pairing of inherited chromosomes in fertilized cells,

allowing the exchange of parental DNA fragments and thus generating new genetic variation in the population [6]. To harness this process for precise gene modification, the most popular strategy is to introduce DNA templates flanked with sequences homologous to the target site into reproductive cells [7]. The first successful report of targeted gene modification in plants was reported in 1988 [8]. Using tobacco protoplasts, a DNA fragment containing part of the neomycin phosphotransferase (NPTII) gene conferring kanamycin resistance was integrated into the targeted genomic locus via HDR, though at a very low frequency ( $0.5\text{--}4.2 \times 10^{-4}$ ). This frequency is at least ten times lower than the one reported for mammalian cells, suggesting that it would be almost impossible to perform gene modification in plant cells without the use of a selectable marker. *Agrobacterium*-mediated gene transformation of plant explants was later used to create heritable gene modifications in *Arabidopsis* whole plants using a partially duplicated beta-D-glucuronidase (GUS) gene as reporter [9]. Successful recombination events were observed in somatic and meristematic cells of the developing shoot apex. This outstanding work also suggested that in the absence of selection, plant somatic recombination frequencies ranged between  $10^{-6}$  and  $10^{-7}$  events/genome.

An important step towards the practical development of HDR in plant cells was reported in 1993, when the meganuclease *I-SceI* was introduced into *Nicotiana glauca* protoplasts for site-specific generation of double strand breaks (DSBs) in the partially duplicated GUS reporter gene [10]. The frequency of extrachromosomal HDR was increased 4.5–10 times when *I-SceI* was co-expressed with the two non-functional GUS genes, strongly suggesting that DSBs promote the incidence of HDR in plants. To pave the way for the use of DSBs as a method for genome manipulation in plants, the frequency of DSB-induced HDR was analyzed in transgenic tobacco calli and *Arabidopsis* seedlings [11, 12]. In both cases, approximately 1 % of the transformants were successively targeted, increasing the frequency of gene modification in excess of 100 times and highlighting the potential of harnessing the DSB repair pathways for targeted gene modification in plants.

The application of site-specific endonucleases for DSB generation also contributed to the development of targeted mutagenesis in plants. In addition to the homology directed repair pathway, DSBs in eukaryotes can also be repaired via the error-prone non-homologous end joining (NHEJ) pathway [13]. It was shown that in the absence of repair templates, the DSBs induced by *I-SceI* within a negative selectable marker gene could be repaired by nucleotide insertions and deletions. Such incorrect DNA repairs resulted in loss-of-function mutations of the toxic marker

gene that facilitated the identification of targeted mutations by screening for survival [14]. However, meganucleases can only recognize a specific DNA sequence, seriously limiting their application for targeted mutagenesis.

Two alternatives to meganucleases for targeted mutagenesis are zinc finger nucleases (ZFNs) and ‘transcription activator-like effector nucleases’ (TALEN). Both are chimeric proteins consisting of an engineered DNA-binding domain and the DNA cleavage domain from the *FokI* restriction endonuclease [15]. Compared to meganucleases, the DNA-binding domains used for target recognition in ZFNs and TALENs are more versatile. Each DNA-binding domain is composed of individual modules that can be engineered to recognize either a specific tri-nucleotide (ZFN) or mono-nucleotide (TALEN). In each case, a number of individual DNA-binding modules are usually assembled to form an array recognizing specific DNA sequences ranging from 12 to 20 nucleotides. [16–18]. The non-specific *FokI* DNA cleavage domain requires dimerization to cut double stranded DNA; therefore, two ZFNs or TALEN proteins are usually designed that each contain a DNA-binding domain targeting DNA sequences on opposite genomic strands [19, 20]. Both ZFNs and TALENs have been shown to generate targeted gene modifications in a number of plant species, but the targeted gene mutagenesis efficiency of ZFN was reported to be lower (1.7–19.6 %) than that of TALEN (30–48 %) [21–25]. Moreover, the specificity of ZFNs was not satisfactory [26]. Although TALENs seem to be superior to ZFNs in some aspects, the vector assembly process for both systems is quite laborious.

### Emergence of the CRISPR/Cas9 system for targeted gene modification

Compared to ZFN and TALEN, the recently developed CRISPR/Cas9 system has established itself as the most efficient and versatile tool available for targeted gene modifications (Table 1). This endonuclease system was derived from the adaptive immune system of bacteria and archaea to fight against the attacks of invasive viruses and plasmids [27, 28]. A typical Type II CRISPR/Cas9 system is composed of a Cas9 endonuclease, capable of cleaving double stranded DNA (dsDNA) and two short RNAs, the CRISPR RNA (crRNA) and the trans-activating crRNA (tracrRNA) for target recognition [29]. In bacterial cells, the tracrRNA hybridizes to the repeat regions of the crRNA precursor directing crRNA processing by RNaseIII [30]. The mature crRNA forms a duplex with the tracrRNA to guide the associated Cas9 protein to its target via base-pairing. The three-component CRISPR/Cas9 system can be simplified by artificially fusing the tracrRNA to the 3' end

**Table 1** Comparison of ZFN, TALEN and CRISPR/Cas9 gene editing technologies

Property/Tools	ZFN	TALEN	CRISPR/Cas9
Type of recognition	Protein–DNA	Protein–DNA	RNA–DNA
Cleavage activity	Paired nickase	Paired nickase	Nuclease
Methylation sensitivity	Sensitive	Sensitive	Insensitive
Module assembly	Complicated	Somewhat	Simple
Off-target effects	More	Less	Variable
Multiplexing	Rarely used	Rarely used	Capable

of crRNA to create a chimeric single guide RNA (sgRNA), streamlining the construction of CRISPR/Cas9 vectors and removing the need for the formation of the RNA duplex *in vivo* [29].

