

Review

# Applications of CRISPR/Cas9 System in Vegetatively Propagated Fruit and Berry Crops

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**Abstract:** Fruit and berry crops, as well as grapes, are important parts of the human diet and, at the same time, significant objects of genetic, breeding, biochemical and nutritional research. Traditional approaches of crop research and improvement are now complemented by effective modern genetic technologies. In this review, we analyze and summarize the achievements in genome editing of fruit, berry crops and grapes. New approaches accelerate the improvement of genotypes for many groups of traits: plant resistance to unfavorable environmental factors, flowering and ripening time, plant architectonics, fruit shelf time and biochemical composition. Genome editing using the CRISPR/Cas9 system has been successfully tested on the most important vegetatively propagated fruit and berry crops (apple, pear, orange, kumquat, grapefruit, banana, strawberry and kiwi) and grapes. About 30 genes of these crops have been used as targets for the introduction of desired mutations using the CRISPR/Cas9 system. The most valuable results are the improvement of important agronomic traits. For 24 genes it has been shown that their knockout can result in the improvement of varieties. In addition, the review pays attention to the comparative analysis of the explant types of vegetatively propagated crops used for the delivery of editing genetic constructs, as well as the comparison of the editing efficiency depending on the variation of the objects used, delivery methods, etc. The article discusses the existing limitations that need to be overcome for a wider application of genomic editing in order to improve varieties of fruit and berry crops, as well as grapes.

**Keywords:** genome editing; CRISPR/Cas9; fruit and berry crops; grape; stress susceptibility; flowering time; plant architectonics; shelf time; plant disease resistance



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## 1. Introduction

The UN General Assembly has declared the 2021 year the International Year of Fruits and Vegetables, urging the world community to pay attention to alterations from healthy nutritional norms that have formed due to the excess of food in some regions of the world and their limitations in others [1]. Fruit and berry cultures, such as apples, grapes, bananas, kiwis, pears, strawberries and citrus, are valuable sources of fiber and nutrients that include easily digestible carbohydrates, organic acids, vitamins, micronutrients, antioxidants and other valuable metabolites [2–7] and constitute an integral part of the food culture and modern agricultural industry [8–14].

The quality of modern fruits and berries and their yield and pathogen resistances are the result of long painstaking work through hybridization, progeny characterization and selection. Before the desired phenotype could be selected, a lot of time passes due to a long juvenile period, open pollination and plant heterozygosity waiting for obtaining new stable varieties of perennial fruit and berry cultures in general and woody plants especially.

Modern breeding methods by means of diagnostic DNA markers usage (marker-assisted selection) or genomic selection based on genetic and genomic data (both based on preliminary studies, such as the detailed genetic maps development, NGS-analysis of genomes, transcriptome, microRNome, etc.) have created conditions for rapid and less labor-intensive improvement of fruit properties and the selection of pathogen and pest-resistant varieties [15]. Next-generation breeding methods for genome fruit and berry cultures editing are being intensively developed today [16].

Since the CRISPR/Cas9 system surpasses earlier editing methods (TALEN, ZNFs and meganucleases) in terms of versatility, simplicity and relative cheapness, it has become possible to widely use genetic editing in agriculture [17]. CRISPR/Cas9 is composed of nuclease Cas9 and sgRNA and functions in the form of a ribonucleotide complex: Cas9 recognizes the PAM (protospacer adjacent motif) and cleaves the target sequence to which the sgRNA is complementary. Nucleases make double-stranded breaks in DNA, which initiate cell repair systems and result in the appearance of deletions or insertions at the site of the break as a result of non-homologous end joining or homology-directed repair mechanisms. If there was a frame shift during the DNA breakage repair, then the gene loses its function [17–19].

CRISPR/Cas9-directed editing gave a unique opportunity for multiplex and polycistronic editing of several targets at the same transformation with relatively high rates [20–22]. This property of the genome editing system is especially valuable for perennial wood plant modifications.

The optimized genome editing techniques provide a unique opportunity to change the properties of commercially viable plant varieties, ensuring their high productivity without the use of redundant treatments with fungicides and pesticides while maintaining the valuable plant variety characteristics. It is hard to overestimate the social, economic and ecological significance of the genome editing techniques for vegetative propagated fruit and berry crops.

