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Development of smart fruit crops by genome editing

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Abstract: Plant genome editing tools as Zinc-Finger Nucleases (ZFNs), Trans Activator-Like Effector Nucleases (TALENs), and Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) associated with Cas proteins are offering new possibilities for crop improvement and new insights for functional genomics. In this review, we discuss (i) the new findings in gene editing technologies, (ii) a comparison between them, and (iii) their applications for genetic analysis and manipulation of fruit crops. Different editing technologies, especially the CRISPR/Cas9 system, were successfully used in fruit crops such as apple, banana, cacao, citrus, grape, kiwifruit, and pear. Experimental designs used to analyze the efficiency of the CRISPR/Cas9 genome editor are presented, including manipulating key genes associated with carotenoid biosynthesis that could allow the development of complete albino and variegated phenotypes in some cultivars. The most recent outcomes of the application of genome-editing tools to improve the quality and yields of fruit crops, such as manipulation of juvenile phase and flowering period, gibberellin biosynthesis and generation of dwarf cultivars, ethylene biosynthesis, fruit ripening and parthenocarpy, development of resistant/tolerant cultivars to numerous pests and diseases are also summarized.

Key words: CRISPR/Cas9, crop improvement, engineered nucleases, fruit crops, genome editing

1. Introduction

Fruit growing is one of the oldest and most important practices in the world. Fruits are essential for a healthy diet, being a substantial source of nutrients and antioxidants, and therefore, the improvement of quality in these crops has gained perpetual interest from growers and researchers. Valuable cultivars and varieties of many fruit crops have been developed by introducing desirable traits through conventional breeding and genetic transformation. Despite their improved qualities, genetically modified (GM) plants have been accepted with restrictions on the market. Even if GM fruits are free from pesticide residues and have more flavor and low-fat content, the consumers are reluctant, and biotechnology companies should find compelling arguments to sell GM foods. In many countries, fruits are not considered staple foods. Thus, the development of new GM fruit crop varieties with a range of novel traits has gained consumer acceptance mainly as luxury products.

The recent development of high-throughput sequencing technologies provided information about genomes and valuable qualities in fruit crops. Moreover, genomes of many plant species have been sequenced (Bolger et al., 2014), which contributed to the deciphering of molecular mechanisms of physiological processes, including flowering, juvenility, ripening, and shelf life.

In addition to the social hurdles, genetic transformation of fruit crops has some technical drawbacks such as multiple restriction sites in the genome ensured by endonucleases, low insertion efficiency of engineered constructs, low efficiency of correct insertion into the chromosomal target site, time-consuming, laborious selection/screening strategies, and the potential adverse mutagenic effects (Capecchi, 2005). RNA interference (RNAi) was developed as a valuable gene knockdown technology to overcome some drawbacks of existing methods. Unfortunately, it also showed disadvantages like incomplete and transient gene knockdown and unpredictable off-target effects (McManus and Sharp, 2002).

The last decade has been marked by the emergence of a new approach that enables direct manipulation of any gene in various cell types and organisms. Known as "genome-editing," the technology is based on the use of engineered nucleases composed of sequence-specific DNA-binding domains fused to a nonspecific DNA cleavage module (Urnov et al., 2010; Carrol, 2011). These engineered nucleases enable efficient and precise genetic modifications

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by double-strand breaks (DSBs) in the targeted DNA.

As effective technologies in genetic engineering, genome editing techniques are used for insertion, substitution, removal, or disruption of DNA sequences. This review focuses on the most recent achievements in genome-editing technologies and discusses their applications in fruit crops for economic and nutritional advantages.

2. Mechanisms of genome editing

The main difference between current technologies and conventional breeding practices based solely on recombination and, to a small extent, on genetic recombination is that genomic editing achieves strict specificity towards the intended DNA target. At first, the artificially engineered nuclease enzymes called molecular scissors (Punwar et al., 2014) such as zinc-finger nucleases (ZFNs) (Carroll, 2011) and the transcription activator-like effector nucleases (TALENs) (Mahfouz et al., 2011; Li et al., 2012) that are capable of generating desired genomic modifications (Shan et al., 2013) have been deployed. The most recent system developed for genome-editing is the clustered regulatory interspaced short palindromic repeat (CRISPR)/Associated Protein-9 Nuclease (CRISPR/Cas9), based on RNA-guided DNA endonucleases that allow precise modification, insertion, or replacement of genes at specific sites. CRISPR/Cas technology is considered the most efficient, cheap, and user-friendly among the genome editing tools (Kaul et al., 2020). All these new technologies for crop improvement allow the modification of any kind of genomic trait (Jaganathan et al., 2018).

