UNDER PEER REVIEW

Review Article

A review on applications of CRISPR/Cas9 in horticultural crops

Abstract

The advent of CRISPR/Cas9 gene editing technology has revolutionized the field of horticulture by offering precise and efficient tools for targeted genetic modifications in various plant species. This abstract explores the multifaceted applications of CRISPR/Cas9 in horticultural crops while considering its pivotal role in addressing climate-related challenges.

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 crops that are more productive 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.

Introduction

Microbes have several defense mechanisms that enable them to resist invasion by plasmids and bacteriophages, among other genome invaders. Utilizing the RNA-directed endonuclease CRISPR-associated (Cas) 9 protein is one such protective tactic. In contrast to prior tools like ZFNs and TALENs, the Cas9 protein, which is derived from the type II CRISPR/Cas system, has been adopted as a flexible tool for genome targeting and engineering. With recent developments, CRISPR/Cas9 technology has become a ground-breaking method for altering the genome in living cells and inspiring novel translational uses in numerous disciplines. The CRISPR/Cas9 technology and its potential applications among horticultural crops, as well as current discoveries in genome engineering, are reviewed in this paper.

Biology of CRISPR/CAS9 system

The CRISPR/Cas9 system is a prokaryotic nucleic acid-based adaptive immune system that enables selected microbes to respond to and eliminate foreign genetic material. The establishment of defensive systems that recognize foreign DNA and defend against genome invaders is encouraged in microbes that have been exposed to foreign genetic material by transduction, conjugation, and transformation. By inserting brief pieces of foreign DNA into the CRISPR area, defence is acquired. The CRISPR area is made up of short, repeating base sequences called spacers that are related to foreign entities like plasmids and bacteriophages in terms of sequence homology. The resistance and susceptibility to phages are altered in the CRISPR locus by the addition and deletion of spacers, respectively. Typically, CRISPR arrays are flanked by AT-rich leader sequences, which are followed by CRISPR arrays that encode Cas proteins.

CRISPR immunity in microbes is acquired through (A) adaptation or spacer acquisition, (B) CRISPR-RNA (crRNA) biogenesis, and (C) target interference. During adaptation phase, invading DNA is spliced into small fragments and incorporated into a CRISPR locus as new spacers that become the memory record of infection. In the crRNA biogenesis phase, CRISPR array is transcribed into precursor CRISPR-RNA (pre-crRNA) followed by maturation to crRNAs, each containing a specific spacer sequence flanked by short RNA sequences. During the interference phase, the crRNA in the Cas9-crRNA-tracrRNA ribonucleoprotein (crRNP) complex base pairs with the corresponding protospacer and stimulates Cas9 for the recognition and destruction of the matching sequence by cleaving

both strands of the target. Cas9 protein cleaves the protospacer at a site that is located 3 bases upstream of the protospacer adjacent motif. The absolute requirement of the PAM sequence for the cleavage of the protospacer excludes the "autoimmune" response within the CRISPR locus as the host locus lacks the PAM sequence.

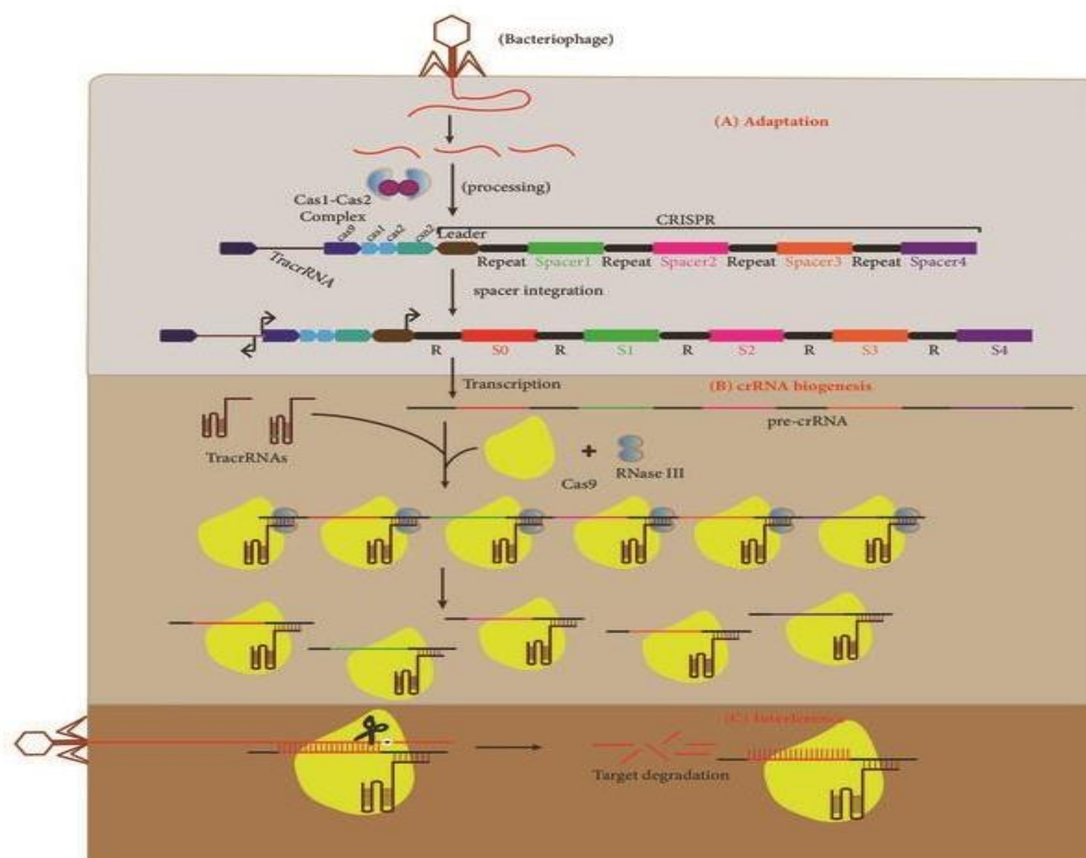

Figure 1. An overview of type II CRISPR/Cas immunity.

