

Review

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Review

Development of Climate Smart Fruit Plants via CRISPR/Cas Genome Editing Systems: A Spatiotemporal Review

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Abstract: Fruit production is an important part of the gross domestic product for many countries around the world especially to those who have a strong focus on agriculture. However, long-term maintenance and yield stability of fruit production may be threatened by the ongoing climate change and its consequences like extended drought periods, heavy rain events, and floodings. Genome editing, with its progressive technological developments, offers opportunities to adapt relevant fruit plant species to new climatic conditions. Among modern genome editing techniques, CRISPR/Cas, in particular, has the potential to support breeding for those fruit plant species with extended breeding cycles, e.g., perennial fruits. In this review, we discuss CRISPR/Cas and other genome editing techniques in detail and how these techniques can be applied to support the breeding of fruit plant species for adaptation to changing climates. The chronological history of CRISPR/Cas9 systems, their associated computational tools, genomic data sources, transformation methods along with their delivery vehicles, quality improvement, environmental-stress resiliency, limitations, and future perspectives will also be discussed with respect to securing future global fruit production.

Keywords: CRISPR/Cas; TALEN; ZFN; fruit plants; genome editing systems

Introduction

Global climate change patterns are one of the main concerns for developing agricultural systems and the food insecurity problem. Greenhouse emissions and the subsequent accumulation of these gases in Earth's atmosphere are one of its leading causes. Even with taking proper measures to control the emissions, the damage that has already been done is going to take millennia to fully recover. All of these abrupt changes had a negative impact on the outputs of agricultural systems all around the world. As a result of droughts and floods, the global food supply is constantly declining (Nunez et al. 2019). Agriculture accounts for about 16.47% of China's total gross domestic product (GDP). Similarly, for India, it's 20.19%, for the United States 5.4%, for Turkey 5.54%, for Mexico 3.89%, and, for Brazil, it's a staggering 29%. All these countries are the top fruit producers globally and a significant portion of their GDP depends upon agriculture and agriculture-related industries. It is of foremost importance to develop fruit crops that are resilient to these environmental changes.

Our current gene editing systems can tackle these climate-related consequences by modifying the fruit plants in such a way that they adapt to the new climate by rendering their effects neutral. The gene editing method involves precise tampering with the DNA that leads to the knockdown of one or a set of genes which mostly leads to the loss of function of a trait. On the contrary, it can also be used to precisely insert our gene of interest that results in knock-in mutants. The well-known and

researched gene editing systems include Zinc Finger Nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and CRISPR/Cas (Khalil 2020). The CRISPR/Cas systems lead the packs in terms of precision, accuracy, and efficiency (Chakrabarti et al. 2019). All of these gene editing systems have paved a streamlined way to develop climate-smart or resilient plants.

Climate-smart fruits have also proven to be the great wall against food insecurity problems worldwide (Kole 2020). As an example, banana has been successfully made resistant to abiotic stress by targeting the semi-dwarfed trait. Here, CRISPR/Cas9 was used to modify five *GA20ox2* homologous genes in banana (Ma04g15900, Ma06g27710, Ma08g32850, Ma11g10500, and Ma11g17210). These gibberellin biosynthesis genes have been knocked out to establish semi-dwarfism which makes the banana plant more resistant to lodging during severe climate conditions (Shao et al. 2020). Worldwide fruit production has increased a little bit since these gene editing systems have gone mainstream development. The latest global fruit production statistics can be visualized in the graph illustrated in Figure 1.