Genome editing of first fruit and berry cultures has been conducted by CRISPR/Cas9 fairly recent [23–25]. This review summarizes the results of CRISPR/Cas9 genome editing studies of different groups of target genes in fruit and berry crops: (1) in order to decrease biotic and abiotic stress susceptibility [19,24–39], (2) change flowering time and plant architectonics [40–45], (3) increase shelf-time [46], (4) study gene functions [43–45,47–49] and (5) optimize CRISPR/Cas9 editing of fruit and berry cultures by using the marker [22,23,32,41,43,44,50–61].

## 2. Optimization of Editing with Marker Gene Usage

The primary experiments usually target marker gene with a bright mutant phenotype, which provides an opportunity to determine gene-editing events at early plant shoot growth stage in order to select optimal gene editing conditions [52,58–64] (Table S1).

Gene *PDS* encodes phytoene desaturase—one of the main carotenoid biosynthesis pathway enzymes that is widely used as a marker for gene editing methods optimization due to bright *PDS* mutant phenotype, which include albino, variegated or pale-green that depends on mutation pattern of transformed plant. Phytoene desaturase catalyzes desaturation of phytoene to  $\zeta$ -carotene, which is further converted to lycopene. The components of the carotenoid biosynthetic pathway interact with a variety of cell metabolites such as abscisic acid, strigolactones, gibberilic acid, chlorophyll, plastoquinones and tocopherol, which regulate development and are involved in plant adaptation to environmental changes [64,65]. The ability to detect mutant phenotype at the early stages of plant growth for quickly checking the effectiveness of the selected conditions is especially valuable in experiments with perennial crops, which include fruit crops, most berry crops and grapes. This approach, based on the use of the marker gene *PDS*, has been successfully applied in the optimization of conditions for the CRISPR/Cas9 editing of plants as grapes, apple trees, bananas, strawberries, kiwi and citrus [22,23,32,41,43,44,50–61] (Table S1).

Using the grape *VvPDS* marker gene (*Vitis vinifera* L.), a key parameter for editing frequencies was determined: a high GC content in the guide RNA sequence, which had a greater effect on mutation rate than the Cas9 expression level [58]. Later, the same authors identified and tested promoters *VvU3*, *VvU6* and *VvUBQ* for the expression of sgRNA and Cas9, the optimal design schemes for multiplex editing of the grape genome using polycistronic tRNA–gRNA constructions (PTG/Cas9) were selected [53]. The regions adjacent to tRNA are recognized and cut by plant P and Z RNases, forming several separate gRNAs, which can direct the nuclease to the different parts of one target gene or to different loci of the genome [22]. In the work of Osakabe et al. a method of non-transgenic edited grapes and apple trees (*Malus domestica* Borkh.) by means of protoplast transformation with ribonucleoprotein particles (RNP) and the conditions for the regeneration of microcalli of both plant species from the obtained edited protoplasts were selected [52] (Table S1).

The apple tree became the first edited plant from the *Rosaceae* family. The use of the *PDS* marker gene revealed the possibility of editing the apple tree genome using shortened gRNAs (18 bp) to obtain a relatively high rate of homozygous mutants (13.6%) [23]. Subsequently, *PDS* usage as an editing marker target gene made it possible to achieve a higher mutation rate (84–90%) [41]. Zhang et al. have shown that in wild apple (unlike grapes) genome integration rate, mutation rates in both one and two *PDS* target sites were higher, while gRNA expression was controlled by separate promoters rather than the PTG/Cas9 expression system [61]. Malabarba et al. demonstrated a high degree of chimerism elimination in apple and pear (*Pyrus communis* L.) shoots with the introduction of an additional stage of transformants regeneration, which may be promising to try on other fruit and berry cultures [41,50] (Table S1).

Naim et al. obtained *PDS1* knockout banana plants (*Musa accuminata* Colla) using a self-cleaving PTG/Cas9 [21,22]. For the expression of Cas9, two CaMV-35S promoters and the promoter of the ubiquitin gene Ubi P were used, which ensured the expression five times higher than with the CaMV-35S promoter. The selected transformants had variegated phenotypes: from albino with a knockout in three alleles to green with insertions/deletions that do not shift the reading frame [22]. Kaur et al. demonstrated the variation in the coding sequences of the *PDS* gene among different banana cultivars. Kaur's work points to the need for sequencing target genes before planning the editing in order to increase specificity and reduce off-target rate [55]. In Ntui et al. research studies, the genomes of two banana lines (AAB) were edited with a high mutation rate: the usage of gRNAs relative to the conserved *PDS* region resulted in the absence of off-target editing; 67–94% of the plants had an albino phenotype due to the editing of all alleles of the *PDS* gene [56] (Table S1).