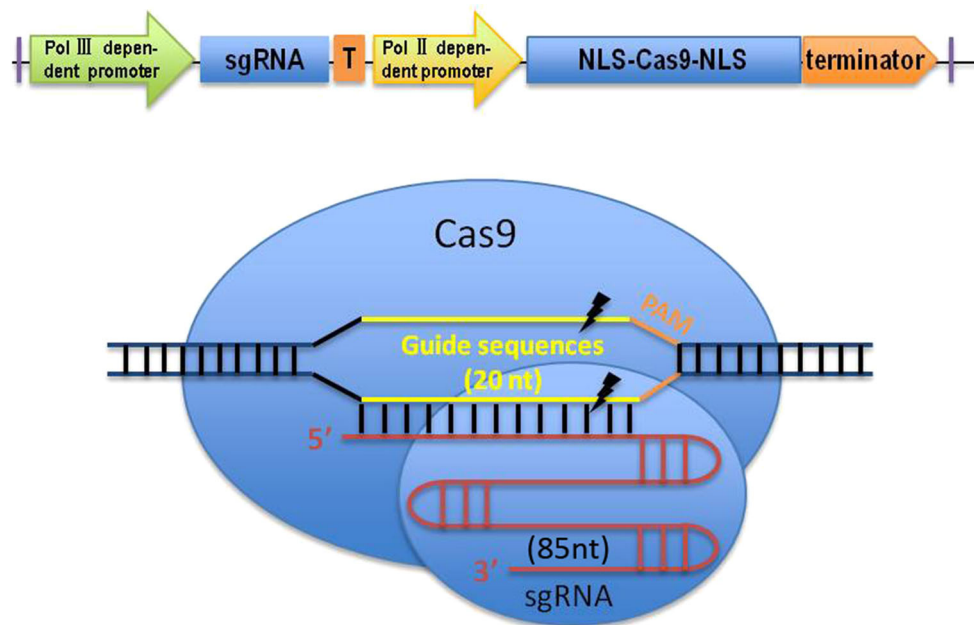
The target specificity of the Cas9 endonuclease is determined by the first 20 nt of the chimeric sgRNA and the presence of a Protospacer Adjacent Motif (PAM), a 3-nt sequence (NGG) located immediately downstream of the sgRNA target site and required for Cas9-mediated DNA cleavage. In the bacterial immune system, the PAM motif serves as a tag allowing Cas9 to discriminate non-infectious “self” from infectious “non-self” [31]. Although every base within the 20 nt guide sequence contributes to the overall specificity, mismatches close to the PAM-distal end of the guide RNA are usually tolerated to a greater degree than those at the PAM-proximal end [29, 32]. The remaining 65 nt of the full length sgRNA (85 nt) form a secondary structure that allows its incorporation into the Cas9 protein. Analysis of the crystal structure of the *Streptococcus pyogenes* Cas9 protein in complex with the sgRNA and target DNA, has revealed the presence of two main lobes: the recognition lobe is responsible for binding the sgRNA:DNA duplex and the nuclease lobe for target cleavage [33]. The nuclease lobe contains two distinct nuclease domains, HNH and RuvC, for cleavage of the complementary and non-complementary strands of the target DNA, respectively. In addition, a carboxyl-terminal domain in the nuclease lobe interacts with the PAM. To produce a functional system in eukaryotic cells, additional sequences such as nuclear localization signals must be artificially added to the original Cas9 gene [34, 35]. Conventionally, sgRNA transcription is controlled by RNA polymerase III dependent promoters, while Cas9 expression is driven by RNA polymerase II dependent promoters (Fig. 1). In some instances, RNA polymerase II dependent promoters, such as the cauliflower mosaic virus (CaMV) 35S promoter, has also been used to drive the expression of sgRNAs in combination with the Nos terminator [36, 37], which suggested the flexibility of sgRNA engineering.

Successful application of the engineered CRISPR/Cas9 system for targeted gene modification was first reported in

mammalian cells [34, 35]. Shortly after the initial report, the CRISPR/Cas9 system was successfully used for gene modification in Arabidopsis, tobacco, rice and wheat [38]. Subsequently, a number of reports highlighted the potential of this system for multiple applications in numerous plant species (Table 2). Most of the developments have been focused on two aspects: the activity of CRISPR/Cas9 system in plant cells and the heritability of the CRISPR/Cas9 induced gene modifications and to some extent, these two factors are correlated.

### CRISPR/Cas9 systems engineered to generate heritable gene modifications in plants

The activity of the CRISPR/Cas9 system is affected by many factors, including the transcript levels of the transgenes, the translational efficiency of Cas9 and the accessibility to the gene targets. The expression of the sgRNA has been traditionally driven by RNA polymerase III dependent promoters, such as the U3 or U6 promoters [39]. In eukaryotic cells, RNA polymerase III transcribes a number of non-coding RNAs, such as the ribosomal 5S rRNA, tRNAs and other small RNAs [40]. These Pol III-transcribed housekeeping genes are expressed in all cell types and under most environmental conditions, therefore reasonably high transcription levels of the sgRNA in transgenic plants can be expected when using their promoters. In addition, the regulatory elements and transcriptional characteristics of these genes are well defined. For example, the transcription of U6 genes is initiated 26 bp downstream of the TATA box and terminated by a small polyT stretch [41]. Using this knowledge, the exact sequence and length of the sgRNA transcripts can be controlled. Importantly, the U6 promoters from dicotyledonous and monocotyledonous plants are exchangeable within classes, but not between classes. For example, sgRNAs driven by an Arabidopsis U6 promoter are efficiently transcribed in tobacco [42, 43], soybean [44, 45], tomato [46], potato [47], petunia [48] and poplar [49], but not in monocotyledonous plants, such as rice and wheat [50]. Nevertheless, endogenous promoters seem to



**Fig. 1** A schematic showing the conventional structure of the CRISPR/Cas9 cassette and the mechanism of action of the engineered CRISPR/Cas9 system. To achieve efficient gene expression in plant cells, a Pol III-dependent promoter and a poly T terminator are used to drive transcription of the sgRNA, while Cas9 transcription is controlled by a Pol II-dependent promoter and a corresponding

terminator. Once transcribed, the 85 nt sgRNA is folded and incorporated into the Cas9 protein, guiding the complex to the targeted gene locus by RNA-DNA base pairing. The DNA cleavage activity of the Cas9 endonuclease complex also requires the presence of a PAM motif located immediately downstream of the 20 nt guide sequence. *T* poly T terminator, *NLS* nuclear localization signal

drive higher levels of sgRNAs transcription than exogenous ones [44]. Strong constitutive RNA Polymerase II dependent promoters have been the most popular choice to achieve high levels of Cas9 expression in plant cells [39]. The CaMV 35S promoter is the most widely used promoter to drive transgene expression in plants, specially dicot species although it is relatively less effective in monocots for which ubiquitin promoters such as the maize ZmUBI have been favored [51, 52]. Multiple studies have shown that the level of promoter activity plays a critical role in the efficiency of the customized CRISPR/Cas9 system for plant gene modification [53–55].

Plant codon optimized Cas9 genes have been used to further increase the expression levels of the CRISPR/Cas9 system with over ten different versions engineered so far in various plant species [56]. Unlike ZFN and TALEN, which were derived from nuclear localized transcription factors, the endonuclease activity of the native Cas9 protein occurs in the cytoplasm to target invading DNA. Therefore, usually one or two nuclear localization signal peptides are fused to the ends of the codon optimized Cas9 proteins to direct their transport into the nucleus [38].

Conventionally, CRISPR/Cas9 expression cassettes are introduced into the plant genome using either Agrobacterium-mediated or biolistic transformation methods. Although both methods can cause integration

of multiple copies of the transgenes, particle bombardment generally results in much higher transgene copy numbers than Agrobacterium-mediated transformation [57]. Multiple integration events, often coupled with inverted repeat T-DNA integration patterns, increase the frequency of transgene silencing [58] and will complicate the removal of the transgenes from the progeny by backcrossing or simple Mendelian segregation. Removal of the transgenes after induction of the intended gene modification is extremely important as it will minimize the chances of non-target effects. In addition, as long as there are no transgenes present, in some cases the progeny of CRISPR/Cas9 edited plants might be classified as non-GM, exempting them from regulatory approval, a fact that has immense commercial implications [59]. As an alternative to the physical integration of the CRISPR/Cas9 sequences into the genome, DSBs could be achieved by transient expression of Cas9 and the sgRNA using non-integrative vectors thus avoiding the GM intermediate step [60, 61]. DNA-free systems have also been used to induce DSBs by introducing preassembled sgRNA and Cas9 ribonucleoproteins directly into the cells [62]. Therefore, heritable gene modifications can be obtained in plants by either stable or non-stable transformation methods as summarized in Table 3.