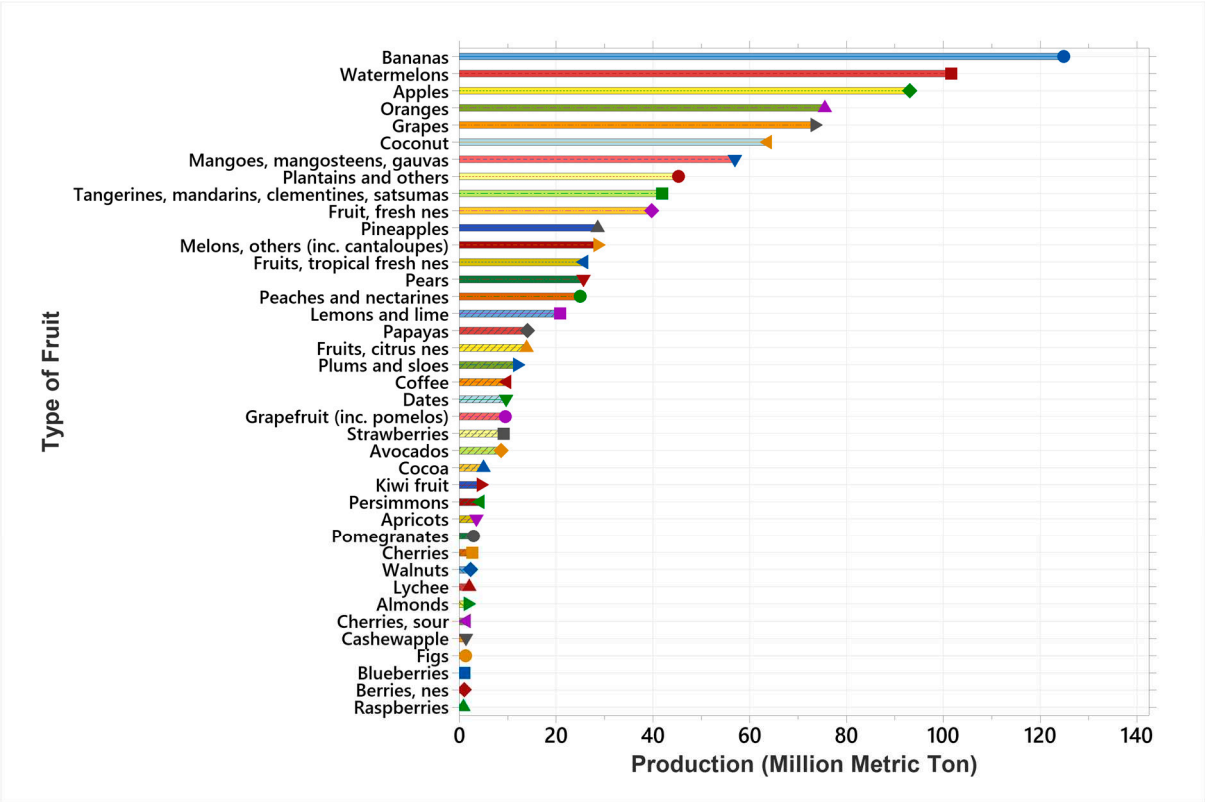


Figure 1. Descriptive statistics of global fruit production from the data taken in year 2020. The graph shows the production in million metric tons including the world’s major fruits like banana, watermelon, apple, and orange, to minor contributors raspberry, fig, and cashew apple.

On the other hand, New Plant Breeding Techniques (NPBTs) are based on highly advanced molecular methods with more precision and accuracy than ever before. Both old and new plant breeding strategies improved concerning quality, quantity, and climate resilience. Similarly, to achieve the targets in fruits, different NPBTs have been utilized, e.g., genome-assisted breeding (GAB), mutation breeding, transgenic breeding, and currently, genome editing plant improvement systems (Salonia et al. 2020; Campoy et al. 2020; A. Brown, Carpentier, and Swennen 2020; Boudichevskaia et al. 2020; Basu 2020; Rugienius et al. 2020; Ramesh, Arunachalam, and Rajesh 2020; Zambounis et al. 2020; Delrot et al. 2020a; Gogorcena et al. 2020; Delrot et al. 2020b).

This review focusses on the recent developments and efforts of these genome editing technologies, especially CRISPR/Cas systems and their applications in the development of climate-smart fruit crops, and to achieve secure future global food production goals with limitations and new opportunities that lie ahead of us.

A Brief History of Genome Editing Systems and their Mechanisms

In the past few years, several advanced genome editing tools have been developed that have paved the way to take genetic engineering to a new level of precision and specificity. These systems have different levels of priority in their utilization in different experiments due to variations and differences in their properties. The journey of genome editing begins with the development of the most notable systems including mega-nucleases and Zinc Finger Nucleases (ZFNs) (Y. G. Kim, Cha, and Chandrasegaran 1996). Then, Transcription Activator-Like Effector Nucleases (TALENs) came into action in 2009 and were identified as an efficient and highly specific genetic engineering tool, honored as “tool of the year” in 2011 (“Method of the Year 2011” 2011; Boch et al. 2009).

The CRISPR/Cas genome editing system was discovered in bacteria and utilized in plant sciences in 2013 (Shan et al. 2013; Nekrasov et al. 2013; Miao et al. 2013; W. Jiang et al. 2013; Xie and Yang 2013; Feng et al. 2013; J. F. Li et al. 2013a). Currently, several advanced versions of the CRISPR/Cas system including cytosine base editor (CBE) that allows C to T conversions, adenine base editor (ABE) that allow A to G conversions, prime editors, CRISPR-Transposases, CRISPR activation (CRISPRa), CRISPR (CRISPRi), Programmable Addition via Site-specific Targeting Elements (PASTE) (Ioannidi et al. 2021), etc., have been developed and are being used for improvement, acceleration of plant breeding programs, and functional characterization of newly identified genes. A general mechanism of restriction enzyme cutting in action and the timeline of all other genome editing systems preceding CRISPR/Cas systems have been given in Figures 2–5 in respective chronological order. Furthermore, a detailed and contrasting comparison of genome editing systems like Oligonucleotide-Directed Mutagenesis (ODM), ZFNs, TALENs, and CRISPR/Cas systems are provided in Table 1.

