Review Article

A Comprehensive Review of CRISPR/Cas9 Based Strategies in Horticultural Crop Modification

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ABSTRACT

The introduction of CRISPR/Cas9 gene editing technology represents a groundbreaking advancement in the realm of horticulture. It provides a precise and effective means for making targeted genetic alterations in a wide range of plant species. This abstract delves into the diverse applications of CRISPR/Cas9 within the domain of horticultural crops, with a particular emphasis on its crucial role in tackling issues related to climate change. This review paper outlines the diverse applications of CRISPR/Cas9 in horticulture, including trait improvement for enhanced stress tolerance, disease resistance, and yield optimization. It highlights specific examples of successful CRISPR-edited horticultural crops and their contributions to climate adaptation. Furthermore, it discusses the potential of CRISPR/Cas9 in accelerating the development of new crop varieties tailored to thrive in changing climatic conditions. Additionally, the ethical and regulatory considerations surrounding the use of CRISPR/Cas9 in horticulture are addressed, as they play a crucial role in determining the broader adoption of this technology. Balancing the benefits of climate-resilient crop development with potential environmental and societal implications remains a critical aspect of its application. In conclusion, the transformative potential of CRISPR/Cas9 in horticultural crop improvement and its role in addressing climate-related challenges. By harnessing the power of genetic editing, horticulturalists can create more productive crops and better equipped to withstand the uncertainties of a changing climate. However, this technology's responsible and ethical use is imperative to ensure its long-term sustainability and benefit to society.

Keywords: Breeding, Mango, physiological problems, disease and insect resistance.

INTRODUCTION

"Microorganisms possess a range of defense mechanisms that empower them to fend off invasions by plasmids, bacteriophages, and other genetic intruders. One such protective strategy involves the utilization of the RNA-directed endonuclease CRISPR-associated (Cas) 9 protein. In contrast to earlier tools like ZFNs and TALENs, the Cas9 protein derived from the type II CRISPR/Cas system has gained widespread adoption as a versatile instrument for directing and modifying genomes" [1]. Recent advancements have elevated CRISPR/Cas9 technology to the status of a groundbreaking method for genome manipulation in living cells, inspiring innovative applications across various disciplines. This paper provides an overview of CRISPR/Cas9 technology and its potential

applications in horticultural crops, along with a review of current developments in genome engineering.

"The CRISPR/Cas9 system represents a nucleic acid-based adaptive immune system found in prokaryotes. It empowers specific microbes to detect and eliminate foreign genetic material" [2]. "Microbes that have been exposed to foreign genetic material through processes such as transduction, conjugation, and transformation are prompted to establish defense mechanisms aimed at recognizing and protecting against invasive DNA. This defense is achieved by incorporating short segments of foreign DNA into the CRISPR region. The CRISPR region consists of concise, repetitive sequences of nucleotide bases called spacers, which exhibit sequence homology with foreign elements like plasmids and bacteriophages. The resistance to and vulnerability to phages are modulated within the CRISPR locus through the addition and removal of spacers, respectively" [3]. In general, CRISPR arrays are bookended by leader sequences rich in AT base pairs, and following these leaders are CRISPR arrays that contain the genetic instructions for Cas proteins. The acquisition of CRISPR immunity in microbes involves three key phases:

(A) Adaptation or spacer acquisition,

(B) CRISPR-RNA (crRNA) biogenesis, and

(C) Target interference.

During the adaptation phase, foreign DNA from invading sources is fragmented and integrated into the CRISPR locus as new spacers, serving as a memory record of the infection. In the crRNA biogenesis phase, the CRISPR array is transcribed into precursor CRISPR-RNA (pre-crRNA), which subsequently matures into crRNAs. Each crRNA comprises a specific spacer sequence flanked by short RNA sequences. In the interference phase, the crRNA within the Cas9-crRNA-tracrRNA ribonucleoprotein (crRNP) complex forms base pairs with the corresponding protospacer. This interaction activates Cas9, leading to the recognition and cleavage of the matching DNA sequence by cutting both strands of the target. The Cas9 protein cleaves the protospacer at a location situated 3 bases before the protospacer adjacent motif (PAM). The presence of the PAM sequence is an essential requirement for protospacer cleavage, preventing an "autoimmune" response within the CRISPR locus, as the host locus lacks the PAM sequence.

CRISPR/CAS9 SYSTEM EXPLOITATION IN GENOMIC ENGINEERING

"The CRISPR/Cas9 system, originally derived from prokaryotes, is a powerful tool for precise and targeted genome editing in living cells. To achieve genome editing, Cas9 nuclease collaborates with the crRNA-tracrRNA duplex to create a double-strand break in the desired DNA target" [5]. "This system introduces innovative methods for modifying genomic DNA both in vivo and in vitro by precisely cleaving the target DNA at specific sites. When compared to older genome editing techniques like ZFN and TALENs, which operate on a similar principle of directing a nuclease to a specific genomic sequence to induce a double-strand break, the CRISPR/Cas9 system offers several advantages. Unlike ZFN and TALEN, where significant protein engineering is required, the CRISPR/Cas9 approach simply necessitates the replacement of the 20-nucleotide guide sequence to target a new site" [6]. Additionally, the CRISPR/Cas9 system enables multiplex genome editing by introducing a mixture of sgRNAs.

