REVIEW

CRISPR/Cas9 System: A Potential Tool for Genetic Improvement in Floricultural Crops

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Abstract

Demand of flowers is increasing with time worldwide. Floriculture has become one of the most important commercial trades in agriculture. Although traditional breeding methods like hybridization and mutation breeding have contributed significantly to the development of important flower varieties, flower production and quality of flowers can be significantly improved by employing modern breeding approaches. Novel traits of significance have interest to consumers and producers, such as fragrance, new floral color, change in floral architecture and morphology, vase life, aroma, and resistance to biotic and abiotic stresses, have been introduced by genetic manipulation. The clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein (Cas) system has recently emerged as a powerful genome-editing tool for accurately changing DNA sequences at specific locations. It provides excellent means of genetically improving floricultural crops. CRISPR/Cas system has been utilized in gene editing in horticultural cops. There are few reports on the utilization of the CRISPR/Cas9 system in flowers. The current review summarizes the research work done by employing the CRISPR/Cas9 system in floricultural crops including improvement in flowering traits such as color modification, prolonging the shelf life of flowers, flower initiation, and development, changes in color of ornamental foliage by genome editing. CRISPR/Cas9 gene editing could be useful in developing novel cultivars with higher fragrance and enhanced essential oil and many other useful traits. The present review also highlights the basic mechanism and key components involved in the CRISPR/Cas9 system.

Keywords CRISPR/Cas9 · Flowering plants · Genetic improvement · Gene expression · NHEJ · sgRNA · Trait improvement

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Abbreviations

CpYGF	*Chiridius Poppei* Yellowish-green fluorescent protein
PDS	Phytoene desaturase
NR	Nitrate reductase
C3H	Coumarate 3-hydroxylase
CH4	Cinnamate 4-hydroxylase
4CL	4-Coumarate: coenzyme A ligase
CCR	Cinnamoyl coenzyme A reductase
IRX	Irregular xylem 5
TfRAD	*Torenia Fournieri* RADIALSIS-like
CAPS	Cleaved amplified polymorphic sequence
DBP	DNA-binding proteins
DFR	Dihydroflavonol-4-reductase
EPH1	EPHEMERAL1
T7E1	T7 endonuclease 1
SSK1	S-locus F-box-interacting SKP1- like 1
CCD4	Carotenoid cleavage dioxygenase 4
F3H	Flavanone 3' –hydroxylase
ACO	1-Aminocyclopropane-1-carboxylate oxidase
DPL	Deep purple
NGS	Next generation sequencing
PCR	Polymerase chain reaction
Gt5GT	Anthocyanin 5-O-glycosyltransferase
Gt3'GT	Anthocyanin 3'-O-glycosyltransferase
Gt5/3'AT	Anthocyanin 5/3'-aromatic acyltransferase
GST	Glutathione S-transferases

Introduction

In ornamentals, various breeding strategies have been used in the past to produce new cultivars. Selection, hybridization, and mutation breeding are all traditional breeding procedures that have been employed since time to produce a variety of colors and forms, crop architecture in the case of foliage plants, and resistance to biotic and abiotic stresses. However, in ornamental plants, traditional methods have several limitations, including the fact that they are time-consuming and that majority of the ornamentals are heterozygous, resulting in polyploidy, and complex genetic components transmission [1, 2]. As a result, new approaches for improving decorative plant production are the need of time. Genome sequencing technology has had a substantial impact on the improvement of flowering crops in recent decades. More than 69 ornamental plants (whole-genome/ draft genome) have been sequenced yet [3]. The nuclear genome of cultivated hybrid rose [4], wild rose [5], scarlet sage [6], petunia [7], sunflower [8], orchids [9], and other ornamentals has been sequenced. Transcriptome data are available for chrysanthemums [10, 11], osmanthus [12], petunia [13], hydrangea [14], lavender [15], and carnation [16]. The chloroplast genome sequence is also available for a few ornamental plants [17]. Genome sequence information on important decorative plant species would be immensely beneficial in ornamental plant breeding and research following the debut of next-generation sequencing (NGS) technologies in 2005. Genetic transformation is an essential requirement for the development and improvement of flowering crops when distinguished with mutation breeding, which delivers a "subtractive" one-point improvement in flowers [18]. Ever since the very first transformation in ornamentals described in 1987, genetic transformation is acknowledged as an important method to develop desirable cultivars [19, 20].

CRISPR/Cas9 is an approach for genetic modification with immense capability and simpler implementation that has accelerated crop improvement research. CRISPR/Cas9 genome-editing technology has been employed in agriculture as well as horticultural crops and found to be effective in crop improvement [20–23]. CRISPR/Cas-based system has progressed to allow for a wide range of applications. These include CRISPR interference (CRISPRi) and CRISPR activator (CRISPRa) gene regulators, as well as development as a base editor, prime editor, and epigenetic editor. It can also be utilized for imaging and chromatin and RNA targeting. Its application has revolutionized a wide range of biological domains, including biomedical and agricultural research. CRISPR has also been created as a diagnostic tool to aid in the detection and screening of both human and plant diseases, and it was even used during the coronavirus (COVID-19) pandemic [24].

CRISPR/Cas9 Genome Editing

Significant improvement has been made in the development of effective approaches for targeted editing of the plant genomes, over the last three decades [25, 26]. Before the introduction of the CRISPR/Cas9 technology in 2013, plant genome engineering relied on sequence-specific nucleases such as zinc finger nucleases (ZENs) and transcription activator-like effectors nucleases (TALENs) [27]. There are two types of genome-editing tools that used double-strand break (DSB) nucleases. MNs (meganucleases), ZFNs, and TALENs come under the first group, that use protein–DNA interactions to enable sequence-specific DNA binding [28, 29], and the second group is further divided into two sub-groups: (a) RNA-guided system (CRISPR/Cas9 and targetrons) [30, 31] and (b) DNA-based-guided systems [structure-guided endonuclease (SGNs), peptide nucleic acids (PNAs), and triplex-forming oligonucleotides (TFOs)] [32–34].