CRISPR/Cas9 System Exploitation in Genomic Engineering

The CRISPR/Cas9 system was derived from prokaryotes and is used for precise and targeted editing of the genome of live cells. The double-strand break within the target DNA required for genome editing is induced by the Cas9 nuclease in conjunction with the crRNA-tracrRNA duplex. The CRISPR/Cas9 system offers novel approaches to engineer genomic DNA both in vivo and in vitro by site-specific cleavage of target DNA. When compared to older genome editing technologies like ZFN and TALENs, which work on the similar premise of training the nuclease to a specific sequence in the genome to cause a double-strand break, the CRISPR/Cas9 system offers a number of advantages. The CRISPR/Cas9 approach requires

only to replace the 20-nucleotide length guide sequence to link it to a new target site, in contrast to ZFN and TALEN which both require extensive protein engineering. By simply introducing a mixture of sgRNAs, the CRISPR/Cas9 system can permit multiplex genome editing.

Mechanism of Specific DNA Cleavage by CRISPR/Cas9

Among the various Cas proteins available, Cas9, a programmable RNA guided endonuclease, is the one most frequently utilised for genome editing. The HNH and RuvC domains, both generate DSB in the target DNA, are two conserved nuclease domains of the Cas9 nuclease. The Cas9 crystal reveals that it includes a bi-lobed structure in which the central nucleic acid recognition (REC) lobe, which is made up of bridge helix, Rec1, and Rec2 domains, collaborates with the NUC lobe to create a channel for the negatively charged sgRNA-target DNA heteroduplex.

In recent years, many studies have been published on genome editing of horticultural plants, including plants with resistance to biotic and abiotic stresses, altered flowering times, improved fruit quality, altered flowers, and altered fruit color. An advantage of genome editing using CRISPR/Cas9 is the possibility of simultaneously editing several target genes. In addition, plants with specified characteristics can be produced much faster as compared with traditional breeding techniques as well as with methods of transgenic plant production. Still, the use of horticultural plants' genome editing has its limitations, such as long juvenile periods for fruit trees, polyploidy, and difficulties in producing homozygous lines.

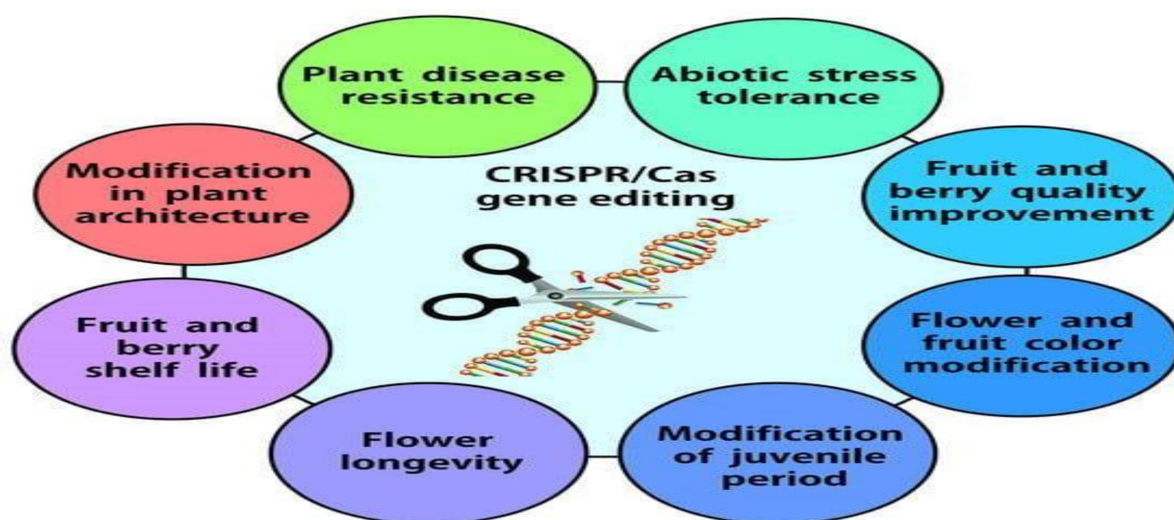

Figure 2. Potential application of CRISPR/Cas9 systems in horticultural crops

Applications of CRISPR/Cas9 in horticultural crops

The high efficiency and precision of CRISPR/Cas9 technology in genome editing motivated researchers to design this system to study the horticultural crop (Ahmar et al., 2020; Biswas et al., 2021). This technology has been applied in enhancing stress resistance, improving fruit quality, and modifying cultivation traits.

Increasing the Resistance of Horticultural Plants to Biotic and Abiotic Stresses

Plants can be susceptible to various diseases caused by pathogens such as bacteria, fungi, and viruses. This deteriorates the development and productivity of plants, which can lead to large losses and increased costs of agricultural products. The CRISPR/Cas technology can greatly contribute to increasing plant resistance to biotic stresses.

Two different approaches are used to create plants resistant to viruses: viral genome editing and editing the genes of plants sensitive to viruses. Viruses usually use host-plant transcription and translation tools. In order to protect plants from viruses, expression of sensitivity genes (S genes) can be disrupted using the CRISPR/Cas technology, e.g., by knocking out translation initiation factors. The CRISPR/Cas9 technology enabled the production of bananas resistant to the endogenous banana streak virus. Mutations were introduced into integrative viral elements, which made it impossible to transcribe and translate viral proteins in banana plants.

