



Application and Future Prospects for CRISPR/Cas9 Genome Editing in Fruit Crops

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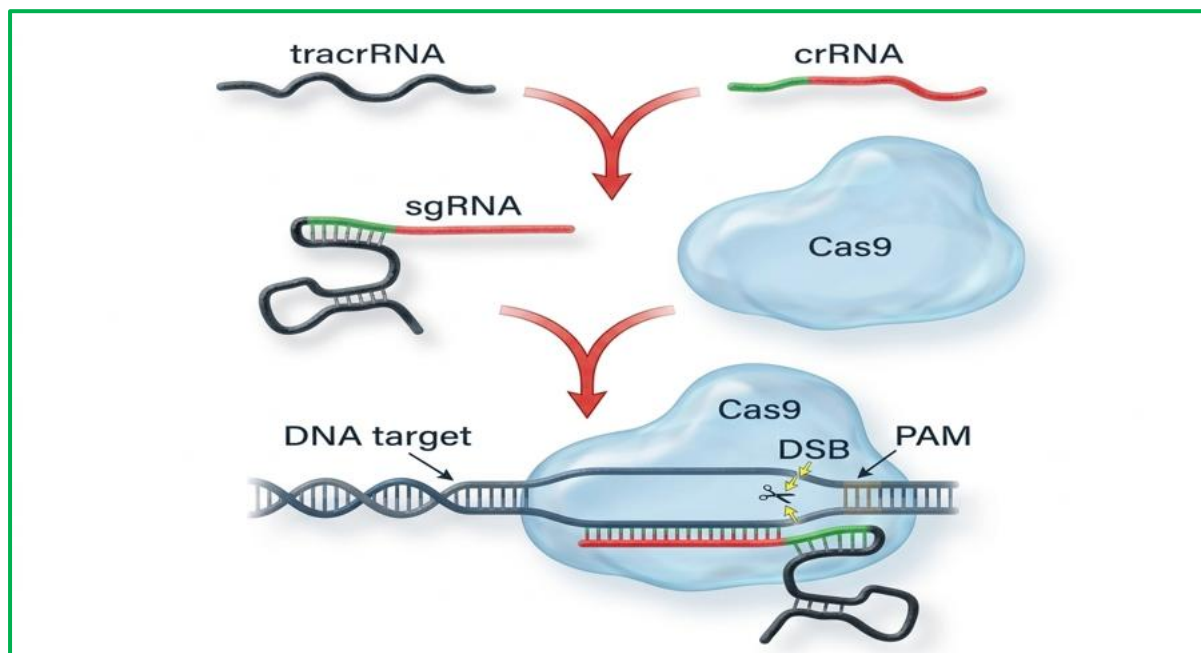
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Fruits and fruit juice supply humans with fibre, useful metabolites, and various nutrients. Many fruit-producing plants are of major economic importance to a number of countries or regions around the world. The fruits most commonly eaten are apple, orange, grape, banana, strawberry, watermelon, kiwifruit and tomato. There has been a long-standing record of research aimed at increasing fruit yield, improving disease resistance, and enhancing various quality characteristics of the fruit. Earlier advances in this area have mostly been made through conventional breeding, which involves selective hybridization, the evaluation of progeny, and propagation. Since the global population is expected to reach 9.7 billion by 2050, demand for fresh horticultural products is likely to increase considerably. The productivity of horticultural crops and their supply chains, however, are becoming more and more restricted by both biotic and abiotic stresses, such as pests, pathogens, drought, and extreme climatic events, all of which result in substantial yield losses and endanger the stability of crops. Postharvest losses make the problem even more serious, since almost 30% of fresh produce is lost as a result of inadequate storage facilities and problems in the supply chain. These methods are slow and require a great deal of labour, and they are constrained by the germplasm available. The field now has the potential for rapid advancement, if not a revolution, thanks to new and exciting molecular genetic techniques, particularly the recently developed CRISPR/Cas9 genome editing tools. Horticultural crop production is nevertheless often faced with a number of biotic and abiotic factors, such as pests, diseases, extreme weather conditions, and soil degradation. Conventional breeding methods have played an important role in improving horticultural crops over the years, but they are generally time-consuming, labour-intensive, and restricted by genetic diversity. Moreover, traditional breeding methods depend heavily on random mutations and recombination, which can cause unwanted genetic variations and extend the breeding process.