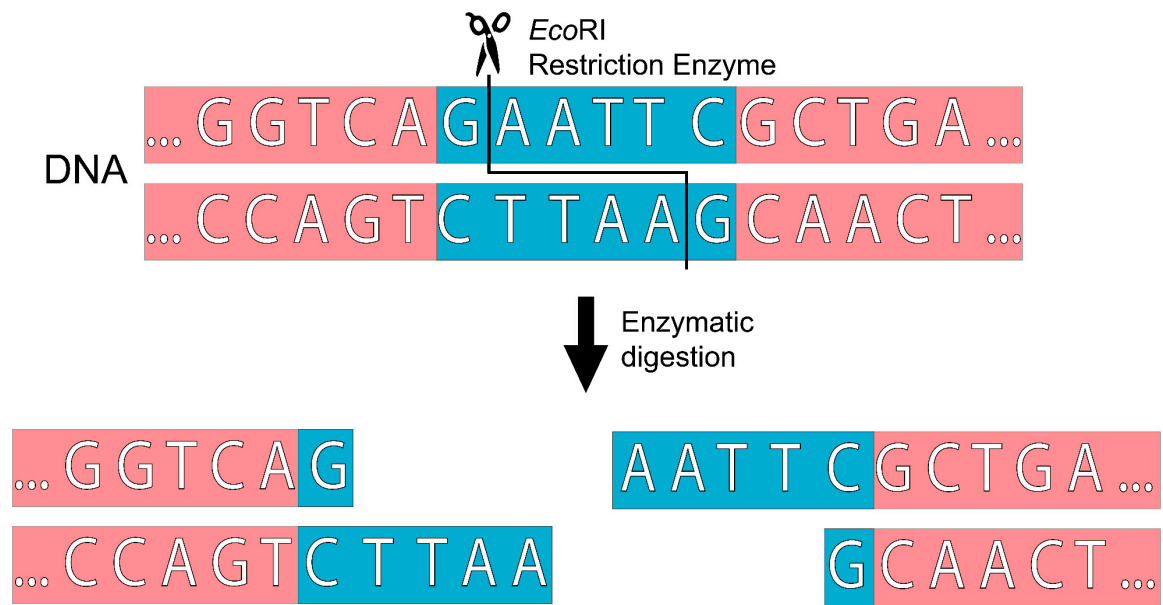


Figure 2. Restriction Enzymes (RE): the first true genome editors (1970).

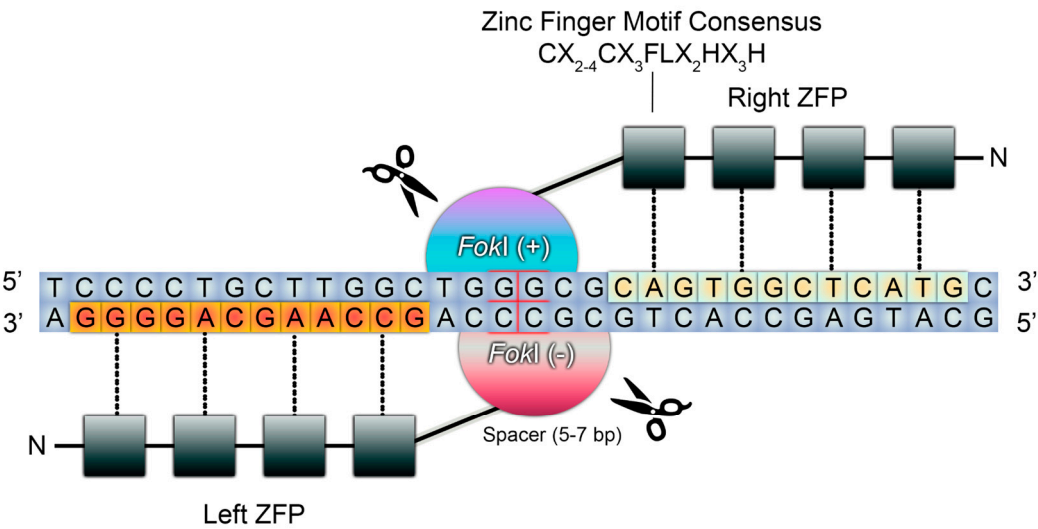


Figure 3. Zinc Finger Nucleases (ZFNs): the masters of recognition (1985).

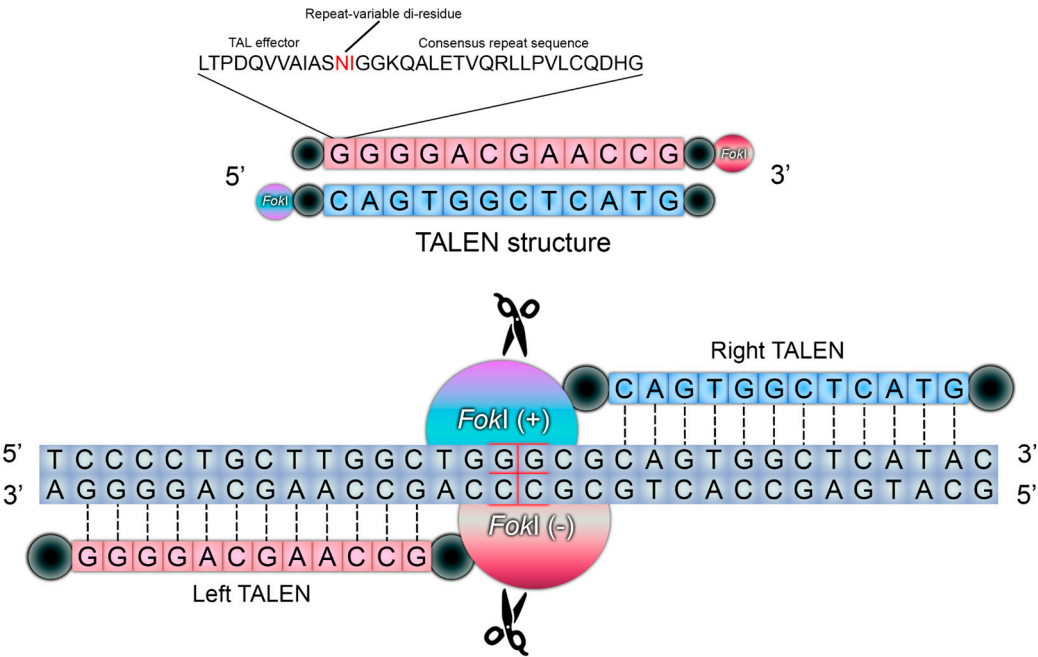


Figure 4. Transcription Activator-Like Effector Nucleases (TALENs): the experts of single nucleotide resolution (2010).