2.1. Zinc-finger nucleases (ZFNs)

ZFNs are engineered nucleases consisting of the DNA-binding zinc-finger (ZF) motifs and the FokI endonuclease (Figure 1a). The recognition target sites consist of two ZF binding sites that flank up to 5-7 bp spacer sequence recognized by the FokI endonuclease cleavage domain. Each ZF recognizes short sequences (3 bp), but it is possible to increase the recognition sequence up to 20 bp by combining 6-8 ZF with specific recognition sites. Three to four ZF domains compile together a ZFN in which each ZF domain contains approximately 30 amino acid residues organized in ββα motifs (Petolino, 2015). The editing method based on ZFN is based on the protein dimer composed of two DNA binding proteins (each heaving 3-6 ZF) with the catalytic domain of the FokI endonuclease, which cleaves the double-stranded DNA. The two ZF proteins recognize two specific DNA sequences and bring the two FokI domains closer together. The dimerization of FokI is mandatory for nuclease activity and is followed by increased specificity of DNA recognition. Moreover, FokI nucleases have been modified to function only as heterodimers to enhance the recognition specificity (Urnov et al., 2010). Due to their efficiency, minimal nontarget effects, and high specificity, ZFNs are valuable genome-editing tools, being suitable for editing diverse crops of interest (Kamburova et al., 2017).

2.2. Transcription activator-like effector nucleases (TALENs)

These artificial nucleases contain a binding domain (TALE) that consists of a series of approximately 32-34 amino acid residue repeats and a FokI DNA cleavage domain (Figure 1b). Each repeat is conserved, except the amino acid in positions 12 and 13, variable di-residues (RVDs), which establish the DNA binding site of TALE. These binding domains can be designed to bind any DNA sequence. The origin of the binding domain is in TAL effectors from *Xanthomonas spp.* TALENs can create DSBs at the target site that can be repaired by NHEJ, introducing small insertions or deletions (Pérez-Quitero et al., 2013). TALENs also require dimerization of the FokI domain, which is similar to ZFNs, but, conversely to a ZF domain, which identifies a DNA triplet, a TALE protein only recognizes a single bp (Dheer et al., 2020).

2.3. Clustered regularly interspaced short palindromic repeats/associated protein (CRISPR/Cas)

The newest technology of genome editing consists of a specialized RNA sequence and a Cas9 enzyme working as molecular scissors to cleave the DNA (Figure 1c). The CRISPR/Cas system confers immunity against viral DNA and RNA in bacteria and archaea, and the mechanism is described in detail by several authors (Charpentier et al., 2015; Rath et al., 2015; Jiang and Doudna, 2017).

The CRISPR/Cas system used for genome editing is comprised of chimeric RNA molecules crRNA (CRISPR-associated RNA) and tracrRNA (transcribed trans-activating crRNA) that are transcribed in a single guide RNA (sgRNA), and the Cas9 protein (Jinek et al., 2012). Different sequences in the genome can be targeted by designed sequences of gRNA (Jiang and Doudna, 2017). The break is repaired by two mechanisms: nonhomologous end-joining (NHEJ) and homology-directed repair (HDR). NHEJ, also known as the "nonhomologous" mechanism, uses different enzymes that join break ends without the need for a homologous template. In most cases, the NHEJ pathway causes indel mutations (insertions/deletions), which often cause the loss of gene function. In contrast, the HDR mechanism requires a homologous sequence for reparation by recombination at the breakpoint (Zhu et al., 2017). The mechanisms of DSB repair are shown in Figure 2.