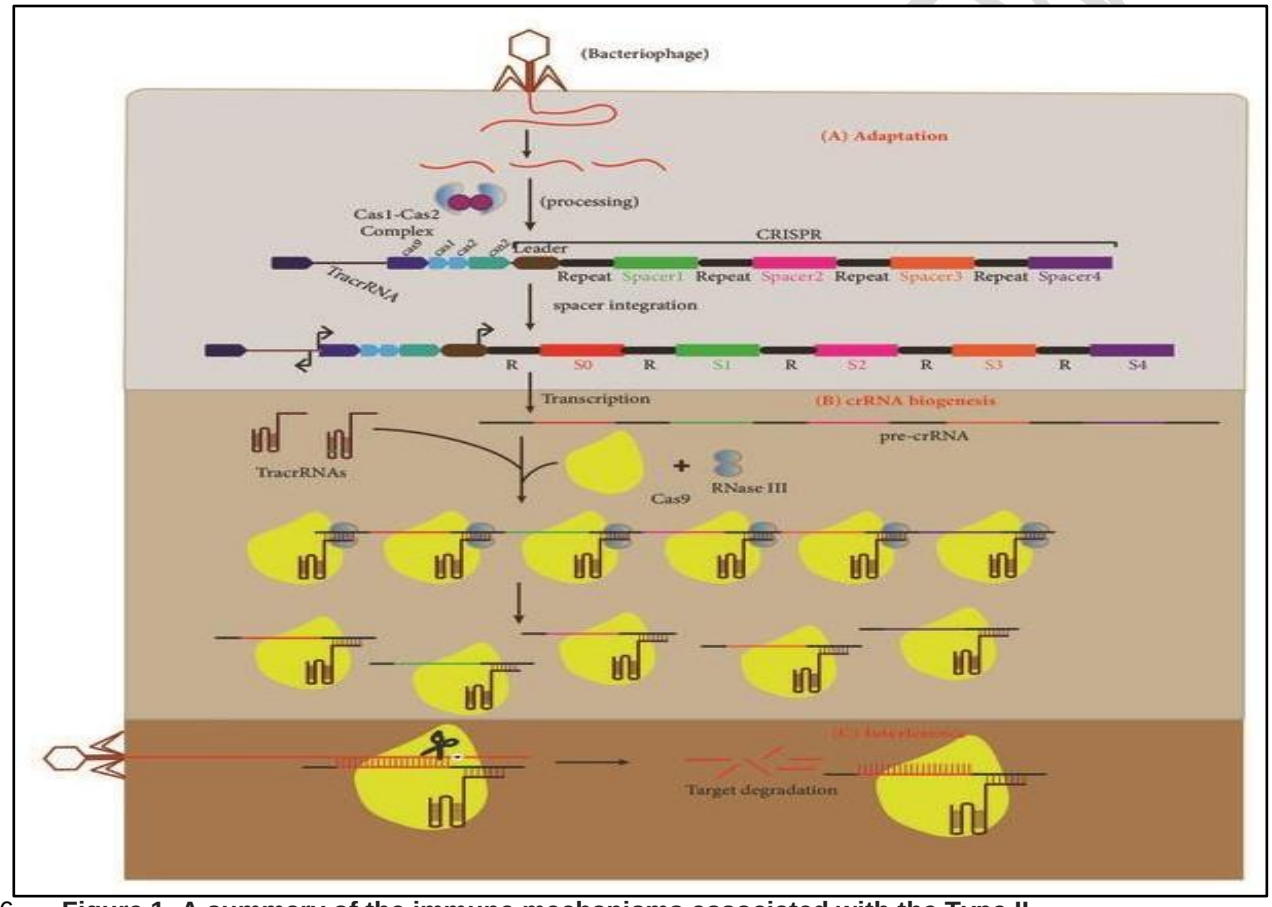

Figure 1. A summary of the immune mechanisms associated with the Type II CRISPR/Cas system. Reference: Lone BA et al. [4].

MECHANISM OF SPECIFIC DNA CLEAVAGE BY CRISPR/CAS9

Among the various Cas proteins available, Cas9 stands out as a programmable RNA-guided endonuclease, and it is the most commonly used for genome editing. Cas9 possesses two conserved nuclease domains, HNH and RuvC, both of which play a role in generating double-strand breaks (DSBs) in the target DNA. "The structure of Cas9, as revealed by crystallography, exhibits a bi-lobed configuration. In this structure, the central

nucleic acid recognition (REC) lobe is comprised of the bridge helix, Rec1, and Rec2 domains. This REC lobe works in concert with the NUC lobe to form a channel through which the negatively charged sgRNA-target DNA heteroduplex can pass" [7].

"In recent years, numerous research studies have been published focusing on genome editing in horticultural plants. These studies encompass a wide range of applications, including the development of plants with enhanced resistance to biotic and abiotic stresses, modifications in flowering times, improvements in fruit quality, alterations in flower characteristics, and changes in fruit color. One notable advantage of employing CRISPR/Cas9 for genome editing in horticultural plants is the capability to edit multiple target genes simultaneously. Additionally, the production of plants with specific desired traits can be achieved much more rapidly compared to traditional breeding methods and even conventional transgenic plant production techniques.Nevertheless, the application of genome editing in horticultural plants does come with certain limitations. Challenges include the extended juvenile periods of fruit trees, issues related to polyploidy (having multiple sets of chromosomes), and difficulties in generating homozygous lines with consistent traits" [8].

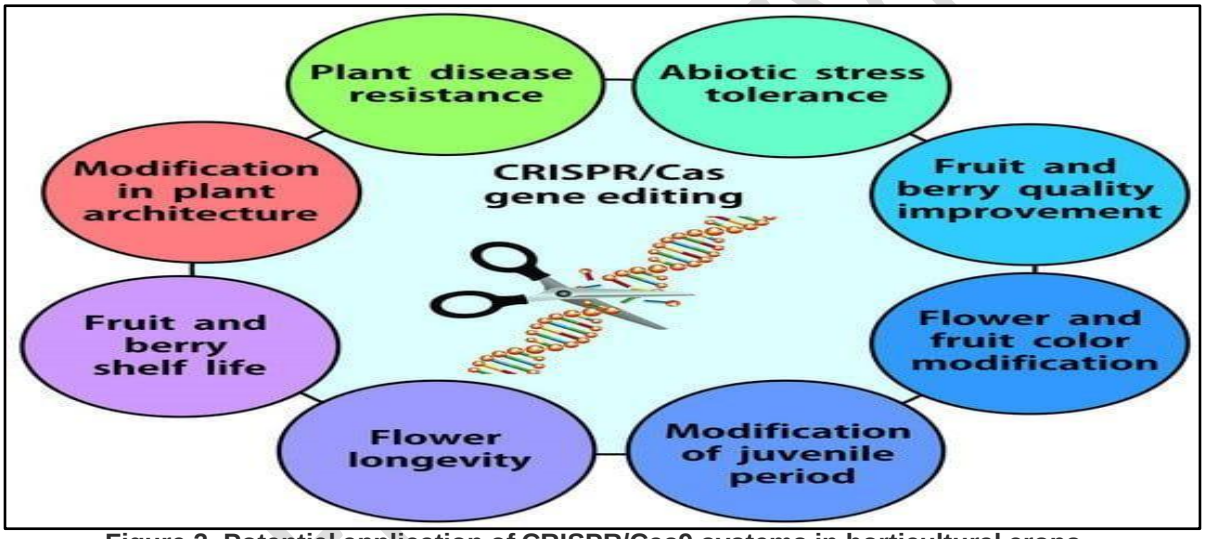

Figure 2. Potential application of CRISPR/Cas9 systems in horticultural crops

APPLICATIONS OF CRISPR/CAS9 IN HORTICULTURAL CROPS

The remarkable efficiency and precision of CRISPR/Cas9 technology in genome editing have inspired researchers to adapt this system for the study of horticultural crops. This technology has found applications in bolstering stress resistance, elevating fruit quality, and altering cultivation characteristics.

Increasing the Resistance of Horticultural Plants to Biotic and Abiotic Stresses

"Plants are susceptible to a range of diseases caused by various pathogens, including bacteria, fungi, and viruses. These infections can adversely affect plant growth and productivity, resulting in significant agricultural losses and increased production costs. The CRISPR/Cas technology offers a valuable tool for enhancing plant resistance to biotic stresses" [9]. "To create plants resistant to viruses, two different strategies are

employed: viral genome editing and editing the genes of plants that are susceptible to viruses. Viruses typically rely on the host plant's transcription and translation machinery. To protect plants from viral infections, the CRISPR/Cas technology can be used to disrupt the expression of sensitivity genes (S genes), such as by knocking out translation initiation factors. An example of the successful application of CRISPR/Cas9 technology is the development of bananas resistant to the endogenous banana streak virus" [10]. Mutations were introduced into integrative viral elements, rendering it impossible for the virus to transcribe and translate its proteins within banana plants. This innovative approach demonstrates the potential for CRISPR/Cas9 to enhance plant resistance to viral pathogens.

