Application and future perspective of CRISPR/Cas9 genome editing in fruit crops

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Abstract Fruit crops, including apple, orange, grape, banana, strawberry, watermelon, kiwifruit and tomato, not only provide essential nutrients for human life but also contribute to the major agricultural output and economic growth of many countries and regions in the world. Recent advancements in genome editing provides an unprecedented opportunity for the genetic improvement of these agronomically important fruit crops. Here, we summarize recent reports of applying CRISPR/Cas9 to fruit crops, including efforts to reduce disease susceptibility, change plant architecture or flower morphology, improve fruit quality traits, and increase fruit yield. We discuss challenges facing fruit crops as well as new improvements and platforms that could be used to facilitate genome editing in fruit crops, including dCas9-base-editing to introduce desirable alleles and heat treatment to increase editing efficiency. In addition, we highlight what we see as potentially revolutionary development ranging from transgene-free genome editing to de novo domestication of wild relatives. Without doubt, we now see only the beginning of what will eventually be possible with the use of the CRISPR/Cas9 toolkit. Efforts to communicate with the public and an emphasis on the manipulation of consumer-friendly traits will be critical to facilitate public acceptance of genetically engineered fruits with this new technology.

INTRODUCTION

Fruits and fruit juice provide fiber, beneficial metabolites, and nutrients to humans. Many fleshy fruit producing crops are major economical drivers for many countries or regions in the world. The most common and widely consumed fruits include apple, orange, grape, banana, strawberry, watermelon, kiwifruit and tomato. There has been a long history of research to improve fruit yield, increase disease resistance, and enhance fruit quality traits. The prior research progress was mainly achieved through conventional breeding via selective hybridization, progeny evaluation, and propagation. These techniques are time-consuming and labor intensive, and they are limited by the available germplasms. With new and exciting molecular genetic techniques, especially the newly-emerged CRISPR/Cas9 genome editing tools, the field stands to see a rapid progress and possibly revolution.

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 (Jinek et al. 2012; Wiedenheft et al. 2012). In bacteria that contain...
the Type II CRISPR/Cas system, such as Streptococcus pyogenes (Jinek et al. 2012; Cong et al. 2013; Gaj et al. 2013; Mali et al. 2013), 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 (Cong et al. 2013; Gaj et al. 2013; Mali et al. 2013; Nekrasov et al. 2013; Shan et al. 2013). 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 double-stranded DNA break (DSB) activates two different DNA repair mechanisms, non-homologous end joining (NHEJ) and homology directed repair (HDR) (Symington and Gautier 2011). 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).

CRISPR/Cas9 toolbox for higher plant genome editing

Until now, successful genome editing mediated by CRISPR/Cas9 was demonstrated in many plant species, including Arabidopsis (Mao et al. 2013), tobacco (Nekrasov et al. 2013), tomato (Brooks et al. 2014), rice (Shan et al. 2013), maize (Xing et al. 2014), wheat (Wang et al. 2014), potato (Wang et al. 2015a), barley (Lawrenson et al. 2015), Brassica (Lawrenson et al. 2015) and others, offering an unprecedented opportunity for functional dissection of genes and direct trait improvement in crop plants. The first report of CRISPR/Cas9 genome editing in plants was in three back-to-back papers in Nature Biotechnology (Li et al. 2013; Nekrasov et al. 2013; Shan et al. 2013). In these reports, successful genome editing was demonstrated in Nicotiana benthamiana and Arabidopsis thaliana leaf protoplasts (Li et al. 2013), Agrobacterium-infiltrated tobacco leaves (Nekrasov et al. 2013), and rice and wheat protoplasts as well as rice plant (Shan et al. 2013). One of the most impressive achievements that soon followed is the simultaneous editing of three MILDEW-RESISTANCE LOCUS (MLO) homoeoalleles in hexaploid bread wheat, leading to powdery mildew resistance in the wheat plant (Wang et al. 2014).

In a standard CRISPR/Cas9 system for plants, the Cas9 nuclease (often codon-optimized for human, Arabidopsis, or monocot crop) is driven by a strong

**Figure 1. Molecular mechanism underlying CRISPR/Cas9 genome editing**

sgRNA is formed by joining the tracrRNA (black) and crRNA (green and red). PAM (mostly NGG) is shown as three orange base paring lines on the target DNA (grey). The location of double stranded breaks is indicated by a pair of yellow arrows.
promoter such as 35S promoter, ubiquitin promoter, or YAO promoter (Feng et al. 2019; Shan et al. 2013; Ma et al. 2015; Yan et al. 2015; Peterson et al. 2016), or tissue-specific promoters such as the egg-cell specific promoter (Wang et al. 2015b). In contrast, the sgRNA is always driven by the U6 promoter transcribed by the RNA Polymerase III. The polycistronic tRNA-gRNA (PTG), where tandem tRNA-gRNA coding units are chained together and cleaved to release individual gRNAs by the endogenous tRNA-processing system, enables simultaneous expression of a large number of gRNAs targeting different genomic loci (Xie et al. 2015). Similarly, a self-cleaving transcript with ribozyme sequences flanking the gRNA also led to simultaneous production of multiple gRNAs. Further, the multi-unit synthetic gRNA genes could be driven by RNA Pol II promoters, allowing tissue-specific expression (Gao and Zhao 2014). Finally, Golden Gate and Gateway cloning methods combined with appropriate vectors also allow for multiplex plant genome editing (Lowder et al. 2018).