Genome editing makes it possible to produce plants resistant to bacterial pathogens. For example, apple protoplasts were transformed by a ribonucleoprotein complex containing the Cas9 nuclease and sgRNA (CRISPR/Cas9 RNPs) into the *DIPM-1, -2*, and *-4* genes encoding negative regulators of resistance to bacterial fire blight of fruit crops caused by *Erwinia amilovora*. The advantage of transient expression was shown, as there are fewer undesirable mutations. Other researchers also carried out a knockout of the *MdDIPM-4* gene in apple plants. Interestingly, foreign DNA was removed from the genome using the FLP/FRT recombination system in the presence of a heat shock.

Citrus canker is known to be caused by *Xanthomonas* bacteria. Citrus mutants (*Citrus sinensis* orange and *C. paradisi* grapefruit) produced by genome editing had a significant tolerance to these pathogens. Citrus plants have the *CsLOB1* gene responsible for sensitivity to a disease caused by the bacteria *Xanthomonas citri* subsp. *citri* . The promoter part of this

gene includes elements for the bacterium's pathogenicity factor PthA4 binding, which leads to the development of symptoms of the disease. The use of CRISPR/Cas9 to modify the binding sites of the PthA4 factor led to a decrease in the bacteria's ability to infect *Citrus sinensis* . Researchers used several vector constructs to modify the promoter region of the *CsLOB1* gene of the Wanjincheng orange variety. Depending on the construct, the frequency of obtained mutations was 11.5–64.7%. As a result, four of the most promising mutant orange lines of canker-resistant citrus fruits were selected. Deletion of the entire binding region of the PthA4 effector in the *CsLOB1* promoter led to a significant resistance of plants to this disease. Similar studies were carried out using not only CRISPR/Cas9 but also another nuclease, Cas12a (Cpf1). Another approach to increasing the resistance of the Wanjincheng orange to bacterial canker was the editing of the *CsWRKY22* gene encoding another transcription factor using CRISPR/Cas9. Genome editing also contributed to producing banana mutants with the *DMR6* gene resistant to banana wilt caused by *Xanthomonas* bacteria.

Fungal pathogens cause numerous diseases in plants. The development of CRISPR/Cas9 technology opened new opportunities for producing plants with a wide range of resistance to diseases caused by pathogenic fungi, e.g., by editing pathogen sensitivity genes. It is known that sensitivity genes in plants facilitate pathogen penetration and infection. For example, the use of CRISPR/Cas9 technology enabled the production of grapevine plants with the knockout of the *MLO-7* gene, which encodes the negative regulator of resistance to powdery mildew, *Erysiphe necator*. Delivery of sgRNA to plants was carried out using RNPs, and the mutation rate was very low (0.1–6.9%). In further studies, the editing protocol using RNPs has been improved. Mutations in three *MLO* genes resulted in grapevine plants with a 77% lower sensitivity to powdery mildew. In addition, by the knockout of the gene encoding transcription factor WRKY52—a negative regulator of the jasmonic acid pathway—grapevine plants with increased resistance to the gray mold *Botrytis cinerea* were obtained. Several sgRNAs were designed to target different sites of the first exon of the *WRKY52* gene, and a mutation in two alleles of the gene was shown to make grapevine plants more resistant to the pathogen compared to mutants in one allele.

Genome editing is often useful for clarifying the role of some genes in the development of a disease or providing protection against it. For example, when knocking out the pathogenesis-related protein 4b (*VvPR4b*) gene, the resistance of grapevines to the downy mildew disease caused by *Plasmopara viticola* decreased. The authors found that

the *VvPR4b* gene encodes the chitinase II-like protein necessary for inhibiting the growth of pathogenic fungus hyphae.

Apple plants can suffer from infection caused by the fungal pathogen *Botryosphaeria dothidea*. Knockout of the negative regulator *CNGC2* gene led to an increase in the resistance of apple calli to this pathogen. Herewith, the content of salicylic acid was noted to increase and the expression of the PR protein gene to be suppressed. However, the choice of the *CNGC2* gene for the knockout is not optimal since mutations in it can lead to undesirable effects, such as reduced fertility.

With the help of transient expression of the CRISPR/Cas9 system, it was possible to obtain sections of leaves and embryos of the cacao plant, *Theobroma cacao,* with increased resistance to infection by the pathogen *Phythophtora tropicalis*. The *TcNPR3* gene, which is a suppressor of the protective response, was chosen as the target of editing. These results confirm the possibility of subsequently producing cacao plants resistant to the disease caused by *Ph. tropicalis*.

A mutation in the *Clpsk1* gene was shown to increase the resistance of watermelon plants to the fungus *Fusarium oxysporum f.* sp. *niveum*. Thus, editing pathogen sensitivity genes in host plant cells using the CRISPR/Cas9 technology can be a fast and reliable approach to creating plants resistant to infections caused by viruses, bacteria, and fungi.

There are a few examples of using the genome editing technology to increase the resistance of horticultural plants to abiotic stresses. For example, using CRISPR/Cas9, a knockout of the watermelon acetolactate synthase (*ClALS*) gene was carried out, which will subsequently allow for the production of watermelons resistant to herbicides. The use of CRISPR/Cas9 base editing of the *ALS* gene as a marker led to the production of *Pyrus communis* L. pear plants resistant to the herbicide chlorosulfuron. A similar editing of the *CsALS* gene in the citrus Carrizo citrange led to the resistance of the obtained mutant plants to the herbicide imazapyr.

Thus, genome editing of horticultural plants using CRISPR/Cas9 technology can be effective for producing plants resistant to various biotic and abiotic stresses. However, it is necessary to achieve mutation stability and investigate comprehensively how gene editing affects varietal characteristics and plant metabolism.

Changing the Agronomic Traits of Fruit and Berry Plants Using Genome Editing

Some studies investigate genome editing in order to change the growth and shape of plants, the ripening time of fruits, modify the color of berries, change the metabolism, and improve the shelf life of fruits.