Overview of CRISPR/Cas9

The CRISPR/Cas9 system was adopted from a defense system in bacteria and archaea, which is used to ward off invading viruses or foreign DNA molecules the invading DNA was destroyed by an RNA-directed process in which the CRISPR RNA (crRNA), together with a trans-activating RNA (tracrRNA), associates and directs Cas nucleases to mediate site-specific cleavage of the invading foreign DNA (Figure 1) and protects the host bacteria. In 2012–2013, the CRISPR/Cas9 system was successfully applied to editing genes of animals and plants with remarkable precision and simplicity. To simplify the system for editing various genomes, the tracrRNA was fused to crRNA to form a synthetic sgRNA (single guide RNA), which greatly facilitates the application of the system (Figure 1). Once the sgRNA-Cas9 complex is introduced into a cell, the sgRNA guides the Cas9 nuclease to cleave its target DNA sequence usually 3 bp upstream of the PAM (Protospacer Adjacent Motif) site.



The resulting sgRNA is formed by joining the tracrRNA (black) and crRNA (green and red). PAM (mostly NGG) is shown as three orange base pairing lines on the target DNA (grey). The location of double stranded breaks is indicated by a pair of yellow arrows. double-stranded DNA break (DSB) activates two different DNA repair mechanisms, non-homologous end joining (NHEJ) and homology directed repair (HDR). NHEJ mediates the direct religation of the broken DNA molecule in the absence of a homologous template, usually leading to insertions, deletions (InDels), or substitutions at the site of DSB. By contrast, HDR, in the presence of a donor DNA sequence, can introduce novel alleles, even correcting existing mutations or inserting new sequence of interests (Budman and Chu 2005; Gong et al. 2005; Zha et al. 2009; Puchta 2017). The CRISPR/Cas9 system offers many advantages in the genetic manipulation of an organism's genome, including ease in assembly, low in cost, and high in efficiency and specificity. To date, the CRISPR/Cas9 system has enjoyed ever-increasing popularity and improvement, propelling its wide applications from medicine to agriculture (Shan et al. 2013; Araki et al. 2014; Abdallah et al. 2015; Ahmad et al. 2018).

Overview of Fruit Crop Genome Editing

Genome editing in banana: Banana is one of the most economically important fruits in the tropics (Picq et al. 2000). It is also the fourth largest food crop in the world (Perrier et al. 2011). Traditional breeding is challenging due to its triploid genome and sterility (Dash et al. 2016). Genetic engineering is perhaps the only way to improve this important staple food and fruit. The single guide RNA targets the conserved domain of two RAS-PDS genes (RAS-PDS1 and RAS-PDS2) in the embryonic cell suspension cultures of banana cv. Rasthali (Kaur et al. 2018). Complete albino and variegated phenotypes were observed in the regenerated plantlets at a 59% rate of mutation. More recently, the multiplexed approach via the polycistronic tRNA-gRNA system was used to target the exon 1 of PDS in the Cavendish cultivar; a 100% mutation rate and triallelic deletions or insertions were found among the 19 regenerated plants whose degree of albino correlated with the genotype (Naim et al. 2018). The study demonstrates that CRISPR/ Cas9 mediated genome editing takes place efficiently in banana. Targeting endogenous genes will be the necessary next step towards the genetic improvement of banana.

Genome editing in citrus: Citrus is one of the most economically important fruit tree crops in the world, while genetic modification of citrus through breeding or genetic engineering is hampered by polyembryony, pollen incompatibility, parthenocarpy, long juvenile period, and recalcitrance to transformation by conventional methods (Talon and Gmitter 2008). In 2014, Jia and Wang reported that the use of *Xanthomonas citri* subsp. *citri* (Xcc)-mediated agroinfiltration for delivery of Cas9 together with a synthetic sgRNA targeting CsPDS gene

resulted in obtained citrus plants with a low percentage of mutated leaves for sweet orange (*Citrus X sinensis*) and Duncan grapefruit (Jia and Wang 2014a, b). The same research group developed transgenic citrus plants with increased resistance to citrus canker by utilizing *A. tumefaciens* -mediated transformation of Duncan grapefruit epicotyl explants. The generated plants contained a CRISPR/Cas9 system targeting the CsLOB1 gene responsible for susceptibility to *Xanthomonas axonopodis* bacteria (Jia et al. 2017b). Another study demonstrated that successful editing of the promoter of the CsLOB1 gene resulted in the generation of homozygous citrus plants resistant to citrus canker (Peng et al. 2017). Thus, the examples of citrus genetic modification demonstrate that editing of the promoter region renders the target gene non-functional and leads to the desired change in plant phenotype. Therefore, it can be concluded that the CRISPR/Cas9 technology can be utilized to fight devastating diseases of citrus.