In 2016, a programmable “base editing” system was developed in mammalian cells (Komor et al. 2016; Gaudelli et al. 2017; Rees and Liu 2018; Yan et al. 2018). A catalytically disabled Cas9 (dCas9) is fused to cytidine deaminase to form the cytosine base editor (CBEs), converting C.G base pair to T.A base pair. Similarly, the adenine base editors (ABEs) converts A.T base pair to G.C base pair. With the sgRNA providing target-specificity, the dCas9-base editors can create point mutations in the target DNA with high specificity and precision. This new technology was quickly adopted in plants, including watermelon (Lu and Zhu 2017; Li et al. 2018a; Ren et al. 2018; Tian et al. 2018).

**OVERVIEW OF FRUIT CROP GENOME EDITING**

*Table 1* summarizes published reports of genome editing in fruit crops. With the exception of tomatoes, most fruit crops have only begun to experiment with this technology. The majority of the reports are on the editing of *Phytoene Desaturase (PDS)* gene. A mutation in the PDS gene reduces photosynthesis and carotenoid biosynthesis and leads to albinism and often death of transgenic shoots. While pds mutants or mutant sectors are easy to identify in T₀ transgenic plants, it is difficult to test the inheritance of the pds mutations due to lethality. Up to now, CRISPR/Cas9-induced mutations inherited through the germline are only reported in few fruit producing species (citrus, tomato, watermelon, grape, and strawberry). The following sections will describe in more detail these achievements.

**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 difficult 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. A single guide RNA was designed to target 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 phenotype among the regenerated plantlets were observed at a 59% mutation rate. More recently, a multiplexed approach via the polycistronic tRNA-gRNA system was used to target the exon 1 of PDS in the Cavendish cultivar; 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 occurs efficiently in banana. Targeting endogenous genes will be the necessary next step toward the genetic improvement of banana.

**Genome editing in citrus**

Citrus is one of the most economically important fruit tree crops in the world, yet genetic modification through breeding or gene manipulation is extremely difficult due to polyembryonic seeds, pollen incompatibility, parthenocarpic fruit, a long juvenile phase, and resistance to transformation by traditional means (Talon and Gmitter 2008). In 2014, Jia and Wang successfully employed *Xanthomonas citri subsp. citri* (Xcc)-facilitated agroinfiltration to deliver Cas9 and a synthetic sgRNA targeting CsPDS into the leaves of sweet orange (*Citrus X sinensis*) and Duncan grapefruit, which led to successful but low mutational rates in infiltrated leaves (Jia and Wang 2014a, b). Later, the same group applied Agrobacterium-mediated transformation to Duncan grapefruit epicotyl explants and...
| Species                        | Cas9                          | Target gene(s) | Target traits                                                      | Cas9 delivery method                           | Efficiency ($T_0$) | Reference                  |
|-------------------------------|-------------------------------|----------------|------------------------------------------------------------------|-----------------------------------------------|-------------------|----------------------------|
| Sweet orange (Citrus sinensis)| 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 (Citrus sinensis)| pYAO:hSpCas9                  | PDS            | Albino phenotype and decrease of carotenoid content              | Agrobacterium-mediated transformation         | 45.5%–75%        | Zhang et al. 2017          |
| Duncan grapefruit (Citrus paradisi) | 35S:: SaCas9            | PDS            | Albino phenotype and decrease of carotenoid content              | Agrobacterium-mediated transformation         | 15.55%–79.67%    | Jia et al. 2017a           |
| Duncan grapefruit (Citrus paradisi) | p1380N-pcoCas9     | CsLOB1 promoter | citrus disease susceptibility gene for citrus canker              | Agrobacterium-mediated transformation         | 14.29%– 81.25%   | Jia et al. 2016            |
| Duncan grapefruit (Citrus paradisi) | p1380N-pcoCas9   | CsLOB1         | citrus disease susceptibility gene for citrus canker              | Agrobacterium-mediated transformation         | 31.58%–89.36%    | Jia et al. 2017b           |
| Duncan grapefruit (Citrus paradisi) | 35S-pcoCas9              | CsLOB1 promoter | citrus disease susceptibility gene for citrus canker              | Agrobacterium-mediated transformation         | 11.5%–64.7%      | Peng et al. 2017           |
| Tomato (Solanum lycopersicum)  | PcUbi4-2(P)::pDe-Cas9         | RIN            | Regulating fruit ripening                                        | Agrobacterium-mediated transformation         | Up to 100% in T1 | Ito et al. 2015            |
| Tomato (Solanum lycopersicum)  | pUbi::pcoCas9               | ORRM4          | RNA editing factors involving in fruit ripening                   | Agrobacterium-mediated transformation         | 36.4%            | Yang et al. 2017           |
| Tomato (Solanum lycopersicum)  | 35S::spCas9                 | ALC            | Ripening of fruit                                                 | Agrobacterium-mediated transformation         | 72.73%           | Yu et al. 2017             |
| Tomato (S. lycopersicum and S. pimpinellifolium) | 35S::hpCas9              | SIWUS, SICLV3   | Fruit size                                                        | Agrobacterium-mediated transformation         |                  | Rodriguez-Leal et al. 2017 |
| Tomato (Solanum pimpinellifolium) | 35S::AtCas9; pCmYLCV::AtCas9 | SP, OVATE, FW2.2, CycB | Domestication traits (morphology, fruit number, nutritional quality) | Agrobacterium-mediated transformation         |                  | Zsogon et al. 2018         |