The main advantage of the system is the specificity that relies on the complementarity between the gRNA and the target sequence. However, off-target activity may occur in some loci with partial complementarity to the gRNA (Sledzinski et al., 2020). NHEJ repair mechanism induces reparations by direct ligation of the broken

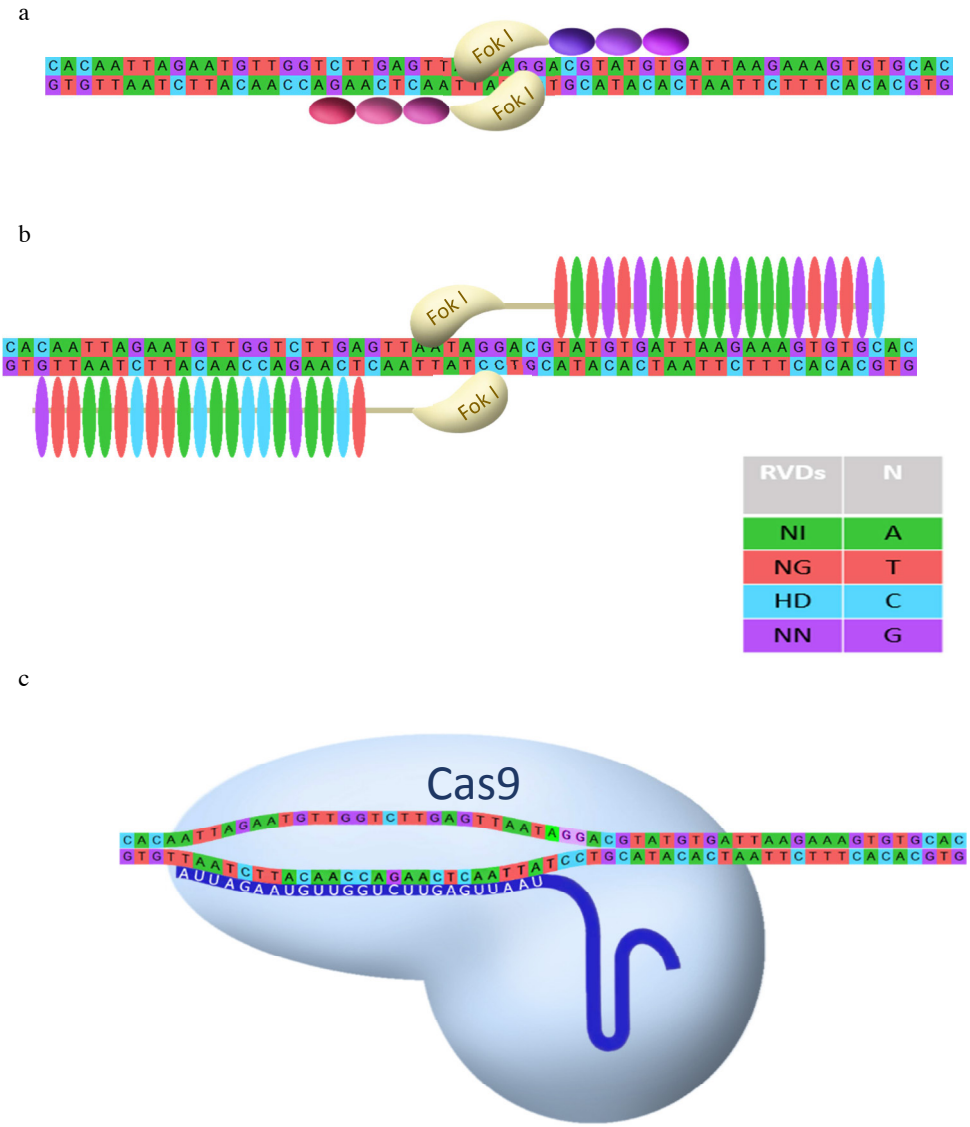

Figure 1. Comparison between genome-editing tools in plants. a) ZFN in complex with target DNA. Each ZF consists of approximately 30 amino acids and contacts 3 pb in the major groove of DNA. b) TALEN in complex with target DNA. Each TALE repeats contain 33–35 amino acids that recognize a single bp via two hypervariable residues (repeat-variable diresidues: RVDs). RVD compositions are indicated. c) CRISPR/Cas9 in complex with target DNA. The Cas9 protein is guided by crRNA, which contains a 20-nt sequence determining target specificity, to cleave the target DNA. The presence of PAM, an NGG sequence directly downstream from the target DNA, is a prerequisite for DNA cleavage by Cas9.

ends, leading to insertions, deletions, or substitutions at the DSB site. HDR acts in the presence of a donor DNA sequence and corrects the existing modifications or inserts new sequences of interest (Puchta, 2017). The integrated transgene is functional in the plant genome and can be expressed (Jaganathan et al., 2018).