The presence of pre-configured CRISPR/Cas9 ribonucleoproteins (RNPs) enables DNA-free genome editing in plants, without the need for codon optimization or highly specialized regulators for expression in host cells. An efficient protoplast transformation by manipulating

the molar ratio of Cas9 and sgRNAs was optimized for grape and apple. The targeted mutagenesis insertion and deletion rate was analyzed with the help of targeted deep sequencing. Direct delivery of CRISPR/Cas9 RNPs to the protoplast system enables targeted gene editing and paves the way to the generation of DNA-free genome edited in plants [35]. Moreover, Cas9 RNPs break the target DNAs shortly after transfection and are thus quickly destroyed in cells, significantly lowering the number of undesired alterations at off-target sites [36, 37]. Preassembled CRISPR/Cas9 ribonucleoproteins can also help circumvent GMO regulations over their release as cultivars since there is no transgene integration [38, 39].

Off-target effects, which are usually caused by unanticipated cleavage at genomic locations that are similar to the target sequences, are one of the key concerns about gRNA design [40]. There are few online off-target prediction tools such as Burrows-Wheeler Alignment Tool (BWA), Bowtie [41], CCTop [42], and Cas-OFFinder [43] that can be used to predict probable off-target sites [44].

To date, the major utilization of the CRISPR/Cas9 technology in plants has been to create gene knockouts. It has also been successfully employed to create gene knockouts in ornamental plants to induce genetic alterations in *Petunia inflate* [45], *Petunia hybrid* [27, 37, 46, 47], *Chrysanthemum morifolium* [48], *Dendrobium officinale* [49], *Torenia* [39], *Ipomoea nil* [50–52], *Lilium longiflorum* and *Lilium pumilum* [53], and *Phalaenopsis equestris* [54]. These reports suggest that CRISPR/Cas9-induced mutagenesis is effective in ornamental plants. The alteration produced is precise [55, 56] and could be passed on to succeeding generations by employing the classic Mendelian segregation [57]. Further in-depth information about CRISPR technology, reports by many research groups [34, 44, 58–60] and may be referred for future research on the subject.

Among several transformed and genetically modified (GM) ornamental plants, few regulatory approvals have been made [61]. Certainly, the regulatory approval process has a direct effect on the economics of product development. Only three ornamental species are recorded in the International Service for the Acquisition of Agribiotech Applications (ISAAA) database as permitted biotech/GM crops: carnation, petunia, and rose. To date, only carnation and rose are allowed in the market of a few nations, conditional to their GMO regulations [61, 62]. Only two GM carnation cultivars are now allowed to be sold as cut flowers in Europe [63]. Many GM crops, both edible and in-edible, are still under research or in limited field trials and are ready for commercialization, despite considerable opposition from some farmers and NGOs being concerned about their safety and effects on biodiversity.

The Fundamental Mechanism of the CRISPR/Cas9 System

Prokaryotes show adaptation and heritable immunity is one of the most exciting findings in microbiology in recent decades [64]. In archaea and bacteria, the CRISPR/Cas9 system has been identified as a component of the immune system that guards the host against invading phages including plasmid DNA [65]. Tandem repetitions of viral genomic segments were discovered in bacterial cells to be consistently interspaced with non-repetitive sequences, a phenomenon known as CRISPR [66]. The sequence of 29-nucleotides, separated by several 32-nucleotide spacer sequences, was discovered first in the *E. coli* genome [67]. Similar sequences were discovered in various bacteria and archaea [21, 68].

By acquiring phage-derived spacer sequences that protect against re-infection, the CRISPR array acts as a repository for previous infections [65]. The Cas9 proteins are encoded by a cluster of genes near the CRISPR array, which controls the three steps of the process: adaptation, CRISPR RNA (crRNA) synthesis, and interference. To offer a memory of infection, bacteria may transfer portions of external DNA into their own genomes during adaptation. When the CRISPR array is translated into two short RNAs, crRNA and trans-activating CRISPR RNA ("tracrRNA"), memory is retained [21, 64] (Fig. 1).

Following infection, the interference machinery recognizes a particular region in the target nucleic acids by complementary base pairing, causing Cas9 enzyme-catalyzed target cleavage [69]. CRISPR/Cas9 system is designed to protect prokaryotes from viruses by undermining the
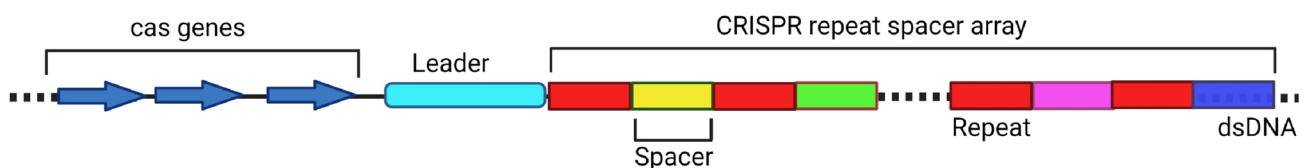
Fig. 1 The CRISPR array serves as memory storage for previously acquired infections. The Cas proteins are encoded by a cluster of genes situated near to the CRISPR array, which controls the three phases of immunity. Non-repetitive sequences were discovered to be frequently interspersed between tandem repeats

selfish, often hostile instructions encoded by invading mobile genomic elements (MGEs). The type II system, which has originated from *Streptococcus pyogenes*, controls the cleavage of DNA at specific site which makes it a good option for genetic modification [70–72]. Several reports have detailed the biology of the CRISPR/Cas9 system, [64, 69, 73, 74].

To create a single molecule, tracrRNA and crRNA can be linked artificially, forming a single-guide RNA (sgRNA) that is complementary to a particular segment of a target sequence (about 20 nucleotides) at a location where a protospacer adjacent motif (PAM) is present [68]. The PAM is a short sequence motif found close to the protospacer on the invading DNA. Cas9 is led by sgRNA and utilizes the PAM sequence to identify the target region and distinguishes between self and non-self nucleotide sequences. The CRISPR locus is protected against self-cleavage by the absence of the PAM sequence in the host genome. In addition, sgRNA includes a "scaffold" sequence required for Cas9 engagement. The sgRNA guides Cas9 and uses the PAM sequence to identify the target location [21, 75].

The CRISPR/Cas9 defense pathway is divided into three steps (Fig. 2). At the time of adaptation cycle, the Cas1–Cas2 complex, which is made up of dual Cas1 dimers and a single Cas2 dimer, a protospacer from intruding viral DNA which is inserted as a new spacer into the host's CRISPR array.