(Continued)
| Species | Cas9 | Target gene(s) | Target traits | Cas9 delivery method | Efficiency ($T_0$) | Reference |
|---------|------|----------------|---------------|---------------------|-------------------|-----------|
| Tomato (*Solanum pimpinellifolium*) | 35S::AtCas9 | SP, SP5G, CLV3, WUS, GGP1 | Domestication traits (morphology, flower and fruit development, ascorbic acid biosynthesis) | Agrobacterium-mediated transformation | | Li et al. 2018d |
| Apple (*Malus prunifolia*) | 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 | 0.1%–6.9% (by targeted deep sequencing) | Malnoy et al. 2016; Osakabe et al. 2018 |
| Apple (semi-dwarfing rootstock cultivar JM2) | 2 × 35S:: fcoCas9 | PDS | Albino phenotype and decrease of carotenoid content | Agrobacterium-mediated transformation | 31.8% | Nishitani et al. 2016 |
| Grapevine (*Vitis vinifera*) | Commercial recombinant Cas9 protein | MLO-7 | Susceptible gene to powdery mildew | Direct delivery of purified CRISPR/Cas9 ribonucleoproteins to the protoplast | 0.1% (by targeted deep sequencing) | Malnoy et al. 2016; Osakabe et al. 2018 |
| Grapevine (*Vitis vinifera*) | 2 × 35S:: Plants codon-optimized Cas9 (pPtC.4) | IdnDH | Biosynthesis of tartaric acid (TA) | Agrobacterium-mediated transformation using embryo callus | 100% | Ren et al. 2016 |
| Grapevine (*Vitis vinifera*) | PcUb14-2(P)::pDe-Cas9 | PDS | Albino phenotype and decrease of carotenoid content | Agrobacterium-mediated transformation | 2.7%–72.2% | Nakajima et al. 2017 |
| Grapevine (*Vitis vinifera*) | 2 × 35S:: Plants codon-optimized Cas9 (Ma et al. 2015) | VWRKY52 | Biotic stress responses | Agrobacterium-mediated transformation | 31% | Wang et al. 2018a |
| Watermelon [*Citrullus lanatus* (Thunb.) Matsum & Nakai] | 2 × 35S:: zCas9 | PDS | Albino phenotype and decrease of carotenoid content | Agrobacterium-mediated transformation | 100% | Tian et al. 2017 |
| Banana (banana cv. Rasthali) | 2 × 35S::pcoCas9 | PDS | Albino phenotype and decrease of carotenoid content | Agrobacterium-mediated transformation | 59% | Kaur et al. 2018 |

(Continued)
| Species                      | Cas9                | Target gene(s) | Target traits                                                                 | Cas9 delivery method                  | Efficiency (T₀) | Reference                |
|------------------------------|---------------------|----------------|-------------------------------------------------------------------------------|----------------------------------------|-----------------|--------------------------|
| Banana (Cavendish cv. Williams) | pUbi::hSpCas9       | PDS            | Albino phenotype and decrease of carotenoid content                           | Agrobacterium-mediated transformation | 100%            | Naim et al. 2018         |
| Kiwifruit (Actinidia Lindl.)  | 35S::SpCas9         | PDS            | Albino phenotype and decrease of carotenoid content                           | Agrobacterium-mediated transformation | 0–8.33%         | Wang et al. 2018b        |
| Kiwifruit (Actinidia Lindl.)  | PTG/Cas9 system (Xie et al. 2015) | PDS            | Albino phenotype and decrease of carotenoid content                           | Agrobacterium-mediated transformation | 65.38%–91.67%   | Wang et al. 2018b        |
| Cucumber (Cucumis sativus L.) | 35S::pcoCas9        | eIF4E          | Resistance against cucumber vein yellowing virus                             | Agrobacterium-mediated transformation | Low             | Chandrasekaran et al. 2016 |
| Cucumber (Cucumis sativus L.) | 35S:: zCas9         | CmWIP1         | inhibitor of carpel development                                               | Agrobacterium-mediated transformation | 64.3%           | Hu et al. 2017           |
| Wild Strawberry (Fragaria vesca) | pAtUBQ10::pcoCas9   | TAA1 ARF8      | Auxin biosynthesis and signaling                                              | Agrobacterium-mediated transformation | 49%–75%         | Zhou et al. 2018         |
| Wild Strawberry (Fragaria vesca) | 35S:: zCas9         | YUCCA10        | Auxin biosynthesis                                                           | Agrobacterium-mediated transformation | 100%            | Feng et al. 2019         |
| Cultivated Strawberry (Fragari × ananassa) | 35S::hSpCas9       | FaTM6          | Anther development                                                           | Agrobacterium-mediated transformation | NA              | Martín-Pizarro et al. 2018 |