Editing with CRISPR/Cas9 of the *MaGA20ox2* genes involved in the regulation of gibberellin biosynthesis led to the production of the semi-dwarf phenotype of banana plants *Musa acuminate* “Gros Michel”. The mutants differed from the original plants by smaller growth but thicker and dark green leaves. The cells of the modified plants differed in their structure from those of wild-type plants. The results of such studies are important for the selection of dwarf banana varieties since tall plants often suffer from strong winds, resulting in large crop losses.

The knockout of one of the strigolactone biosynthesis (*VvCCD8*) genes in *Vitis vinifera* 41B grapevine plants led to increased branching of shoots compared to wild-type plants. Strigolactones are plant hormones that inhibit the growth of axillary buds. Through the use of CRISPR/Cas9, it was possible to find the key role of the *VvCCD8* gene in the control of shoot branching. Subsequently, it is intended to investigate other mechanisms for the regulation of the architecture of shoots in grapevines.

With the help of the CRISPR/Cas9 technology, it was possible to obtain strawberry fruits with the colour of berries changed from red to white. To do this, the authors used a knockout of the *RAP* (reduced anthocyanins in petioles) gene encoding the glutathione S-transferase enzyme involved in binding anthocyanins to facilitate their transport from the cytosol to the vacuole. Editing the *RAP* gene can be promising for producing strawberry varieties with white berries that are popular among consumers.

Genome editing using CRISPR/Cas9 is an effective tool for improving the nutritional properties of fruits and berries. For example, bananas with an increased content of β-carotene were obtained by editing the lycopene epsilon-cyclase (*LCYε*) gene. In fruits of the obtained mutant lines, the content of β-carotene increased sixfold, while the content of lutein and α-carotene significantly decreased.

There are few attempts to carry out gene editing using CRISPR/Cas9 in red raspberry, *Rubus idaeus* L., to obtain plants with an improved phenotype. One of the studies knocked out the flavone 3-hydrolase (*F3'H*) gene, encoding one of the key enzymes of

flavonoid biosynthesis. Another target for editing was the *MYB-16*-like gene, which is a possible regulator of prickle formation in raspberries. However, in both cases, researchers faced the difficulty of regenerating plants from the obtained raspberry calli.

Mutation in the *GlBG1* β-glucosidase gene led to a decrease in seed size in watermelon (*Citrullus lanatus*) and improved their germination by reducing the content of abscisic acid. It was shown that this gene can play a role in regulating the size of seeds and their germination, which is a very important trait for use in watermelon breeding.

With the help of gene knockout using CRISPR/Cas9, it was possible to study the work of some genes regulating fruit ripening in plants valuable to humans as well as to extend the shelf life of these fruits. For example, bananas were obtained by editing the 1-aminocyclopropane-1-carboxylate oxidase 1 (*MaACO1*) gene involved in ethylene biosynthesis. The resulting plant lines produced fruits of a smaller size and with a much extended ripening time (60 days instead of 21 days for control bananas), which positively affected their storage. In addition, the content of vitamin C in the edited banana fruits increased. Other researchers used CRISPR/Cas9 to knock out the *CmNAC-NOR*, *CTR1*-like, and *ROS1* genes involved in the regulation of fruit ripening in *Cucumis melo cantalupensis* melon, which led to the appearance of fruits with delayed ripening and a long shelf life.

Thus, the reviewed works showed the feasibility of using the CRISPR/Cas9 technology to change various parameters of horticultural plants, such as to improve their taste qualities and fruit color, to change the ripening and storage periods, as well as their growth characteristics.

Changing Flower Color and Shape, Flowering Time, and Flower Longevity

Some studies consider editing the plant genome using the CRISPR/Cas9 technology to change the flowering time, flower longevity, and shape and color of flowers in horticultural plants.

Gene editing using the CRISPR/Cas9 technology has been successfully used in wild and cultivated strawberry plants to clarify the function of various genes in the development of flowers and fruits. Some of the first genes to be edited were *FveARF8* and *FveTAA1*, involved in auxin synthesis, as auxins are known to be important for the formation of

strawberries. Homozygous strawberry *FveARF8* gene mutants were large and grew faster as compared with control plants. Mutations in other strawberry genes (*FaTM6* and *FveSEP3*) led to abnormal development of petals, anthers, and pollen grains, as well as to parthenocarpy and an incorrect fruit phenotype. Thus, the role of these genes in the development of strawberry flowers and berries has been shown.

With the help of genome editing, it is possible to make changes in the flowering processes of fruit plants. For example, apple and pear plants with the knockout of the *TFl1* flowering repressor gene were obtained. The authors observed early flowering in 93% of the obtained apple tree lines and only in 9% of the pear plants. In kiwi plants, *Actinidia chinensis*, the role of the *AcCen4*, *AcCen*, and *SyGl* genes in slowing down the flowering processes was elucidated using genome editing. Thus, there is a possibility of obtaining horticultural plants with earlier flowering, which will lead to a reduction in fruit harvest times.

The *CENTRORADIALIS* (*CEN*) gene in blueberry plants, *Vaccinium corymbosum* L., was edited. The authors expected that the knockout of this gene would lead to precocious flowering, as was noted for *TFl1*/*CEN*-like genes in apple, pear, and kiwi. However, attempts to affect the flowering of blueberry plants by editing the *CEN* gene failed. In addition, mutant plants lagged far behind in growth as compared with control plants. Further analysis of the progeny of edited blueberry plants is proposed to explain the role of mutations in the *CEN* gene in the development of the dwarf phenotype.