The CRISPR array is subsequently transcribed into a long pre-crRNA, which is then processed into mature crRNAs by Cas9 proteins or, in certain cases, cellular RNases, each of which contains a transcribed spacer and a portion of the repeat sequence, thereafter the crRNAs form ribonucleoprotein complexes. During the interference phase, mature crRNAs drive Cas9 nucleases to the relevant foreign DNA by using complementary base pairing to identify the invading DNA. The invading genome is destroyed when the target sequence is cleaved. Multi-Cas9 protein complex acts as an interference mechanism in class 1 systems, class 2 systems, on the other hand uses one Cas9 protein to cleave the target [64]. Endonuclease cleaves DNA in a specific region of the cell, causing homology-directed repair (HDR), non-homologous end joining (NHEJ) or microhomology-mediated end joining (MEEJ) [57, 76]. HDR is a genetic modification that enables the results precision editing of repair templates [77]. MMEJ is an error-prone repair approach that combines insertions and deletions with micro homologous sequence placement inside damaged ends prior to joining [78]. Because NHEJ lacks a DNA repair template, it is also prone to errors, which frequently results in inactivation mutations [79, 80]. NHEJ is the most prevalent kind of DSB repair, which is

Fig. 2 The three stages of the CRISPR-Cass9 defense system are as follows: Cas complex gets the protospacer from invading viral DNA during the adaptation stage and incorporates it as a new spacer into the host's CRISPR array. The CRISPR array is subsequently translated into a lengthy pre-crRNA, Cas9 proteins then convert into mature crRNAs. With the aid of Cas9 proteins, these crRNAs generate RNP complexes. During the interference stage, Cas nucleases are directed by mature crRNAs, to the relevant foreign DNA using complementary base pairing to detect the invading DNA. The invading genome is eliminated when the target sequence is cleaved
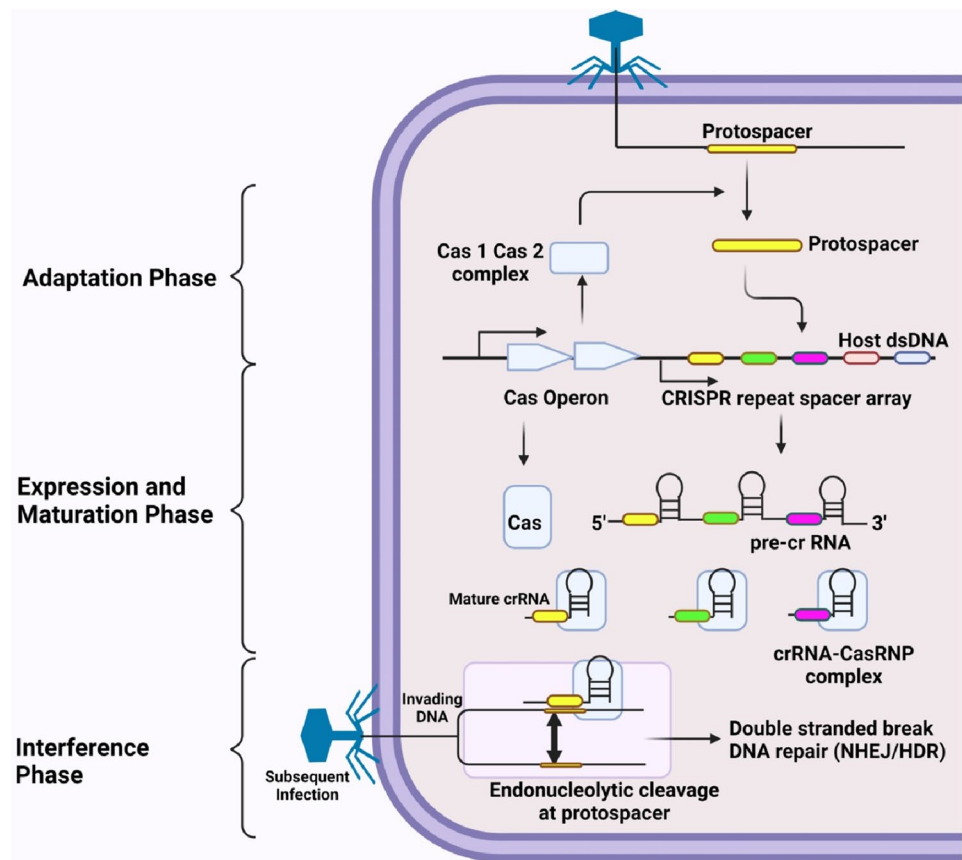
defined by the fusion of the broken ends, might be resulting in one or more random nucleotides insertions or deletions (indels), leading to partial repair and mutations [81]. NHEJ is primarily responsible for DSB repair in somatic cells [27, 82]. When DSBs are repaired, NHEJ typically results in gene deletion or protein function impairment. Chemically stabilized double-stranded oligo-deoxy-nucleotides (dsODNs) donors with 5-phosphorylated ends can likewise be used to drive targeted insertion via NHEJ [83, 84]. The target has a low mutation frequency owing to the difficulty in adjusting the artificial DNA repair template [21, 85, 86].

Approaches to Applying CRISPR/Cas9 System in Plants

Major Web-Based Resources

One of the most fundamental elements is to guide precise changes in the genomic sequences in the desired way, which is highly dependent on sgRNA-directed identification of targeted DNA sequences. CRISPR-PLANT, CRISPR-P, and CRISPR-GE are online sgRNA design resources for plants. These tools usually provide empirical CRISPR/Cas vectors and methods that are highly valuable for wet lab investigations. By gathering sgRNA data from large-scale CRISPR/Cas research, several web-accessible databases have been developed. These platforms not only provide useful resources for sgRNA selection, but also highlight the major parameters that influence sgRNA efficacy and specificity, allowing for further sgRNA design optimization [44].