A comparison of different genetic constructs was carried out with kiwi fruit (*Actinidia deliciosa* A. Chev.), and the advantage of using PTG/ Cas9 constructs compared to the standard method using an individual promoter for each gRNA was shown. With PTG/ Cas9 usage, a 10-fold increase in mutation rate was observed due to the tRNA sequence also acting as a transcription enhancer of the RNA polymerase III-initiated promoters. In addition, due to the hydrolysis of a single transcript by RNases and the simultaneous release of two gRNAs in ratios close to equimolar, the authors obtained large deletions: that result was especially valuable for the stable knockout lines selection that will not be able to reverse [60].

Similar results were obtained by Huang et al. on citrus (*Citrus × sinensis* (L.) Osbeck), showing a higher PTG/Cas9 constructs efficiency for *PDS* knockout. As a result, biallelic mutations with a 44.4% rate and homozygous mutations with a 11.1% rate were obtained, and the possibility of obtaining large deletions (15 kb) using CRISPR/Cas9 was demonstrated [32]. Zhu et al. used a marker gene knockout to test editing conditions on another citrus crop kumquat (*Fortunella margarita* (Lour.) Swingle), comparing the mutation rates, the regeneration of explants and mutations inheritance in the T1 generation. In this work, the kumquat genome was first sequenced and characterized. It was shown that due to the short juvenile period (8 months compared to 5–10 years of other citrus fruits), stable monoembryony and close phylogenetic relationship with other cultivated citrus fruits (es-

pecially mandarin), kumquat can be successfully used as a model for other plants relative to studying the *Citrus* genus [59].

The above-mentioned results suggest that the delivery of several gRNAs using PTG/Cas9 vectors is more efficient for genome editing of such fruit and berry crops as grapes and kiwis, unlike apple tree [21,22,53,60,61] (Table S1). Thus, for different fruit and berry crops, the *PDS* marker gene usage for genome editing experiments made possible both to optimize the gRNA/Cas9 expression and selection/regeneration steps and to change the ratio of chimeric/homozygous plants.

### 3. CRISPR/Cas9 for Increasing Disease and Pest Resistance

One of the most important goals of genome editing is to decrease the susceptibility of crop plants to fungal, viral and bacterial pathogens, which brings not only an obvious economic profit but also contributes to human health and environment.

Gray mold, powdery mildew and downy mildew are the main diseases of grapes, which reduce grape yields and create the need for annual preventive treatments of vineyards with fungicides, which not only spoil the ecology of the regions but also negatively affects the quality of the harvested berries. Downy mildew, powdery mildew and grape gray mold are caused by *Plasmopara viticola* (Berk. and M.A.Curtis), Berl. and De Toni, *Erysiphe necator* (Schwein.) and Burrill and *Botrytis cinerea* Fr., respectively. Infection triggers the cell response to biotic stress mechanisms through the ethylene/jasmonate (JA) and the salicylic acid signaling pathways (SA) [66]. In response to biotic stress and in order to limit the spread of infection, a number of expression changes are initiated followed by biochemical and histological alterations in the plant, including the release of phenolic and peroxide compounds and the formation of deposits of callose, papillae and lignin at the site of infection [66,67].

Over the past 20 years, many transgenic grape varieties have been obtained with overexpression of homologous and heterologous components of cell response to biotic stress: pathogen-associated proteins (VvNPR1.1; RCC2; RIP; Chi 11; VpPR4-1; Vvtl-1; VqTLP29; VaTLP; VpPR10.1), antimicrobial peptides (*Xenopus laevis* Mag2), transcription factors (VvWRKY2; VpWRKY3; VvWRKY33; VvbZIP60; VvDOF3; VvTIFY9; VvZPF11), secondary stress-associated metabolites (Vst1; STS) and genes associated with cell defense (VqJAZ4; pPGIP; nag70; VpEIRP1; VvSNAT2; RPW8.2; VpPUB23; VaPUB; VaHAESA), which were characterized by false mildew and gray mold decreased sensitivity. Nevertheless, the use of these varieties in the industry is associated with additional time and material costs to verify the safety of plant integration into ecosystems and human consumption [68–70]. Due to the safety, speed and low cost for new varieties with pathogen resistance to enter the market, the use of CRISPR/Cas9-mediated genome editing has an undeniable competitive advantage.