High efficiency editing in kiwifruit is achieved with the PTG/Cas9 system: Kiwifruit (*Actinidia* Lindl.) is an economically important fruit species that contains high vitamin C content and dietary fiber (Huang et al. 2013). Genome editing in kiwifruit was achieved through both the standard CRISPR/Cas9 system and the more powerful polycistronic tRNA-sgRNA cassette (PTG)/Cas9 system (Xie and Yang 2013; Xie et al. 2015). The *PDS* gene of Kiwifruit was edited in callus-derived kiwifruit plantlets with the PTG/Cas9 system at 10-fold higher efficiency than the traditional CRISPR/Cas9 system (Wang et al. 2018b). In addition, large chromosome fragment deletions in kiwifruit were generated with the paired-sgRNAs of PTG/Cas9 system, suggesting that the PTG/Cas9 is a powerful tool in kiwifruit genome editing and quality improvement.

Genome editing in grape and apple through direct delivery of RNPs into protoplasts: Grape (*Vitis vinifera* L.) is another economically important fruit crop that can be either fresh or processed into wine, jams, juices and even medicines. The whole genome sequence of grape (*V. vinifera* L.) was reported and released in 2007 (Jaillon et al. 2007) and the grape transformation using embryonic suspensions was established as early as the year of 1993 (Hebert et al. 1993). In 2016, a research group successfully edited the Lidonate dehydrogenase gene (*IdnDH*) in the biosynthesis of tartaric acid (Ren et al. 2016). A CRISPR/Cas9 construct, consisting two sgRNAs, were transformed into the ‘Chardonnay’ embryonic suspension cells by way of agrobacterium that resulted in 100% mutational efficiency. A second report concerning the editing of *Vitis vinifera* phytoene desaturase (*VvPDS*) resulted in albino leaves with efficiencies ranged from 0-86% (Nakajima et al. 2017). However, it was found that older leaves possessed a higher ratio of mutant cells, probably due to the accumulation of DSBs or decreased DSBs repair in older leaves. A very recent study edited the *VvWRKY52* transcription factor, which participated in biotic stress response (Wang et al. 2018a). The proembryonal masses derived from embryo calli were infected with agrobacterium that contains the CRISPR/Cas9 construct. The fifteen out of twenty-two first generation transgenic lines were carrying biallelic mutations, and four of them showed increased resistance to *Botrytis cinerea*.

Rapid and low-cost methods for mutation screen and detection are needed: Robust, low-cost, high throughput detection methods of CRISPR/Cas9-induced mutations are highly desirable, especially when the efficiency of induction is low. The most common approaches for detecting the target genotype are direct DNA sequencing of PCR amplicons, T7 Endonuclease diagnostic analysis of HDR (Guschin et al. 2010) or High-resolution Melting (HRM) analysis (Samarut et al. 2016). Direct DNA sequencing may be costly, for a large number of plants. The T7 Endonuclease or hermithood’s approach are less reliable and often require optimization and/or expensive reagents. Illumina sequencing is also expensive for the task of detecting mutations. “SHERLOCK” (specific high-sensitivity enzymatic reporter unlocking) is a new Cas-based platform for quick detection of SNPs (single nucleotide polymorphism). Indeed, the method not only detects, but quantifies the target content and allows simultaneously testing several targets in one sample. The use of lateral flow strips allows inexpensive and rapid detection of mutations by simply reading the test strip, like a pregnancy

test (Gootenberg et al. 2018; Myhrvold et al. 2018). Therefore, “SHERLOCK” seems promising for on-site use to screen fruit trees for genetic modifications.

Challenges and Future Prospects of Crispr-cas9 in Horticulture Crops

Despite the great potential of the CRISPR-Cas9 technology to improve horticultural crops, there are several limitations. The first major limitation is the regulatory framework since different countries adopt different policies towards genome-edited crops. While some countries like the US adopt a more relaxed approach towards GMO products, others take a cautious approach; thus, making genome-edited food subject to strict regulations in Europe (Singh & Shashikant, 2024). This limitation has hindered the commercialization of many genome-edited crops and food products by biotech companies.

Besides the regulatory limitations, CRISPR has several off-target effects that could be detrimental to the food supply and the environment. Currently, the best way to reduce these limitations is through the use of modern computers to better understand and predict the effect of each edit made on the plant genome. However, there is a need to verify whether the recent developments in bioinformatics and modified CRISPR-Cas9 procedures have been tested and proven efficient in different horticultural crops.