Transformation Method

The majority of CRISPR/Cas9 studies have used *Agrobacterium*-mediated transformation to introduce exogenous DNA into ornamental plants. A key advantage of *Agrobacterium*-mediated transformation is its higher incidence of single transgene insertion. Moreover, several of the most often employed *A. tumefaciens* strains in plant transformation (e.g., *AGL1, C58C1, GV3101, LBA4404,* and *EHA105*) show enhanced resistant tissue tolerance or greater adaptability to the preferred plant species, which can boost transformation efficiency. The hypervirulent strains *EHA105, AGL1,* and *LBA4404* are likely to be the outcome of increased *vir* gene induction. These strains are employed to transform recalcitrant or monocot plants, whereas milder strains are utilized to transform non-recalcitrant dicotyledonous plants [87]. In plants that are sensitive to *Agrobacterium*-mediated leaf-disk transformation, sequential transformation is an alternative for Cas9-mediated genome alteration. Because the significantly larger size of Cas9 expression cassette (usually greater than 5 kb), manipulation in destination vectors is difficult. To overcome this problem, sequential transformation method of introducing Cas9 and sgRNA expression cassettes sequentially into plants makes CRISPR/Cas9-mediated genetic changes smoother. The efficiency of CRISPR/Cas9-mediated targeted genome mutagenesis is unaffected by genome size [27]. Although recent CRISPR/Cas9 toolkits, based on Golden Gate ligation or Gibson Assembly, have made it easier to assemble numerous sgRNA expression cassettes [88, 89], sequential transformation retains some advantages, including improved transformation capabilities.

The transmission of CRISPR/Cas9 nucleases through *Agrobacterium tumefaciens* allows for the continuous expression of CRISPR complexes, leading to a variety of chimera genotypes in tissues or organisms. In petunia, Yu et al. [37] used a Cas9 RNP-mediated transformation technique and discovered no chimeric genotypes in every regenerated plant based on next-generation sequencing data, as reported previously by Woo et al. [38]. Cas9 RNPs cleave the target DNAs as they are transected and then decay quickly in protoplasts, resulting in genotypes that are homozygous or heterozygous instead of being chimeric. When using the CRISPR/Cas9 system to target a particular gene, orthologous or paralogous genes that are found frequently in plant speciation must be examined. A full change in flower color was achieved in petunia by simultaneously targeting both loci of F3H (A and B) with CRISPR/Cas9 RNPs [37].

Due to the high percentage of chimeras and low efficiency of transformation, only a few *Lilium* transformations have been reported. For the first time, Yan et al. [53] used *L. pumilum* embryogenic callus as a target material for *Agrobacterium*-mediated transformation. Each embryogenic cell of the callus can develop into a somatic embryo; it could diminish or eliminate chimeras if used as explants for *Agrobacterium-mediated* transformation. Although the transformation cycle is quite long, cell proliferation and transformation efficiency are high when embryogenic callus is employed as a recipient, and chimeras are rare. On the other hand, despite the disadvantages of low transformation efficiency and a high number of chimeras, using vegetative tissue scales as a transformation recipient is a reasonably easy operation that takes far less time [53].

Plant protoplasts offer a flexible system for DNA-free genome editing. Protoplasts are plant cells without cell walls that provide a useful transient system for evaluating the efficacy of gene-editing reagents before moving forward with a more labor-intensive transformation process [90, 91]. PEG-mediated transfection and electro-transfection are two popular ways of delivering plasmid DNA, in vitro transcripts, or RNPs into protoplasts for CRISPR cassette transient expression. Once successful protoplast isolation, transfection, and regeneration have been established in a plant species, it could be used as a high-throughput platform for gene editing [92]. The usage of the protoplast technology can

be advantageous because a high level of transgenic expression could be achieved [76, 93]. The protoplast transient expression system has been demonstrated to be an effective tool for CRISPR/Cas9 genetic modification in plants. A robust mechanism aids Cas9 transfection in *Petunia hybrida*. Cas9 and gRNA complex-mediated genetic manipulation through plasmid vector elicited target alteration frequencies of 21 and 13% in rice and maize protoplast systems, respectively, after 72 and 48 h post-transfection [76, 94]. Within 24 h of transfection, almost comparable mutation frequencies (20%) were generated in the petunia protoplast system, demonstrating that the direct delivery of RNPs is more successful than the vector-based approach. In animal cell lines, direct administration of recombinant Cas9 proteins resulted in genetic variation ranging from 16 to 72% [36]. Differences in mutation rates between plant and animal systems could be attributed to experimental differences. Direct administration of recombinant Cas9 protein caused specific mutations in *Arabidopsis*, rice, and tobacco protoplasts with 17 to 23% efficiency, which was comparable with previous findings of Subburaj et al. [46] in petunia protoplasts [38]. One of the significant advantages of employing direct Cas9 protein administrations is the ability to induce mutations quickly and precisely [36, 38].

The introduction of CRISPR/Cas9 components via floral dip method is both cost-effective and simple. This is the most widely used approach for genome editing in *Arabidopsis*. Due to limited flower and seed production, floral-dip-mediated administration of CRISPR/Cas9 components is confined to a few plants such as *Arabidopsis*, flax, and tomato [95]. The key benefit of this delivery method was that it did not necessitate the use of a plant tissue culture facility.

Co-delivery of developmental regulators (DRs) with CRISPR reagents via *Agrobacterium* can expedite and improve gene-editing efficiency in plants. Developmental regulators are genes involved in dictating meristem identity in plants. Ectopic expression of DRs in plants has resulted in somatic embryogenesis [96]. This phenomenon was leveraged to induce de novo meristems in somatic tissues by injecting *Agrobacterium* cultures co-delivering DRs and gene-editing cassettes directly into soil-grown plants [92].

Heritable changes have traditionally been achieved through persistent expression of CRISPR cassettes and regeneration of transgenic progenies. Tobacco rattle virus (TRV) [97], Barley stripe mosaic virus (BMSV) [24], and Sonchus yellow net rhabdovirus (SYNV) [98] are autonomously replicating viral vectors delivered into plants via *Agrobacterium* that provide an alternative for heritable gene-editing in plants.

Nanotechnology has made significant advances during the last decade especially in relation to plant genetic engineering. Recent reports have highlighted the importance of nanomaterials such as carbon dots, carbon nanotubes (CNTs), and mesoporous silicon nanoparticles (MSNs) to deliver biomolecules such as DNA, RNA, RNPs, and proteins [99]. In plants, nanoparticle-mediated administration of DNA and proteins into nuclear and chloroplast genomes has been achieved [100, 101].

Biolistics, also known as particle bombardment, is a popular method for plant transformation especially in plants that are not amenable to *Agrobacterium* infection. However, random incorporation of DNA at multiple genomic loci and labor-intensive production of explants such as calli or immature embryos are two major limitations of biolistic delivery. In cereal crops, however, bombarding RNPs instead of plasmid DNA was effectively demonstrated to produce marker-free gene-edited plants [102–104].

