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Review

CRISPR-Cas Genome Editing for Horticultural Crops Improvement: Advantages and Prospects

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Abstract: Horticultural plants, in particular fruit trees, berry crops, and ornamentals, are valuable objects for studying their genetic and biochemical properties. Along with traditional methods of studying these plants, modern molecular genetic technologies are emerging, in particular genome editing using CRISPR/Cas9 nucleases. In this review, we have analyzed modern advances in genome editing of horticultural plants. To date, it has become possible to improve many plant characteristics using this technology, e.g., making plants resistant to biotic and abiotic stress factors, changing the time of flowering and fruit ripening, changing the growth characteristics of plants, as well as the taste properties of their fruits. CRISPR/Cas9 genome editing has been successfully carried out for many horticultural plants. Dozens of genes from these plants have been modified by means of genome editing technology. We have considered the main ways of delivering genetic constructs to plants as well as limitations that complicate the editing of target genes. The article reviews the prospects of using genome editing to improve the valuable properties of plants important to humans.

Keywords: genome editing; CRISPR/Cas9; horticultural crops; plant disease resistance; herbicide resistance; flowers longevity; flowers color changes; fruits and berries improvement

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1. Introduction

Increasing the yield and pathogen resistance of vegetable, fruit, and berry crops is an urgent problem of modern agriculture and horticulture. Conventional breeding methods are usually very time-consuming. Novel plant breeding techniques have been intensively developed recently, in particular genome editing using CRISPR/Cas technology. Prior to the discovery of CRISPR/Cas9 nucleases, other methods—TALEN, ZNFs, and meganucleases—had been used for gene editing in plants [1–3]. However, CRISPR/Cas technology proves to be simple, reproducible, and cheaper for use in plant biotechnology.

CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) are short palindromic DNA repeats regularly arranged in groups and separated by spacers with unique sequences [4]. They are special genomic loci found in the chromosomes of bacteria and archaea and are necessary for protection against bacteriophages [5]. CRISPR/Cas9 is a ribonucleotide complex consisting of a Cas9 nuclease and an RNA guide (single guide RNA, sgRNA) [6]. The Cas9 nuclease recognizes a special site in the host DNA, called PAM (protospacer adjacent motif), and cuts the target sequence, to which the sgRNA is complementary [7]. The nuclease introduces double-stranded breaks in the DNA, which initiate a DNA repair system of two types: nonhomologous end joining (NHEJ) and homology-dependent repair (HDP) [8,9]. Resulting deletions or insertions in the target gene at the site of the breaks usually lead to the loss of the gene's functions. There are several types and classes of CRISPR/Cas systems [10–12]. The most convenient and often used system for genome editing is the CRISPR/Cas system with an effector nuclease, Cas9 (class II and type II) from the bacterium *Streptococcus pyogenes* [13]. Recently, other, more advanced

variants of Cas9 nucleases began to be used [14,15], as well as other types of nucleases, e.g., Cas12a (formerly Cpf1) (class II and type V) [16–18] and Cas13 (type VI) [19].

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 [20–29] (Figure 1). 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. The aim of this review is to analyze the published investigations which use the CRISPR/Cas technology for gene editing of horticultural plants and ornamentals. We searched articles on this topic using a combination of the key words "CRISPR" and "plant name". The search was carried out in the titles, abstracts, and key words of articles indexed in the PubMed, Scopus, and Google Scholar databases. This review gives a comprehensive analysis of research into the genome editing of horticultural plants using CRISPR/Cas9 and discusses the prospects of obtaining, by means of this technology, new improved varieties of fruit and berry crops as well as ornamentals that are of value in horticulture.