"Genome editing techniques have opened the door to the production of plants that exhibit resistance to bacterial pathogens. For instance, apple protoplasts were genetically transformed using a ribonucleoprotein complex containing the Cas9 nuclease and sgRNA (CRISPR/Cas9 RNPs) to target the DIPM-1, -2, and -4 genes, which encode negative regulators of resistance to bacterial fire blight in fruit crops caused by *Erwinia amylovora"* [11]. "One significant advantage of this approach lies in the transient expression of the editing components, which results in fewer unintended mutations. In another study, researchers conducted a knockout of the MdDIPM-4 gene in apple plants. Notably, they successfully removed foreign DNA from the genome using the FLP/FRT recombination system, which was triggered by a heat shock treatment" [12]. These advancements highlight the potential of genome editing to enhance plant resistance to bacterial pathogens and the development of more resilient agricultural crops.

"Citrus canker, which is caused by *Xanthomonas bacteria*, has been effectively addressed through genome editing. Mutant varieties of citrus, such as *Citrus sinensis* (orange) and *C. paradisi* (grapefruit), were created using genome editing techniques, resulting in significant tolerance to these pathogens. In citrus plants, the CsLOB1 gene is responsible for susceptibility to the disease caused by *Xanthomonas citri* subsp. *citri* bacteria. The promoter region of this gene contains elements that facilitate the binding of the bacterium's pathogenicity factor PthA4, leading to the development of disease symptoms. CRISPR/Cas9 was employed to modify the binding sites of the PthA4 factor, reducing the bacteria's ability to infect Citrus sinensis. Researchers utilized various vector constructs to edit the promoter region of the CsLOB1 gene in the Wanjincheng orange variety" [13]. "Depending on the construct used, the frequency of mutations obtained ranged from 11.5% to 64.7%. Consequently, four of the most promising mutant orange lines with resistance to citrus canker were selected. Complete deletion of the binding region of the PthA4 effector in the CsLOB1 promoter resulted in significant resistance of plants to the disease. Similar studies were conducted not only with CRISPR/Cas9 but also with another nuclease, Cas12a (Cpf1). Another strategy for enhancing resistance in Wanjincheng orange against bacterial canker involved editing the CsWRKY22 gene, which encodes another transcription factor, using CRISPR/Cas9. Genome editing also played a role in creating banana mutants with resistance to banana wilt caused by *Xanthomonas* bacteria, by targeting the DMR6 gene" [14]. These examples demonstrate how genome editing can effectively combat bacterial diseases in important horticultural crops.

Fungal pathogens pose a significant threat to plants, causing various diseases. The advent of CRISPR/Cas9 technology has ushered in new possibilities for developing plants with broad resistance to diseases caused by pathogenic fungi. This resistance is often achieved by editing genes responsible for plant sensitivity to these pathogens, as these sensitivity genes play a role in facilitating pathogen penetration and infection. For instance, CRISPR/Cas9 technology was utilized to create grapevine plants with a knockout of the MLO-7 gene, which encodes a negative regulator of resistance to

powdery mildew caused by *Erysiphe necator*. Delivery of sgRNA to the plants was accomplished using RNPs (ribonucleoproteins), and initially, the mutation rate was relatively low (0.1–6.9%). Subsequent studies, however, refined the editing protocol using RNPs, resulting in mutations in three MLO genes that conferred grapevine plants with a significantly lower sensitivity to powdery mildew, up to a 77% reduction. In addition to powdery mildew resistance, grapevine plants with increased resistance to the gray mold *Botrytis cinerea* were developed by knocking out the gene encoding the transcription factor WRKY52, which acts as a negative regulator of the jasmonic acid pathway. Multiple sgRNAs were designed to target different sites in the first exon of the WRKY52 gene. Mutations in both alleles of the gene were found to enhance grapevine plants' resistance to the pathogen compared to mutants with changes in just one allele [15]. These examples illustrate how CRISPR/Cas9 technology can be harnessed to bolster plant defenses against fungal pathogens, contributing to disease resistance in important crops.

Genome editing is a valuable tool for elucidating the roles of specific genes in disease development and providing protection against these diseases. For instance, when the pathogenesis-related protein 4b (VvPR4b) gene was knocked out, grapevines' resistance to downy mildew disease caused by *Plasmopara viticola* decreased. Researchers discovered that the VvPR4b gene encodes a chitinase II-like protein critical for inhibiting the growth of pathogenic fungus hyphae. In the case of apple plants susceptible to infection by the fungal pathogen *Botryosphaeria dothidea*, knocking out the negative regulator CNGC2 gene resulted in increased resistance of apple calli to this pathogen. This resistance boost was associated with an increase in salicylic acid levels and the suppression of PR (pathogenesis-related) protein gene expression. However, it's worth noting that choosing the CNGC2 gene for knockout might not be optimal, as mutations in this gene can lead to undesirable effects, such as reduced fertility. By transiently expressing the CRISPR/Cas9 system, researchers successfully obtained leaf and embryo sections from cacao plants (*Theobroma cacao*) with enhanced resistance to infection by the pathogen *Phythophtora tropicalis*. They targeted the TcNPR3 gene, which acts as a suppressor of the protective response. These results demonstrate the potential for developing cacao plants resistant to diseases caused by *Ph. tropicalis* in subsequent studies [16].

Mutation in the Clpsk1 gene has been demonstrated to enhance the resistance of watermelon plants to the fungus *Fusarium oxysporum* f. sp. *niveum*. Therefore, employing CRISPR/Cas9 technology to edit pathogen sensitivity genes in host plant cells represents a rapid and reliable approach for developing plants resistant to infections caused by viruses, bacteria, and fungi [17]. Several instances highlight the use of genome editing technology to enhance the resistance of horticultural plants to abiotic stresses. For instance, CRISPR/Cas9 was employed to knockout the watermelon acetolactate synthase (ClALS) gene, setting the stage for the future production of herbicide-resistant watermelons. Similarly, CRISPR/Cas9 base editing of the ALS gene served as a marker for generating *Pyrus communis* L. pear plants resistant to the herbicide chlorosulfuron. A comparable approach involved editing the CsALS gene in Carrizo citrange citrus, resulting in herbicide-resistant mutant plants. In summary, genome editing of horticultural plants using CRISPR/Cas9 technology holds promise for creating plants resistant to various biotic and abiotic stresses. Nonetheless, ensuring the stability of mutations and conducting comprehensive investigations into how gene editing impacts varietal characteristics and plant metabolism are essential considerations [18].