Pollen transformations by *Agrobacterium* and biolistic method bypasses tissue culture regeneration step but frequently results in pollen with poor viability [105, 106]. Furthermore, pollen-tube conversions can lead to chimerism [107]. While pollen magnetoreception has made progress in addressing these issues [106], it is still limited to dicots [108]. Nanoparticles as delivery engines for plant genome editing are also promising [109], further progress is needed to make plant gene editing easier [92].

CRISPR/Cas9 Specificity

Watanabe et al. [50] observed that roughly 75% (24/32) of transgenic plants were biallelic mutants (mutations that occur on both alleles of a single gene) at the locus of interest in one generation, thus the CRISPR/Cas9 system has been found to be an extremely effective and useful tool for next-generation breeding. Moreover, the efficacy of editing is dependent on sgRNA [56, 57], and researchers are working to improve sgRNA design to boost its activity [110]. Using an optimization tool, the editing efficacy in *Ipomoea nil* can be boosted further [50, 111, 112]. One-third of stable transgenic plants suggest that CRISPR/Cas9 system mutation frequency in *Ipomoea nil* is significantly higher compared to higher plants [113]. Hoshino et al. [114] presented a high-quality genome sequencing of *Ipomoea nil* that enables the creation of sgRNA intended to prevent targeted mutagenesis. The application of CRISPR/Cas9 technology on *Ipomea nil* could provide a useful model for learning some of the key features including flower color, shape, and floral lifespan.

Kui et al. [49] demonstrated that the CRISPR/Cas9 system is capable of precisely inserting mutations into targets. However, compared with the efficacy of different targets in each candidate gene, they discovered differed mutation rates of these target sites and some targets to be completely immutable. One reason could be a difference in the greater chromatin structure of certain target regions. Some target locations on the chromosomes may be tightly folded, whereas

others may be more loosely folded, resulting in a variable Cas9 protein and sgRNA complex accessibility. An appropriate expression system to drive Cas9 and sgRNA is necessary for the initial utilization of genome editing in plants. A higher mutation frequency may result from the use of more effective target locations. The effectiveness of genome editing appeared to vary among callus clones; it is likely that the higher amount of Cas9 expression tends to boost mutation efficiency, as seen in rice calli [48, 115].

The ability to perform targeted deletions between two Cas9 cut sites is a key feature of CRISPR/Cas9 technology. The capability of the system was originally tested using transient systems [116, 117]. The efficiency of fragment deletion was also shown to be adversely linked with the distance between two paired gRNA/Cas9 cut sites employing transient systems [118]. Other parameters that include cell type and cell division phase, length of deletion, the efficiency of various sgRNAs, orientation and base structure of two paired sgRNAs, and target locations and context may contribute to effective deletion [57]. After identifying genome-edited transgenic plants, T-DNA containing Cas9 can be separated through selfing or crossing, and the progeny plants that are Cas9-free can be analyzed [45].

Mutation Detection

PCR/restriction enzyme assay, TaqMan qPCR test, T7 Endonuclease I, and high-resolution melting (HRM) analysis are available to evaluate the effect of genetic mutation [119]. As compared to the above approaches, sequencing analysis is preferred for evidence-based genome editing. The novel genetic engineering methods are much more accurate than the basic tools of genetic engineering developed earlier.

Applications of CRISPR/Cas9 System in Floriculture

Flower color is among the most essential characteristics of commercial flower production and it is mostly produced from betalains, carotenoids, and flavonoids [63, 120]. Flower pigmentation had first been suppressed by inserting antisense chalcone synthase (CHS) or dihydroflavonol-4-reductase (DFR) genes into transgenic lines, which produced flower alterations in 0–89% of transgenic lines; however, the extent of color lightening differed between lines [121]. Floral color suppression was next achieved using RNAi-targeted *CHS* or *ANS* (*anthocyanidin synthase*) genes, revealing that RNAi was a more effective (more than 50%) strategy for producing a stable white flower color phenotype in torenia [122, 123]. RNAi has also been used to block *F3H* gens in torenia plants, resulting in white blooms [39, 124].

Nowadays, desired flower colors have been generated through the application of genetic modification techniques, Fig. 3. In this context, Watanabe et al. [51] used CRISPR Cas9-mediated mutagenesis to modify color in higher plants by modifying the carotenoid cleavage dioxygenase (*CCD*) gene in *Ipomea nil* and obtained pale yellow petals mutant (55.5%) plants. In a previous study, Watanabe et al. [50] targeted *dihydroflavonol-4-reductase-B* (*DFR-B*) gene, which encodes an anthocyanin biosynthetic enzyme, and observed changes in stem color throughout the initial phase of plant tissue culture. Because of biallelic changes in the Cas9 cleavage site in DFR-B, 75% of transgenic plants developed anthocyanin-free white flowers, with single base insertion or deletion of more than two bases. Nishihara et al. [39] used the CRISPR/Cas9 system to detect color changes in the *Torenia fournieri* from blue to white (approximately 80%) due to genetic variation of *flavone 3-hydrolase* (*F3H*), which encodes the critical enzyme for flavonoid biosynthesis. Su et al. [125] also explored *T. fournieri*, and observed that the abnormal expression of *TfCYC2* (CYCLOIDEA) or *TfRAD1* (RADIALIS) disturbed the asymmetric corolla pigmentation pattern, resulting in severely dorsal flowers, which ensures that the CYC-RAD module controls petal shape and corolla pigmentation. When *TfCYC*2 was down regulated or *TfRAD*1 was knocked out, dorsal petal identity was lost. The CRISPR/Cas9 technique was utilized to modify the *phytoene desaturase* (*PDS*) gene, which is a key enzyme in the production of carotenoids and necessary for chlorophyll biosynthesis. Zhang et al. [27] altered petunias using a CRISPR/Cas9 construct that targeted PDS and obtained a 55–87% albino phenotype. Also in *Lilium pumilum* (DC Fisch) and *Lilium lonfiflorum* (White heaven), the *LpPDS* gene was knocked out, and the mutants were observed to be completely albino, albino green, and pale yellow. The overall mutation rates for Fisch and White heaven cultivars were 69.57% and 63.64%, respectively [53]. Tasaki et al. [126] targeted genes in the *Japanese gentian* including *anthocyanin 5/3'-aromatic acyltransferase* (*Gt5/3'AT*), *anthocyanin 5-O-glycosyltransferase* (*Gt5GT*), and *anthocyanin 3'-O-glycosyltransferase* (*Gt3GT*) and concluded that for the production of blue flowers, glycosylation, following acylation of the 3'-hydroxy B-ring group in delphinidin aglycone is required. Tasaki et al. [127] also further described a type of *glutathione S-transferases* (*GST*) indicating that *GST1* is a gene that is involved in anthocyanin movement in gentian flowers and is required for gentiodelphin accumulation.