Thus, in the work of Wang et al., grape transformants resistant to gray mold were obtained by the *WRKY52* gene editing. *WRKY52* encodes the transcription factor of the cell's response to biotic stress, a negative regulator of the JA pathway [19]. Later, the same authors have shown high specificity of CRISPR/Cas9 technology for grapevine editing by whole genome sequencing of edited *VvWRKY52* and *VvbZIP36* T0 plants [39]. *VvbZIP36* encodes basic leucine zipper transcription factor, which increases drought tolerance of grape by the regulation of abscisic acid pathway and stress response genes [71]. (Table S1). Grapes are one of the first crops that were modified by direct delivery of guide RNA in ribonucleic acid particles (RNP): This is how regenerants of grapes with the *MLO7* gene mutation were obtained, but the mutation rates were quite low 0.1–6.9 % [24]. This technique was improved by Osakabe et al., who managed to obtain a successful protocol for delivery and genome editing using RNP, which takes only 2–3 weeks, while the standard protocol for agrobacterial transformation with a plasmid for CRISPR/Cas9 editing and selection takes 2–3 months [52]. It is assumed that the transmembrane proteins *Mlo* are the negative regulators of the cell defense mechanisms through vesicular transport and actin cytoskeleton reorganization in response to the biotic stress. *MLO*-mediated

powdery mildew resistance is associated with H<sub>2</sub>O<sub>2</sub> accumulation and host cell death, which result in the formation of papillae and cell wall apposition (CWA) on the *MLO* mutations' background [35,72] (Table S1). It was shown that grape of the 'Brachetto' variety with the triple knockdown *mlo7*, *mlo6* and *mlo11* had a 77% reduction in susceptibility to powdery mildew [73]. *MLO* genes mutations often have pleiotropic effects: necrotic leaf spots, accumulation of peroxides and phenolic conjugates on barley, reduced yields and premature senescence of *Arabidopsis thaliana*, barley and wheat mutants and slow growth of pepper. Nevertheless, in strawberries, apples and grapes, the mutant pleiotropic effects were not described [72–78].

In grapes, *Xylella fastidiosa* Wells. bacterial infection (XF) (Pierce's disease/ PD) and Red Blotch Disease Viral Infection (GRBV) are accompanied with anthocyanin accumulation regulated through the activity of the MYBA7 transcription factor. The first symptom of GRBV viral disease in grapes is the appearance of red spots on the leaves. The GRBV is carried by sap-sucking insects that develop in the plants' xylem, and as the disease develops in grapes, delayed fruit ripening, acid accumulation and a decrease in the concentration of sucrose and anthocyanins in berries are observed [27,79]. The XF pathogenesis in grapes is associated with inorganic phosphate level decrease, the accumulation of anthocyanins and abscisic acid [80]. It is known that phosphate, sugar and ultraviolet light regulate the accumulation of anthocyanins through miR828 and the transacting small interfering RNA locus 4 (*TAS4*). In grapes, this process is regulated through the formation of small interfering RNAs *TAS4a/b/c*, which are differentially expressed and target *MYBA5*. Sunitha and Rock selected *TAS4* and *MYBA7* gene knockout lines [27] (Table S1). The regenerated plants showed no apparent anthocyanins accumulation, possibly due to the presence of repeated loci *TAS4c* and *MYBA5/6* or to the absence of inductive environmental stress conditions. The absence of obvious visible pigment changes in the edited plants made it difficult to test the role of anthocyanins in the mechanisms of resistance/ tolerance to PD and GRBV; nevertheless, further study of the transformant shoots seems to be very promising for the selection of PD and GRBV resistant plants. Previously, the inhibition of XF growth by phenolic compounds has been shown, although the level of accumulation of polyphenols in response to infection can vary greatly among different cultivars [81].