**Table 2** Summary of the adaption of CRISPR/Cas9 system for targeted gene modification in various plant species

Species	CRISPR/Cas9 system	Transformation methods	References
Arabidopsis	DNA-free	Protoplast transfection	[62]
	Constitutive	Protoplast transfection	[43, 70, 72]
	Constitutive	Agroinfiltration	[43, 140]
	Constitutive	Agrobacterium mediated in planta	[55, 70–72, 95, 105, 106, 141, 142]
	Meristem specific	Agrobacterium mediated in planta	[55, 119, 120]
	Germline specific	Agrobacterium mediated in planta	[112, 116]
Tobacco	DNA-free	Protoplast transfection	[62]
	Constitutive	Protoplast transfection	[43]
	Constitutive	Agroinfiltration	[36, 42, 140, 142]
	Constitutive	Agroinfiltration followed by tissue regeneration	[42]
	Viral-based	Agroinfiltration	[61, 84, 132]
	Viral-based	Agroinfiltration followed by tissue regeneration	[132]
Rice	DNA-free	Protoplast transfection	[62]
	Constitutive	Protoplast transfection	[50, 101, 109, 140, 142, 143]
	Constitutive	Agrobacterium-mediated	[54, 70, 72, 79, 104, 106, 109, 128, 142, 143]
	Constitutive	Biolistic-mediated	[50]
Wheat	Constitutive	Protoplast transfection	[50]
	Constitutive	Agrobacterium-mediated	[36]
	Constitutive	Biolistic-mediated	[81]
MAIZE	Constitutive	Protoplast transfection	[102, 105, 144, 145],
	Constitutive	Agrobacterium-mediated	[102, 105, 145, 146],
	Constitutive	Biolistic-mediated	[146]
Barley	Constitutive	Agrobacterium-mediated	[103]
Soybean	Constitutive	Protoplast transfection	[44]
	Constitutive	Hairy root transformation	[44, 45, 53, 75, 77]
	Constitutive	Agrobacterium-mediated	[53]
	Constitutive	Biolistic-mediated	[75, 76]
Tomato	Constitutive	Hairy root transformation	[147]
	Constitutive	Agrobacterium-mediated	[46, 148]
	Viral-based	Protoplast transfection	[133]
	Viral-based	Agrobacterium-mediated	[133]
Potato	Constitutive	Agrobacterium-mediated	[47, 149]
	Viral-based	Agrobacterium-mediated	[47]
Petunia	DNA-free	Protoplast transfection	[150]
	Constitutive	Agrobacterium-mediated	[48]
<i>Brassica oleracea</i>	Constitutive	Agrobacterium-mediated	[103]
Lettuce	DNA-free	Protoplast transfection followed by tissue regeneration	[62]
Sorghum	Constitutive	Agrobacterium-mediated	[140]
Populus	Constitutive	Agrobacterium-mediated	[49]
Sweet organe	Constitutive	Agroinfiltration	[37]
<i>Medicago truncatula</i>	Constitutive	Hairy root transformation	[45]
Liverwort	Constitutive	Agrobacterium-mediated	[151]



**Table 3** Summary of the applicable strategies used to generate heritable gene modifications in plants

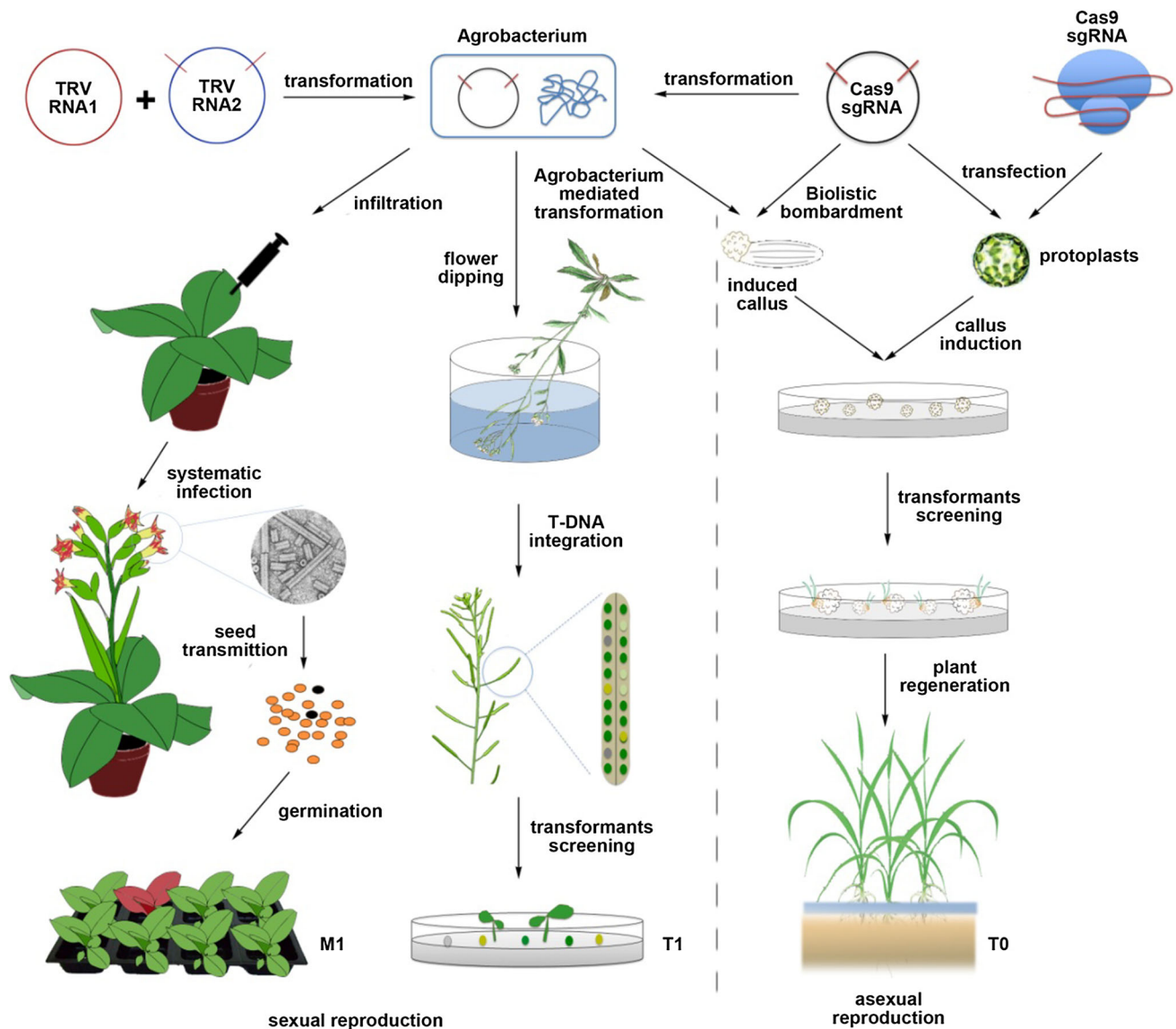
CRISPR/Cas9 systems	Genetic materials	Expressional regulation	Plant materials	Transformation methods	sgRNA multiplexing	Heritable modifications
Integrative	T-DNA based	Constitutive, spatial-temporal or inducible	Seedlings (flowering or root tissues)	Agrobacterium-mediated	Tandem repeats	Via fertilization
	T-DNA based	Constitutive or inducible	Cultured embryonic cells	Agrobacterium-mediated	Tandem repeats or mixture	Via tissue regeneration
	Plasmid-based or linearized DNA	Constitutive or inducible	Cultured embryonic cells	Biolistic or microinjection	Mixture	Via tissue regeneration
Non-integrative	Plasmid-based or linearized DNA	Constitutive	Protoplasts	PEG or electroporation	Mixture	Via tissue regeneration
	Virus-based	Rep binding motifs or viral promoter	Seedlings (leaf tissues)	Leaf infiltration	Mixture	Via fertilization or tissue regeneration
	DNA-free (mRNA or ribonucleoprotein)	Naked or chemical modified	Protoplasts	PEG or electroporation	Mixture	Via tissue regeneration