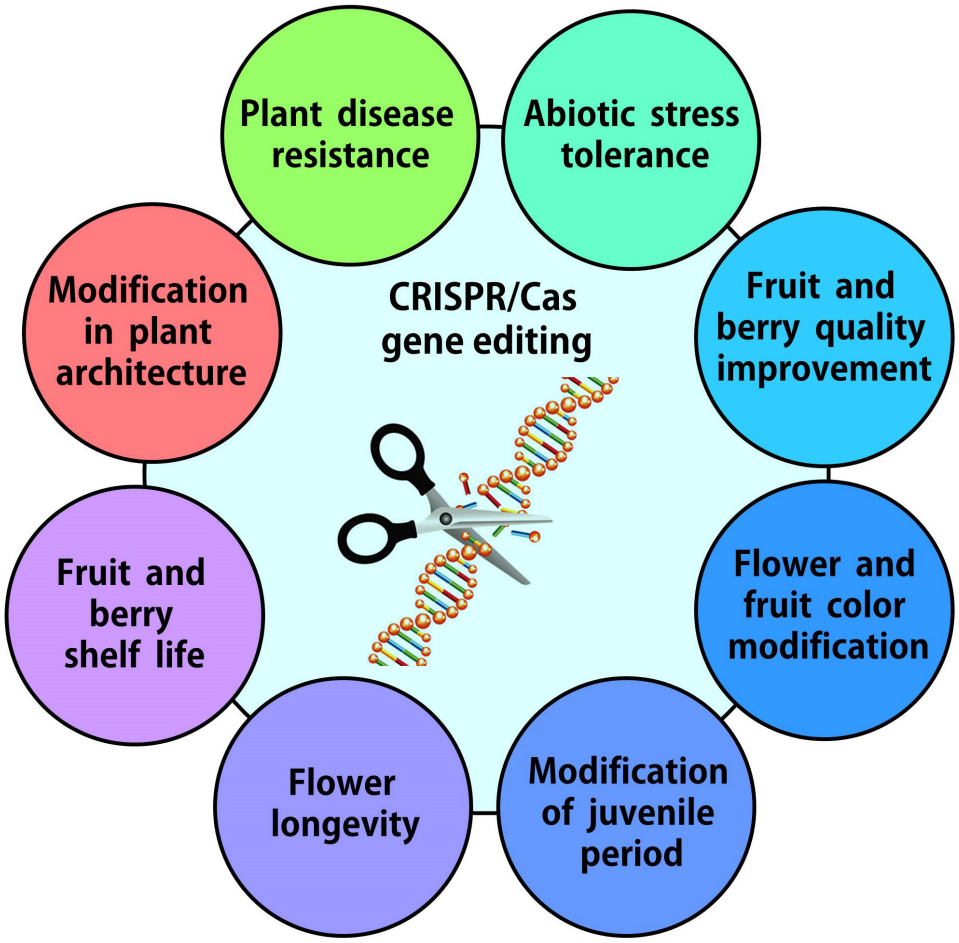

Figure 1. Traits of fruit and berry crops, as well as ornamentals, that can be improved by genome editing.

2. Methods of Delivery of CRISPR/Cas Components to Plant Cells and Optimization of Genome Editing Conditions

Genomic editing experiments usually begin with choosing a target gene. There are several ways of causing gene mutations in plants by means of the CRISPR/Cas9 system—gene

knockout by insertion or deletion of nucleotides in the editing site, insertion of a foreign fragment, and single-base substitutions by deamination of DNA nitrogenous bases [30]. Depending on the task to be solved, the most suitable sites for editing in the plant genome are analyzed with the help of special programs, and the sgRNA design is developed. Furthermore, the editing of off-target sites should be reduced to a minimum; for this, the most conservative DNA sites in the target gene are usually chosen. Then special vector constructs carrying the Cas nuclease and sgRNA genes are formed.

There are several ways to deliver CRISPR/Cas components to plant cells. Frequently, vector constructs with these components are created, and the bacteria *Agrobacterium tumefaciens* or *A. rhizogenes* are used to genetically transform plants. Many binary vectors containing sgRNA and Cas nuclease genes have been created. These genes are most often inserted under the control of promoters of the cauliflower mosaic virus 35S RNA, ubiquitin gene, or U6 and U3 promoters of the RNA polymerase III [31,32]. The disadvantage of this editing technique is the occurring constitutive expression of the sgRNA and Cas nucleases, which can lead to incorrect editing. The way out of this situation can be transient expression of CRISPR/Cas components. In this case, two different vector constructs are often used. The vector containing the sgRNA is used for stable transformation of the plant; the vector with the Cas nuclease gene is used for transient expression. However, the efficiency of gene editing with stable transformation of plants is usually much higher than that with transient expression, due to the permanent expression of the nuclease.