Efficient delivery of genome-editing components remains a technical hurdle, especially in species with complex genomes or strong cell walls that hinder genetic transformation. Conventional methods such as *Agrobacterium*-mediated transformation and particle bombardment have limitations in terms of efficiency and tissue specificity. Emerging techniques, including nanoparticle-based delivery and ribonucleoprotein (RNP) approaches, offer promising alternatives but require further optimization for large-scale application (Safdar & Safdar, 2024a). Public perception and consumer acceptance are also critical factors influencing the future of CRISPR-edited horticultural crops. While genome editing is often considered distinct from traditional genetic modification, misconceptions persist, leading to skepticism about its safety and ethical implications. Transparent communication, public engagement, and education efforts are essential to fostering a better understanding of the benefits of CRISPR technology in enhancing crop resilience, nutritional quality, and sustainability (Seriki & Otoikhila, 2022).

Table 1. Summary of CRISPR/Cas9 genome editing in fruit crops

Species	Cas9	Target Gene(s)	Target Traits	Cas9 Delivery Method	Efficiency (T0) (%)	Reference
Sweet orange (<i>Citrus sinensis</i>)	35S: s_pyogenes Cas9	PDS	Albino phenotype and decrease of carotenoid content	Xcc-facilitated agroinfiltration delivery	3.2–3.9%	Jia and Wang 2014b
Sweet orange (<i>Citrus sinensis</i>)	pYAO: hSpCas9	PDS	Albino phenotype and decrease of carotenoid content	<i>Agrobacterium</i> -mediated transformation	45.5–75%	Zhang et al. 2017
Duncan grapefruit (<i>Citrus paradisi</i>)	35S:SaCas9	PDS	Albino phenotype and decrease of carotenoid content	<i>Agrobacterium</i> -mediated transformation	15.55–79.17%	Jia et al. 2017a
Duncan grapefruit (<i>Citrus paradisi</i>)	p1301-N-pcoCas9	CsLOB1 promoter	Citrus disease susceptibility gene for citrus canker	<i>Agrobacterium</i> -mediated transformation	14.29–81.25%	Jia et al. 2016
Duncan grapefruit (<i>Citrus paradisi</i>)	p1301-N-pcoCas9	CsLOB1	Citrus disease susceptibility gene for citrus canker	<i>Agrobacterium</i> -mediated transformation	31.58–89.36%	Jia et al. 2017b

Duncan grapefruit (<i>Citrus paradisi</i>)	35S-pcoCas9	CsLOB1 promoter	Citrus disease susceptibility gene for citrus canker	Agrobacterium-mediated transformation	11.57–64.71%	Peng et al. 2017
Apple (<i>Malus domestica</i>)	Commercial recombinant Cas9 protein	DIPM-1, DIPM-2, DIPM-4	Resistance to fire blight disease	Direct delivery of purified CRISPR/Cas9 ribonucleoproteins to the protoplast	0.18–6.9% (by targeted deep sequencing)	Malnoy et al. 2016; Osakabe et al. 2018
Sweet orange (<i>Citrus sinensis</i>)	35S: s_pyogenes Cas9	PDS	Albino phenotype and decrease of carotenoid content	Xcc-facilitated agroinfiltration delivery	3.2–3.9%	Jia and Wang 2014b
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Grapevine (<i>Vitis vinifera</i>)	2×35S: plants codon-optimized Cas9 (pP1C4)	IdnDH	Biosynthesis of tartaric acid (TA)	Agrobacterium-mediated transformation using embryo callus	100%	Ren et al. 2016
Grapevine (<i>Vitis vinifera</i>)	pCUBI4-2(P): pDe-Cas9	VvWRKY52	Albino phenotype and decrease of carotenoid content	Agrobacterium-mediated transformation	2.1%–77.2%	Nakajima et al. 2017
Grapevine (<i>Vitis vinifera</i>)	2×35S: plants codon-optimized Cas9 (Ma et al. 2015)	VvWRKY52	Biotic stress responses	Agrobacterium-mediated transformation	31%	Wang et al. 2018a

Conclusion

Genetic improvement of fruit crops. Its successful application in crops such as banana, citrus, kiwifruit, grape and other horticultural crops demonstrates its potential to improve important traits including disease resistance, fruit quality, nutritional value, productivity, and shelf life. Despite its great potential, the application of CRISPR/Cas9 in fruit crops is still limited by several challenges, including difficulties in transformation and regeneration, complex genomes, long juvenile periods, off-target effects, regulatory issues, and the need for rapid and low-cost mutant screening. Improved CRISPR systems, base and prime editing, RNP-based delivery, and other advanced delivery methods are expected to overcome many of these limitations. In the future, the development of transgene-free edited plants, efficient screening methods, consumer-friendly traits, and greater public awareness will be important for the commercialization and acceptance of genome-edited fruit crops. CRISPR/Cas9 therefore has considerable potential to accelerate fruit crop improvement and contribute to sustainable horticultural production, healthier fruits, improved resilience to environmental stresses, and the development of novel designer fruit varieties.