Some researchers used genome editing to study genes involved in the regulation of aging, as well as in changing the color of the corollas of ornamental flowers, such as petunia, lily, chrysanthemum, ipomoea, gentian, torenia, and orchid. For example, the *PhACO1* gene involved in the regulation of ethylene biosynthesis was edited in petunia cultivar “Mirage Rose” plants. This led to the appearance of petunia plants with reduced ethylene synthesis and longer flower longevity. The wilting of flowers was also slowed down by knocking out the *EPH1* gene, which is a regulator of petal senescence, in Japanese morning glory (*Ipomoea nil*, “Violet”) plants. There are many studies that have focused on changing the color of flower corollas in ornamentals. It became possible to change the color of the flowers in *Ipomoea nil* plants by knocking out the dihydroflavonol-4-reductase (*DFR*) and carotenoid cleavage dioxygenase 4 (*CCD4*) genes. Other investigators carried out a knockout of the flavone 3-hydrolase (*F3'H*) gene encoding one of the key enzymes of flavonoid biosynthesis.

As a result, the flowers of *Torenia fournieri* changed color from pale blue to white. Mutagenesis of the *PDS* gene encoding the key enzyme of carotenoid synthesis led to the production of mutants *Lilium longiflorum* and *L. pumilum* with chimeric phenotypes with altered flower coloration. There are few studies of orchids using CRISPR/Cas9 gene editing .

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*↑	Bari et al., 2019, 2021
Tomato (*Solanum lycopersicum*)	*TYLCV CP, Rep*	T	*TYLCV*↑	Tashkandi et al., 2018
Banana (*Musa nana* Lour.)	*BSOLV, eBSOLV*	T	*eBSV*↑	Tripathi et al., 2019
Cucumber (*Cucumis sativus* L.)	*Eif4e*	N	*CVYV*, *ZYMV*, and *PRSV-W*↑	Chandrasekaran et al., 2016
Citrus (*Citrus reticulata* Blanco)	*CsLOB1* promoter	T	Canker↑	Peng et al., 2017; Jia and Wang, 2020
Apple (*Malus × domestica*)	*MdDIPM4*	L	Fire blight↑	Pompili et al., 2020
Grape (*Vitis vinifera* L.)	*VvMLO3*	T	Powdery mildew↑	Wan et al., 2020
Tomato (*Solanum lycopersicum*)	*SlPMR4*	T	Powdery mildew↑	Martínez et al., 2020
Cacao (*Theobroma cacao*)	*TcNPR3*	T	*P. tropicalis*↑	Fister et al., 2018
Responding to abiotic stress				
Tomato (*Solanum*	*SlARF4*	T	Salinity, osmotic	Bouzroud et al.,

Crop	**Target genes**	**Transgenic background**	**Traits**	**References**
lycopersicum)			stress↑	2020
Tomato (*Solanum lycopersicum*)	*SlHyPRP1*	T	Salinity stress↑	Tran et al., 2020
Tomato (*Solanum lycopersicum*)	*SlLBD40*	T	Drought stress↑	Liu et al., 2020a
Tomato (*Solanum lycopersicum*)	*SlMAPK3*	T	Drought stress↓	Wang et al., 2017
Tomato (*Solanum lycopersicum*)	*SlCBF1*	T	Chilling stress↓	Li et al., 2018b
Improvement of fruit quality				
Tomato (*Solanum lycopersicum*)	*SlGAD2, SlGAD3*	T	GABA content↑	Nonaka et al., 2017
Tomato (*Solanum lycopersicum*)	*SlGABA-TP1, SlGABA-TP2, SlGABA-TP3, SlSSADH, SlCAT*	T	GABA content↑	Li et al., 2018a
Potato (*Solanum tuberosum* L.)	*StPPO2*	T	Browning of tubers↓	González et al., 2021
Eggplant (*Solanum melongena* L.)	*SmelPPO4, SmelPPO5, SmelPPO6*	N	Browning of fruit cut surface↓	Maioli et al., 2020
Potato (*Solanum tuberosum* L.)	*St16DOX*	T	Bitter↓	Nakayasu et al., 2018
Tomato (*Solanum lycopersicum*)	*SlCLV3, SlSP, SlS* promoter	N	Fruit size, inflorescence branching and plant architectures modified	Rodríguez et al., 2017

Crop	Target genes	Transgenic background	Traits	References
Tomato (*Solanum lycopersicum*)	*SlSGR1, SlLCY-E, SlBlc, SlLCY-B1, SlLCY-B2*	T	lycopene content↑	Li et al., 2018c
Tomato (*Solanum lycopersicum*)	*SlPL*	T	Shelf life↑	Uluisik et al., 2016
Tomato (*Solanum lycopersicum*)	*SlPL, SlPG2a, SlTBG4*	T	Shelf life↑	Wang et al., 2019
Tomato (*Solanum lycopersicum*)	*SlALC*	F	Shelf life↑	Yu et al., 2017
Improvement of cultivation traits				
Banana (*Musa nana* Lour.)	*MaGA20ox2*	T	Semi-dwarf	Shao et al., 2020
Tomato (*Solanum lycopersicum*)	*SlGAI*	T	Dwarf	Tomlinson et al., 2019
Tomato (*Solanum lycopersicum*)	*SlMAPK20*	T	Defective post-meiotic pollen development	Chen et al., 2018
Tomato (*Solanum lycopersicum*)	*SlMS10*	N	Male Sterility	Jung et al., 2020
Apple (*Malus × domestica*)	*MdFTL1.1*	T	Early flowering	Charrier et al., 2019
Pear (*Pyrus* L.)	*PcFTL1.1*	T	Early flowering	Charrier et al., 2019
Kiwifruit (*Actinidia Chinensis*)	*AcCEN4, AcCEN*	T, N	Early flowering and compact plant architecture	Erika et al., 2019
Tomato (*Solanum lycopersicum*)	*SlBOP1, SlBOP2, SlBOP3*	T	Early flowering and Inflorescence	Xu et al., 2016