Some laboratories have constructed virus-based vectors for plant genome editing [33–35]. The vectors are delivered to plants by agroinfiltration or biolistic-transformation techniques. Sometimes protoplast transformation using PEG or electroporation is used to deliver vector constructs, as well as ribonucleoproteins (RNPs) containing the purified Cas nuclease and sgRNA. For example, the genomes of grapevine and apple plants were edited by the transformation of protoplasts using RNPs based on Cas9 and sgRNA [20]. Since the foreign DNA in this case was not inserted in the plant genome, the specificity of gene editing was higher. Similar experiments were carried out to optimize the editing of the phytoene desaturase (*PDS*) gene in banana protoplasts [36].

After editing the plant genome using CRISPR/Cas technology, it is necessary to detect plant lines in whose DNA the required mutations occurred. There are several approaches to this. For example, marker green or red fluorescent proteins can be used [37–39]. In other cases, bleaching or mosaic coloring of leaves as a result of the impairment of the function of the *PDS* gene encoding one of the enzymes of the carotenoid biosynthesis pathway can serve as an indirect confirmation of genome editing. This approach has been extensively applied in experiments on gene editing of many fruit and berry crops, such as apple, pear, grapevine, banana, citrus, and strawberry [24,25,32,40–42]. However, more exact signs of gene editing in plants are needed. For this, specially developed PCR methods and whole genome sequencing are often used [43].

Many investigators optimized methods of genome editing of fruit and berry crops by using the *PDS* gene. For example, the main parameters for increasing the frequency of mutations in *Vitis vinifera* L. plants were determined; the main factor proved to be not the level of expression of the Cas9 nuclease, but the high GC content in the sgRNA sequence [31]. In addition, the choice and use of host plant promoters for expression of sgRNA and Cas9 are important [32,44]. An important factor is also the possibility of regenerating edited plants, e.g., from transformed protoplasts or microcalli. The use of the *PDS* gene helped optimize the routes for delivering vector constructs to plants, making more advanced vectors, and developing plant regeneration techniques. In addition, in some cases it became possible to obtain more stable homozygous mutant plant lines. Some examples of editing fruit and berry crops, as well as ornamentals, are given in Tables 1 and 2, and the chronology of their production is shown in Figure 2.

Table 1. CRISPR/Cas9 gene editing in fruit and berry plants.

Plant	Targeted Gene	Trait	References
Apple *Malus domestica*	*DIPM-1, 2, 4* *MdDIPM4*	Fire blight disease resistance	[20,45]
	MdPDS *Md/PcPDS*	Photobleaching, albinism	[25,42,46]
	CNGC2	Resistance to *Botryosphaeria dothidea*	[47]
Banana *Musa Whilliams cv. Cavendish*	*PDS*	Photobleaching, albinism, dwarfing	[48]
Musa acuminata	*PDS*	Photobleaching, albinism	[40,49]
Musa balbisiana	*BSOLV*	Banana streak virus resistance	[23]
Musa acuminata 'Gros Michel'	*MaGA20ox2*	Semi-dwarf phenotype	[26]
Musa acuminata	*MaACO1*	Fruit ripening delay, extended shelf life	[27]
Musa spp.	*LCYε*	Sixfold enhancement of β-carotene content in fruits	[50]
	DMR6	Banana *Xanthomonas* wilt resistance	[51]
Blueberry *Vaccinum corymbosum*	*CEN*	Dwarfism, lack of precocious flowering	[52]
Cacao *Theobroma cacao*	*TcNPR3*	Resistance to *Ph. tropicalis*	[53]
Carrizo citrange *Citrus sinensis* L. Osb. x *Poncirus trifoliata* L. Raf.	*CsALS*	Herbicide resistance	[29]
Coffee *Coffea canephora*	*CcPDS*	Photobleaching, albinism	[54]
Grapefruit *Citrus paradisi*	*CsLOB1*	Citrus canker resistance	[55–58]
Grapes *Vitis vinifera* L.	*IdnDH*	Failure of tartaric acid biosynthesis	[59,60]
	PDS	Photobleaching, albinism	[31,32,61]
	WRKY52	*Botrytis cinerea* resistance	[62]
	MLO-7	Resistance to powdery mildew	[20]
	VvMLO3, VvMLO4	Resistance to powdery mildew	[63]
	VvPR4b	Sensitivity to downey mildew	[64]
	VvCCD8	Highly branched phenotype	[65]
Kiwifruit *Actinidia chinensis*	*AcPDS*	Photobleaching, albinism	[66]
	AcCen4, AcCen	Compact growth, terminal flowering	[67]
	AcCen4, SyGl	Rapid flowering	[28]
Kumquat *Fortunella hindsii*	*FhPDS*	Photobleaching, albinism	[24]
	FhCCD4b	No mutant phenotype	
	FhDUO1		
	FhNZZ	Leaf curling, longer pedicel length	[68]
Melon *Cucumis melo*	*CmPDS*	Photobleaching, albinism	[69]
	CmNAC-NOR, CTR1-like, ROS1	Shelf life	[70,71]
Orange *Citrus sinensis* Wanjincheng	*CsWRKY22*	Delayed citrus canker symptoms	[72]
Papaya *Carica papaya* L.	*CpPDS*	Photobleaching, albinism	[73]
Pear *Pyrus communis* L.	*MdTFL1, Pc TFL1*	Early flowering	[25]
Pyrus bretschneideri	*Md/PcALS*	Herbicide resistance	[42]
	PbPAT14	Dwarf yellowing phenotype	[74]
Pomegranate *Punica granatum* L.	*PgUGT84A23, PgUGT84A24*	Change of phenolic metabolites	[75]
Red raspberry *Rubus idaeus* L.	*F3′H*	No mutant phenotype	[76]
Strawberry *Fragaria vesca*	*FveARF8*	Dwarfism	[77]
	FveTAA1		
	FvPDS	Photobleaching, albinism	[78]