CHANGING THE AGRONOMIC TRAITS OF FRUIT AND BERRY PLANTS USING GENOME EDITING

Numerous studies are exploring genome editing techniques to bring about changes in plant growth, shape, fruit ripening times, berry color, metabolic processes, and the shelf life of fruits. For instance, the editing of MaGA20ox2 genes, which play a role in regulating gibberellin biosynthesis, using CRISPR/Cas9 technology resulted in the development of semi-dwarf banana plants from the *Musa acuminate* "Gros Michel" variety. These mutants exhibited distinct characteristics compared to the original plants, featuring reduced growth but thicker and darker green leaves. Furthermore, the cellular structure of the modified plants differed from that of the wild-type plants. The findings from such studies hold significance, especially in the context of selecting dwarf banana varieties, as tall plants are often susceptible to damage from strong winds, which can lead to substantial crop losses [19].

The disruption of one of the strigolactone biosynthesis genes (VvCCD8) in Vitis vinifera 41B grapevine plants resulted in increased shoot branching when compared to wild-type plants. Strigolactones are plant hormones that typically inhibit the growth of axillary buds. The application of CRISPR/Cas9 technology proved instrumental in uncovering the pivotal role of the VvCCD8 gene in controlling shoot branching. This discovery paves the way for further investigations into additional mechanisms governing shoot architecture in grapevines. Additionally, CRISPR/Cas9 technology was successfully used to alter the berry color of strawberry fruits, transitioning them from red to white. This transformation was achieved through the knockout of the RAP (reduced anthocyanins in petioles) gene, which encodes the glutathione S-transferase enzyme responsible for binding anthocyanins and facilitating their transport from the cytosol to the vacuole. Editing the RAP gene holds promise for producing strawberry varieties with white berries, a feature popular among consumers [20].

Genome editing using CRISPR/Cas9 technology has proven to be a powerful tool for enhancing the nutritional characteristics of fruits and berries. For example, researchers have successfully edited the lycopene epsilon-cyclase (LCYε) gene to increase the β-carotene content in bananas. This genetic modification resulted in a sixfold increase in β-carotene levels while causing a significant decrease in the content of lutein and α-carotene in the mutant fruit lines. In the case of red raspberries (*Rubus idaeus* L.), there have been limited attempts to perform gene editing using CRISPR/Cas9 to achieve plants with improved phenotypes. One study targeted the flavone 3-hydrolase (F3′H) gene, which encodes a key enzyme in flavonoid biosynthesis. Another focus of editing was the MYB-16-like gene, which is a potential regulator of prickle formation in raspberries. However, in both cases, researchers encountered challenges in regenerating plants from the edited raspberry calli. Furthermore, the mutation in the GIBG1 β-glucosidase gene led to a reduction in seed size in watermelon (*Citrullus lanatus*) and improved germination rates by decreasing the abscisic acid content. This gene was found to play a significant role in regulating seed size and germination, making it an important trait for use in watermelon breeding [8]. These examples illustrate the potential of CRISPR/Cas9 for modifying fruit and berry characteristics to enhance their nutritional value and other desirable traits.

Gene knockout through CRISPR/Cas9 technology has enabled the study of genes that regulate fruit ripening in economically valuable plants and has also contributed to extending the shelf life of these fruits. For instance, in the case of bananas, editing the 1-aminocyclopropane-1-carboxylate oxidase 1 (MaACO1) gene, which is involved in ethylene biosynthesis, resulted in plant lines that produced smaller fruits with significantly extended ripening times (60 days as opposed to 21 days in control bananas). This prolonged ripening

period has a positive impact on fruit storage, and it was accompanied by an increase in the vitamin C content of the edited banana fruits. In a similar vein, other researchers applied CRISPR/Cas9 to knock out genes such as CmNAC-NOR, CTR1-like, and ROS1, which play roles in regulating fruit ripening in *Cucumis melo cantalupensis melons*. This led to the development of melons with delayed ripening and an extended shelf life [21]. These studies highlight the versatility of CRISPR/Cas9 technology in altering various aspects of horticultural plants, including enhancing taste qualities and fruit color, modifying ripening and storage periods, and influencing growth characteristics.

Changing Flower Color and Shape, Flowering Time and Flower Longevity

Several studies have explored the potential of using CRISPR/Cas9 technology to edit the genomes of horticultural plants with the aim of altering characteristics such as flowering time, flower longevity, and the shape and color of flowers. In the context of wild and cultivated strawberry plants, CRISPR/Cas9 has proven successful in shedding light on the functions of various genes involved in flower and fruit development. Among the genes targeted for editing in strawberries were *FveARF8 and FveTAA1*, which play roles in auxin synthesis. Auxins are known to be crucial for the formation of strawberries. Homozygous mutants of the strawberry FveARF8 gene exhibited larger size and faster growth compared to control plants. Mutations in other strawberry genes, including *FaTM6 and FveSEP3*, resulted in abnormal development of petals, anthers, and pollen grains, as well as parthenocarpy (development of seedless fruit) and incorrect fruit phenotypes [22]. These studies have provided valuable insights into the functions of these genes in the development of strawberry flowers and berries.

Genome editing techniques have made it possible to manipulate the flowering processes of fruit plants. For instance, apple and pear plants were modified with the knockout of the TFl1 flowering repressor gene. This genetic alteration resulted in early flowering in a significant proportion of the obtained apple tree lines (93%) and a smaller percentage of pear plants (9%). In kiwi plants, specifically *Actinidia chinensis*, researchers used genome editing to study the roles of genes such as AcCen4, AcCen, and SyGl in slowing down the flowering processes [23]. These studies demonstrate the potential to develop horticultural plants with accelerated flowering, which could lead to a reduction in the time required for fruit harvest.

Efforts to edit the *CENTRORADIALIS (CEN)* gene in blueberry plants (*Vaccinium corymbosum* L.) with the expectation of inducing precocious flowering, similar to the effects observed in TFl1/CEN-like genes in apple, pear, and kiwi, did not yield the desired results. Instead, the attempts to influence the flowering of blueberry plants by editing the CEN gene were unsuccessful. Moreover, the mutant plants exhibited significant growth lag compared to the control plants. To gain a deeper understanding of the implications of mutations in the CEN gene for the development of the dwarf phenotype, further analysis of the progeny of edited blueberry plants is recommended [24]. This research underscores the complexities and unique characteristics of gene editing outcomes in different plant species.

Researchers have harnessed genome editing techniques to investigate genes associated with the regulation of aging and to alter the color of ornamental flower corollas in various plant species, including petunia, lily, chrysanthemum, ipomoea, gentian, torenia, and orchid. For example, in petunia cultivar "Mirage Rose" plants, the PhACO1 gene, which is involved in regulating ethylene biosynthesis, was edited. This resulted in petunia plants with reduced ethylene synthesis and extended flower longevity. Slowing down flower wilting was achieved by knocking out the EPH1 gene, a regulator of petal senescence, in Japanese morning glory (Ipomoea nil, "Violet") plants. Numerous studies have focused on altering the

color of flower corollas in ornamental plants. In Ipomoea nil plants, the color of the flowers was changed by knocking out the dihydroflavonol-4-reductase (DFR) and carotenoid cleavage dioxygenase 4 (CCD4) genes. Another approach involved knocking out the flavone 3-hydrolase (F3′H) gene, which encodes a key enzyme in flavonoid biosynthesis. This led to a color change in the flowers of Torenia fournieri from pale blue to white. Mutagenesis of the PDS gene, which encodes the key enzyme in carotenoid synthesis, resulted in the production of chimeric phenotypes with altered flower coloration in Lilium longiflorum and L. pumilum mutants.