Crop	**Target genes**	**Transgenic background**	**Traits**	**References**
			simplification	
Tomato (*Solanum lycopersicum*)	*SlMBP21*	T	*Jointless-2* phenotype	Maria et al., 2017
Tomato (*Solanum lycopersicum*)	*SlARF7*	T	Parthenocarpy	Hu et al., 2018
Tomato (*Solanum lycopersicum*)	*SlAGL6*	T	Facultative parthenocarpy	Chen et al., 2017
Watermelon (*Citrullus lanatus*)	*ClBG1*	T	Watermelon seed size↓and seed germination rate↑	Wang et al., 2021
Cucumber (*Cucumis sativus* L.)	*CsWIP1*	N	Monoecious flowers	Hu et al., 2017

Table 2. Applications of CRISPR/Cas9 in ornamental plants

Plant	Targeted Gene	Trait	References
Chrysanthemum moriflorium	*CpYGFP*	Fluorescence	Kishi-Kaboshi et.al. 2017
Dendrobium officinale	*C3H*, *C4H*, *4CL*, *CCR*, *IRX*	No mutant phenotype	Kui et al 2017
Japanese gentians *Gentiana scabra x G. triflora*	*Gt5GT*, *Gt3'GT*, *Gt5/3'AT*	Flower color change	Tasaki et al 2019
	GST1	Flower color change	Tasaki et al 2020
	EPH1	Flower longevity	Takahashi et al 2022
Japanese morning glory *Ipomoea nil*	*DFR-B*	Flower color change	Watanabe et al 2017
	CCD4	Flower color change	Watanabe et al 2018
	EPH1	Flower longevity	Shibuya et al 2018
Lilium longiflorum, *L. pumilum*	*LpPDS*	Photobleaching, albinism	Yan et al 2019
Petunia *Petunia hybrida*	*PDS*	Photobleaching, albinism	Zhang et al 2017
	NR	Deficiency in nitrate assimilation	Subburaj et al 2016
		Flower longevity	
	ACO1	Absence of corolla tube venation	Xu et al 2020
	AN4	Self-incompatibility	Zhang et al 2021
P. inflata	*PiSSK1*		Sun et al 2018
Phalaenopsis equestris	*MADS8*, *MADS36*, *MADS44*	Long juvenile period	Tong et al 2020
Poinsettia *Euphorbia pulcherrima*	*F3'H*	Change of the bract color from red to reddish orange	Nitarsaka et al 2021
Torenia fournieri	*TfRAD1*	Abnormal shape and color of flowers	Su et al 2017
		Pale blue flowers	
	F3H		Nishihara et al 2018

Conclusion and future perspectives

The CRISPR/Cas9 systems are widely applied in horticultural crops for breeding and trait improvement. The CRISPR/Cas system optimization accelerates its application in more crops. Selection of the most active interspecies *U6* or *U3* promoters for driving sgRNAs expression and the tissue-specific and strong promoters for driving Cas9 expression should improve editing efficiency. The SpCas9 variants and orthologs that recognize different PAMs broaden the genome-wide range of target sites. The STU-Cas9 systems are much more simplified, compact, and easy to operate for multiple gene editing. Furthermore, efficient sgRNA design is also required for high editing efficiency. CRISPR/Cas9 vector delivery methods are dependent on the horticultural species, and the emergence of nanoparticles-based transformation provides a choice. All the strategies make the CRISPR/Cas system a highly efficient, accurate, simple, and easy-to-use technology.

The CRISPR/Cas system still has limitations, preventing it from broad application. For instance, it is difficult for most horticultural woody crops to get homology T-DNA free mutants through segregation because of the long juvenility to reach sexual maturity, and some species are incompatible. To get transgene-free edited plants, a gene edit system containing a heat-shock inducible FLP/FRT recombined with CRISPR/Cas9 expression cassettes was constructed. The heat-shock inducible FLP/FRT recombination system excises the exogenous DNA after gene editing (Pompili et al., 2020). This methodology likely represents a promising method for transgene-free breeding.

The tissue-culture-based plant regeneration efficiencies are very low and time-consuming for some horticultural crops. Recently, a group developed a strategy that combines the development regulators (DRs), such as maize *Wuschel2* (*Wus2*) and *Arabidopsis SHOOT MERISTEMLESS* (*STM*) genes, with the gene-editing cassette and introduced into tobacco seedlings with *Agrobacterium*, obtaining *de novo* meristem and target gene-modified shoots from seedlings (Maher et al., 2020). Another study indicated that the chimeric protein GROWTH REGULATING FACTOR 4 (GRF4) and its co-factor GRF INTERACTING FACTOR 1 (GIF1) significantly increase regeneration efficiency in wheat and *Citrus*. The combined GRF4-GIF1 with CRISPR/Cas9 achieves 93.7% genetic transformation efficiency with 33.3% gene editing efficiency in wheat (Debernardi et al., 2020). These two methods are expected to enable the diffusion and application of gene-editing technology in various horticultural plants by enhancing the efficiency of genetic transformation. Hence, with

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optimization and modification of the CRISPR/Cas9 system, its advantages in terms of simplicity and efficiency will be maximized and allowing its application in research and breeding of horticultural crops in the future.

References

Alquézar, B.; Bennici, S.; Carmona, L.; Gentile, A.; Peña, L. Generation of transfer-DNA-free base-edited citrus plants. *Front. Plant Sci.* **2022**, *13*, 835282.

Bari, V. K., Nassar, J. A., Kheredin, S. M., Gal-On, A., Ron, M., Britt, A., ... & Aly, R. (2019). CRISPR/Cas9-mediated mutagenesis of CAROTENOID CLEAVAGE DIOXYGENASE 8 in tomato provides resistance against the parasitic weed Phelipanche aegyptiaca. *Scientific reports*, *9*(1), 11438.

Chandrasekaran, J., Brumin, M., Wolf, D., Leibman, D., Klap, C., Pearlsman, M., ... & Gal-On, A. (2016). Development of broad virus resistance in non-transgenic cucumber using CRISPR/Cas9 technology. *Molecular plant pathology*, *17*(7), 1140-1153.