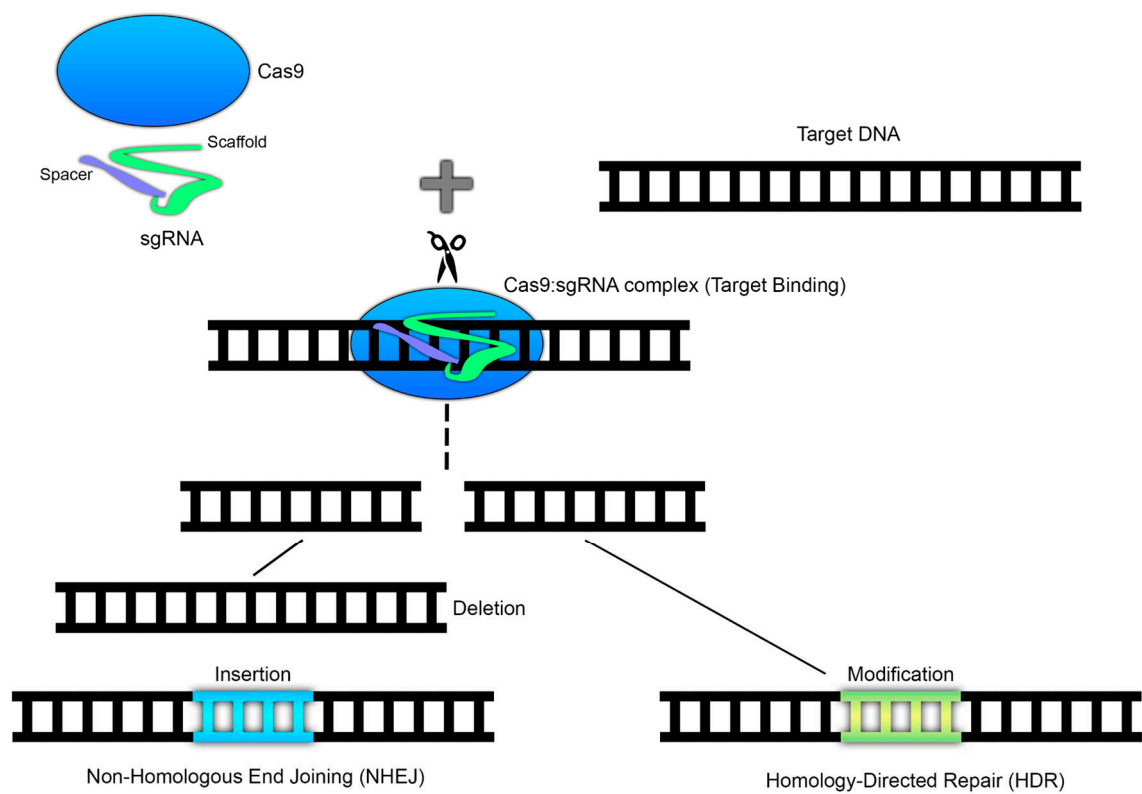


Figure 5. Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) / CRISPR-associated protein 9 (Cas9): the revolution of precision genome editing (2012).

Table 1. Comparison of genome editing techniques in plants.

Properties	ODM	ZFNs	TALENs	CRISPR/Cas9	Reference(s)
Multiplexing Capability	Difficult	Difficult	Difficult	Possible	(Mao et al. 2013)
					(Noman, Aqeel, and He 2016)
Mutation Rate	Medium	High	Medium	Low	(Gaj, Gersbach, and Barbas 2013)
Mode of Action	Conversion within the target region	Breaks in target DNA	Breaks in target DNA	Breaks in target DNA	(Mao et al. 2013)

	(sense strand directed)	(Double strand)	(Double strand)	(Double strand)	
Cloning	Not required	Necessary	Necessary	Not Required (Usually)	(Sauer et al. 2016)
Target Sequence Length [bp]	70-86	26-34	26-57	20-22	(F. Chen et al. 2017)
Targeting Efficiency	Relatively high	High	High	Very high	(Gaj, Gersbach, and Barbas 2013)
Components	Chimeraplast	Zn finger <i>FokI</i> nuclease domain (non-specific)	TALE DNA-binding <i>FokI</i> nuclease domain (non-specific)	Cas9 proteins, sgRNA	(V. Kumar and Jain 2015)
Structural Protein Composition	Non-protein	Proteins (dimeric)	Proteins (dimeric)	Protein (monomeric)	(J. F. Li et al. 2013b)
Catalytic Domains	Catalytic domain absent	Restriction endonuclease <i>FokI</i>	Restriction endonuclease <i>FokI</i>	HNH and RuvC	(Sauer et al. 2016)
Large-Scale Libraries	Difficult	Difficult	Very Difficult	Possible	(Hsu et al. 2013)
Engineering Steps for Protein	Not required	Required	Required	Required	(Cho et al. 2013)