While there have been numerous studies in various ornamental plants, there have been relatively few studies on orchids using CRISPR/Cas9 gene editing [25]. These studies showcase the versatility of genome editing in modifying flower characteristics for ornamental purposes.

Table 1. Applications of CRISPR/Cas9 technology in different horticultural crops

Crop	Target genes	Transgenic background	Traits	References
Responding to biotic stresses				
Tomato (*Solanum lycopersicum*)	*SlCCD8, SlMAX1*	T	*P. aegyptiaca*↑	[26]
Tomato (*Solanum lycopersicum*)	*TYLCV CP, Rep*	T	*TYLCV*↑	[27]
Banana (*Musanana* Lour.)	*BSOLV, eBSOLV*	T	*eBSV*↑	[28]
Cucumber (*Cucumis sativus* L.)	*Eif4e*	N	*CVYV, ZYMV*, and *PRSV-W*↑	[29]
Citrus (*Citrus reticulata* Blanco)	*CsLOB1* promoter	T	Canker↑	[30-31]
Apple (*Malus* × *domestica*)	*MdDIPM4*	L	Fire blight↑	[32]
Grape (*Vitisvinifera* L.)	*VvMLO3*	T	Powdery mildew↑	[33]
Cacao (*Theobroma cacao*)	*TcNPR3*	T	*P. tropicalis*↑	[34]
Responding to abiotic stress				
Tomato (*Solanumlycopersicum*)	*SlHyPRP1*	T	Salinity stress↑	[35]
Tomato (*Solanumlycopersicum*)	*SlLBD40*	T	Drought stress↑	[36]
Tomato (*Solanumlycopersicum*)	*SlMAPK3*	T	Drought stress↓	[37]
Tomato (*Solanumlycopersicum*)	*SlCBF1*	T	Chilling stress↓	[37]
Improvement of fruit quality				
Tomato (*Solanumlycopersicum*)	*SlGAD2, SlGAD3*	T	GABA content↑	[38]
Tomato (*Solanumlycopersicum*)	*SlGABA-TP1, SlGABA-TP2, SlGABA-TP3, SlSSADH, SlCAT*	T	GABA content↑	[37]
Potato (*Solanumtuberosum* L.)	*StPPO2*	T	Browning of tubers↓	[39]
Eggplant (*Solanummelongena* L.)	*SmelPPO4*, *SmelPPO5*, *SmelPPO6*	N	Browning of fruitcut surface↓	[40]
Potato (*Solanumtuberosum* L.)	*St16DOX*	T	Bitter↓	[41]
Tomato (*Solanumlycopersicum*)	*SlCLV3, SlSP,*	N	Fruit size,	[42]

	SlS promoter		inflorescence branching and plant architecturesmodified	
Tomato(*Solanum lycopersicum*)	*SlSGR1, SlLCY-E, SlBlc, SlLCY-B1, SlLCY-B2*	T	lycopene content↑	[43]
Tomato(*Solanum lycopersicum*)	*SlPL*	T	Shelf life↑	[44]
Tomato(*Solanum lycopersicum*)	*SlPL, SlPG2a, SlTBG4*	T	Shelf life↑	[26]
Tomato(*Solanum lycopersicum*)	*SlALC*	F	Shelf life↑	[36]
Improvement of cultivation traits				
Banana (*Musanana* Lour.)	*MaGA20ox2*	T	Semi-dwarf	[45]
Tomato (*Solanum lycopersicum*)	*SlGAI*	T	Dwarf	[46]
Tomato (*Solanum lycopersicum*)	*SlMAPK20*	T	Defective post-meiotic pollen development	[47]
Tomato (*Solanum lycopersicum*)	*SlMS10*	N	Male Sterility	[48]
Apple (*Malus* × *domestica*)	*MdFTL1.1*	T	Early flowering	[49]
Pear (*Pyrus* L.)	*PcFTL1.1*	T	Early flowering	[49]
Tomato (*Solanum lycopersicum*)	*SlARF7*	T	Parthenocarpy	[50]
Tomato (*Solanum lycopersicum*)	*SlAGL6*	T	Facultative parthenocarpy	[51]
Watermelon (*Citrullus lanatus*)	*ClBG1*	T	Watermelon seed size↓and seed germination rate↑	[52]
Cucumber (*Cucumis sativus* L.)	*CsWIP1*	N	Monoecious flowers	[53]

Table 2. Applications of CRISPR/Cas9 in ornamental plants

Plant	Targeted Gene	Trait	References
Chrysanthemum moriflorium	*CpYGFP*	Fluorescence	[54]
Dendrobium officinale	*C3H*, *C4H*, *4CL*, *CCR*, *IRX*	No mutant phenotype	[47]
Japanese gentians *Gentiana scabra x G. triflora*	*Gt5GT*, *Gt3'GT*, *Gt5/3'AT*	Flower color change	[55]
	GST1	Flower color change	[55]
	EPH1	Flower longevity	[56]
Japanese morning glory *Ipomoea nil*	*DFR-B*	Flower color change	[57]
	CCD4	Flower color change	[58]
	EPH1	Flower longevity	[59]
Lilium longiflorum, *L. pumilum*	*LpPDS*	Photobleaching, albinism	[60]
Petunia *Petunia hybrida*	*PDS*	Photobleaching, albinism	[61]
	NR	Deficiency in nitrate	[62]

		assimilation	
		Flower longevity	
	ACO1	Absence of corolla tube venation	[63]
	AN4	Self-incompatibility	[64]
P. inflata	*PiSSK1*		[65]
Poinsettia *Euphorbia pulcherrima*	*F3'H*	Change of the bract color from red to reddish orange	[66]
Torenia fournieri	*TfRAD1*	Abnormal shape and color of flowers Pale blue flowers	[67]
	F3H		[68]

FUTURE PERSPECTIVES

While the CRISPR/Cas system has proven to be a valuable tool in genome editing, it does have certain limitations that restrict its widespread application, especially in horticultural woody crops. One significant challenge is the difficulty of obtaining transgene-free mutants through segregation, primarily due to the long juvenile period required for these crops to reach sexual maturity, as well as issues of incompatibility in some species. To address this challenge and achieve transgene-free edited plants, researchers have developed a gene editing system that incorporates a heat-shock-inducible FLP/FRT recombination system alongside CRISPR/Cas9 expression cassettes. This heat-shock-inducible FLP/FRT recombination system is capable of excising exogenous DNA after the gene editing process [69]. This innovative methodology holds promise as a means of facilitating transgene-free breeding in horticultural woody crops, addressing one of the limitations of the CRISPR/Cas system in such species.