*note: only selective tomato genome editing studies are included in Table 1.*
created six transgenic lines that contain a CRISPR/Cas9 construct targeting the CsLOB1 gene controlling susceptibility to citrus canker, a disease caused by bacterium Xanthomonas axonopodis. The resulting plants showed varying degrees of resistance to the citrus canker (Jia et al. 2017b). Another effort successfully edited the promoter of CsLOB1, which rendered the resulting homozygous plants resistant to citrus canker (Peng et al. 2017). Hence, genome-editing of the promoter region proves to be an effective method in disrupting target gene expression. These encouraging developments illustrate that the CRISPR/Cas9-mediated editing will help combat devastating diseases affecting citrus including citrus greening.

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 eaten fresh or used for making wine, jam, juice, and even medicine. Whole-genome sequence of grape (V. vinifera L.) was reported and released in 2007 (Jaillon et al. 2007), and grape transformation using embryonic suspensions was established as early as 1993 (Hebert et al. 1993). In 2016, a group successfully edited the L-idonate dehydrogenase gene (IdnDH) in the biosynthesis of tartaric acid (Ren et al. 2016). A CRISPR/Cas9 construct containing two sgRNAs was transformed into the ‘Chardonnay’ embryonic suspension cells via agrobacterium, yielding 100% mutational efficiency. A second report targeting Vitis vinifera phytoene desaturase (VvPDS) showed albino leaves at efficiencies ranging from 0–86% (Nakajima et al. 2017). Interestingly, older leaves possess 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 plays a role in biotic stress response (Wang et al. 2018a). Proembryonal masses derived from embryo calli were infected with agrobacterium containing the CRISPR/Cas9 construct. Fifteen of 22 first generation transgenic lines carried biallelic mutations, and four of these showed increased resistance to Botrytis cinerea.

Transgene-free genome editing is highly desirable to facilitate commercialization of genetically modified grapes. To knockout MLO-7, a susceptible locus to powdery mildew, direct delivery of purified CRISPR/Cas9 ribonucleoproteins (RNPs) into grapevine protoplasts was demonstrated (Malnoy et al. 2016). The RNPs were introduced into protoplasts via the PEG-mediated transformation and subsequently led to the editing of MLO-7 without involving any foreign DNA. However, the mutagenesis efficiency was only 0.1% to 6.9%. Recently, an improved protocol was established for the direct delivery of CRISPR/Cas9 RNPs into grapevine protoplasts (Osakabe et al. 2018). The complete protocol of the direct delivery takes only 2–3 weeks, and whole plants can be subsequently generated from edited protoplasts. Therefore, grape is becoming a pioneering fruit crop in the arena of transgene-free genome editing.

Apple is another major fruit crop in the world. Efficient genome editing was shown in first generation young transgenic plants of semi-dwarfing rootstock cultivar JM2. The PDS gene was precisely modified and a variety of albino phenotypes from clear white to pale green, to variegated young shoots were observed (Nishitani et al. 2016). The white sectors can be used to indicate when and where successful editing occurs. Direct delivery of RNPs into apple leaf protoplasts was shown possible, and edited mutations in the DIPM-1, -2, and -4 genes were detected by deep sequencing of pooled protoplast DNA (Malnoy et al. 2016). However, microcalli generation from apple protoplasts will need to be established before transgene-free apple plants are attainable (Osakabe et al. 2018).
Transgene-free edited plants in cucumber, watermelon and strawberry through segregation of the T-DNA vector

Cucumber (Cucumis sativus L.), often consumed as a vegetable, is a botanical fruit. The CRISPR/Cas9 technology was used to disrupt elf4E (eukaryotic translation initiation factor 4E). Homozygous and transgene-free T3 elf4E mutant progeny exhibited broad viral resistance (Chandrasekaran et al. 2016). A more efficient CRISPR/Cas9 system was recently developed by driving gRNA with a cucumber U6 promoter (Hu et al. 2017). This system was used to knockout CsWIP1, an inhibitor of carpel development in cucumber. Transgene-free and homozygous T2 CsWIP1 mutant progeny developed female and hermaphrodite flowers only and significantly more female flowers when compared with wild type. The mutant cucumber is generated from a commercially valuable inbred line and will be valuable for heterosis breeding (Hu et al. 2017).