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. *Front. Plant Sci.* **2019**, *10*, 40.

Gao, Q.; Luo, H.; Li, Y.; Liu, Z.; Kang, C. Genetic modulation of *RAP* alters fruit coloration in both wild and cultivated strawberry. *Plant Biotechnol. J.* **2020**, *18*, 1550–1561.

Hu, C.; Sheng, O.; Deng, G.; He, W.; Dong, T.; Yang, Q.; Dou, T.; Li, C.; Gao, H.; Liu, S. CRISPR/Cas9-mediated genome editing of *MaACO1* (aminocyclopropane-1-carboxylate oxidase1) promotes the shelf life of banana fruit. *Plant Biotechnol. J.* **2021**, *19*, 654–656.

Hu, Y.; Zhang, J.L.; Jia, H.G.; Sosso, D.; Li, T.; Frommer, W.B.; Yang, B.; White, F.F.; Wang, N.; Jones, J.B. Lateral organ boundaries1 is a disease susceptibility gene for citrus bacterial canker disease. *Proc. Natl. Acad. Sci. USA* **2014**, *111*, 521–529.

Jia, H.; Orbović, V.; Wang, N. CRISPR-LbCas12a-mediated modification of citrus. *Plant Biotechnol. J.* **2019**, *17*, 1928–1937.

Kaur, N.; Alok, A.; Shivani, K.P.; Kaur, N.; Awasthi, P.; Chaturvedi, S.; Pandey, P.; Pandey, A.; Pandey, A.K.; Tiwari, S. CRISPR/Cas9 directed editing of lycopene epsilon-cyclase modulates metabolic flux for beta-carotene biosynthesis in banana fruit. *Metab. Eng.* **2020**, *59*, 76–86.

Khadgi, A. Uncovering genetic and transcriptomic regulation of prickle development in red raspberry. A dissertation presented to the Faculty of the Graduate School of Cornell University in partial fulfillment of the requirements for the Degree of Doctor of Philosophy. *Cornell Theses Diss.* **2020**, *12*, 158.

Kishi-Kaboshi, M.; Aida, R.; Sasaki, K. Generation of gene-edited *Chrysanthemum morifolium* using multicopy transgenes as targets and markers. *Plant Cell Physiol.* **2017**, *58*, 216–226.

Kui, L.; Chen, H.; Zhang, W.; He, S.; Xiong, Z.; Zhang, Y.; Yan, L.; Zhong, C.; He, F.; Chen, J.; et al. Building a genetic manipulation tool box for orchid biology: Identification of constitutive promoters and application of CRISPR/Cas9 in the orchid, *Dendrobium officinale*. *Front. Plant Sci.* **2017**, *7*, 2036.

Leisen, T.; Bietz, F.; Werner, J.; Wegner, A.; Schaffrath, U.; Scheuring, D.; Willmund, F.; Mosbach, A.; Scalliet, G.; Hahn, M. CRISPR/Cas with ribonucleoprotein complexes and transiently selected

telomere vectors allows highly efficient marker-free and multiple genome editing in *Botrytis cinerea*. *PLoS Pathog.* **2020**, *16*, e1008326.

Malnoy, M.; Viola, R.; Jung, M.H.; Koo, O.J.; Kim, S.; Kim, J.S.; Velasco, R.; Kanchiswamy, C.N. DNA-Free genetically edited grapevine and apple protoplast using CRISPR/Cas9 ribonucleoproteins. *Front. Plant Sci.* **2016**, *7*, 1904.

Miller, S. Gene Editing of Red Raspberry (*Rubus ideaus* L.) with CRISPR/Cas9 Knocking out F3'H. Master's Thesis, Norwegian University of Life Sciences, Ås, Norway, 2019. Available online: **http://hdl.handle.net/11250/2608590** (accessed on 13 May 2019).

Naim, F.; Dugdale, B.; Kleidon, J.; Brinin, A.; Shand, K.; Waterhouse, P.; Dale, J. Gene editing the phytoene desaturase alleles of Cavendish banana using CRISPR/Cas9. *Transgen. Res.* **2018**, *27*, 451–460.

Nakajima, I.; Ban, Y.; Azuma, A.; Onoue, N.; Moriguchi, T.; Yamamoto, T.; Toki, S.; Endo, M. CRISPR/Cas9-mediated targeted mutagenesis in grape. *PLoS ONE* **2017**, *12*, 0177966.

Nishihara, M.; Higuchi, A.; Watanabe, A.; Tasaki, K. Application of the CRISPR/Cas9 system for modification of flower color in *Torenia fournieri*. *BMC Plant Biol.* **2018**, *18*, 331.

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. *Sci. Rep.* **2016**, *6*, 31481.

Nitarska, D.; Boehm, R.; Debener, T.; Lucaciu, R.C.; Halbwirth, H. First genome edited poinsettias: Targeted mutagenesis of flavonoid 3'-hydroxylase using CRISPR/Cas9 results in a colour shift. *PCTOC* **2021**, *147*, 49–60.

Ntui, V.O.; Tripathi, J.N.; Tripathi, L. Robust CRISPR/Cas9 mediated genome editing tool for banana and plantain (*Musa* spp.). *Curr. Plant Biol.* **2020**, *21*, 100128.

Osakabe, Y.; Liang, Z.; Ren, C.; Nishitani, C.; Osakabe, K.; Wada, M.; Komori, S.; Malnoy, M.; Velasco, R.; Poli, M.; et al. CRISPR-Cas9-mediated genome editing in apple and grapevine. *Nat. Protoc.* **2018**, *13*, 2844–2863.

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 *CsLOB1* promoter in citrus. *Plant Biotechnol. J.* **2017**, *15*, 1509–1519.

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. *Hortic. Res.* **2016**, *3*, 16016.