Tissue-culture-based plant regeneration processes can be challenging and time-consuming, particularly for certain horticultural crops. However, recent developments have sought to improve the efficiency of genetic transformation and gene editing in these crops. One strategy involves combining development regulators (DRs) such as maize *Wuschel2 (Wus2)* and Arabidopsis SHOOT MERISTEMLESS (STM) genes with gene-editing cassettes. These constructs are introduced into tobacco seedlings using Agrobacterium, resulting in the de novo formation of meristems and the production of target gene-modified shoots from seedlings [70]. This approach offers promise for enhancing the genetic transformation efficiency in various horticultural plants. Another method leverages the chimeric protein GROWTH REGULATING FACTOR 4 (GRF4) and its co-factor GRF INTERACTING FACTOR 1 (GIF1) to significantly increase regeneration efficiency in wheat and Citrus. When combined with the CRISPR/Cas9 system, this GRF4-GIF1 approach achieved a remarkable 93.7% genetic transformation efficiency and a 33.3% gene editing efficiency in wheat [71]. These innovative techniques are expected to facilitate the adoption of gene-editing technology in a wide range of horticultural plants by improving the efficiency of genetic transformation. By optimizing and modifying the CRISPR/Cas9 system, researchers aim to maximize its advantages in terms of simplicity and efficiency, ultimately enabling its broader application in research and breeding of horticultural crops in the future.

CONCLUSION

CRISPR/Cas9 systems find extensive application in horticultural crops, contributing to breeding and enhancing desired traits. Optimizing the CRISPR/Cas system facilitates its broader utilization across various crops. The selection of highly active interspecies U6 or U3 promoters to drive sgRNA expression, coupled with the use of tissue-specific and robust promoters for Cas9 expression, enhances editing efficiency. Additionally, SpCas9 variants and orthologs recognizing diverse PAM sequences expand the scope of genome-wide target sites. The STU-Cas9 systems, known for their simplicity and compactness, offer ease of operation for multiple gene editing tasks. Effective sgRNA design is essential to achieve superior editing results. Delivery methods for CRISPR/Cas9 vectors vary depending on the horticultural species, with the emergence of nanoparticle-based transformations providing an alternative option. These collective strategies combine to make the CRISPR/Cas system a highly efficient, precise, straightforward, and user-friendly technology.

COMPETING INTERESTS

Authors have declared that no competing interests exist for this manuscript.

AUTHORS' CONTRIBUTIONS

All authors read and approved the final manuscript for galley proofing."

REFERENCES

1. Afzal, S., Sirohi, P., & Singh, N. K. (2020). A review of CRISPR associated genome engineering: application, advances and future prospects of genome targeting tool for crop improvement. *Biotechnology letters*, *42*, 1611-1632.
2. Lone, B. A., Karna, S. K. L., Ahmad, F., Shahi, N., & Pokharel, Y. R. (2018). CRISPR/Cas9 system: a bacterial tailor for genomic engineering. *Genetics research international*, *2018*.
3. Laanto E, Hoikkala V, Ravantti J, Sundberg LR. Long-term genomic coevolution of host-parasite interaction in the natural environment. Nature communications. 2017;8(1):111.
4. Lone BA, Karna SK, Ahmad F, Shahi N, Pokharel YR. CRISPR/Cas9 system: a bacterial tailor for genomic engineering. Genetics research international. 2018.
5. Serif M, Dubois G, Finoux AL, Teste MA, Jallet D, Daboussi F. One-step generation of multiple gene knock-outs in the diatom Phaeodactylum tricornutum by DNA-free genome editing. Nature Communications. 2018;9(1):3924.
6. Altenbuchner J. Editing of the Bacillus subtilis genome by the CRISPR-Cas9 system. Applied and environmental microbiology. 2016;82(17):5421-7.
7. Liu L, Chen P, Wang M, Li X, Wang J, Yin M, Wang Y. C2c1-sgRNA complex structure reveals RNA-guided DNA cleavage mechanism. Molecular cell. 2017;65(2):310-22.
8. Rukavtsova EB, Zakharchenko NS, Lebedev VG, Shestibratov KA. CRISPR-Cas genome editing for horticultural crops improvement: advantages and prospects. Horticulturae. 2022;9(1):38.
9. Mushtaq M, Bhat JA, Mir ZA, Sakina A, Ali S, Singh AK, Tyagi A, Salgotra RK, Dar AA, Bhat R. CRISPR/Cas approach: A new way of looking at plant-abiotic interactions. Journal of plant physiology. 2018;224:156-62.
10. Uranga M, Daròs JA. Tools and targets: The dual role of plant viruses in CRISPR–Cas genome editing. The Plant Genome. 2023;16(2):e20220.
11. Arora L, Narula A. Gene editing and crop improvement using CRISPR-Cas9 system. Frontiers in plant science. 2017;8:1932.

12. Arora L, Narula A. Gene editing and crop improvement using CRISPR-Cas9 system. Frontiers in plant science. 2017;8:1932.
13. Jia H, Orbović V, Wang N. CRISPR-LbCas12a-mediated modification of citrus. Plant biotechnology journal. 2019;17(10):1928-37.
14. Tripathi L, Ntui VO, Tripathi JN. Application of genetic modification and genome editing for developing climate-smart banana. Food and Energy Security. 2019;8(4):e00168.
15. Li C, Cao S, Wang K, Lei C, Ji N, Xu F, Jiang Y, Qiu L, Zheng Y. Heat shock protein HSP24 is involved in the BABA-induced resistance to fungal pathogen in postharvest grapes underlying an NPR1-dependent manner. Frontiers in Plant Science. 2021;12:646147.
16. Mishra R, Mohanty JN, Mahanty B, Joshi RK. A single transcript CRISPR/Cas9 mediated mutagenesis of CaERF28 confers anthracnose resistance in chilli pepper (*Capsicum annuum* L.). Planta. 2021;254(1):5.
17. Zhang M, Liu Q, Yang X, Xu J, Liu G, Yao X, Ren R, Xu J, Lou L. CRISPR/Cas9-mediated mutagenesis of Clpsk1 in watermelon to confer resistance to Fusarium oxysporum f. sp. niveum. Plant cell reports. 2020;39:589-95.
18. Karkute SG, Singh AK, Gupta OP, Singh PM, Singh B. CRISPR/Cas9 mediated genome engineering for improvement of horticultural crops. Frontiers in plant science. 2017;8:1635.
19. Shao X, Wu S, Dou T, Zhu H, Hu C, Huo H, He W, Deng G, Sheng O, Bi F, Gao H. Using CRISPR/Cas9 genome editing system to create MaGA20ox2 gene-modified semi-dwarf banana. Plant Biotechnology Journal. 2020;18(1):17.
20. Ren C, Guo Y, Kong J, Lecourieux F, Dai Z, Li S, Liang Z. Knockout of VvCCD8 gene in grapevine affects shoot branching. BMC plant biology. 2020;20(1):1-8.
21. Pujol, M., & Garcia-Mas, J. (2023). Regulation of climacteric fruit ripening in melon, recent advances and future challenges. *Journal of Experimental Botany*, erad256.
22. Yao JL, Kang C, Gu C, Gleave AP. The roles of floral organ genes in regulating Rosaceae fruit development. Frontiers in Plant Science. 2022;12:644424.
23. Nerva L, Dalla Costa L, Ciacciulli A, Sabbadini S, Pavese V, Dondini L, Vendramin E, Caboni E, Perrone I, Moglia A, Zenoni S. The role of Italy in the use of advanced plant genomic techniques on fruit trees: state of the art and future perspectives. International Journal of Molecular Sciences. 2023;24(2):977.
24. Vaia G, Pavese V, Moglia A, Cristofori V, Silvestri C. Knockout of phytoene desaturase gene using CRISPR/Cas9 in highbush blueberry. Frontiers in Plant Science. 2022;13:1074541.
25. Watanabe K, Oda-Yamamizo C, Sage-Ono K, Ohmiya A, Ono M. Alteration of flower colour in Ipomoea nil through CRISPR/Cas9-mediated mutagenesis of carotenoid cleavage dioxygenase 4. Transgenic research. 2018;27:25-38.
26. Bari VK, Nassar JA, Kheredin SM, Gal-On A, Ron M, Britt A, Steele D, Yoder J, Aly R. CRISPR/Cas9-mediated mutagenesis of Carotenoid Cleavage Dioxygenase 8 in tomato provides resistance against the parasitic weed *Phelipanche aegyptiaca*. Scientific reports. 2019;9(1):11438.
27. Tashkandi M, Ali Z, Aljedaani F, Shami A, Mahfouz MM. Engineering resistance against Tomato yellow leaf curl virus via the CRISPR/Cas9 system in tomato. Plant signaling & behavior. 2018;13(10):e1525996.
28. Tripathi JN, Ntui VO, Ron M, Muiruri SK, Britt A, Tripathi L. CRISPR/Cas9 editing of endogenous banana streak virus in the B genome of Musa spp. overcomes a major challenge in banana breeding. Communications Biology. 2019;2(1):46.
29. Chandrasekaran J, Brumin M, Wolf D, Leibman D, Klap C, Pearlsman M, Sherman A, Arazi T, Gal-On A. Development of broad virus resistance in non-transgenic cucumber using CRISPR/Cas9 technology. Molecular plant pathology. 2016;17(7):1140-53.