Watermelon (Citrullus lanatus) is the second species in the Cucurbitaceae family with successful genome editing (Tian et al. 2017; 2018). By first targeting CIPDS gene with standard CRISPR/Cas9, almost all the transgenic watermelon plants harbor CIPDS mutations, indicating almost 100% editing efficiency. Subsequently, dCas9-mediated base-editing was used to specifically mutate Pro190 of the acetolactate synthase (ALS) gene. Specifically, the researchers infected cotyledon of an elite inbred variety ZG94 with Agrobacterium containing a construct expressing dCas9 fused with the cytidine deaminase. Although 45 of 199 T0 transgenic plants harbor C to T mutations at the Pro190 of ALS, all are heterozygous. However, some T1 progeny plants not only became homozygous for the edited mutation, but also lost the dCas9 transgene. These transgene-free homozygous als mutant plants were shown to be resistant to the herbicide Tribenuron (Tian et al. 2018). This is a very exciting development using the dCas9-base editing platform.

Diploid wild strawberry (Fragaria vesca) has emerged as a new model system for the garden strawberry (Fragaria x ananassa), which is octoploid (Shulaev et al. 2011). Recently, Zhou et al. (2018) reported successful application of CRISPR/Cas9 in F. vesca. In this study, dual sgRNA cassettes were driven by an Arabidopsis U6 promoter (AtU6-26) and a Fragaria vesca U6 promoter (FveU6-2), respectively. T0 generation transgenic F. vesca lines exhibited a mutation efficiency of 49%-75% in the two targeted genes FveARF8 and FveTAAT1, both acting in auxin pathway. Interestingly, analysis of T1 progeny plants revealed new mutations generated in T1, with even a higher efficiency (83%) than the T0 generation (49%). The increased editing rate in T1 resulted in half of the T1 plants harboring large fragments deletion between the two sgRNA target sites. Some of these T1 homozygous mutant plants lost the transgene and became transgene-free. Further, the arf8 mutant seedlings showed faster growth than the wild type seedling (Zhou et al. 2018). Another recent study targeted the auxin biosynthesis gene FveYUC10, showing 100% efficiency in the mutation rate (Feng et al. 2019). However, homozygous FveYUC10 mutants did not exhibit any morphological phenotype.

Garden strawberry F. ananassa is octoploid and a hybrid. CRISPR/Cas9 was used to edit the FaTM6 MADS box gene, encoding a putative ortholog of Arabidopsis APETALA3 (Martín-Pizarro et al. 2018). The mutant FaTM6 knockout lines failed to develop any fruit due to a lack of fertile anthers (Figure 2), indicating that the FaTM6 MADS box gene plays a key role in anther development in strawberry. This result is very exciting as editing octoploid genome was not as difficult as previously anticipated. As such, the result suggests that the CRISPR/Cas9 technology will be invaluable for the genetic improvement of commercial strawberry.

Genome editing in tomato, pioneering in trait improvement and de novo domestication

Tomato (Solanum lycopersicum L.) is typically classified as a vegetable. However, tomato has been a model for studying fleshy fruit development and fruit ripening for decades (Vrebalov et al. 2002; Giovannoni 2004). CRISPR/Cas9 genome editing was used to investigate gene function as shown in ARGONAUTE7 (SIAGO7) (Brooks et al. 2014), SHORT ROOT (SHR) (Ron et al. 2014), RIPENING INHIBITOR (RIN) (Ito et al. 2015; 2017), RNA Editing Factor SIORMR4 (Yang et al. 2017), SIMAPK3 in drought tolerance (Wang et al. 2017), auxin signaling SlIAA9 in parthenocarpic fruit (Ueta et al. 2017), and PROCERA, a DELLA protein, in fruit development (Tomlinson et al. 2019). High mutation frequency (83.56%) was observed at SIPDS (phytoene desaturase) and SIPIF4 (Phytochrome interacting factor) in the T0 lines, and mutations were stably transmitted to the T1 and T2 generations (Pan et al. 2016).
Double and higher order knockouts created by CRISPR/Cas9 indicate great promise in overcoming redundancy and polyploidy in the study of fruit crops. For instance, Li et al. (2018c) designed a multiplex pYLCRISPR/Cas9 system targeting five key genes in the γ-aminobutyric acid (GABA) shunt in tomato. They obtained 53 genome-edited plants, including single to quadruple mutants. The GABA contents in edited mutants were significantly enhanced, setting an example of metabolic pathway engineering. In another study, a CRISPR/Cas9 generated double mutant tomato of Slddm1a Slddm1b (DECREASE IN DNA METHYLATION) showed pleiotropic vegetative and reproductive phenotypes associated with loss of DNA methylation in the heterochromatic and euchromatic transposons (Corem et al. 2018). The double Slddm1a Slddm1b mutants provide important insights into genome-wide chromatin methylation and DDM function.