Key Aspects of Fruit Plant Modification through Genome Editing Systems

Generally, genome editing technology can be used mainly for any of the following two purposes. First, for plant modification or the development of their breeding material, and second is to characterize the function of genes in plants enabling also indirect plant improvement if the

characterized genes are considered in conventional breeding. However, there are several key steps involved in CRISPR/Cas experiments to fulfill any of the mentioned goals. To go for either option, in fruits, the first step is the availability of the gene sequence of the target species. In this regard, genomes of various fruit species are freely available and can be used according to our needs and objectives. An exhaustive list of the genomes of major fruit species and their accompanying details have been supplied in Table S1 (Supplementary File 1) (Alioto et al. 2020; Velasco et al. 2010; F. Jiang et al. 2019; Dash and Rai 2016; Argout et al. 2010; Lantican et al. 2019; Xiao et al. 2017; Scalabrin et al. 2020; Al-Mssallem et al. 2013; Chagné et al. 2014; Mori et al. 2017; Mittal et al. 2020; Q. gang Zhu et al. 2019; Huang et al. 2013; Arumuganathan and Earle 1991; Ming et al. 2008; Verde et al. 2013; Wu et al. 2013; Ming et al. 2015; H. Xu et al. 2018; Liu et al. 2020; Luo et al. 2020; J. Wang et al. 2020; Q. Xu et al. 2012; Jaillon et al. 2007; Martínez-García et al. 2016; He et al. 2013). The second step involves the selection of target gene mining from the genome and then a specific region to be targeted by the CRISPR/Cas genome editing system (H. Li et al. 2020; Yi Zhang et al. 2018; Tabassum et al. 2021).

One of the ways to identify the target gene for plant improvement is a sound literature review. Different susceptibility or sensitivity-causing genes can be identified from available literature, partly transferred from other plant species, and can be targeted by a CRISPR/Cas approach in fruits for their improvement. The transferability of candidate genes for genome editing in a selected plant species appears to be particularly promising if functional domains of the related protein match in the source and target species. For this purpose, functional domains can be identified based on the gene or amino acid sequences with freely available online tools and analyzed with respect to their function such as CRISPRscan, CCTop, and Cas-OFFinder. A detailed list of modern mainstream single guide RNA (sgRNA) designing tools has been provided in Table 2. Moreover, target genes can also be identified through differential transcriptome analysis of plants under certain stress treatments. Afterward, the sgRNAs are cloned into a suitable CRISPR/Cas vector. Then, that vector is transformed into the targeted plant species through direct or indirect transformation methods. Finally, CRISPR/Cas-edited mutant plants are screened and further processed till the possible commercialization step. This whole procedure may vary from 3-5 years, depending on the species and transformation method (Gogorcena et al. 2020; Delrot et al. 2020a; Zambounis et al. 2020; Ramesh, Arunachalam, and Rajesh 2020; Rugienius et al. 2020; Basu 2020; Boudichevskaia et al. 2020; A. Brown, Carpentier, and Swennen 2020; Campoy et al. 2020; Delrot et al. 2020b).

Table 2. CRISPR/Cas sgRNA Designing Tools.

<i>Tool</i>	<i>Web Address</i>	<i>Reference(s)</i>
<i>CRISPRscan</i>	http://www.crisprscan.org/	(Moreno-Mateos et al. 2015)
<i>CHOPCHOP</i>	https://chopchop.cbu.uib.no/	(Montague et al. 2014)
<i>WU-CRISPR</i>	https://crisprdb.org/wu-crispr/	(Wong, Liu, and Wang 2015)
<i>CRISPRdirect</i>	http://crispr.dbcls.jp/	(Naito et al. 2015)

CRISPR <i>MultiTargeter</i>	http://www.multicrispr.net/	(Prykhozhij et al. 2015)
<i>E-CRISP</i>	http://www.e-crisp.org/E-CRISP/	(Heigwer, Kerr, and Boutros 2014)
<i>CCTop</i>	https://cctop.cos.uni-heidelberg.de/	(Stemmer et al. 2015)
<i>Cas-OFFinder</i>	http://www.rgenome.net/cas-offinder/	(Bae, Park, and Kim 2014)

Delivery of CRISPR/Cas constructs in fruit plants through direct and indirect transformation methods

CRISPR/Cas systems have caused a paradigm shift from ZFNs and TALENs through their flexibility and ease of use in many complex and diverse scenarios. The dispersion of these site-specific nuclease systems has repurposed many biological research areas. Target discovery of disease and associated mechanisms, transcriptional trans-modulation, and the development of transgenic hybrid species of plants are the most prominent of them. One of the primary hurdles that researchers have to deal with is the delivery of the developed CRISPR/Cas constructs into the host of interest (Lino et al. 2018). Over the years, many methods have been developed and improved to deal with CRISPR/Cas payload delivery according to the varying needs of the situation and hence diverse methodologies and protocols. These advanced methods have merits and demerits in terms of efficiency and other important relevant factors.