Different types of CRISPR/Cas systems have distinct molecular mechanisms for DNA targeting (Makarova et al., 2011; Chylinski et al., 2014). Bioinformatic analysis of different Cas proteins showed that Cas9 was previously identified as OG3513, Csx12, Cas5, or Csn1 and acted as a multifunctional protein containing two nuclease domains: RuvC, which is the catalytic site (Makarova et al., 2006) and Nuc, which is responsible for the regulation of the substrate DNA (Li et al., 2018c). The CRISPR/Cas9 system was used both to manipulate cells in living organisms and in cell cultures (Lemmon et al., 2018).

3. New tools for plant genome editing

Based on the high diversity of Cas proteins, the CRISPR/Cas systems have been classified into two classes and six types with multiple subtypes. Types I, III, and IV belong

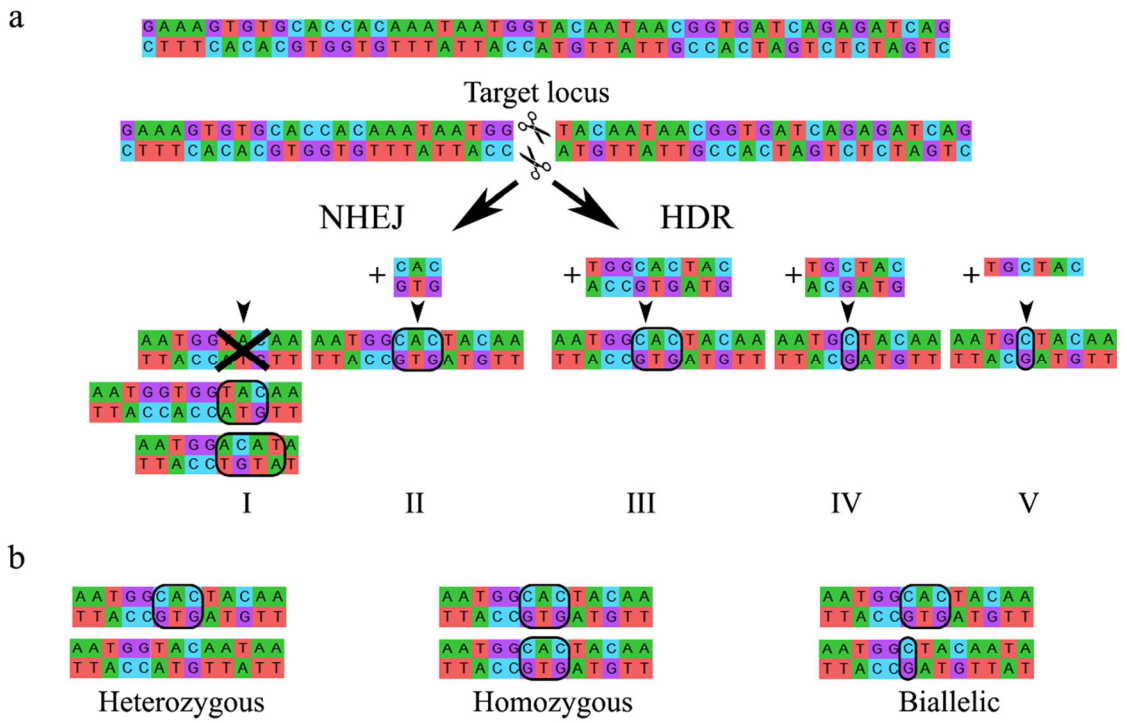

Figure 2. Genome editing at target locus. a) Site-specific nucleases introduce double-strand breaks where gene modification is acquired by two repair pathways. Nonhomologous end joining (NHEJ) generates gene knockout (I) by deletion, insertion or inversion, in the absence of donor DNA, and gene insertion (II) when integrates donor DNA by compatible ends. Homology dependent repair (HDR) results in gene insertion (III) when integrates donor DNA based on homology regions and gene correction when assimilates a small change provided as either double-stranded (IV) or single-stranded DNA (V). b). Gene modifications in diploid plants. Adapted from Zhu et al., 2017.