Yu et al. [37] used Cas9-ribonucleoprotein delivery to create a petunia mutant line with mutations across both *F3H* genes, resulting in a pale purple-pink flower color. Only the *f3ha-f3hb* mutant plants had visible changes, pale purplish-pink flower color, while the rest, including plants with a single-copy gene knockout, had purple-violet flowers that resembled wild-type petunia. Chib et al. [128] established
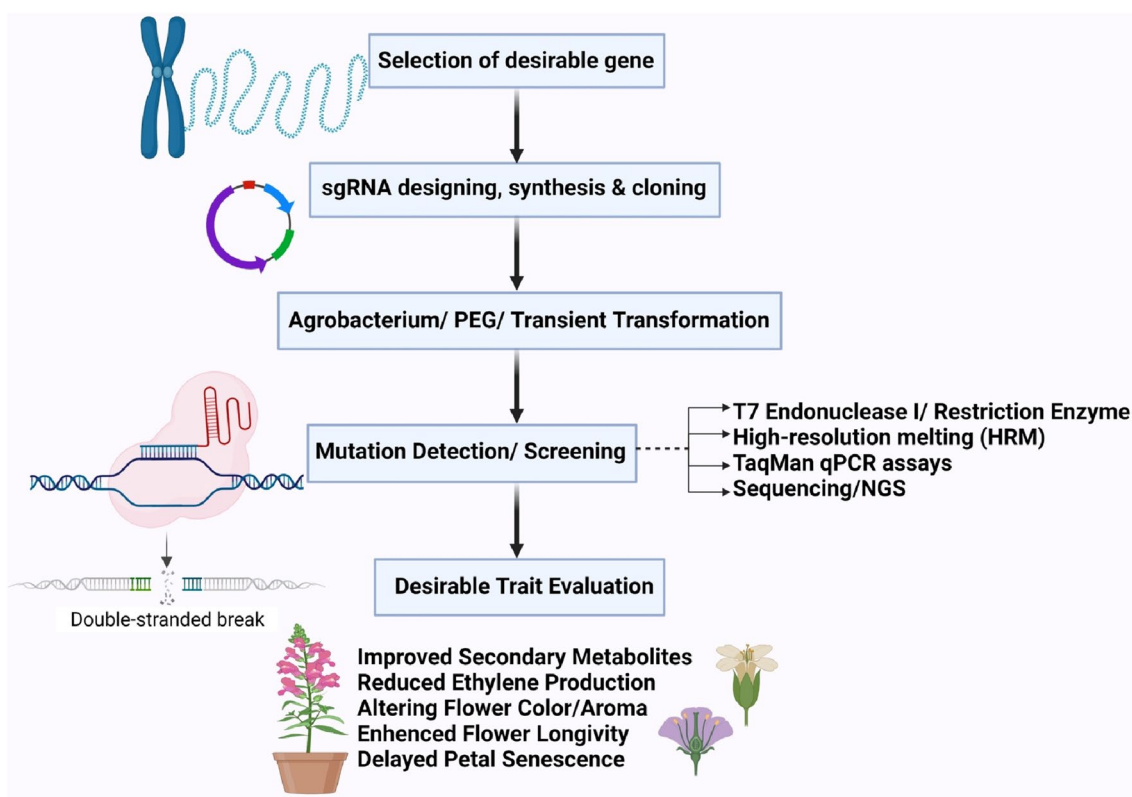
Fig. 3 CRISPR/Cas9 genome editing application for ornamental plants improvement is depicted schematically

a CRISPR/Cas9-based system for future research advancements in saffron (*Crocus sativus* L.). Zhang et al. [129] presented evidence for redefining DPL's role (DPL: THE R2R3-MYB transcription factor, which is connected to anthocyanin, has been linked to the regulation of corolla tube venation) in Petunia plants. The disappearance of the vein-associated anthocyanin pattern above the abaxial surface of the flower bud was produced by the CRISPR/Cas9-mediated mutation at *DPL*, but not corolla tube venation, suggesting that *DPL* had no influence over corolla tube venation development. Nitarska et al. [130] utilized the CRISPR/Cas9 system to knock out *flavonoid 3'-hydroxylase* (*F3'H*) from red blooming poinsettias (*Euphorbia pulcherrima*) cultivar 'Christmas Eve,' expecting plants with orange bracts and strong pelargonidin accumulation. The enzyme F3'H is required for the synthesis of cyanidin-type anthocyanins, which give poinsettia bracts their red color. Despite the fact that F3'H was not totally inactivated, transgenic plants' bract color changed from vivid red to brilliant reddish-orange, and cyanidin levels reduced considerably as compared to wild type. The *F3'H* encodes a significant enzyme in the flavonoid/anthocyanin synthesis pathway and has been mentioned as a potential target in floral color engineering.

Flower longevity is among the most important characteristics of attractive flowering plants. The majority of the flowering plants show a decrease in floral lifetime due to increased ethylene production [131]. Previously, Liu et al. [132] used virus-induced silencing to silence suspected ethylene signaling components EIL1 and EIL2 in petunia. They also observed that the *EIL* genes are functionally redundant and that down regulating the two genes affects floral bloom lifespan. However, *EIL* gene silencing resulted in no phenotypic changes in transgenic plants other than increased flower longevity, demonstrating that *EIL* genes have a specific function [132, 133]. Flower lifetime can be increased by reducing ethylene production, which can be reduced by targeting a critical enzyme in the ethylene metabolism [*1-aminocyclopropane-1-carboxylate oxidase* (*ACO*)] [134, 135]. Xu et al. [47] targeted *1-aminocyclopropane-1-1carboxylase* (*PhACO*) genes (*PhACO1, PhACO3,* and *PhACO4*) and observed that *PhACO1*-edited mutant lines produced significantly less ethylene and had significantly longer flower lifespan. In another study, Xu et al. [136] modified the *PhACO3* and *PhACO4* genes in petunia cv. Mirage Rose through CRISPR/Cas9 editing showed a reduction in ethylene and extended flower longevity. EPHEMERAL1 (*EPH1*) is a NAC transcription factor and is thought to be a key regulator of Japanese Morning Glory (*Ipomoea nil*) petal senescence. Petal senescence was delayed after

targeted mutagenesis of the *EPH1* gene [52]. Ethylene is also thought to be an essential seed germination regulator. Naing et al. [137] observed that three distinct petunia mutants (*phaco1, phaco3,* and *phaco4*) revealed considerably lower germination percentage, along with delayed germination time and seedling growth when compared to wild-type plants. To reduce the negative impacts of ethylene suppression on seed germination and plant growth, it is critical to reevaluate when modifying the ethylene production route to maintain postharvest flower quality.