CRISPR/Cas9 was also used to study the function of long non-coding RNA (lncRNA), dissect cis-regulatory sequence, and initiate HDR in tomato. IncRNA1459 is a ripening-related lncRNA found in tomato (Zhu et al. 2015). Small Indel mutations were created with CRISPR/Cas9 and the resulting homozygous mutants of IncRNA1459 exhibited delayed ripening process (Li et al. 2018b). Second, Rodriguez-Leal et al. (2017) reported a CRISPR/Cas9-based mutagenesis system targeting the promoters of genes that regulate fruit size and inflorescence architecture. The resulting promoter alleles provide a continuum of variations in gene activity. By segregating away the Cas9 transgene in the following generation, the novel promoter alleles were stabilized, providing beneficial quantitative variations for breeding. Third, the ability to apply HDR in fruit crop would greatly expand the genome editing toolkit, including introduction of novel alleles or correction of existing mutant alleles. alc (alcobaca) is a naturally occurring long-shelf life allele in tomato; the molecular basis of the alc mutation is a Val to Asp change at the residue 317 of the NOR (Non-ripening) gene (Casals et al. 2012). The alc allele was successfully introduced into tomato M82 strain in the absence and presence of the repair template via CRISPR/Cas9 (Yu et al. 2017). Although the replacement efficiency was low (7.69%), a heterozygous alc T₀ plant was obtained that segregated some homozygous alc mutants in T₁. The homozygous mutants, some lost the T-DNA construct, showed increased storage performance without sacrificing other fruit characteristics (Figure 3).

De novo domestication of wild species is a new breeding concept propelled by the CRISPR/Cas9 technology (Zsogon et al. 2017). It was proposed that monogenic and domestication-related traits could be directly introduced into wild relatives of crops through genome editing. These wild relatives carry polygenic traits such as disease or drought resistance, which are
too diffuse to map or manipulate through conventional breeding. On the contrary, de novo domestication of wild species via genome editing could be fast and highly effective. Recently two different groups applied multiplex genome editing to target four or six domesticated-trait loci in *Solanum pimpinellifolium*, the putative wild ancestral progenitor of tomato (Li et al. 2018d; Zsogon et al. 2018). The engineered *S. pimpinellifolium* showed increased fruit size, improved shoot architecture and fruit ripening, as well as increased fruit lycopene accumulation, while maintaining the wild species’ disease resistance and salt tolerance. This pioneering work in tomato provides the first glimpse of what is to come in the era of genome editing.

**DISCUSSION**

**Challenges for genome editing in fruit crops**

With the completion of genome sequencing of more and more fruit-bearing crop species, the understanding of their genome structure, gene pathways, and gene function is paving the way for genome editing of economically important traits. Of particular interest for fruit crops are the traits in biotic or abiotic stress response, shelf life, flavor and nutritional value, plant architecture, and flowering time. However, polyploidization is found in many fruit species (Chen 2007; Emshwiller et al. 2009; Wood et al. 2009) such as apple, banana, garden strawberry, watermelon, plum, and kiwifruit. The genome complexity including heterozygosity and polyploidy contributes to the difficulties in applying genome editing to these species as many more copies of the genes need to be mutated to have a desired phenotype. Second, many fruit crops lack efficient transformation methods, severely limiting delivery of the CRISPR/Cas9 constructs. Third, low editing efficiency and somatic mosaics of CRISPR/Cas9-induced mutations may pose difficulty in identifying heritable mutations. Fourth, the long juvenile stage for many fruit trees make it difficult to experiment and optimize genome editing. Further, it takes a long time to obtain germline-transmitted homozygous mutations or next generation segregants. Fifth, public resistance to GMO suggests that robust transgene-free genome editing methods need to be developed for fruit crops.

**Methods for increasing editing efficiency are highly desirable**

To achieve homozygous or biallelic or multiallele mutations within the same generation, high efficiency editing is desirable. In addition to properly designing sgRNA and expressing Cas9 and sgRNA under strong or tissue-specific promoters, multiplex gRNA design such as the polycistronic tRNA-gRNA (PTG) (Xie et al. 2015) allows simultaneous targeting of multiple regions of the same gene or functionally-redundant genes. Recently, short heat stress (SHS) was reported to be effective in increasing editing efficiency (LeBlanc et al. 2018).
Arabidopsis, four 30 h treatments at 37°C was shown to increase mutation rate by five-folds in somatic tissues and up to 100 folds in the germline. A similarly increased mutagenesis rate was observed in citrus plants after heat treatment (seven times of 37°C for 24 h). The mechanism of increased mutation rate results from higher spCas9 (Cas9 from Streptococcus pyogenes) activity at 37°C as well as 3-fold increase in the gRNA expression level (LeBlanc et al. 2018). It will be important to explore the effect of SHS in other fruit crops and optimize heat treatment schemes for different species.

Off-target effect can be minimized by stringent sgRNA design or use of highly specific Cas nucleases

Targeting specificity was recently investigated comprehensively using whole genome sequencing (WGS) of Cas9 and Cpf1 edited rice plants as well as a large number of control plants (Tang et al. 2018). Significantly, the work showed that most mutations in edited plants are generated by the tissue-culture process not by CRISPR. They argued that off-target can be avoided by designing gRNA with high specificity. Their WGS analysis did not find off-target mutations for 14 of 15 gRNAs tested in both T0 and T1 plants. The Cas-OFFinder program (Bae et al. 2014) should be used to minimize off-target effects by making sure that the highest scored potential off-target sites have at least a 2-nt mismatch in the seed sequence.