Another fruit crop that is losing yield because of diseases is the apple tree. Zhou et al. knocked out the cyclic nucleotide-gated ion gene (*CNGC2*) and showed the increasing resistance of apple callus cells to the fungal infection *Botryosphaeria dothidea* (Moug. ex Fr. Ces. and De Not.) and demonstrated a concomitant constitutive increase in salicylic acid (SA) concentration and suppression of *PR* gene expression. Thus, the authors demonstrated that the protein *CNGC2* acted as a negative regulator of *B. dothidea* resistance. At the same time, *CNGC2* mutations have some pleiotropic effects and, in addition to resistance to the pathogen, resulted in decrease in fertility, impaired pollen tube formation, and shortened anthers; therefore, as a target for development of pathogen-resistant apple varieties, they are not the optimal choice [29] (Table S1).

For the apple tree, the method of delivering Cas9 and guide RNA in RNP particles was used: the genes *DIPM-1*, *DIPM-2* and *DIPM-4*, encoding receptor protein kinases, were knocked out [24]. *DIPM* interacted with the effector protein DspA/E of the bacteria *Erwinia amylovora* Burrill, which causes bacterial burns of apple and other *Rosaceae* [82]. Pompili et al. obtained knockout of the *MdDIPM-4*, while foreign DNA was eliminated from the genome during heat shock by using the FLP/FRT recombination system, which is a promising approach for obtaining edited plants to accelerate and simplify the integration of a successful cultivar into industry [28] (Table S1).

Subtropical banana (*Musa* L.) and citrus crops (*Citrus* L.) are other types of fruit crops with great economic importance. In order to make bananas *M. accuminata* Cavendish (genome AAA) resistant to pathogens, the hybridizations with the diploid bananas *M. balbisiana* (genome BB) and its derivatives, which are distinguished by their endurance, strong root system and resistance to biotic and abiotic stresses, are used. A significant limitation of hybridization using *M. balbisiana* and its derivatives is the frequent infection of B-genome plants with

the banana streak virus (BSV), which results in the plant's death. Tripathy et al., by using the CRISPR/Cas9 editing, introduced mutations in integrative viral elements, making it impossible to transcribe and/or translate functional viral proteins [30,31].

The main pathogen of citrus, which significantly reduces yield, is a bacterial ulcer caused by the pathogen *Xanthomonas citri* subspecies *citri* (Xcc). *CsLOB1* gene products (LATERAL ORGAN BOUNDARIES 1) are the transcription factors involved in the regulation of plant tissue formation and the main targets for editing and selection of Xcc-resistant citrus fruits [83]. In orange, *CsLOB1* is known to have three alleles; the EBEPthA4 regulatory element was found in promoter regions of two of them. EBEPthA4 element is recognized by the main effector Xcc, which results in the *CsLOB1* expression activation and triggers the infection. Peng et al. obtained Xcc-resistant plants by knocking out the EBEPthA4 and TATA-box of the *CsLOB1* gene in orange [33] (Table S1). Similar results were published earlier on Duncan's grapefruit (*Citrus paradisi* Macfadyen): The authors demonstrated complete resistance to Xcc in plants biallelic for the *LOB1* mutation. The identified heterogeneity of the promoter region in grapefruit, which is a hybrid of a pomelo and an orange, is also interesting [25] (Table S1). The main transcription factors of the citrus immune response to Xcc infection are WRKY22 and WRKY29. The expression level of WRKY22 is significantly different in the sensitive «Newhall» orange variety, which is characterized by a high level of expression of this TF, and in resistant calamondin (*Citrus microcarpa*) with low levels of expression [84]. Wang et al. obtained an orange with significantly reduced sensitivity relative to Xcc by using CRISPR/Cas9 to obtain chimeric mutants *CsWRKY22*. The use of different gRNAs showed that there was no correlation between the on-score parameter and editing efficiency, while successful gRNAs had higher GC-content [34] (Table S1). *Citrus* genomes often contain several allelic variants of target genes; in order to select the optimal scheme for highly efficient editing, it is desirable to sequence the target genes and use several gRNAs [85].

Thus, editing the genome using the CRISPR/Cas9 system is an effective tool for obtaining various fruit crops plants resistant to pathogens; however, the stability of modification and the impact of each mutation on plant metabolism and its varietal characteristics are promising research areas.