The initiation and growth of flowers are important stages in the life cycle of an ornamental plant. CRISPR/Cas9 system-based genome editing can improve various floral traits for profit [61]. Orchids are the members of the *Orchidaceae* family, the second largest family of flowering plants, and have significant commercial importance on a global scale [138]. However, very little genome sequencing data on orchid species are available such as *Dendrobium catenatum* [9], *Dendrobium officinale* [139], *Gastrodiaelata* [140], *Phalaenopsis aphrodite* [141], and *Phalaenopsis equestris* [142]. Kui et al. [49] targeted five genes in the orchid family's lignocellulose biosynthesis pathway (*9C3H, 4CL, C4H, CCR, and IRX*) and revealed that the CRISPR/Cas9 system may cause mutation at a rate of 10–100% for each target. Tong et al. [54] successfully produced several MADS-box mutants (97.9%) in the orchid *Phalaenopsis equestris;* however Semiarti et al. [143] observed low transformation efficiency (0.96%) in *Phalaenopsis anabilis* targeting, but genome editing using the CRISPR/Cas9 system is complex and challenging due to the huge size of the genome and higher genomic ploidy levels, as well as a higher amount of repetitive sequences. There is only one study in which the CRISPR/Cas9 technology was applied to Chrysanthemum [48]. CRISPR/Cas9 system targeted *Chiridius poppei* yellowish-green fluorescent protein gene (*CpYGFP*) and induced mutation efficiency of 0–28.9%. In the *Petunia hybrida* protoplast system, Subburaj et al. [46] demonstrated site-directed mutagenesis via direct administration of pure Cas9 protein preassembled with guide RNA to efficiently create mutations in *nitrate reductase* (*NR*) genes. The use of CRISPR/Cas9 to create frameshift indel mutations in *PiSSK1* (S = locus F-box-interacting SKP-like 1) gene in *Petunia inflata* demonstrates the gene's involvement in self-incompatibility and supports the notion that SLF-containing SCF complexes are essential for compatible pollination [45].

The CRISPR/Cas9 system has evolved into a versatile and cutting-edge tool that allows scientists to manufacture any desirable alterations in plants voluntarily, allowing plant mutants to be created. Unlike traditional genetic approaches that require extensive breeding cycles, CRISPR alters a desired feature in a site-specific manner in a few generations [37]. To the best of our knowledge, this report includes all of the CRISPR/Cas9 research on ornamental plants that have been reported to date, Table 1.

Conclusion

CRISPR/Cas9 technology has been accepted to be effective in genome editing to achieve desirable characteristics in flowering crops. Genome editing in a targeted manner of genes that regulate desirable traits like flowering promotion, both by raising the number of flowers and altering flowering time and longevity, color spectrum, aromas, and creation of innovation in flower structure can help to develop desirable genotypes that can fulfill the void of modern demands and also, be fruitful for the investors or producers. It can be concluded that editing specific genes is an excellent way to enhance the characteristics of existing floriculture plants and allows us to make the most of the usage of ornamental plants for global competitiveness.

However, there are a few key drawbacks to this cutting-edge technology that must be addressed. Plant tissue culture-based gene transformation is critical for CRISPR/Cas9 based genome editing in plants. *Agrobacterium*-mediated gene transformation is currently the most efficient technique to obtain genome editing events, although it is confined to a small number of plant species. Hence, establishing a new transformation process that does not require plant regeneration is critical. Although plant tissue culture-based approaches can produce genome-edited plants, various difficulties, such as induced mutations or somaclonal changes during plant tissue culture, as well as the lengthy process, have hampered CRISPR/Cas9 technology's applicability. Thus, finding a delivery approach that is not dependent on tissue culture could progress the CRISPR/Cas9 genome editing.

The most common method of administering gene-editing reagents is through *Agrobacterium*, which has a narrow host range, and some plant species are resistant to transformation by *Agrobacterium*. The use of *A. rhizogenes*, which can substantially minimize the time between reagent administration and mutation evaluation, as well as expand the spectrum of species changed are two further ways to circumvent the regeneration process [92, 144, 145].

Off-target effects are a major concern with CRISPR/Cas9 genome editing as it may have an impact on precise breeding by changing other essential agricultural features. When Cas/sgRNA genes are incorporated into the genome, they become permanently produced in the cells, potentially causing off-target effects. Off-target effects may be reduced if the Cas9/sgRNA genes are not introduced into the plant genome and only reside in the target cells for a short duration [24]. Off-target effects can also be reduced by designing high-fidelity sgRNA and using the correct Cas9 enzymes and