Table 1. *Cont.*

Plant	Targeted Gene	Trait	References
	FvMYB10, *FvCHS*, *FvUF3GT*, *FvLDOX*	Changes in anthocyanin synthesis	
	PDS	Photobleaching, albinism	
	RAP	White berries	[41]
	FveSEP3	Alteration in flowers, abnormal berries	[79]
Strawberry *Fragaria vesca, F. x ananassa*	*FaTM6*	Abnormal petals, anthers, pollen grains and berries	[80]
	PDS	Photobleaching, albinism	[81]
Sweet orange *Citrus sinensis*	*PDS*	Photobleaching, albinism	[41]
	CsLOB1	Citrus canker resistance	[39,82,83]
Walnut *Juglans regia*	*JrPDS*	Photobleaching, albinism	[22]
	JrWOX11	Reduced adventitious root formation and vegetative growth	[84]
Watermelon *Citrullus lanatus*	*ClPDS*	Photobleaching, albinism	[85]
	ClALS	Herbicide resistance	[86]
	Clpsk1	Resistance to *Fusarium oxysporum f.* sp. *niveum*	[87]
	GlBG1	Decreased seed size and promoted seed germination	[88]
	ClCOMT1	Decreased melatonin content	[89]
			[90]

Table 2. CRISPR/Cas9 gene editing in ornamentals.

Plant	Targeted Gene	Trait	References
Chrysanthemum moriflorium	*CpYGFP*	Fluorescence	[91]
Dendrobium officinale	*C3H*, *C4H*, *4CL*, *CCR*, *IRX*	No mutant phenotype	[92]
Japanese gentians *Gentiana scabra x G. triflora*	*Gt5GT*, *Gt3′GT*, *Gt5/3′AT*	Flower color change	[93]
	GST1	Flower color change	[94]
	EPH1	Flower longevity	[95]
Japanese morning glory *Ipomoea nil*	*DFR-B*	Flower color change	[96]
	CCD4	Flower color change	[97]
	EPH1	Flower longevity	[98]
Lilium longiflorum, *L. pumilum*	*LpPDS*	Photobleaching, albinism	[99]
Petunia *Petunia hybrida*	*PDS*	Photobleaching, albinism	[100]
	NR	Deficiency in nitrate assimilation	[101]
		Flower longevity	
	ACO1	Absence of corolla tube venation	[102]
	AN4	Self-incompatibility	[103]
P. inflata	*PiSSK1*		[104]
Phalaenopsis equestris	*MADS8*, *MADS36*, *MADS44*	Long juvenile period	[105]
Poinsettia *Euphorbia pulcherrima*	*F3′H*	Change of the bract color from red to reddish orange	[106]
Torenia fournieri	*TfRAD1*	Abnormal shape and color of flowers	[107]
		Pale blue flowers	
	F3H		[108]