to class 1, while types II, V and VI belong to class 2 (Koonin and Makarova, 2019). Discovered in *Prevotella* and *Francisella*, class II CRISPR has a type V effector (Cpf1 or Cas12a) (Malzahn et al., 2019) that can be designed to cleave specific DNA sequences (Ma et al., 2018). It targets T-rich motifs and does not require the tracrRNA to form a mature crRNA. Cpf1 represents a valuable alternative to Cas9 due to its capacity to induce DSBs and to process RNA and DNA (Safari et al., 2019). As a valuable nuclease, Cpf1 generates staggered ends (Ding et al., 2018), enabling genome manipulation (Li et al., 2018b). Cfp1 allows precise gene knockout (Gaudelli et al., 2018), insertion or deletion of DNA sequences, base substitutions, and development of "prime editing" that can insert new sequences into a DNA site, expanding the applications of genome editing (Anzalone et al., 2019).

Another CRISPR/Cas system was recently identified in bacteriophages, suggesting that the CasΦ enzyme could also be used for genome editing in plants. It shows higher target recognition capabilities compared with Cas9 and Cas12a and has half of their molecular weight. Moreover, the CasΦ enzyme uses the same active site for processing mature crRNA and cleavage of foreign nucleic acids (Pausch et al., 2020).

4. Comparison between editing tools

Until 2013, the most used genome editing tools were ZFNs (Kim et al., 1996) and TALENs (Christian et al., 2010). Both function as dimers and have a DNA-binding domain that gives the sequence specificity. Despite the elaborate design of different ZFNs (Sander et al., 2011), many performant ZFNs were developed (Ramirez et al., 2008). TALENs design is easier, but homologous recombination in vivo may occur due to the highly repetitive sequences (Holkers et al., 2013).

The most valuable CRISPR-Cas technology is based on the CRISPR type II from *Streptococcus pyogenes* (Jinek et al., 2012) due to its simplicity, efficiency, and versatility. This system consists of a monomeric protein Cas9 and a chimeric gRNA of 20 nucleotides that could recognize and modify different targets.

ZFNs can theoretically target any DNA sequence, but in practice, the choice of targets is limited. Nevertheless, functional ZFNs can be prepared using available databases (Kim et al., 2009). TALENs targets are limited by the need for a thymidine residue at the first position (Doyle et al., 2012). There are also many designed TALENs available, but unfortunately, not all of them work efficiently in vivo, and thus, they must be validated experimentally (Hwang et al., 2013).

In contrast, the CRISPR/Cas9 system needs only the presence of the PAM (protospacer adjacent motif) downstream of the target sequence and the proper gRNA sequences to avoid off-target cleavage due to imperfectly matching spacer sequences. Specific gRNA sequences were designed by in silico analysis of nuclear genome sequences from important crops (Xie et al., 2014). A comprehensive comparison between the genome editing system tools was provided by Bortesi and Fischer (2015).

It is known that the CRISPR/Cas9 system could achieve high mutation rates in plants, in some instances higher than those obtained with ZFNs and TALENs (Lozano-Juste and Cutler, 2014), and the target efficiency is higher with CRISPR/Cas9 than with TALENs (Liang et al., 2014). On the other hand, CRISPR/Cas9 activity is dependent on the delivery methods and the cell type (Li et al., 2013). Generally, gRNAs and Cas9 were incorporated into plant cells by different methods: *Agrobacterium*-based transformation of T-DNA regions, viral vectors, PEG-mediated transformation (protoplasts), biolistic approach (callus), nanoparticles (Kaul et al., 2020). The most popular methods are transformation mediated by *Agrobacterium* (Ali et al., 2015), but the transformation with geminiviral DNA replicons enhanced gene targeting efficiencies by one to two-fold, in contrast to traditional *Agrobacterium* transformation. Nanoparticle-mediated delivery systems have been successfully adopted in plants, decreasing the frequency of unwanted changes (Kaul et al., 2020).

Another difference between ZFNs, TALENs, and CRISPR/Cas9 systems is that CRISPR/Cas9 can cleave methylated DNA in human cells (Hsu et al., 2013). Even if this aspect was not studied in plants, it could be assumed as possible. Due to the high percentage of methylated CpG/CpNpG sites in plants (Vanyushin and Ashapkin, 2011), the CRISPR/Cas9 technology is suitable for monocots that have high genomic GC content, such as rice (Miao et al., 2013). Conventional TALENs cannot cleave DNA sequences containing 5-methylcytosine, but the repeat that recognizes cytosine can be replaced with a repeat that recognizes thymidine (Valton et al., 2012).