The components of CRISPR/Cas delivery systems have been categorized (Lino et al. 2018) into two groups:

1. Cargo

2. Delivery Vehicle

Cargo has been sub-divided (Lino et al. 2018) and has exploited the following three approaches:

- I. DNA plasmid encoding a sgRNA and Cas protein
- II. Cas translocation-mediating mRNA and a separate sgRNA
- III. Ribonucleoprotein (RNP) complex (sgRNA + Cas protein)

The mediation of the above three cargoes is done by the delivery vehicles along with affecting factors like the usability of system utilizing both the *in vivo* and *in vitro* conditions. The system's usability is a very important factor to consider while dealing with a cargo-mediating delivery vehicle. For example, molecular compatibility and cross-checking between cargo and the delivery vehicles are very important in the case of negatively charged oligonucleotides compared to the positively charged Cas9 proteins. Cas9:sgRNA RNPs exhibit positive and negative partial charge, which may also be affected by pH. Moreover, the concentration of Cas proteins can be mediated by using DNA instead of using recombinant protein as a direct input (Sun et al. 2015). Considering all the factors, it is still impossible to predict the number of effectively interacting Cas units in a specific time frame of a given assembly system.

The properties and expansive details of all the up-to-date delivery methods along with their important respective properties have been provided in Table S2 (Supplementary File 2) (Matano et al. 2015; Ousterout et al. 2015; J 1995; Crispo et al. 2015; Raveux, Vandormael-Pournin, and Cohen-

Tannoudji 2017; Dong et al. 2015; Guan et al. 2016; Maggio et al. 2016; Maddalo et al. 2014; Tabebordbar et al. 2016; Truong et al. 2015; Platt et al. 2014; Roehm et al. 2016; Koike-Yusa et al. 2013; Mout et al. 2017; Ebina et al. 2013; Kennedy et al. 2014; Schwank et al. 2013; Horii et al. 2014; Zuris et al. 2014; Sun et al. 2015; Axford, Morris, and McMurry, n.d.; D’Astolfo et al. 2015; Bates and Kostarelos 2013; Nakamura et al. 2012; Su et al. 2017; Gonzalez Porras et al. 2016; Teng et al. 2016; Brito et al. 2008). Apples and grapevines are one of those fruit crops that have been successfully edited by exploiting vector-free transformation methods (Malnoy et al. 2016). The *Agrobacterium*-mediated transformation has also been used as a delivery method in apple, banana, cacao, citrus, grapevine, kiwi, and strawberry (Nishitani et al. 2016b; Naim et al. 2018; Fister et al. 2018b; F. Zhang et al. 2017; Nakajima et al. 2017; Z. Wang et al. 2018b; Zhou, Wang, and Liu 2018).

Exploiting the CRISPR/Cas system in fruit plants for biotic and abiotic stress management

Though the work of genome editing is challenging in fruits, there are certain applications of the CRISPR/Cas genome editing system that have been successful in modifying fruit crops for various purposes. For example, many fruits have been targeted via the CRISPR/Cas system and improved their response to various environmental stresses, yield, and quality to meet the global need for food and nutrition. The successfully modified fruit plant species include apple, banana, kiwi, fig, pear, orange, and papaya. All of these have been modified for one or more than one gene. A detailed list of CRISPR-edited fruits along with their loci, their type of modification, mode of transformation, and plant parts under modification have been provided in Table 3. Although *Agrobacterium*-mediated transformation and RNP transformation methods pave the way to the development of genome-edited mutants in terms of identification, these are indistinguishable from any other mutants produced by mean of other genetic modification methods like physical (UV-rays, X-rays, gamma rays, and ion beams) and chemical methods (alkylation agents, base analogs, and acridine dyes).

Table 3. CRISPR/Cas genome editing in fruits. The plants are transformed with *Agrobacterium*-mediated transformation protocols.

<i>Fruits Crop</i>	<i>Loci being Targeted</i>	<i>Type of Modification(s)</i>	<i>Tissue for Modification</i>	<i>Reference(s)</i>
<i>Apple</i>	<i>uidA</i>	Activity of β-glucuronidase	Leaf tissue	(Peer et al. 2015)
	<i>PDS</i>	Biosynthesis of carotenoids	Leaf tissue	(Nishitani et al. 2016a)
	<i>IdnDH</i>	Tartaric acid biosynthesis	Leaf tissue	(Osakabe et al. 2018)
	<i>DIPM</i> (1, 2, and 4)	Resistance to fire blight	Protoplast	(Malnoy et al. 2016)
<i>Banana</i>	<i>MaPDS</i>	Biosynthesis of carotenoids	Cell suspension (embryogenic)	(Kaur et al. 2018)