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 Biotechnol. J.* **2020**, *18*, 845–858.

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 Biol.* **2020**, *20*, 47.

Ren, C.; Lin, Y.; Liang, Z. CRISPR/Cas genome editing in grapevine: Recent advances, challenges and future prospects. *Fruit Res.* **2022**, *2*, 7.

Ren, F.; Ren, C.; Zhang, Z.; Duan, W.; Lecourieux, D.; Li, S.; Liang, Z. Efficiency optimization of CRISPR/Cas9-mediated targeted mutagenesis in grape. *Front. Plant Sci.* **2019**, *10*, 612.

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Shao, X.; Wu, S.; Dou, T.; Zhu, H.; Hu, C.; Huo, H.; He, W.; Deng, G.; Sheng, O.; Bi, F.; et al. Using CRISPR/Cas9 genome editing system to create *MaGA20ox2* gene-modified semi-dwarf banana. *Plant Biotechnol. J.* **2020**, *18*, 17–19.

Shibuya, K.; Watanabe, K.; Ono, M. CRISPR/Cas9-mediated mutagenesis of the *EPHEMERAL1* locus that regulates petal senescence in Japanese morning glory. *Plant Physiol. Biochem.* **2018**, *131*, 53–57.

Su, S.; Xiao, W.; Guo, W.; Yao, X.; Xiao, J.; Ye, Z.; Wang, N.; Jiao, K.; Lei, M.; Peng, Q.; et al. The cycloidea-radialis module regulates petal shape and pigmentation, leading to bilateral corolla symmetry in *Torenia fournieri* (*Linderniaceae*). *New Phytol.* **2017**, *215*, 1582–1593.

Subburaj, S.; Chung, S.J.; Lee, C.; Ryu, S.-M.; Kim, D.H.; Kim, J.-S.; Bae, S.; Lee, G.-J. Site-directed mutagenesis in *Petunia* x *hybrida* protoplast system using direct delivery of purified recombinant Cas9 ribonucleoproteins. *Plant Cell Rep.* **2016**, *35*, 1535–1544.

Sun, L.; Kao, T. 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 Reprod.* **2018**, *31*, 129–143.

Takahashi, S.; Yoshida, C.; Takahashi, H.; Nishihara, M. Isolation and functional analysis of *EPHEMERAL1-LIKE* (*EPH1L*) genes involved in flower senescence in cultivated Japanese gentians. *Int. J. Mol. Sci.* **2022**, *23*, 5608.

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. *Sci. Rep.* **2019**, *9*, 15831.

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 Biol.* **2020**, *20*, 370.

Tong, C.-G.; Wu, F.-H.; Yuan, Y.-H.; Chen, Y.-R.; Lin, C.-S. High-efficiency CRISPR/Cas-based editing of *Phalaenopsis* orchid MADS genes. *Plant Biotechnol. J.* **2020**, *18*, 889–891.

Tripathi, J.N.; Ntui, V.O.; Ron, M.; Muiruri, S.K.; 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. *Commun. Biol.* **2019**, *2*, 46.

Varkonyi-Gasic, E.; Wang, T.; Cooney, J.; Jeon, S.; Voogd, C.; Douglas, M.J.; Pilkington, S.M.; Akagi, T.; Allan, A.C. Shy Girl, a kiwifruit suppressor of feminization, restricts gynoecium development via regulation of cytokinin metabolism and signalling. *New Phytol.* **2021**, *230*, 1461–1475.

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 Biotechnol. Rep.* **2019**, *13*, 501–510.

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 Biotechnol. J.* **2018**, *16*, 844–855.

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*. *Sci. Rep.* **2017**, *7*, 10028.

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 Res.* **2018**, *27*, 25–38.

Xing, S.; Jia, M.; Wei, L.; Mao, W.; Abbasi, U.A.; Zhao, Y.; Chen, Y.; Cao, M.; Zhang, K.; Dai, Z.; et al. CRISPR/Cas9-introduced single and multiple mutagenesis in strawberry. *J. Genet. Genom.* **2018**, *45*, 685–687.

Xu, J.; Kang, B.C.; Naing, A.H.; Bae, S.J.; Kim, J.-S.; Kim, H.; Kim, C.K. CRISPR/Cas9-mediated editing of 1-aminocyclopropane-1-carboxylate oxidase1 enhances *Petunia* flower longevity. *Plant Biotechnol. J.* **2020**, *18*, 287–297.

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. *Int. J. Mol. Sci.* **2019**, *20*, 2920.

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*. *Hortic. Res.* **2021**, *8*, 116.

Zhang, B.; Yang, X.; Yang, C.; Li, M.; Guo, Y. Exploiting the CRISPR/Cas9 system for targeted genome mutagenesis in *Petunia*. *Sci. Rep.* **2016**, *6*, 20315.

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 Rep.* **2020**, *39*, 589–595.

Zhou, H.; Bai, S.; Wang, N.; Sun, X.; Zhang, Y.; Zhu, J.; Dong, C. CRISPR/Cas9-Mediated Mutagenesis of *MdCNGC2* in apple callus and VIGS-mediated silencing of *MdCNGC2* in fruits improve resistance to *Botryosphaeria dothidea*. *Front. Plant Sci.* **2020**, *11*, 575477.

Zhou, J.; Wang, G.; Liu, Z. Efficient genome-editing of wild strawberry genes, vector development, and validation. *Plant Biotechnol. J.* **2018**, *16*, 1868–1877.

Zhu, C.; Zheng, X.; Huang, Y.; Ye, J.; Chen, P.; Zhang, C.; Zhao, F.; Xie, Z.; Zhang, S.; Wang, N. Genome sequencing and CRISPR/Cas9 gene editing of an early flowering Mini-Citrus (*Fortunella hindsii*). *Plant Biotechnol. J.* **2019**, *17*, 2199–2210.