The main practical advantage of CRISPR/Cas9 compared to ZFNs and TALENs is the ease of multiplexing by simultaneously targeting multiple sites (Li et al., 2013). Multiplexing could be used to induce multiple deletions or inversions in different sites on the same chromosome (Li et al., 2013; Zhou et al., 2014), requiring only the monomeric Cas9 protein and any number of different sequence-specific gRNAs. In contrast, multiplex editing with ZFNs or TALENs requires different dimeric proteins, specific for each target site.

Another advantage of the CRISPR/Cas system is that the research community provides access to plasmids (nonprofit repository-Addgene) and web tools for selecting gRNA sequences (http://cbi.hzau.edu.cn/cgi-bin/CRISPR, http://www.genome.arizona.edu/crispr/, http://www.rgenome.net/cas-offinder, http://www.e-crisp.org/E-CRISP/index.html) that contributed to the rapid development of various applications (Bortesi and Fischer, 2015).

Despite the many advantages of the CRISPR/Cas9 technology, one of its shortcomings is the occurrence of off-target mutations (Cong et al., 2013; Hsu et al., 2013), but it was shown that they are influenced by numerous parameters, such as the target site recognition and designing of sgRNAs, the frequency of HDR-mediated repair and inactivation of Cas9 by anti-CRISPR proteins (Kaul et al., 2020). Different algorithms allow computer programs to precisely identify unique target sequences and possible off-target sites in the genomes of targeted organisms (Cong et al., 2013; Gerashchenkov et al., 2020). The sgRNA-Cas9 complex can tolerate several mismatches in the PAM-distal region, but mutation of the bases at positions 8-13 at the PAM-proximal end of the spacer along with the first base at the 5' end are intolerable for DNA cleavage (Jinek et al., 2012; Cong et al., 2013; Hsu et al., 2013; Anderson et al., 2015; Doench et al., 2016). It was proved that the sgRNAs should be designed with high precision to reduce the off-target effects. Strategies such as the addition of two guanidine residues at the 5′ end of the gRNA (Cho et al., 2014) or a truncated chimeric single guide RNAs (tru-sgRNAs) of 17 nucleotides were shown to reduce off-target mutations (Fu et al., 2014). Thus, the length, mismatches, and GC content of gRNAs are essential factors that regulate off-target effects (Kaul et al., 2020). Besides, anti-CRISPR (Acr) proteins inactivate the CRISPR's molecular scissors. More than 50 Acr proteins have been characterized, but the essential functions of these proteins remain ambiguous (Dolgin, 2019).

5. Application of genome editing in fruit crops

Recent data showed that genome editing tools have significant effects on plant biotechnology in general and on fruit crops as well. These technologies allow the manipulation of several genes without genetic transformation, and thus, such plants might be considered nontransgenic plants. Editing tools offer the opportunity to develop improved fruit crops that could be accepted even in countries where genetically modified crops are restricted. Moreover, genome-editing technologies provide high-quality products that are almost impossible to be produced by using traditional breeding methods (Hussain et al., 2018).

ZFNs and TALENs were used in *Arabidopsis* and tobacco plants as model organisms (Wright et al., 2005; Zhang et al., 2010) and then in different crops (Cantos et al., 2014; Shan et al., 2015; Butler et al., 2016), but

their employment in fruit crops is limited. Most of the applications on fruit crops such as apple, banana, cacao, citrus, grape, kiwifruit, and pear were developed with CRISPR/Cas9 technology (Erpen-Dalla Corte et al., 2019). Although the CRISPR/Cas9 technology was used in different crops for applications such as NHEJ-mediated gene knockout, HDR-mediated gene replacement, gene targeting and rearranging, base editing, prime editing, single-cell genome engineering, germline engineering, genome editing for a single trait, multiplexing of genes for trait stacking, molecular farming (genetic alteration of agricultural merchandise, manufacture of biopharmaceuticals), plant domestication, metabolic engineering, research in fruit crops is rather limited (Kaul et al., 2020). Nevertheless, several experiments were performed to optimize the CRISPR/Cas9 technology for fruit cultivars (Ahmar et al., 2020), and different physiological mechanisms were targeted. Among them, chlorophyll and carotenoid production (Qin et al., 2007), juvenile phase and flowering period (Nishikawa, 2013; Varkonyi-Gasic et al., 2019), fruit ripening (Parkhi et al., 2018), or resistance to diseases and pests were considered. The CRISPR/Cas9 editing technology applications for gene targeting in various fruit crops are presented in Table.