Kiwifruit	<i>eBSV</i>	Viral pathogenesis control	Explant (epicotyl)	(Tripathi et al. 2019)
	<i>acPDS</i>	Biosynthesis of carotenoids	Leaf tissue	(Z. Wang et al. 2018a)
	<i>CsPDS</i>	Biosynthesis of carotenoids	Leaf tissue	(Jia and Nian 2014)
	<i>DMR6</i>	Resistance to Huanglongbing disease	Explant (epicotyl)	(“Regulation of Citrus <i>DMR6</i> via RNA Interference and CRISPR/Cas9-Mediated Gene Editing to Improve Huanglongbing Tolerance,” n.d.)
Fig	<i>uidA</i>	Activity of β -glucuronidase	Leaf tissue	(Peer et al. 2015)
Wanjincheng Orange	<i>CsLOB1</i> (promoter Sequence)	Resistance against citrus canker	Explant (epicotyl)	(Peng et al. 2017a)
Pear	<i>TFL1</i>	Early flowering	Cell suspension (embryogenic)	(Charrier et al. 2019)
Coffee	<i>CcPDS</i>	Biosynthesis of carotenoids	Cell suspension (meristematic)	(Breitler et al. 2018)

<i>Cacao</i>	<i>TcNPR3</i>	Enhanced defense response	Leaf tissue	(Fister et al. 2018a)
<i>Grapevine</i>	<i>VvPR4b</i>	Downy mildew resistance	Proembryogenic mass (PEM) cells	(M. Y. Li et al. 2020)
	<i>VvMLO3</i>	Powdery mildew	Leaf tissue	(Wan et al. 2020)
<i>Papaya</i>	<i>cp</i>	Resistance against Papaya ringspot virus	immature zygotic embryos	(Fitch et al. 1992)

CRISPR/Cas genome editing is also being used to produce and develop those varieties of fruits that are resistant to multiple biotic stress situations posed by the environment. For example, papaya has been made resistant to its infamous pathogen named *papaya ringspot virus* (PRSV). The virus uses insects mainly aphids as a transmission vector. The biolistic transformation approach was exploited to develop the transgenic 'SunUp' cultivar from the original 'Sunset' cultivar by utilizing genes isolated from a closely-related Hawaiian papaya strain (Fang et al. 2020). To achieve the desirable yellow flesh of papaya that the new cultivar was lacking, it was further crossed with a non-engineered 'Kapoho' cultivar. The resulting cultivar was also resistant to the papaya ringspot virus (Fitch et al. 1992; Conover 1964). Similarly, *Citrus* species as one of the more economically important fruit plant groups are susceptible to citrus canker. Targeted modifications at the 5'-regulatory region have been made in the effector-binding element (EBEPthA₄) of the *Citrus sinensis* *Lateral Organ Boundaries 1* (*CsLOB1*) gene that is responsible for the susceptibility. When the promoter of the gene was disrupted, it was observed that the overall resistance was improved. Complete resistance was achieved by completely deleting the EBEPthA₄ promoter sequence from both *CsLOB1* alleles (Jia et al. 2017a; Peng et al. 2017b).

Furthermore, through CRISPR/Cas-mediated gene disruptions, cacao has been made resistant to the fungus *Phytophthora tropicalis*, grapevine against *Botrytis cinerea*, and grapefruit against citrus canker (Jia et al. 2017b, 2016; X. Wang et al. 2018). Furthermore, Bayoud disease in date palms can also be tackled successfully using CRISPR/Cas manipulation of multiple date palm genomic loci through the universal tRNA-based approach (Sattar et al. 2017; Jaganathan et al. 2018). Plant productivity and survival are negatively impacted by abiotic stresses posed by the environment, especially and increasingly by climate change. Many genes have been identified to regulate the adaptive mechanisms related to abiotic stresses like heat, cold, salinity, and drought (Bressan, Bohnert, and Zhu 2009). For example, pear (*Pyrus communis*) has been modified to produce multiple abiotic stress tolerances at once. This has been made possible by the overexpression of the apple-derived *spermidine synthase* gene (*MdSPDS1*) modulated by the application of *Agrobacterium*-mediated transformation that alters the titers of polyamines in pear fruits (Wen et al. 2008). Similarly, apple has been made tolerant to cold, salt, and drought stress by overexpressing its *MdCIPK6L* gene that encodes specific CIPKs (CBL-interacting protein kinases) (Y. Wang et al. 2016; Kaur, Awasthi, and Tiwari 2020).

CRISPR/Cas genome editing has been used to control the browning in apple fruits. The browning of the apple's flesh happens when the underlying phenolic compounds are oxidized by polyphenol oxidases (PPOs). An approach was successful to develop multiple varieties of apples by

using a CRISPR/Cas system to silence the genes mediating the activity of PPOs (Butiuc-Keul et al. 2022). Similarly, as a proof of concept of the great potential of the CRISPR/Cas system, Pinkglow™ pineapple was developed by modifying the carotenoid synthesis pathway through the expression of the tangerine (*Citrus reticulata*) *PSY* gene. The pink color is due to the subsequent accumulation of lycopene that is formed as an intermediate during general carotenoid synthesis. In tomatoes, enhancements have been made to their floral architecture and fruit size through mobile *CLV3* peptide (regulates floral stem cells) promoter edited by CRISPR/Cas (Rodríguez-Leal et al. 2017).