30. Peng A, Chen S, Lei T, Xu L, He Y, Wu L, Yao L, Zou X. Engineering canker-resistant plants through CRISPR/Cas9-targeted editing of the susceptibility gene Cs LOB 1 promoter in citrus. Plant biotechnology journal. 2017;15(12):1509-19.
31. Jia H, Orbović V, Wang N. CRISPR-LbCas12a-mediated modification of citrus. Plant biotechnology journal. 2019;17(10):1928-37.
32. Pompili V, Dalla Costa L, Piazza S, Pindo M, Malnoy M. Reduced fire blight susceptibility in apple cultivars using a high-efficiency CRISPR/Cas9-FLP/FRT-based gene editing system. Plant biotechnology journal. 2020;18(3):845-58.
33. Wang L, Chen S, Peng A, Xie Z, He Y, Zou X. CRISPR/Cas9-mediated editing of CsWRKY22 reduces susceptibility to Xanthomonas citri subsp. citri in Wanjincheng orange (*Citrus sinensis* (L.) Osbeck). Plant Biotechnology Reports. 2019;13:501-10.
34. Fister AS, Landherr L, Maximova SN, Guiltinan MJ. Transient expression of CRISPR/Cas9 machinery targeting TcNPR3 enhances defense response in Theobroma cacao. Frontiers in plant science. 2018;9:268.
35. Vu TV, Sivankalyani V, Kim EJ, Doan DT, Tran MT, Kim J, Sung YW, Park M, Kang YJ, Kim JY. Highly efficient homology-directed repair using CRISPR/Cpf1-geminiviral replicon in tomato. Plant Biotechnology Journal. 2020;18(10):2133-43.
36. Zhang M, Liu Q, Yang X, Xu J, Liu G, Yao X, Ren R, Xu J, Lou L. CRISPR/Cas9-mediated mutagenesis of Clpsk1 in watermelon to confer resistance to *Fusarium oxysporum* f. sp. *niveum*. Plant cell reports. 2020;39:589-95.
37. Wang X, Tu M, Wang D, Liu J, Li Y, Li Z, Wang Y, Wang X. CRISPR/Cas9-mediated efficient targeted mutagenesis in grape in the first generation. Plant biotechnology journal. 2018;16(4):844-55.
38. Nonaka S, Arai C, Takayama M, Matsukura C, Ezura H. Efficient increase of ɣ-aminobutyric acid (GABA) content in tomato fruits by targeted mutagenesis. Scientific reports. 2017;7(1):7057.
39. Zhao X, Jayarathna S, Turesson H, Fält AS, Nestor G, González MN, Olsson N, Beganovic M, Hofvander P, Andersson R, Andersson M. Amylose starch with no detectable branching developed through DNA-free CRISPR-Cas9 mediated mutagenesis of two starch branching enzymes in potato. Scientific reports. 2021;11(1):4311.
40. Maioli A, Gianoglio S, Moglia A, Acquadro A, Valentino D, Milani AM, Prohens J, Orzaez D, Granell A, Lanteri S, Comino C. Simultaneous CRISPR/Cas9 editing of three PPO genes reduces fruit flesh browning in Solanum melongena L. Frontiers in plant science. 2020;11:607161.
41. Nakayasu M, Akiyama R, Lee HJ, Osakabe K, Osakabe Y, Watanabe B, Sugimoto Y, Umemoto N, Saito K, Muranaka T, Mizutani M. Generation of α-solanine-free hairy roots of potato by CRISPR/Cas9 mediated genome editing of the St16DOX gene. Plant Physiology and Biochemistry. 2018;131:70-7.
42. Rodriguez-Ortega WM, Martinez V, Rivero RM, Camara-Zapata JM, Mestre T, Garcia-Sanchez F. Use of a smart irrigation system to study the effects of irrigation management on the agronomic and physiological responses of tomato plants grown under different temperatures regimes. Agricultural Water Management. 2017;183:158-68.
43. Zhu C, Zheng X, Huang Y, Ye J, Chen P, Zhang C, Zhao F, Xie Z, Zhang S, Wang N, Li H. Genome sequencing and CRISPR/Cas9 gene editing of an early flowering Mini-Citrus (Fortunella hindsii). Plant biotechnology journal. 2019;17(11):2199-210.