#### 4. Genome Editing for Changing Time of Flowering, Plant Architectonics and Shelf Life of Fruits

Along with creating pathogen-resistant varieties, genome editing provides an opportunity to change the architectonics of crops, which can improve crop yields and adaptability to environmental changes. For example, Ren et al. used CRISPR/Cas9 editing to knockout the *CCD8* gene, which encodes a component of the biosynthesis of strigolactone hormones from carotenoids [40,86]. Selected *Vvccd8* mutants were characterized by increased shoot branching [40] (Table S1). The weak lodging and high height of banana plants results in significant yield losses in the event of storms and typhoons. The creation of dwarf varieties, without reducing yield and quality of the fruit, can save plantations and crops in unfavorable climatic conditions and simplify the collection of fruits. Mutations in genes encoding the signaling components and gibberellin biosynthetic pathways often have a dwarf phenotype [87]. Shao et al. obtained mutants of the banana cultivar 'Gros Michel' for two genes, *Ma04g15900* and *Ma08g32850*, with reduced height. The *Ma04g15900* mutant was characterized by an altered spectrum of gibberellins and an irregular structure of the stratum corneum and epidermal cell layers [42].

The second group of target genes includes potential repressors of flowering and growth of fruit and berry plants. For example, Charrier et al. edited apple and pear plants, knocking out the *TFL1* gene encoding the flowering repressor protein (Table S1). The edited plants showed earlier flowering times. Interestingly, for *TFL1* editing of both apples and pears, the same gRNA was used [41].

In Varkonyi-Gasic et al. studies on kiwi, a double knockout of *AcCEN* and *AcCEN4* genes, the main candidates of vegetative growth and flowering control, was obtained, which resulted in the production of four kiwi lines, three of which had compact bush shapes

and early flowering, and one line was characterized only by early flowering. Pollination of these lines resulted in three lines of seeds with 100% germination [44]. Subsequently, the authors continued to study the influence of the *CEN* and *AcCEN4* genes and the *SyGl* (*Shy Girl*) gene on the kiwi dioecy. The authors analyzed the phenotype of F1 plants obtained by self-pollination of the T0 kiwi with *CEN4 SyGl* double knockout, which gave precocious, small vines. The authors found that *SyGl* affects the cytokinin profile and was involved in the regulation of cytokinin biosynthesis pathway gene expression. These results indicate that *SyGl* acts as a suppressor of feminization in kiwifruit and could potentially serve as an accelerator of breeding in outcrossing horticultural woody plants. In addition, this work is one of the few that demonstrates the successful inheritance of the CRISPR/Cas edited genes and the desired traits in F1 perennial woody plants [45].

Hu et al. have edited banana genome at *MA-ACO1* loci, which encodes a key component of the ethylene biosynthetic pathway; the plants were characterized by extended shelf-time [46,88]. Six banana lines were selected and analyzed: the edited plants had 15% smaller size and 5–14% smaller fruit length and weight. Two of the six lines were planted in the open field, and the time of their full ripening was analyzed. The control line berries have ripened in 21 days, and the edited bananas were still bright yellow upon the 60th day, some with green peel. At the same time, the treatment with the growth regulator ethephon resulted in an almost complete ripening time restoration of edited fruits lines, which is convenient for controlled banana ripening. The fruits obtained from *MA-ACO1* edited lines were also characterized by increased content of vitamin C [46] (Table S1).

These studies have shown the high potential of using the CRISPR/Cas9 system to change the flowering time, ripening and shelf life of fruits, as well as to modify the architectonics of the plant in order to reduce losses and render easier crop harvesting.

## 5. CRISPR/Cas9-Based Knockout Implementation in Reverse Genetics Studies

The CRISPR/Cas9 system has become a revolutionary technology in reverse genetics.

Thus, the L-idonate dehydrogenase (*VvL-IdnDH*), which is involved in the accumulation of tartaric acid (TA) in grape berries and leaves, was studied [51] (Table S1). TA is the main organic acid of grapes, accumulated during the first 4 weeks of ripening. TA is not utilized during fermentation, creates an acidic pH up to 3–3.5 and determines the resistance of grapes to microorganisms, potentiates ripening and determines the taste of wine and its oxidative characteristics [89] (Table S1). CRISPR/Cas9 was also used for confirmation of the role of GRAS-domain transcription factor *VaPAT1* in JA-biosynthesis and cold stress response of grape *Vitis amurensis* [47]. In grapes, the *PR4* gene (pathogenesis-related) was edited, and its role in *P. viticola*-associated powdery mildew pathogenesis was defined. *PR4* encodes chitinase, a chitin-binding protein with DNase activity that inhibits pathogen hyphae growth and has antifungal activity [26] (Table S1).