Limitations and their possible solutions in engineering climate-smart fruit plants

One of the major concerns that limit the efficacy of CRISPR/Cas systems are undesirable off-target effects, meaning unintended editing events. They can be caused by PAM (protospacer adjacent motif) sequences and sgRNA binding sites besides the intended target when implemented *in vivo*. *In silico* techniques are readily utilized to precisely predict these off-target cleavages but are limited by the exceptionally complex interaction of epigenetic modifications that are almost impossible to predict with the current technological standards. Moreover, these programs are limited to the examination of only homologous genes (Yee and Yee 2016; H. C. Yang and Chen 2018; Suleiman, Saedi, and Muhaidi 2021; Zischewski, Fischer, and Bortesi 2017). High-throughput NGS or genome-wide next-generation sequencing can contribute to reducing off-target effects by designing extremely target-specific sgRNAs using existing gene sequences. If off-targets cannot be completely excluded, gene sequences can at least be used to predict possible off-targets, which can then be checked for unintended editing via DNA sequencing. One of the strategies that can be applied directly to a CRISPR/Cas system is reducing the functional time frame of activity as well as target locus alterations and enhancing the specificity of nuclease cleavages. The newly engineered CRISPR-associated proteins like Sniper-Cas9, HF-Cas9, eSpCas9, and HypaCas9 exhibit on-target specificity with great efficiency reducing the off-target effects (Hu et al. 2018; J. K. Lee et al. 2018; J. S. Chen et al. 2017; J. Lee et al. 2019; Davis et al. 2015). Online tools such as Cas-OFFinder and CCTop can be utilized to predict potential off-targets involved in the intended CRISPR/Cas approach (Bae, Park, and Kim 2014; Stemmer et al. 2015).

Furthermore, the availability of PAM sequences at the locus of interest is limited. This restriction can be circumvented by using variants of CRISPR-associated proteins such as Cas12a and SpCas9 or alternative PAMs with lower efficiency. The indel target specificity can be further enhanced by exploiting artificial intelligence-mediated predictions and analysis (H. K. Kim et al. 2018; Kleinstiver et al. 2015; Gao et al. 2017). To obtain single point mutations without the induction of DNA double strand breaks, base editors are exploited because of their unmatched efficiency. These include cytosine and adenine base editors that convert C-G base pairs to T-A pairs and A-T pairs to G-C pairs, respectively (Gaudelli et al. 2017; Komor et al. 2016; Rees and Liu 2018; Y. Yang et al. 2021). Other prominent limitations hindering the efficacy of CRISPR/Cas systems are mosaicism, RNA instability, and Cas-associated immunogenicity (P. Kumar et al. 2020). The mosaicism problem can be tackled through the proper optimization of the transformation pathway being used for the induction of faster editing. RNA instability can be corrected by avoiding RNase contaminations in the process. Furthermore, it has been shown that the Cas proteins-associated immunogenic response can be reduced by the induction of two consecutive mutations in the epitope anchor residues (Ferdosi et al. 2019).

Conclusions and Future Perspectives

The original prokaryotic defense system against bacteriophages, i.e., modern CRISPR/Cas technology, holds virtually infinite potential and clings tightly to an immensely positive future outlook. It has already affected and turned the face of global food insecurity by a mile due to its easiness of use in developing multiple climate-smart fruit crops. Through the precision and accuracy of CRISPR/Cas technology, researchers can implement the features of *C₄*-plants in *C₃*-plants to cope with the yield losses due to the deficiency in the overall photosynthesis rate, especially in warmer climates (Cui 2021; N. J. Brown et al. 2011). Although, the underlying mechanisms of *C₄*-plants are

highly complex (Sedelnikova, Hughes, and Langdale 2018; Furbank 2016; H. Zhu, Li, and Gao 2020; J. H. Lee et al. 2020; Yingxiao Zhang et al. 2019), recent important developments have paved the way for the new C₄-plants to emerge (Lundgren et al. 2016; Newell et al. 2010; Brutnell et al. 2010). Moreover, CRISPR biosynthetic pathway modification is already being applied to fruits like apples, bananas, citrus, pineapple, pear, fig, and kiwi to introduce novel mechanisms like carotenoid biosynthesis as a proof-of-concept and regulation in the activities of different enzymes. These modifications lead the way in achieving resistance against climate change by enhancing the biotic and abiotic stress tolerance profile in fruit crops (South et al. 2019; Narayanan et al. 2019; Taylor et al. 2019). The recent advances in base and prime editing have demonstrated that the capacity and scope of CRISPR/Cas technology are still expanding (Grünwald et al. 2020; Lin et al. 2020; Anzalone et al. 2019).

Furthermore, a lot of potential of CRISPR/Cas systems has been exploited to make fruits resistant to biotic and abiotic stresses of the environment along with enhancing their overall nutritional value. All of these developments are ultimately necessary especially when the world population is rising at an alarming rate along with climate change. To get the most out of these revolutionary genome editing technologies, the general population also needs to be guided about the long-term benefits and perks of using climate-smart fruits generated through CRISPR/Cas and other systems provided that their concerns are justified. Maybe in the distant future, this technology morphs into a new form with its current precise, accurate, and efficient editing and without its limitations, which will surely help to use the uncharted and gigantic genetic resources of plants.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org. Supplementary File 1: Major Fruits and their Genome Sequence Information. Supplementary File 2: Common Features of Delivery Vehicles for CRISPR/Cas system

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Data Availability: The authors declare that the data supporting the findings of this study are available within the paper and its Supplementary Information files. Should any raw data files be needed in another format they are available from the corresponding author upon reasonable request

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