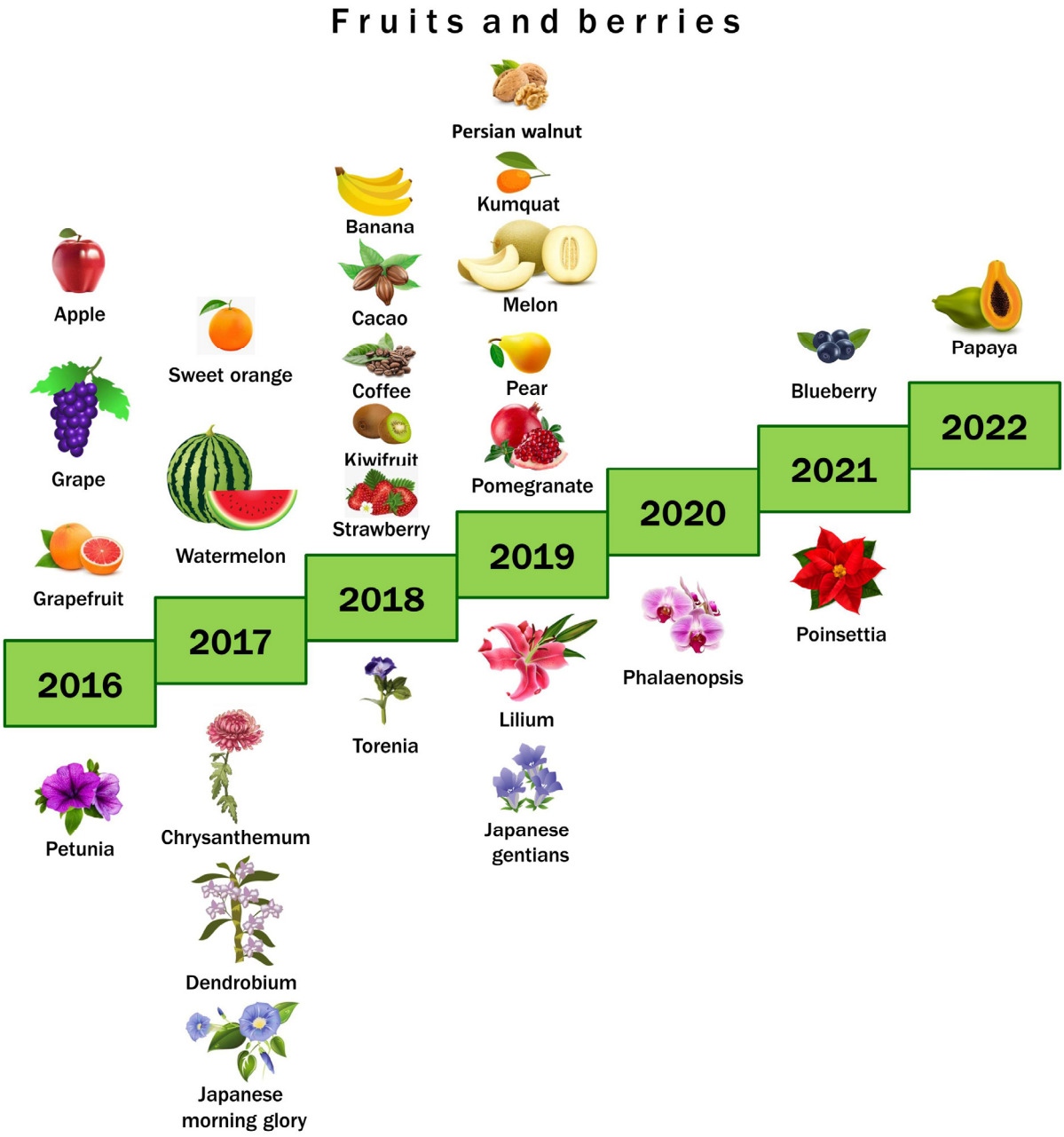

Figure 2. Chronology of producing fruit and berry crops and ornamentals with new traits by gene editing using the CRISPR/Cas9 technology.

3. 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 [23]. 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* [20]. 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 [45].

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 [22,58]. Citrus plants have the *CsLOB1* gene responsible for sensitivity to a disease caused by the bacteria *Xanthomonas citri* subsp. *citri* [109]. 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 [110]. 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* [22]. 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) [111]. 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 [72]. Genome editing also contributed to producing banana mutants with the *DMR6* gene resistant to banana wilt caused by *Xanthomonas* bacteria [51].