Strawberries (*Fragaria vesca* L.) serve as a model object for studying the effect of auxins on fruit development due to the surface localization of seeds on an overgrown floral receptacle. As early as 1950, it was demonstrated that if the ovary is removed after fertilization, the receptacle ceased to develop, but it resumed by means of auxins during the growth of the receptacle. Sequencing of total RNA confirmed the activation of genes of the auxin biosynthesis pathway in achenes after fertilization [90]. It is not surprising that the genes of the auxin biosynthesis pathway were the first genes to be edited in strawberries. Thus, in 2018, the first CRISPR/Cas9 editing work of *FveARF8* and *FveTAA1* (encode components of the auxins biosynthetic pathway) was published. For the expression of guide RNA, the *AtU6-26* and *FveU6-2* promoters were used. In the T0 generation, high editing efficiency (49–75%) was shown, which became even higher in T1 possibly due to the introduction of new mutations in 83% of the plants by the preserved Cas9. Some plants became homozygous at T1, lost Cas9 and had stabilized mutation at T0, which is especially valuable for further safe agricultural industry integration of edited plants. Homozygous *FveARF8* mutant plants were characterized by rapid growth and larger size compared to control plants [43]. Another target gene for editing was *FveYUC10*, which

encodes a flavin-containing monooxygenase that transforms indole-3-pyruvic acid into indole-3-acetic acid, the main auxin [48,91] (Table S1). Mutant lines were characterized by a 40% reduced free auxin, but the development and morphology of plants and fruits did not change, which may indicate either functional redundancy of the *YUC* genes in the *F. vesca* genome or the existence of a feedback loop or an alternative biosynthesis pathway that can compensate the loss of *FveYUC10* [92]. Martín-Pizarro et al. edited, for the first time, the octaploid strawberry for the MADS box gene *FaTM6* orthologous to *APETALA3* *A. thaliana*. Mutant lines had decreased the amount of reduced pollen and altered flower and anther morphology: short and intense green petals and small and dark anthers. The receptacle did not grow on mutant plant lines, which indicated the key role of *FaTM6* in anther development of garden strawberry [93]. The main point that authors managed to demonstrate was the possibility of successfully editing octaploid strawberry by using CRISPR/Cas9.

## 6. CRISPR/Cas9 Plant Genomes Editing Limitations and Prospects

CRISPR/Cas9 genome editing technology has significantly simplified and accelerated the study of gene functions and the process of improving valuable varieties of fruit and berry plants due to the accuracy, speed and low cost; however, the CRISPR/Cas9 editing approach has a number of limitations that are necessary to consider.

First, the following are the limits of the CRISPR/Cas9 system itself.

1. The need for the PAM sequence presence in the target region, which may be absent in the desired genome region. The use of modified Cas9p depending less on the PAM sequence seems to be a promising approach. For example, SpRY nuclease has been tested on monocotyledonous rice and dicotyledonous Dahurian larch (a coniferous tree) [94].

2. The need of gRNA/Cas9 expression optimization for a plant or plant variety, which is especially important for multiplex and polycistronic editing. It is necessary to look for new selective markers because of the duration of plant editing experiments and small number of convenient markers with bright mutant phenotype, which would make possible fast optimization of editing, selection and regeneration steps. The widely used *PDS* is not a perfect choice: albino mutations result in the death of plant cells, so the mutation frequency, the ratio of their types in a chimeric and heterogeneous cell populations, can be underestimated and is difficult to compare with the frequencies in other studies [54] (Table S1).

3. The heterogeneity of mutations: Editing can occur with the formation of a heterozygous (one mutant allele), biallelic (mutant alleles but different mutations), chimeric (a mixture of different mutation profiles) or homozygous edited plants, with the most desirable homozygous state (all alleles are edited in the same way) that is not often achieved. Plants are often edited with chimeric and biallelic lines formation due to editing with nuclease at different stages of development of the transformed line due to the retention of active nuclease, which indicates the necessity to use inducible rather than constitutive promoters for the expression of nucleases [28,29,33,41,43,48].