Table 1 Progress of CRISPR/Cas9 genome editing in ornamental plants

Plant species	Target gene	Gene function	Transformation method	Material transformed	Edited genome trait Results	Mutation efficiency (%)	Mutation detection methods	References
Populus tomentosa Carr. (clone 741)	PtoPDS	Key enzyme in carotenoid biosynthesis	Agrobacterium-mediated	Leaf	Albino phenotype was observed	51.7%	PCR, sanger sequencing	[147]
Chrysanthemum Morifolium(Sei-Marin)	CpYGFP	Express yellowish-green fluorescent protein (marker)	Agrobacterium-mediated	Leaf	Disruption in fluorescence protein	0–28.9%	PCR, DNA sequencing	[48]
Petunia Hybrid (Madness)	NR	Facilitate nitrogen uptake & nitrate metabolism	PEG-mediated	Protoplast	Deficiency in nitrate assimilation	5.30–17.83%	T7E1 assay, PCR, Illumina sequencing	[46]
Petunia hybrid (Mitchell Diploid)	PDS	Key enzyme in carotenoid biosynthesis	Agrobacterium-mediated	Leaf	Albino phenotype	55.6–87.5%	PCR, DNA sequencing	[27]
Dendrobium officinale	C3H, C4H, 4CL, CCR, IRX	Lignocelluloses biosynthesis pathway	Agrobacterium-mediated	Protocorm	Reduced ligno cellulose biosynthesis	10% to 100%	PCR, DNA sequencing	[49]
Torenia fournieri	TfRAD1	Regulate petal shape & corolla pigmentation	Agrobacterium-mediated	Leaf sections	Flowers with abnormal shape & pigmentation	ND	PCR, DNA sequencing	[125]
Petunia inflate	PiSSK1	SCF's subunit, degrade non-self S-RNases non-self S-RNases	Agrobacterium-mediated	ND	Self-incompatibility	50%	PCR, DNA sequencing	[45]
Ipomoea nil (Violet)	DFR-B	Anthocyanin biosynthesis enzyme	Agrobacterium-mediated	Immature embryo	Anthocyanin-less stems, leaves & flowers	75%	CAPS analysis, DNA sequencing	[50]
Torenia fournieri L. (Crown Violet)	F3H	Flavonoid biosynthesis	Agrobacterium-mediated	Leaf	Pale blue (almost white) flowers	80%	PCR, Sanger & next-generation sequencing	[39]
Ipomoea nil (Violet)	EPH1	Key regulator of petal senescence	Agrobacterium-mediated	Immature embryo	Delay in petal senescence	N D	CAPS analysis, DNA sequencing	[52]
Ipomoea nil (AK77/ Morning glory)	CCD	Carotenoid degradation	Agrobacterium-mediated	Immature embryo	White petals to turn pale Yellow	55.5%	CAPS analysis, DNA sequencing	[51]
Lilium longiflorum (White Heaven)	LlPDS	Key enzyme of carotenoid synthesis	Agrobacterium-mediated	Tissue culture seedling scales	Chimeric mutations that are completely albino, albino, pale green & pale yellow	4%	GUS assay, PCR & Southern blot	[53]
Lilium pumilum (DC. Fisch)	LpPDS	Key enzyme of carotenoid synthesis	Agrobacterium-mediated	Callus	Chimeric mutations that are completely albino, albino, pale green & pale yellow	29.17%	GUS assay, PCR & Southern blot	[53]

Table 1 (continued)

Plant species	Target gene	Gene function	Transformation method	Material transformed	Edited genome trait Results	Mutation efficiency (%)	Mutation detection methods	References
Japanese Gentian (Albireo)	Gt5GT, Gt3'GT, Gt5/3'AT	Encodes anthocyanin (vivid blue flowers)	Agrobacterium-mediated	Leaf	Pale red violet, dull pink, and pale mauve flowers	0.07%	PCR, illumina NGS	[126]
Japanese Gentian (Albireo)	GST1	Anthocyanin accumulation in petals	Agrobacterium-mediated	–	Reduced anthocyanin in petals, white and pale blue flower	–	Sanger, NGS	[127]
Phalaenopsis amabilis	PDS3	Regulate enzyme in carotenoid synthesis	Agrobacterium-mediated	Protocorms	Albino leaf	0.96%	PCR	[143]
Phalaenopsis equestris	MADS	Encodes DBP for flower initiation & development	Agrobacterium-mediated	Protocorms	MADS-null mutants (Long juvenile period)	97.9%	PCR, sequencing	[54]
Petunia hybrida (Mirage Rose)	ACO1	Ethylene production linked to flower senescence	PEG-mediated	Protoplast	Ethylene production reduced & enhanced flower longevity	31.5%	PCR, deep sequencing	[47]
Petunia hybrid (Madness Midnight)	F3'H	Synthesis of anthocyanidin & flavonol pigments (purple violet flower)	PEG-mediated	Protoplast	Pale purplish-pink flower color	11.9%	PCR, illumina sequencing	[37]
Euphorbia pulcherrima (Poinsettia)	F3'H	Formation of cyanidin-type anthocyanins	Agrobacterium-mediated	Internode stem	Flower color changed from vivid red to vivid reddish-orange	24%	PCR illumina sequencing	[130]
Petunia hybrida (Mirage Rose)	ACO3, ACO4	Ethylene production linked to flower senescence	PEG-mediated	Protoplast	Ethylene production reduced & enhanced flower longevity	34.32%	PCR, deep sequencing	[136]
Petunia hybrid (Mitchell Diploid)	DPL	Regulate corolla tube venation	Agrobacterium-mediated	ND	Absence of the Vein associated anthocyanin pattern (redefine the prime regulator of corolla venation)	ND	PCR, sanger sequencing	[129]
Petunia hybrid (Mirage Rose)	ACO1, ACO3, ACO4	Seed germination	PEG- mediated	Protoplast	Delay and inhibition of seed germination	–	–	[137]

genome editing tools. According to Zhang and Zhang [146], combining the proofreading enzyme with the Cas9 enzyme will repair any error induced by an off-target event.

Future Perspective

Many ornamental plants are highly heterozygous, have huge genome size, polyploidy, higher chromosome number, long lifespan, self-sterility, or incapacity to produce seeds which indicate a major obstacle to using conventional breeding methods. Nowadays, the floriculture industry needs additional and more cultivars with superior characteristics such as flowering promotion, both by increasing the number of flowers and changing flowering time, floral longevity, color spectrum, aromas, and creation of innovation in flower structure.

CRISPR/Cas9 technology, which first appeared in early 2013, has been more popular in the field of genome editing due to its simplicity of use, economic, and wide applicability. We would like to mention here that there is no report available on increasing essential oil traits with the fragrance that is used in the cosmetic industry. Rose contains a fragrant and essential oil, and the nuclear genome of the hybrid rose plant [4] and wild rose [5] has been sequenced. This information can be exploited further. Advances in functional studies on key genes involved in the scent of flowers and essential oils may depend on the development of mutation technology with CRISPR/Cas9 that can lead to more efficient results, so CRISPR/Cas9-based genome editing study must be carried out to fill the gap of scent and essential oil-based flower crops. Deep knowledge about the tools of the CRISPR/Cas9 system, particularly strategies that enable the development of non-transgenic ornamentals using advanced delivery systems, will contribute to the emergence of better qualitative traits, and bring about creative ideas for competitive and sustainable flower production around the world. Therefore, non-transgenic gene-editing technologies could provide us with new varieties with augmented traits and could fulfill the void of modern demands, and also, be fruitful for the investors or producers.

Declarations

Conflict of interest There is no conflict of interest with any one either within the institute or outside the institute.

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