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* [20]. 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 [60]. Mutations in three *MLO* genes resulted in grapevine plants with a 77% lower sensitivity to powdery mildew [21]. 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 [62,112]. 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 [63]. 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 [47]. 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* [53]. 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* [88]. 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 [87]. 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 [42]. 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 [29].

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.

4. 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" [26]. 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 [65]. 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 color 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 [79]. 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 [50]. 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 [76]. Another target for editing was the *MYB-16*-like gene, which is a possible regulator of prickle formation in raspberries [113]. 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 [89]. 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 [27]. 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 [69–71].

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.

5. 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 [77]. 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 [80,81]. 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 [25]. 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 [28,67]. 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 [52]. 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 [25,28,67]. 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 [52].

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 [91–108]. For example, the *PhACO1* gene involved in the regulation of ethylene biosynthesis was edited in petunia cultivar "Mirage Rose" plants [102]. 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 [98]. 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 [96,97]. Other investigators carried out a knockout of the flavone 3-hydrolase (*F3′H*) gene encoding one of the key enzymes of flavonoid biosynthesis [108]. 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 [99]. There are few studies of orchids using CRISPR/Cas9 gene editing [92,105].

Thus, the feasibility of using the CRISPR/Cas9 technology to change various parameters of horticultural plants, such as flowering, flower coloring, and the flowering period of plants, has been shown.

6. Limitations in the Use of CRISPR/Cas9 in Genome Editing of Horticultural Plants and Further Prospects

Although CRISPR/Cas9 genome editing technology has greatly accelerated the production of improved varieties of horticultural plants, there are still a number of limitations and difficulties when using this method. There are difficulties in obtaining edited offspring of fruit and berry crops, as these plants reproduce vegetatively. In addition, it is necessary to take into account the long juvenile period for fruit trees, polyploidy, and the difficulty of obtaining homozygous lines. There are few cases of obtaining garden plants of the T1 generation, e.g., kumquat *CCDb4* mutants [24], strawberry *FveARF8* mutants [77], as well as kiwi plants with early flowering due to the knockout of the *AcCEN* and *SuGl* genes [28].

For some fruit and berry crops, methods of transformation and regeneration of edited plants need to be developed and optimized. For example, special protocols with an additional stage of regeneration were developed for apple and pear trees in order to obtain plants without chimerism [25,42].

In addition, new marker genes for the selection of plants with mutations should be searched for because mutations in the frequently used *PDS* gene can lead to abnormal and non-viable phenotypes. It is not always that the use of CRISPR/Cas9 for genome editing leads to homozygous plants; chimeric and biallelic lines emerge more often because the nuclease in transformed plants continues to work at all stages of their development. To overcome these disadvantages, it is necessary to use not only constitutive but also inducible promoters.

Recently, new methods of editing the plant genome using modified nucleases have been developed. A disadvantage of the CRISPR/Cas9 system is that only the gene knockout can often be performed because the nuclease performs mainly *indel*-like mutations. In addition, the Cas9 nuclease strongly depends on the PAM sequence, and sometimes, because of this, it is not always possible to edit the gene. In these cases, modified nucleases can be used that do not strongly depend on the PAM sequence, e.g., Cas9-NG, xCas9, and SpRY [114–117]. The use of more precise nucleases, such as the Cas12 (Cpf1) nuclease from *Francisella novicida*, can be more promising than Cas9. Some researchers have shown the effectiveness of the Cas12 nuclease for genome editing of citrus fruits [18,111]. However, a comparison of the use of Cas9 and Cas12a for editing the beta-glucuronidase (*gusA*) marker gene in blueberry plants did not lead to the same results [118]. Despite the fact that Cas12 showed a high efficiency for editing various plant species [119,120], it was not possible to achieve the same effect for blueberries. Recently, several highly efficient CRISPR/Cas9-

based systems for editing a number of genes in pear microcalli have been developed [121]. The Cas12a and Cas12b nucleases tested in the same studies led to low editing efficiency. The authors suggest that gene editing using various nucleases depends on the type of plant. Researchers have also developed other variants of Cas12 nucleases. For example, the use of the temperature-tolerant variant of the ttLbCas12a nuclease for editing the *LOB1* gene proved more effective than the Cas9 nuclease and led to the production of canker-resistant pummelo (*Citrus maxima*) plants [122]. There have been a few studies of successful editing of the plant genome using nucleases from other microorganisms and phages—Cas13 and CasΦ [19,123,124].