### Heritable gene modifications obtained via sexual propagation using integrative CRISPR/Cas9 systems

Two different strategies are usually applied to generate heritable gene modifications in plants, depending on the method used for plant reproduction (Fig. 2). Generally speaking, the production of new offspring can be accomplished via sexual or asexual reproduction methods [63]. Sexual reproduction produces offspring by the fusion of gametes involving two fundamental processes: meiosis, which rearranges fragments of genomic DNA and halves the number of chromosomes, and fertilization, which restores the chromosome to the pre-meiosis number. The offspring of sexually reproduced plants are genetically different from their parents due to the recombination of homologous chromosomes [63]. Theoretically, to generate heritable gene modifications via sexual reproduction, targeted gene modifications should occur either in gametes during or after meiosis, or in zygotes during fertilization. In plants, however, somatic cells within the meristems can differentiate into reproductive cells opening the door to alternative strategies aiming to achieve targeted gene modification in meristematic cells during vegetative growth [64, 65].

*Agrobacterium*-mediated transformation is the prevalent method for genetic modification in plants, being efficient for a wide spectrum of plant species [66]. Although *Agrobacterium*-mediated transformation usually involves a laborious tissue culture step, the development of in planta transformation methods in *Arabidopsis thaliana* incredibly simplified the process making this species the preferred model for genetic studies over the last 20 years [67, 68]. The use of in planta transformation for CRISPR/Cas9

delivery has been widely adopted for the routine and efficient generation of heritable gene modifications via sexual reproduction [69]. Using constitutive promoters for Cas9 expression, such as the CaMV 35S, targeted gene modifications have been achieved in T1 Arabidopsis plants at a frequency of 30–84 % although the frequency was reduced to 20–54 % in the T2 progeny [70, 71]. In most cases, only 15–25 % of the mutations present in the T2 generation were also found in the T1 generation, suggesting a high degree of chimerism in T1 plants and the possibility that many of the somatic mutations were not inherited. In contrast, use of the AtUBQ1 promoter for Cas9 expression resulted in a mutagenesis frequency of 74–92 % in T1 plants without a noticeable reduction in the subsequent generation (58–96 %) with a relatively large proportion of the T2 mutations (37–66 %) present in the T1 generation [71, 72]. Although the mutagenesis efficiency of these two CRISPR/Cas9 systems is not comparable due to the difference in target sites, the AtUBQ1 promoter appears to be more efficient than the CaMV 35S promoter for generating heritable gene mutations. These differences might be attributable to the expression characteristics of each promoter as there is evidence suggesting that even though both are considered to be constitutive, neither the CaMV 35S promoter nor the AtUBQ1 promoter are uniformly expressed in plants. Studies using the GUS reporter gene have shown that the expression patterns of these two promoters are different in tobacco transgenic lines with the AtUBQ1 promoter generating strong staining in pollens and ovules of immature flowers, whereas adjacent vascular tissues showed lower staining levels. In contrast, weak staining was observed in pollens of CaMV 35S transgenic lines, whereas the vascular tissues exhibited the strongest



**Fig. 2** Methods used to generate heritable gene modifications in plants by CRISPR/Cas9. To obtain heritable gene modifications by CRISPR/Cas9, at least three different transformation methods can be used. In planta transformation by flower dipping is the method of choice for Arabidopsis. Heritable gene modifications can be created in the T1 generation by early expression of CRISPR/Cas9 in egg cells. Targeted gene modification is also possible using viruses as vehicles for CRISPR/Cas9 delivery, although only those that can infect meristematic tissues or be transmitted through seeds are suitable for

signal [73]. In addition, the CaMV 35S promoter was unable to drive gene expression in young zygotic embryos [74]. The differences observed in reproductive cells suggest that the AtUBQ1 promoter might be more suitable than the CaMV 35S promoter to generate heritable gene modifications in plants like Arabidopsis. Besides, it was also found that neither promoter generated homozygous, bi-allelic or heterozygous mutants in the T1 generation, with all of the analyzed T1 mutants being

this application. Tissue regeneration from genetically modified calli is suitable for most crop species with delivery being provided by plasmids or preassembled ribonucleoproteins. Both Agrobacterium-mediated and biolistic methods can be used to deliver the CRISPR/Cas9 DNA constructs into calli, whereas the preassembled CRISPR/Cas9 complex can only be delivered into protoplasts by cell transfection before calli induction. In all cases, homozygous mutants can be obtained in the first transformed generation; however, the frequency is far higher in plants regenerated from protoplasts

chimeras (i.e. individuals containing a mix of mutated and non-mutated cells). In such plants, the mutations induced by the incorporated CRISPR/Cas9 cassette occurred after the division of zygotes, explaining why the genotypes of the targeted sites are not uniform within the same individual plant. However, mutants with all zygotic types were identified in the T2 generation at variable frequencies. In the absence of the Cas9 gene, progeny from T2 mutants segregated according to the Mendel's law, whereas in the

presence of Cas9, new mutation types were obtained from heterozygous, chimeric and wild type seedlings, but not from homozygous or bi-allelic ones, suggesting that the CRISPR/Cas9-induced gene mutations in Arabidopsis were faithfully inherited [71]. In summary, the ideal scenario to obtain stably heritable gene modifications using integrative CRISPR/Cas9 systems, is to remove the CRISPR/Cas9 cassette as soon as possible from the transgenic lines. But even if the transgenes are not removed, the specificity of CRISPR/Cas9 system for target recognition will prevent it from introducing new mutations in the modified gene loci.

### Heritable gene modifications obtained via asexual propagation using integrative CRISPR/Cas9 systems

For most crop species, the in planta transformation method is not feasible and it is necessary to regenerate transgenic plants from explants via asexual reproduction [66]. To perform targeted gene modification in those species, both *Agrobacterium*-mediated transformation and the biolistic particle system can be used to deliver the CRISPR/Cas9 cassette into the plant cells (Fig. 2). In these cases, the heritability of the targeted gene modifications almost equals the mutagenesis frequency of transformed cells. Assuming that no cellular toxicity is conferred by the induced gene modification, the gene-edited seedlings will be regenerated from transformed cells without frequency change. To maximize expression levels, and the mutagenesis efficiency of the CRISPR/Cas9 system, the endogenous U6 and the CaMV 35S promoters have been routinely used to drive sgRNA and Cas9 transcription, respectively, while high genetic transformation efficiency has been achieved by the use of suitable transformation vectors, *Agrobacteria* strains and explant materials [56].