Another way to reduce off-target is to use high fidelity Cas9 nucleases such as the “enhanced specificity” SpCas9 (eSpCas9) (Slaymaker et al. 2016), high-fidelity SpCas9-HF1 (Kleinstiver et al. 2016), hyper-accurate Cas9 (Hypacas9) (Chen et al. 2017), and xCas9 (Hu et al. 2018). These engineered Cas9 forms were designed and tested in human cells; their utility and specificity in plants are still unknown. One recent report revealed low editing efficiency of xCas9 in rice (Wang et al. 2019), which may limit its application in plants.

Developing transgene-free edited fruit crops

Developing transgene-free edited fruit crops is critical to winning consumer acceptance. One way to achieve this is through segregating away the transgene in the next generation once editing is attained. One example is the alc (alcoholic) tomato discussed earlier. To facilitate the removal of transgenes, fluorescent protein like mCherry can be driven by a seed-specific promoter and placed in the binary vector to allow easy and fast visual screen of transgene-free seeds at the next generation (Yu and Zhao 2019). However, segregating away transgenes is not always practical; many fruit trees require several years of juvenile growth before reaching sexual maturity. Another issue is that asexual reproduction is needed for many crops, so as to maintain their hybrid genotype, as is the case in garden strawberry. Therefore, introduction of gRNA and Cas9 in a Ribonucleoprotein (RNP) complex directly into host cells could lead to genome editing without involving any foreign DNA as the RNPs are subsequently degraded. The success of this approach has been demonstrated in the protoplasts of Arabidopsis, tobacco, lettuce, and rice (Woo et al. 2015; Kanchiswamy 2016) and more recently in grape and apple (Malnoy et al. 2016; Osakabe et al. 2018). After direct delivery of CRISPR/Cas9 RNPs into the grapevine and apple protoplasts to conduct precise genome editing, microcalli could be generated from grapevine protoplasts, leading to transgene-free grapevine. However, regeneration of plants from apple protoplasts has been difficult. Therefore, direct delivery of RNPs into protoplasts may not be widely applicable to fruit crops due to recalcitrant to regeneration from protoplasts. Efforts are needed to develop efficient regeneration systems from protoplasts as well as effective and inexpensive screening methods to uncover plants with mutated alleles.

Other approaches that transiently express CRISPR/Cas9 components from non-integrating foreign DNA could be achieved through viral infection, particle bombardment, or agrobacterium-mediated transient expression in the host leaves, calli, immature embryos, or other easier to regenerate host tissues (Cermak et al. 2015; Chen et al. 2018; Liang et al. 2018). The resulting tissues could regenerate more easily into seedlings. However, screening is necessary to identify mutant plants via sequencing or PCR. In the viral-based systems, Cas9-sgRNA was introduced into Nicotiana benthamiana through tobacco rattle virus (TRV) (Ali et al. 2015) or Cabbage Leaf Curl virus (CaLCuV) (Yin et al. 2015). Circular Geminiviral Replicons (GVRs) were developed to facilitate transient expression of sequence-specific nucleases (ZFNs, TALENs, CRISPR/Cas9) and donor templates that induce DSBs as well as HDR, leading to highly efficient editing (Baltes et al. 2014). GVRs were soon demonstrated to mediate high-efficiency gene editing in both dicot tomato (Cermak et al. 2015; Dahan-Meir et al. 2018)
and monocot hexaploid wheat (Gil-Humanes et al. 2017). Given that GVRs greatly enhance DNA-donor-template mediated homologous recombination, an added advantage is that mutant alleles can be repaired, and novel and beneficial alleles can be introduced, offering tremendous flexibility in the type of edits in the genome.

Biolistic delivery of CRISPR/Cas9 in vitro transcripts, RNPs, or DNA constructs into bread wheat immature embryos appears an excellent method that overcomes reliance on protoplasts (Zhang et al. 2016; Liang et al. 2018). This method not only eliminates random insertion of T-DNA into the genome but also reduces off-target effects. The wheat embryos formed calli which regenerated into seedlings and desirable mutants were identified through pooling and sequencing. Even easier than the biolistic delivery is the transient expression of CRISPR/Cas9 via agrobacterium-mediated infiltration of tissues (Chen et al. 2018), where the infiltrated tobacco leaves were shown to induce editing at the target PDS gene. However high throughput screening is necessary to identify transformants due to low editing efficiency. Further development of the above-mentioned methods will be necessary.