To develop more efficient genome editing, new improved enzymes are emerging, as are new systems for editing and simultaneous activation of gene transcription, such as CRISPR-Act3.0 and CRISPR-Combo [121,125,126]. For several plant species (*Arabidopsis*, poplar, rice, tomato, and pear), it was demonstrated that the use of such systems increased the frequency of editing as well as accelerated the regeneration, reproduction, and flowering of edited plants.

Many researchers use various online tools to develop sgRNA sequences. A new program, CROPSR, for genome-wide design and evaluation of sgRNA sequences for CRISPR experiments has recently been proposed [127]. CROPSR should with great efficiency assist researchers in conducting experiments on editing plant genes using CRISPR/Cas9 technology. This program is useful for experiments with polyploid genomes as well as gene regions with a high A/T content.

Thus, despite some limitations in the use of genome editing on horticultural plants, there are prospects for faster production of improved varieties of fruit and berry crops, as well as ornamentals valuable for horticulture.

7. Legal Regulation of Growing Plants Produced by Genome Editing Technology

One of the topical challenges is the need to develop a unified regulatory framework for the introduction of genome-edited plants into agriculture and horticulture. Various countries have worked out laws regulating the commercial use of such plants, but a unified mechanism does not exist. For example, in 2018, the USDA decided not to regulate the cultivation of plants with an edited genome, as in most cases they do not differ from plants obtained through conventional breeding. In most countries of North and South America (the USA, Canada, Argentina, Chile, Colombia, and others), there are no special restrictive laws regarding genetically edited plants and products from them [128,129]. On the whole, plants produced by modern technologies can be approved in these countries based on their new traits, not their production method. What is more, approaches without introducing a genetic construct into the plant genome (DNA-free) are often used to produce gene-edited plants. RNPs consisting of a Cas nuclease and sgRNA can be used for this [20]. The resulting plants can no longer be classified as genetically modified, as they do not contain foreign genes in the genome. In addition, at the usual transformation using vector constructs, as a result of a number of crosses, edited plants can have no such genes in the genome. Thus, plants with an edited gene often do not differ from plants with useful mutations obtained as a result of conventional breeding. Obviously, there should be no obstacles to the commercial use of such plants, as they should not be subject to the laws adopted for transgenic plants.

However, in some countries genetically edited plants are also perceived in the same way as genetically modified plants. For example, in 2018, the European Court of Justice extended the European Union (EU) legislation for genetically modified plants to edited plants; thus, a restriction on their use in the EU was introduced [130,131].

The sources available to us have no data on field trials of fruit and berry crops with an edited genome, unlike other plants important for agriculture. For example, several field trials are known for such genetically edited crops as rice; rapeseed; peanuts (China); tomatoes (USA, China); corn; sugarcane (USA); camelina; and cabbage (UK) [132–134]. None of the conducted field trials showed any environmental damage from genome-edited

plants. However, there have been almost no field trials in the EU countries since 2018. Some countries, e.g., China, are introducing new rules for regulating the cultivation and use of genetically edited plants [135]. These rules are more conservative than in the US, but relatively mild as compared with the EU. At the beginning of 2022, an application was submitted to the Ministry of Agriculture of China for the use of powdery mildew-resistant wheat cultivars created using genome editing. Work on the creation of this wheat was started back in 2014, and great success has been achieved since then [136,137].

However, in many countries, the status of plants with an edited genome has not yet been resolved and is in the process of discussion. Nevertheless, a number of countries are planning to approve the cultivation and commercial use of such plants in the near future.

8. Conclusions

To date, the effectiveness of genome editing in horticultural plants using CRISPR/Cas9 technology has been confirmed. Genome modification has been successfully carried out for many fruit, berry, and ornamental plants. Dozens of genes from these plants have been modified using genome editing in order to make plants resistant to biotic and abiotic stress factors, to change the time of flowering and ripening of fruits, and to improve the growth characteristics of plants as well as the taste properties of their fruits. Modified enzymes for developing more efficient genome editing appear, as well as new improved systems for editing and simultaneous activation of gene transcription, which is relevant for the creation of new varieties of horticultural plants. A thorough analysis of plant species obtained under field testing conditions with traits valuable to humans is required to enable their subsequent commercial use.

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