Six different studies have used the CRISPR/Cas9 system to perform targeted gene modifications in soybean, five of which used *Agrobacterium rhizogenes*-mediated gene transformation for quick detection of CRISPR/Cas9-induced gene mutations in hairy roots [44, 45, 53, 75–77]. This bacterium can infect a number of dicotyledonous plants upon wounding and generate “composite plants”, i.e., plants with wild-type shoots and transgenic roots, by transferring its T-DNA to the plant genome [78]. Considering that each transgenic hairy-root represents an independent transformation event, high numbers of transformants can be obtained and analyzed with this method. One study developed a soybean codon optimized CRISPR/Cas9 system to perform targeted gene editing in hairy root of both soybean and *Medicago truncatula* [45]. Another reported targeted mutagenesis with efficiencies as high as 95 % using a combination of the *M. truncatula* U6.6 polymerase III promoter to drive sgRNA transcription and the double-enhanced CaMV 35S promoter for Cas9

expression in hairy roots [75]. Two other studies suggested that the soybean endogenous U6 promoter is more effective than the Arabidopsis U6 promoter to generate targeted gene modifications in soybean [44, 53]. The rest showed that two endogenous soybean genes can be targeted simultaneously using only one AtU6 promoter-regulated sgRNA module [77]. Although hairy root transformation allows for the quick identification of CRISPR/Cas9-induced gene modifications and facilitates optimization of the system, regeneration of whole plants from transgenic hairy roots is difficult, making stable transformation the method of choice to obtain heritable mutations in soybean. Therefore, biolistic-mediated transformation was applied to deliver the CRISPR/Cas9 system into soybean somatic embryo cultures [75, 76]. These embryonic cell cultures were usually obtained from non-zygotic immature plant tissues and can be cultured to form undifferentiated cell masses before tissue regeneration. Jacobs et al. showed that only 5 of the 31 recovered transgenic lines contained a complete Cas9 gene, but continuous Cas9 expression leads to additional mutations during the development of somatic embryos [75]. Li et al. reported that a high mutagenesis frequency at the targeted gene loci (up to 76 %) was achieved by using a CRISPR/Cas9 system driven by soybean elongation factor gene promoter (EF1A2). Targeted gene integration was also achieved at a frequency of 3.8–4.6 % and the integrated genes were successfully transmitted to the T1 generation and segregated according to Mendelian laws, demonstrating the feasibility of the CRISPR/Cas9 system in soybean [76]. Besides, *Agrobacterium tumefaciens*-mediated transformation was also used to target the phytoene desaturase (PDS) gene in soybean. The expected dwarf and albino phenotype was observed in adventitious buds regenerated from the inoculated cotyledon node [53]. Together, these studies demonstrated the feasibility of obtaining heritable gene modification using integrative CRISPR/Cas9 systems.

A more detailed study of the heritability of CRISPR/Cas9-induced gene mutations was performed in rice using a constitutive promoter to drive Cas9 expression and *Agrobacterium*-mediated transformation of somatic embryos [79]. Targeted gene mutagenesis was detected at an average frequency of 44.4 % in the T0 generation and the presence of homozygous, bi-allelic, heterozygous and chimeric individuals confirmed. Notably, 3.8 % of the analyzed T0 plants were homozygous, suggesting that targeted gene modifications in these seedlings occurred before the first round of embryonic cell division and the mutation types of the two gene alleles happened to be the same. In contrast, Arabidopsis studies using Cas9 driven by constitutive promoters and *Agrobacterium*-mediated in planta transformation produced only chimeras in the first generation, perhaps reflecting the fact that this



transformation method targets the ovule and therefore the only chance of obtaining homozygous and bi-allelic mutants with this transformation method is by inducing mutations before the first division of the zygote.

Polyloid crop species pose an additional challenge as their genomes are larger and highly repetitive compared to diploid species. A recent report described the application of TALEN and CRISPR tools in bread wheat, an allohexaploid in which most of the genes have three similar but not identical copies [80, 81]. While TALEN was used to simultaneously edit all three homoeoalleles that encode the MILDEW-RESISTANCE LOCUS (TaMLO) protein, CRISPR constructs targeted just one allele. Genetic transformation of a winter wheat variety was performed using immature embryos via particle bombardment. Twenty seven mutations were identified in 450 independent T0 transgenic TALEN lines (6.0 %), with only one line containing heterozygous mutations for all the three homoeoalleles. The mutagenesis frequency induced by CRISPR/Cas9 was very similar to that observed in TALEN lines with four mutations generated in the TaMLO-A1 allele out of 72 T0 transgenic lines (5.6 %). Two homozygous *mlo* triple mutants lacking the TALEN transgene cassette were obtained by self-pollination and segregation in two generations. As expected, the triple mutants exhibited broad-spectrum resistance to powdery mildew [81].

#### **Heritable gene modifications obtained via asexual propagation using non-integrative CRISPR/Cas9 systems**

Integration of the CRISPR/Cas9 coding sequences into the plant genome is not an absolute requirement to perform in vivo gene modification. In animals, in vitro transcribed RNAs encoding Cas9 and sgRNA can be directly injected into zygotes to generate homozygous and biallelic mutants with high frequency [82]. However, the plant cell wall provides a strong physical barrier that handicaps the application of mechanical approaches such as microinjection, electroporation and PEG-mediated DNA transfection. To overcome this obstacle, plant protoplasts can be used as the target for transfection with the added advantage that the delivered cargos can be DNA, RNA or even proteins. In the case of CRISPR/Cas9, the delivery of preassembled ribonucleoproteins rather than DNA plasmids can appease concerns about non-intended incorporation of plasmid fragments into the host genome [62, 83]. Using this delivery method, targeted mutagenesis was achieved at a frequency ranging from 8.4 to 44 % in transfected *Arabidopsis thaliana*, tobacco, lettuce and rice protoplasts. The resulting gene modifications were maintained after the regeneration of microcalli from transfected protoplasts and

the overall mutation frequency in lettuce calli was up to 46 %. It was also shown that the targeted gene modifications present in regenerated plants can be transmitted to the germline and inherited by their offspring. Although promising, the main drawback of this approach is that regeneration of plants from protoplasts is feasible in only a few plant species.

#### **Heritable gene modifications obtained via sexual propagation using non-integrative CRISPR/Cas9 systems**

As an alternative, viral vectors have been used to attempt gene modification using non-integrative expression of CRISPR/Cas9 in host cells. Systemic infection of *N. benthamiana* leaves with tobacco rattle virus (TRV) has been used to deliver sgRNA by incorporating it into a RNA2 replicon-based vector [84]. TRV is a bipartite, positive-sense RNA virus and its RNA2 genome can be modified to carry foreign gene fragments (usually less than 2–3 Kb) into plant cells by agroinoculation. The incorporated sgRNAs was under the control of the pea early browning virus promoter to facilitate its transcription from the virus replicon. Systemic expression of the engineered sgRNA modules was achieved by co-inoculating the TRV1- and TRV2-carrying *Agrobacterium* strains into 3-week-old plant leaves [85]. However, this viral system does not have the capacity to include the Cas9 gene due to the large size of Cas9 gene, and thus the TRV inoculation must be performed on stable transgenic plants expressing the Cas9 protein. Targeted gene mutagenesis was detected 10 days post-inoculation in inoculated and systemic leaves at a frequency of 56 and 30 %, respectively. Notably, two seedlings carrying targeted gene mutations were recovered from the progeny suggesting that the virus-based system is able to generate heritable gene modifications in plants. In a similar approach, another study attempted to use the bipartite begomovirus Cabbage Leaf Curl virus (CaLCuV) as a vector for sgRNA delivery [61]. CaLCuV is a DNA virus in the Geminiviridae family that replicates in the nucleus and encodes seven proteins in two separate genomes. T-DNA vectors based on these two genomes have been developed to deliver foreign genes into plant cells by agroinoculation [86, 87]. In order to adapt the viral replicons for targeted gene modification in host plants, the AtU6-sgRNA expression module was constructed in the genome A-based vector to inoculate Cas9-expressing transgenic plants along with the genome B-based vector. Although mutagenesis frequency of the endogenous PDS gene in systemically infected leaves was up to 85 %, no heritable mutations were produced in the offspring of inoculated plants [61]. A possible explanation for this apparent disparity is that the CaLCuV virus does not move

to all plant tissues and often exists in mature cells which do not divide, whereas TRV has a low chance to be transmitted to the offspring through the germline [88]. Therefore, to induce heritable gene modifications using the CaLCuV-based system it is necessary to regenerate plants from systemically infected tissues.