Application of various new Cas9 platforms for basic research of higher plants

To identify candidate genes for genetic manipulation to achieve desirable traits, it is necessary to gain better understanding of gene function through CRISPR-based reverse genetics. Programmable dCas9-based systems offer increasingly diverse tools for investigations of gene function in higher plants. By mutating the catalytic domains of Cas9, the deactivated Cas9 (dCas9) can be fused to a variety of functional domains and then targeted to a specific genomic region by the gRNA (Xu et al. 2018). For example, dCas9-VP64 and dCas9-SRDX were respectively shown to activate or repress endogenous genes in Arabidopsis and tobacco (Lowder et al. 2015; Lowder et al. 2018) (Figure 4A, B). This gene activation or repression system enables

![Figure 4](image_url)

**Figure 4.** Illustration of a few types of programmable dCas9-based systems, where dCas9, under the guidance of specific gRNA, brings different enzymatic or regulatory activities to precise genomic locations. (A) dCas9 is fused to a repressor domain (brown hexagon) to repress Gene X expression. (B) dCas9 is fused to an activator domain (orange circle) to up-regulate the expression of Gene X. (C) dCas9-9 is fused to an epigenetic modifier (green start) such as DNA methyltransferase to modulate local chromatin. The red pins represent DNA methylation. (D) dCas9 is fused to a base editor (blue square) to change a specific nucleotide within a short range.
not only highly specific regulation of endogenous genes but also multiplexed activation or repression of endogenous genes. When sgRNAs targeting multiple genes are simultaneously introduced into a plant, multiple endogenous genes could be simultaneously activated by recruiting the same dCas9-VP64 fusion protein as was demonstrated in mammals (Cheng et al. 2013). This feature is highly desirable for manipulating multiple steps in a metabolic pathway or multiple members of the same gene family. In another application, dCas9 can be fused to base editing enzymes such as cytidine deaminase to induce specific missense mutations at the targeted genomic region (Figure 4C). A successful application was reported in creating herbicide-resistant watermelon as described earlier (Tian et al. 2018). In addition, dCas9 can be fused to chromatin modifying enzymes including DNA methyltransferase, histone deacetylase, and others to epigenetically modulate transcription at specific genomic locus (Xu et al. 2018) (Figure 4D).

As more efficient methods of introducing sgRNA into plant cells are developed, large scale functional genomics using sgRNA libraries could be carried out as demonstrated in mammalian cell systems (Parnas et al. 2015), where pooled sgRNAs libraries were introduced into bone-marrow-derived dendritic cells collected from Cas9-expressing mice and genes regulating immune responses were identified. In plants, the library of sgRNAs could be introduced as RNPs, or via agrobacterium-mediated transformation, or viral vectors. The Cas9-expression plants could be first established and used to provide the protoplasts, calli, or embryos.

New types of Cas such as Cpf1 and xCas9 are also offering new options and flexibility in both basic research and application. Cpf1 recognizes a T-rich PAM, enable editing of AT-rich regions. Further, Cpf1 cuts with a 5’ overhang, creating sticky ends that could be exploited to insert sequences of interests through complementation and ligation (Zetsche et al. 2015; Tang et al. 2017). xCas9 is an engineered Streptococcus pyogenes Cas9 (SpCas9) capable of recognizing a broader range of PAM sequences, including NG, GAA and GAT (Hu et al. 2018). xCas9 also shows greater DNA specificity than Cas9, without necessarily any “trade-off” between DNA specificity and PAM compatibility. Together Cpf1 and xCas9 offer new solutions for fruit crops by overcoming the problems of poor candidate sequence, greatly expanding potential target sites in the genome.

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 needed, especially when the mutation rate is low. Traditional methods for detecting genotype include direct sequencing of PCR fragments, T7 Endonuclease diagnostic analysis (Guschin et al. 2010), or High Resolution Melting (HRM) analysis (Samarut et al. 2016). Direct DNA sequencing can be expensive if large numbers of plants are screened. The T7 Endonuclease or the HRM methods are not as robust and require optimization, and/or expensive reagents. Illumina sequencing is still expensive for detecting mutations. “SHERLOCK” (specific high-sensitivity enzymatic reporter unlocking) is a new Cas-based platform for rapid detection of SNPs (single nucleotide polymorphism). It not only detects but also accurately quantifies the target content and can simultaneously test multiple target sites in a sample. The employment of lateral flow allows inexpensive and fast detection simply with a paper strip (Gootenberg et al. 2018; Myhrvold et al. 2018). Hence “SHERLOCK” holds potential for field application such as screening genetic modifications in fruit trees growing in the field.

FUTURE DIRECTIONS AND REMARKS
CRISPR/Cas9-based genome editing is a breakthrough technology for basic and applied research of animal and plant. The CRISPR/Cas9 system is currently being developed and applied toward engineering traits of fruit crops. However, due to the difficulties of transformation, complex genomes, slow growth cycles, and a lack of genomic information, it remains a long and arduous task to apply CRISPR/Cas9 to most fruit crops. The rapid development of new and improved CRISPR/Cas9-based tools and delivery systems may help overcome some of the barriers in horticultural crops. Methods that reduce the need for multi-generation cycles or that increase editing efficiency are also highly desirable. Fast and low-cost mutant screening is an area of urgent need. Targeting cis-elements to alter gene...
regulation is a clever way to generate allelic series as demonstrated in citrus LOB1 gene and tomato CLV3 gene. Strategies that aid in the production of transgene-free edited fruit crops will be vital to consumer acceptance and commercialization. In addition, one can never over emphasize the importance of public dialog on genome editing and investment on consumer-friendly traits such as healthier products and longer shelf-life. Given what is already been demonstrated in tomato, de novo domestication and designer fruit is not far from reality.

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