44. Uluisik S, Chapman NH, Smith R, Poole M, Adams G, Gillis RB, Besong TM, Sheldon J, Stiegelmeyer S, Perez L, Samsulrizal N. Genetic improvement of tomato by targeted control of fruit softening. Nature biotechnology. 2016;34(9):950-2.
45. Shao X, Wu S, Dou T, Zhu H, Hu C, Huo H, He W, Deng G, Sheng O, Bi F, Gao H. Using CRISPR/Cas9 genome editing system to create MaGA20ox2 gene-modified semi-dwarf banana. Plant Biotechnology Journal. 2020;18(1):17.
46. Li Z, Tomlinson AC, Wong AH, Zhou D, Desforges M, Talbot PJ, Benlekbir S, Rubinstein JL, Rini JM. The human coronavirus HCoV-229E S-protein structure and receptor binding. Elife. 2019;8:e51230.
47. Kui L, Chen H, Zhang W, He S, Xiong Z, Zhang Y, Yan L, Zhong C, He F, Chen J, Zeng P. Building a genetic manipulation tool box for orchid biology: identification of constitutive promoters and application of CRISPR/Cas9 in the orchid, *Dendrobium officinale*. Frontiers in plant science. 2017;7:2036.
48. Malnoy M, Viola R, Jung MH, Koo OJ, Kim S, Kim JS, Velasco R, Nagamangala Kanchiswamy C. DNA-free genetically edited grapevine and apple protoplast using CRISPR/Cas9 ribonucleoproteins. Frontiers in plant science. 2016;7:1904.
49. Charrier A, Vergne E, Dousset N, Richer A, Petiteau A, Chevreau E. Efficient targeted mutagenesis in apple and first time edition of pear using the CRISPR-Cas9 system. Frontiers in Plant Science. 2019;10:40.
50. Hu C, Sheng O, Deng G, He W, Dong T, Yang Q, Dou T, Li C, Gao H, Liu S, Yi G. CRISPR/Cas9-mediated genome editing of MaACO1 (aminocyclopropane-1-carboxylate oxidase 1) promotes the shelf life of banana fruit. Plant Biotechnology Journal. 2021;19(4):654.
51. Tong CG, Wu FH, Yuan YH, Chen YR, Lin CS. High-efficiency CRISPR/Cas-based editing of Phalaenopsis orchid MADS genes. Plant biotechnology journal. 2020 Apr;18(4):889.
52. Zhou J, Wang G, Liu Z. Efficient genome editing of wild strawberry genes, vector development and validation. Plant biotechnology journal. 2018 Nov;16(11):1868-77.
53. Hu Y, Zhang J, Jia H, Sosso D, Li T, Frommer WB, Yang B, White FF, Wang N, Jones JB. Lateral organ boundaries 1 is a disease susceptibility gene for citrus bacterial canker disease. Proceedings of the National Academy of Sciences. 2014;111(4):E521-9.
54. Kishi-Kaboshi M, Aida R, Sasaki K. Generation of gene-edited Chrysanthemum morifolium using multicopy transgenes as targets and markers. Plant and Cell Physiology. 2017;58(2):216-26.
55. Tasaki K, Higuchi A, Watanabe A, Sasaki N, Nishihara M. Effects of knocking out three anthocyanin modification genes on the blue pigmentation of gentian flowers. Scientific Reports. 2019;9(1):15831.
56. Tasaki K, Yoshida M, Nakajima M, Higuchi A, Watanabe A, Nishihara M. Molecular characterization of an anthocyanin-related glutathione S-transferase gene in Japanese gentian with the CRISPR/Cas9 system. BMC Plant Biology. 2020;20:1-4.
57. Watanabe K, Kobayashi A, Endo M, Sage-Ono K, Toki S, Ono M. CRISPR/Cas9-mediated mutagenesis of the dihydroflavonol-4-reductase-B (DFR-B) locus in the Japanese morning glory Ipomoea (Pharbitis) nil. Scientific reports. 2017;7(1):10028.
58. Watanabe K, Oda-Yamamizo C, Sage-Ono K, Ohmiya A, Ono M. Alteration of flower colour in Ipomoea nil through CRISPR/Cas9-mediated mutagenesis of carotenoid cleavage dioxygenase 4. Transgenic research. 2018;27:25-38.
59. Shibuya K, Watanabe K, Ono M. CRISPR/Cas9-mediated mutagenesis of the EPHEMERAL1 locus that regulates petal senescence in Japanese morning glory. Plant Physiology and Biochemistry. 2018;131:53-7.

60. Yan R, Wang Z, Ren Y, Li H, Liu N, Sun H. Establishment of efficient genetic transformation systems and application of CRISPR/Cas9 genome editing technology in Lilium pumilum DC. Fisch. and *Lilium longiflorum* White Heaven. International journal of molecular sciences. 2019;20(12):2920.
61. Zhang B, Xu X, Huang R, Yang S, Li M, Guo Y. CRISPR/Cas9-mediated targeted mutation reveals a role for AN4 rather than DPL in regulating venation formation in the corolla tube of *Petunia hybrida*. Horticulture research. 2021;8.
62. Subburaj S, Chung SJ, Lee C, Ryu SM, Kim DH, Kim JS, Bae S, Lee GJ. Site-directed mutagenesis in Petunia× hybrida protoplast system using direct delivery of purified recombinant Cas9 ribonucleoproteins. Plant cell reports. 2016;35:1535-44.
63. Xu J, Kang BC, Naing AH, Bae SJ, Kim JS, Kim H, Kim CK. CRISPR/Cas9-mediated editing of 1-aminocyclopropane-1-carboxylate oxidase1 enhances Petunia flower longevity. Plant biotechnology journal. 2020;18(1):287-97.
64. Zhang B, Yang X, Yang C, Li M, Guo Y. Exploiting the CRISPR/Cas9 system for targeted genome mutagenesis in petunia. Scientific Reports. 2016;6(1):20315.
65. Sun L, Kao TH. CRISPR/Cas9-mediated knockout of PiSSK1 reveals essential role of S-locus F-box protein-containing SCF complexes in recognition of non-self S-RNases during cross-compatible pollination in self-incompatible Petunia inflata. Plant Reproduction. 2018;31:129-43.
66. Nitarska D, Boehm R, Debener T, Lucaciu RC, Halbwirth H. First genome edited poinsettias: targeted mutagenesis of flavonoid 3′-hydroxylase using CRISPR/Cas9 results in a colour shift. Plant Cell, Tissue and Organ Culture (PCTOC). 2021;147(1):49-60.
67. Su S, Xiao W, Guo W, Yao X, Xiao J, Ye Z, Wang N, Jiao K, Lei M, Peng Q, Hu X. The CYCLOIDEA–RADIALIS module regulates petal shape and pigmentation, leading to bilateral corolla symmetry in *Torenia fournieri* (Linderniaceae). New Phytologist. 2017;215(4):1582-93.
68. Nishihara M, Higuchi A, Watanabe A, Tasaki K. Application of the CRISPR/Cas9 system for modification of flower color in Torenia fournieri. BMC Plant Biology. 2018;18(1):1-9.
69. Pessina S, Lenzi L, Perazzolli M, Campa M, Dalla Costa L, Urso S, Valè G, Salamini F, Velasco R, Malnoy M. Knockdown of MLO genes reduces susceptibility to powdery mildew in grapevine. Horticulture Research. 2016;3.
70. Nishitani C, Hirai N, Komori S, Wada M, Okada K, Osakabe K, Yamamoto T, Osakabe Y. Efficient genome editing in apple using a CRISPR/Cas9 system. Scientific reports. 2016;6(1):31481.
71. Gao Q, Luo H, Li Y, Liu Z, Kang C. Genetic modulation of RAP alters fruit coloration in both wild and cultivated strawberry. Plant biotechnology journal. 2020;18(7):1550-61.