### Target specificity of the CRISPR/Cas9 system in plants

Target specificity is arguably the most important feature of the CRISPR/Cas9 technology and multiple studies have been performed to systematically examine the specificity of the CRISPR/Cas9 system both in vivo and in vitro [32, 89–91]. In general, the nucleotides in the PAM and the PAM-proximal sgRNA sequence are crucial for target recognition while PAM-distal sequences can tolerate some, but not many, mismatches. However, the number and position of tolerable mismatches between the DNA target and the guide sequence seems to be target-dependent, making it difficult to define simple rules for sgRNA design based on the available data from these studies [92]. In addition to the positional effects, a trade-off between specificity and activity has been found in mammalian cells, where shorter guide RNAs can be more specific than longer ones and high concentrations of CRISPR/Cas9 complex can cleave off-target sites that are not usually cleaved when the complex concentration is low [32, 90, 93]. To minimize the incidence of off-target effects, a nickase version of Cas9 (Cas9n) was engineered by mutating its N terminal RuvC-like nuclease domain. In order to produce DSBs with Cas9n it is necessary to express two different sgRNAs complementary to opposite strands of the target site, effectively extending the target recognition site from 20 to 40 nt [91, 94]. Application of this strategy in mammalian cells was reported to reduce the off-target activity by 50–1500 folds without sacrificing on-target cleavage efficiency [94]. However, use of the nickase in Arabidopsis resulted in a 740-fold reduction of NHEJ-mediated mutagenesis compared to the regular CRISPR/Cas9 system but similar HDR frequencies [95].

In plants systems, the potential off-target effects of the CRISPR/Cas9 system have been analyzed using two strategies. The first strategy uses the BLASTN algorithm to predict candidate off-target sites by sequence similarity and later determines the existence of mutations on the predicted off-target candidates. Although the parameters used for candidate selection are somewhat hard to determine due to the lack of strong rules to predict mismatch tolerance of the CRISPR/Cas9 system, a number of webtools have been developed to assist the prediction of sgRNAs off-targets based on the number and position of potential mismatches in plant genomes [96–98]. The second approach is to

perform whole genome re-sequencing followed by evaluation of the genome editing outcomes using analytic tools [98, 99]. Employing these strategies, no off-target effects were detected in Arabidopsis T1 and T2 plants obtained using the Agrobacterium-mediated flower dipping method [71, 100]. A limited number of off-target mutations were identified in rice, soybean, *Brassica oleracea*, barley and maize transgenic plants produced via either biolistic or Agrobacterium-mediated transformation of cultured cells [44, 50, 79, 101–103]. The observed differences in off-target effects among species could be the result of differences in the expression levels of the CRISPR/Cas9 components, which is also correlated with the dosage of CRISPR/Cas9 reagents delivered into plant cells. In summary, the CRISPR/Cas9 system seems to have very low incidence of off-target effects in plants and even in the cases when they occurred, they could be easily removed by backcrossing.

### Developing multiplex CRISPR/Cas9 systems in plants

Compared to Zinc-finger and TAL-effector proteins, the innate multiplex crRNA array makes the recognition of multiple DNA targets much simpler for CRISPR/Cas9 systems. The use of CRISPR/Cas9 to simultaneously edit two endogenous gene loci was first reported in mammalian cells by co-expressing either two crRNAs or two sgRNAs [34, 35]. However, the dual-crRNA constructs seemed to be less efficient than the sgRNA constructs for generating targeted mutagenesis in plants probably due to the inefficient processing of the mature crRNA by the tracrRNA in vivo [104]. A more efficient way to achieve multiplex gene modifications in plants via agrobacterium-mediated methods is to assemble multiple sgRNA modules into one binary vector for co-expression in transgenic plants [105–108]. Using this approach, homozygous individuals with multiple targeted mutations were successfully identified in the progenies of Arabidopsis, rice and maize transformants, suggesting that these multiple mutations are heritable. Alternatively, multiple targeting can be achieved in plants by adopting an interstitial tRNA architecture for sgRNA expression, with up to eight individual sgRNAs precisely excised from a single transcript by the plant native tRNA processing machinery [109]. Using this approach, very high efficiencies (up to 100 %) have been reported to simultaneously mutagenize four different loci in stable transgenic rice plants, although the possible quadruple mutants were not presented. A major concern when designing multiplex gene editing systems is to reduce the risk of triggering the plant gene silencing pathways, which are usually activated by highly repetitive DNA sequences. To reduce the repetitiveness of sgRNA

modules, different RNA Polymerase III-dependent promoters have been used to drive the expression of the multiple sgRNAs [105–107].

In addition, multiplexing can also be achieved at the initial stage by providing multiple sgRNAs cloned into separate vectors or transcribed as sgRNAs in vitro for simultaneous delivery by biolistic bombardment or cell transfection [43, 50]. Individual plants with heritable multiple mutations can be generated by tissue regeneration afterwards. In summary, selection of the ideal strategy for multiple gene modification largely depends on the plant species and the transformation methods available for the generation of transgenic lines.