4. The limitation of online instruments accuracy: Unfortunately, the results of the selection and analysis of the used gRNAs in different online instruments do not always correlate with each other and with the experimental results (Table S1). To overcome this limitation, it is necessary to use several analysis tools and several gRNAs per target.

5. The Cas9p fidelity: CRISPR/Cas9 predominantly makes indel mutations and is commonly used for gene knockouts. Recently, a series of editors based on CRISPR/Cas9 has been developed, which can perform precise genome manipulation base editing systems for transition or transversion substitutions using a combination of Cas9 nickase and cytidine/adenosine deaminase or Cas9 nickase, cytidine deaminase and uracil-DNA-glycosylase, respectively. In addition, technologies for gene prime editing systems using DNA or RNA as donors have been developed [95,96]. Moreover, the use of another accurate nucleases, such as Cpf1p, seems to be rather promising [97].

6. The frequent integration of *Agrobacterium* vector into the plant genome: The need to use an inducible system for the elimination of the vector from the genome in order to obtain a non-transgenic modified plant, for example, FRT/ FLP [28].

Second, the limitations of CRISPR/Cas9 delivery systems are as follows.

The use of direct delivery of RNP has significantly reduced the time required for obtaining edited plants; however, unfortunately it did not solve the problem of low mutation rates [52]. The frequency of vector penetration into the cell can be increased by reducing vector size and/or by optimizing the vector capacity. For these purposes, the PTG/Cas9 systems [22,32,60,98] and self-cleaving linkers 2A [23,52] are already commonly used. It may also be promising to use the more compact Cas $\Phi$  nuclease isolated from *Biggiephage* clade viruses [99] and new delivery vectors such as the recombinant *Rhabdovirus*. The use of the latter made it possible to obtain single and multiplex editing of the tetraploid tobacco genome without antibiotics selection with a mutation frequency of more than 90%, of which 57% were tetra-allelic inherited mutations [100].

Third, the selection and regeneration limitations are as follows.

In the vast majority of the reviewed studies, nearly scant information of plant regeneration has been published. It indirectly indicates the need to optimize the stage of plant regeneration in order to increase both the viability of transformants and the frequency of selected homozygous mutated shoots, which can be achieved through alternative selection methods [50,101–105] and the introduction of additional stages of regeneration [50,105].

Finally, the limitations by the biological characteristics of the object of editing are as follows.

Due to the high degree of genome heterozygosity, polyploidy, endoreduplication, chimerism and differences in the metabolism of fruit and berry plants, it is necessary to check the absence of SNPs in the areas of planned editing by sequencing the target gene of the cultivar and select gRNAs in order to the most conservative regions of the gene. It is equally important to assess changes in plant morphology, cell morphology, intracellular structures and key metabolites pattern in order to characterize the pleiotropic effects of the mutation [56,106,107]. Nowadays, there are few studies analyzing CRISPR/Cas9 edited T1 lines and F1 perennial fruit and berry plants in general and wood plants in particular. The majority of the published studies characterize transgenic cell mass and T0 plant lines (Table S1). Some of rare examples of characterized edited T1 plants include *CCDb4* mutants of mini-citrus kumquat (52) and *FveARF8* fast growing mutants of strawberry [43]. It is important to note that all fruit, berry crops and grape are predominantly vegetatively propagated due to long juvenile periods, open pollination, high heterozygosity and concomitant difficulties in stabilization of the valuable trait in the elite background of variety. That is why there are very few examples of F1 generation analysis of CRISPR/Cas9 modified plants: Varkoney-Gasik et al. edited *AcCEN4 SyGl*, which had resulted in precocious, small vines of kiwi with early flowering that could self-pollinate and produce fast-flowering offspring [45].

## 7. Conclusions

Thus, the effectiveness of CRISPR/Cas9 genome editing of vegetatively propagated fruit and berry crops, as well as grapes, has already been confirmed in a sufficient number of plants (strawberries, grapes, apple trees, pears, kiwi, bananas, oranges, kumquat, grapefruit, etc.), despite the existing methodological limitations that require optimization. Involving more crops in projects for genetic editing of various target genes will optimize existing protocols and, thus, will facilitate the integration of the resulting lines and varieties of fruit and berry crops, as well as grapes, into production.

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