### Improving the heritability of CRISPR/Cas9-induced gene modifications

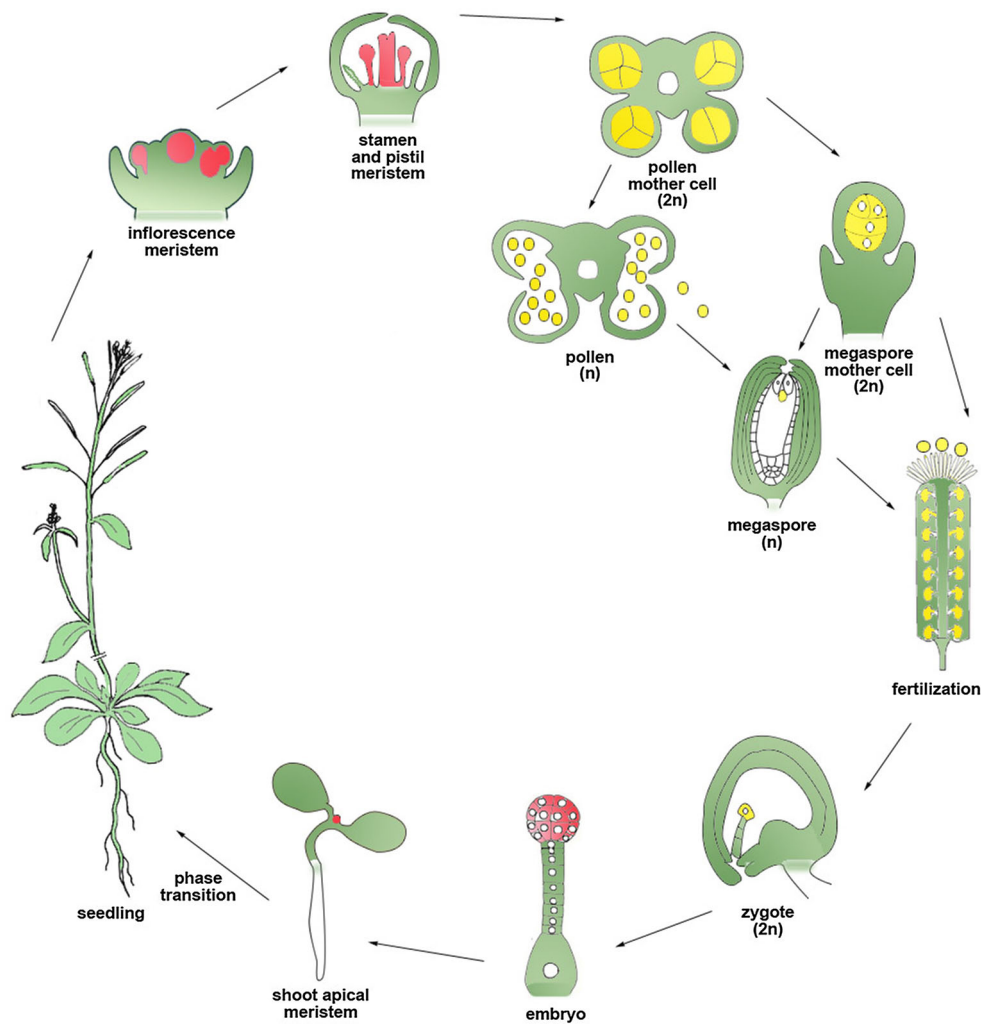
In CRISPR/Cas9 systems developed for stable transformation via sexual reproduction, the heritability of CRISPR/Cas9-induced gene modifications is strongly affected by the sgRNA and Cas9 promoter activities in reproductive cells. Two types of promoters can be used to improve heritability by optimizing expression of Cas9 in reproductive cells (Fig. 3). Germline-specific promoters are specifically expressed during gamete formation from gametocytes. Gametes are mature haploid cells generated from the successive division of diploid gametocytes. In flowering plants, the male gamete is produced inside the pollen grain and the female gamete is produced inside the embryo sac of the ovule. The Arabidopsis *SPOROCTELESS/NOZZLE (SPL)* gene is a central regulator of cell division and differentiation in both anther and ovule [110, 111]. Early expression of this gene was observed in both anther and ovule primordia, but its transcription is much stronger in pollen mother cells (PMCs) than in megaspore mother cells (MMC). Expression of Cas9 in the genomic context of the *SPL* gene was able to constrain the activity of the CRISPR/Cas9 system to germline cells [112]. Compared to a constitutively expressed CRISPR/Cas9 system, both the abundance and the diversity of heritable gene modifications were increased by this germline specific CRISPR/Cas9 system. However, due to the preferential expression of the *SPL* promoter in PMCs, only heterozygous mutants were obtained in the T2 population. Considering the large quantity of pollen cells in a single individual plant, application of this germline specific system will facilitate the screening of specific mutation types in offspring derived from a limited number of transgenic plants.

In addition to the *SPL* promoter, the same study reported the use of Arabidopsis *DD45* and tomato *LAT52* promoters to attempt germline specific gene modifications in Arabidopsis. The *DD45* gene encodes an egg-cell-specific

protein expressed in zygotes and early embryos [113, 114], while the tomato *LAT52* gene is transcribed during the late stage of pollen development [115]. While the mutagenesis frequency of these two systems in the T1 population was comparable, the *DD45* system produced more mutations in the T2 population, suggesting that it was more efficient than the *LAT52* system in generating heritable gene modifications. Moreover, the presence of bi-allelic and homozygous gene modifications in the T1 population of the *DD45* system indicated that targeted gene modifications were successfully induced in both alleles of the targeted genes before the first cell division of zygotes, though at a low frequency (1 of 36 for one of the targeted loci and 2 of 36 for the other). Interestingly, this study used the *NOS* terminator in the Cas9 genetic construct in combination with each promoter and a previous report had suggested that the choice of terminator had a strong influence on the efficiency in producing non-mosaic T1 mutants when using egg cell-specific promoters [116]. According to Wang et al., the mutagenesis efficiency of the CRISPR/Cas9 system when using the *DD45/EC1.2* promoter to drive Cas9 could be sharply improved by the *rbcS-E9t* terminator compared to the *NOS* terminator [116]. The *EC1.2* system was reported to produce homozygous *etc2 try cpc* triple mutants at a frequency of 8.3 % in T1 plants and the resulting mutations could be reliably passed to T2 plants; shortening the time needed to produce homozygous or bi-allelic mutants to one generation. In addition, the frequency of homozygous T1 triple mutants was further increased to 17 % by adding an additional enhancer to the native *EC1.2* promoter. These studies clearly illustrate the importance of the regulatory elements used in the Cas9 expression cassette and the possibility to improve performance by manipulating them. Further improvements using similar strategies are still achievable as extensive expression studies of angiosperm male and female gametogenesis have provided a stock of additional regulatory elements yet to be tested [117, 118].

Besides germline-specific promoters, meristem-specific promoters are logical candidates to improve the heritability of targeted gene modifications induced by stably transformed CRISPR/Cas9 systems. All aerial organs in higher plants are generated from meristematic tissues in shoot apices, therefore any mutation induced in shoot meristematic cells can produce mutated somatic sectors in the plant throughout shoot development. Some of those sectors can develop into floral primordia and lead to the transmission of gene modifications through the gametes. A number of meristem-active promoters, such as *INCURVATA2 (ICU2)*, *APETALA (API)*, *YAOZHE (YAO)*, and *Histon H4 (AtH4)* have been used to drive the expression of Cas9 in Arabidopsis [55, 100, 119, 120]. All of these systems were highly efficient in generating targeted gene

**Fig. 3** Optimal expression patterns to generate heritable gene modifications in Arabidopsis with the CRISPR/Cas9 system. During the plant life cycle, heritable gene modifications can be generated in dividing cells at the vegetative and reproductive growth stages. These dividing cells can be classified into two classes, meristematic cells (*red*) and germline cells (*yellow*). The meristematic cells including young embryo cells, shoot apical meristem, inflorescence meristems, floral meristems, stamen and pistil meristems can differentiate into germline cells. Generally speaking, the earlier the mutations are induced, the more uniform the mutation types are. Germline cells include ancestor cells that will give rise to pollen and megaspores as well as zygotes (egg cells after fertilization). Mutations induced in either of the two gametes can only generate heterozygotes in the progeny, whereas those induced in zygotes will produce homozygous or bi-allelic mutants



modifications in dividing cells of T1 plants and heritable gene mutations were obtained in the T2 populations, but the severity and time of onset of mutations in T1 plants were different for each system. The *ICU2* and *YAO* promoters are highly expressed in proliferating cells, including those in meristems, primordia, vegetative shoot, inflorescences and flowers [121–123]. Early expression of these two genes was identified in egg cells and young embryos after fertilization, therefore the resulting T1 mutants were usually chimeras produced during vegetative growth. In contrast, *AP1* encodes a homeobox protein involved in the establishment of floral meristem [124], thus T1 mutations will be created after the transition from vegetative growth to reproductive growth. In summary, the earlier and broader the induction of mutagenesis occurs in the meristematic tissues of T1 plants, the more progeny with heritable gene mutations will be obtained.

Although a number of tissue-specific promoters have been tested to improve the heritability of CRISPR/Cas9-induced targeted gene modifications in plants, most efforts

were focused on the Cas9 gene rather than the sgRNA. An innovative approach has used ribozyme-flanked sgRNAs (RGRs) as part of the CRISPR/Cas9 system [125]. Primary RGR transcripts undergo self-catalyzed cleavage to generate the desired gRNA containing 2',3'-cyclic phosphate and 5'-hydroxyl modified termini. It has been suggested that such an RNA structure may be more stable in vivo because some nucleases require the 5'-terminal phosphate group for specific cleavage [126]. Application of this approach in yeast and mammalian cells resulted in high cleavage activity both in vitro and in vivo but it has not been tested in plants thus far [125, 127].

In the case of stable transformation via asexual reproduction, the heritability of CRISPR/Cas9-induced gene modifications can also be affected by the length of the culture period in addition to the expression level and transformation efficiency. Considering that the promoters routinely used to drive the expression of the CRISPR/Cas9 system in transformed cells are already very strong, any improvements will need to address the



remaining variable, culture length. In fact, extension of the culture period increased the proportion of mutated cells in transformed rice and soybean calli [53, 128]. However, prolonged tissue culture also increases the incidence of somaclonal mutations and decreases regeneration ability, which may hamper its application for crop improvement.

The heritability of targeted gene modifications in virus-based systems largely depends on the virus species used for delivery. In the case of TRV, this virus can infect roots and move to aerial parts of several plant species in *Solanaceae*, but can also infect meristematic tissues and achieve seed transmission, whereas many other virus species cannot [88, 129, 130]. TRV can infect pollen and fertilized flowers and can then enter the seed through the megaspore mother cell and egg, or through the pollen mother cells and pre-meiotic pollen allowing the delivery of sgRNA and the generation of heritable gene modification in gametes [131]. However, most viruses cannot infect the meristems and therefore alternative strategies need to be devised. The DNA replicons of Geminiviral species can be modified to carry the CRISPR/Cas9 system in plants although the reformed replicon cannot achieve systemic infection [132]. In compensation, *Agrobacterium* or particle bombardment can be used to deliver the viral vector to cells. This geminivirus-based system takes advantage of the virus replication initiator protein (Rep) to amplify the CRISPR/Cas9 coding sequences or DNA repair templates to very high copy numbers in plant cells increasing the probability of obtaining heritable gene modifications via homology-directed repair. The geminivirus-based system has been applied to generate precise gene modifications in tomato and potato [47, 133]. In this application, a strong promoter was inserted upstream of the anthocyanin synthesis gene along with an antibiotic selection marker to allow the screening of recombinant cells. The frequency of targeted gene replacement was as high as  $\sim 10\%$  and whole plants recovered from these recombinant cells segregated according to Mendel's laws. However, compared to the conventional T-DNA vector approach, the geminivirus-based system seemed to be less efficient for generating targeted mutagenesis in potato [47]. The reduction of targeted mutations supports the use of the geminiviral vector system for promoting HR purposes rather than NHEJ-induced mutagenesis.

## Future prospects

### The development of CRISPR/Cas9 systems used for genetic screening

Over the past thirty years, forward genetics approaches have been the primary choice for gene functional studies.

Random mutagenesis approaches using chemical or physical agents were extensively used to produce large mutant populations that were subsequently screened for phenotypes of interests. However, the work load of this approach is enormous and is only applicable to a few self-pollinated plant species. With the emergence of the CRISPR/Cas9 system, targeted gene mutagenesis in plants is becoming routine and the development of methods to generate genome-scale knockout libraries is now conceivable and has already been reported in bacteria and mammalian cells [50, 134]. The nature of these libraries can be controlled as it is determined by the combination of sgRNA inputs, a huge advantage over random mutagenesis. If used for genetic screening using negative or positive selection strategies, they can strongly facilitate the validation of candidate genes obtained from "Omics" approaches. In Arabidopsis, constitutively expressed CRISPR/Cas9 systems usually generate chimeras in the first generation of transgenic plants with homozygous or biallelic plants not available until the T2 generation, thus increasing the complexity of genetic screenings [71, 112]. In contrast, germline specific, especially egg-cell specific systems, produce multiple homozygous and biallelic mutations in the T1 generation, saving labor and time. However, the available egg-cell specific systems still generate a high proportion of chimeras in the T1 generation, which may hamper its application for large scale genetic screenings, underpinning the need for novel promoters exhibiting early and continuous expression in egg-cells.

### The development of CRISPR/Cas9 systems for crop breeding

To generate heritable gene modifications in crop species, both the integrative and the non-integrative CRISPR/Cas9 approaches can be applied. In integrated systems, the transcription level of CRISPR/Cas9 in somatic embryos during tissue regeneration is critical and new promoter combinations could increase the efficiency of the system to obtain heritable modifications. In addition, it is important to remove the CRISPR/Cas9 sequences from the genome by conventional crossing to produce varieties with a "clean" background, a time consuming procedure especially for asexually propagated crops. These limitations could be overcome by using non-integrative CRISPR/Cas9 systems. Integration of the *Agrobacterium* T-DNA into the plant chromosome requires the activity of the VirD2 protein and strains lacking the C-terminal  $\omega$  domain of VirD2 are unable to integrate the T-DNA into the plant genome [135]. Tobacco cells infected with the  $\omega$  mutant strain, show transient expression of a GUS gene contained in the T-DNA for at least 2 days after infection, although such a short time might not be enough for the CRISPR/Cas9



system to induce mutations. An interesting alternative is the introduction of preassembled CRISPR/Cas9 complexes into protoplasts [62]. Transient expression of these ribonucleoproteins in plant cells can induce heritable gene mutations with an efficiency directly related to the transfection rate. The relative proportions and dosage of the synthesized sgRNA and Cas9 proteins could be finely adjusted before transfection in order to achieve maximal efficiency. The microcalli regenerated from mutated cells are either heterozygous or biallelic, therefore the probability of obtaining heritable gene modifications equals the overall mutagenesis frequency in transfected protoplasts. The main drawback of this approach is the difficulty to regenerate whole plants from protoplasts. Future modifications in the nature of the components of the CRISPR/Cas9 system could further increase efficiency with over 600 Cas9 orthologues identified so far, some of which show improved properties for targeted gene modifications in mammalian cells [136].

Targeted gene modification in plants is rapidly becoming routine owing to the rapid development of multiple CRISPR systems. These powerful tools can help to create a large variety of mutations in otherwise inaccessible genes, and can simultaneously target multiple loci or produce large fragment rearrangements, thus accelerating plant breeding programs. The generated genetic modifications are stably inherited and any introduced transgenes can be easily removed by segregation or backcrossing. In many cases, crops engineered using CRISPR/Cas9 are virtually indistinguishable from plants in which the mutation might have happened naturally and may not be regulated as GMO plants. In fact, the US Department of Agriculture has recently decided that a CRISPR/Cas9 engineered mushroom in which a polyphenol oxidase gene had been mutagenized does not require special regulation [59]. This mushroom is the first CRISPR/Cas9-edited crop exempted from regulatory approval. Several previous examples of crops bypassing regulatory approval were obtained using other gene-editing techniques such as ZFN and TALEN systems [137]. This is definitely encouraging news to scientists trying to apply the CRISPR/Cas9 system to other crops. Moreover, it was shown that mutations in the centromere-specific histone H3 variant CENH3 could be harnessed for the induction of haploids in Arabidopsis and several crop species [138, 139]. On self-pollination, these induced haploids can produce normal diploid seeds. The possibility of combining the CRISPR/Cas9 technology with this centromere-mediated genome elimination method for plant genetic engineering is certainly exciting. Considering that the production of new varieties with stacked traits can be accomplished in one generation by simultaneously editing multiple gene loci by CRISPR/Cas9, the speed of genetic research and crop breeding could undergo

a quantum leap. We anticipate that widespread adoption of the CRISPR/Cas9 technology will revolutionize basic and applied research in plant biology.

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**Compliance with ethical standards**

**Conflict of interest** The authors declare that they have no conflicts of interest with respect to this work.

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