5.1. Manipulation of the carotenoid biosynthesis pathway

Optimization of the CRISPR/Cas9 technique was used by targeting the phytoene desaturase (*PDS*) gene encoding an enzyme involved in carotenoid biosynthesis. Mutations of this gene influence chlorophyll and carotenoid synthesis and the induction of the albino phenotype (Qin et al., 2007). In banana, the complete albino and different variegated phenotypes were obtained by targeting the conserved region of two *PDS* genes (Kaur et al., 2018). Clear albino phenotype by editing the *PDS* gene was also obtained in strawberry cultivars (Wilson et al., 2019). Similar results were obtained in Carrizo citrange (Zhang et al., 2017), apple (Nishitani et al., 2016; Charrier et al., 2019), grapes (Nakajima et al., 2017), kiwifruit (Wang et al., 2018b), pear (Charrier et al., 2019), watermelon (Tian et al., 2017), and kumquat (Zhu et al., 2019).

5.2. Manipulation of juvenile phase and flowering period

Many perennial fruit crops show a long juvenile period followed by an extended and variable nonflowering period. A long juvenile period is a significant disadvantage for developing new cultivars through traditional breeding (Nishikawa, 2013). Juvenility is induced and maintained by a high level of terminal flowering (TFL) protein that inhibits the expression of flowering proteins, such as the Flowering Locus T (FT), Leafy (LFY), and Apetala1 (AP1) (Pillitteri et al., 2004). By CRISPR/Cas9 technology, the *TFL1* gene was targeted by different gRNAs in apple and pear (Charrier et al., 2019). Early flowering was observed in 93% of the transgenic apple plants targeted in the *MdTFL1.1* gene, despite the single mismatch between the gRNA1 and the target. In pear, a lower rate of the mutated phenotype (9%) was observed in edited plants targeted in the *PcTFL1.1* gene, most probably because both *PcTFL1.1* and *PcTFL1.2* genes should be edited to release the floral repression (Charrier et al., 2019). CRISPR/Cas9 system was also used in kiwifruit to insert mutations in the *AcCEN4* and *AcCEN* genes, which transformed the perennial plants having a long juvenile period into plants with rapid flowering and fruit development (Varkonyi-Gasic et al., 2019).

5.3. Fruit quality

CRISPR/Cas9 technology was used to improve fruit quality by targeted mutagenesis of genes encoding the ripening inhibitor (*RIN*), lycopene desaturase (*LD*), pectate lyase (*PL*), SlMYB12 and CLAVATA3 transcription factors (*CLV3*) that affect fruit ripening, fruit bioactive compounds, fruit texture, fruit coloration, and fruit size (Xu et al., 2020). Inhibition of ethylene biosynthesis by gene editing also plays an essential role in the fruit-ripening process (Wang et al., 2018b). In tomato, early fruit ripening was obtained by editing several genes, such as those responsible for transcription factors Apetala2a (*AP2a*), Non-Ripening (*NOR*), and Fruitfull (*FUL1*/*TDR4* and *FUL2*/*MBP7*) (Parkhi et al., 2018). It was further shown that ethylene production was reduced in *RIN*-deficient fruits obtained by CRISPR/Cas9 technology, and the synthesis of volatile substances and carotenoids was reduced as well (Li et al., 2020).

Interesting results were obtained by editing the *NOR* gene with CRISPR/Cas9 technology. It was observed that the spontaneous *NOR* mutant fruits were green, while the edited *NOR* mutant exhibited earlier ripening and orange phenotypes due to CRISPR/Cas9-mediated mutagenesis that was followed by delayed or partial immature phenotypes (Wang et al., 2020). Moreover, fruit ripening is also associated with epigenetic modification. The DNA cytosine methylation in the plant genome regulates gene expression and stabilize the genome in response to different stress factors (Chen et al., 2018). *SlDML2* knockout mutants were obtained using the CRISPR/Cas9 system, which inhibits fruit ripening (Zhou et al., 2019). Degradation of plant cell walls leading to softening and even death of plant tissues was decreased by editing the Pectate lyases (*PL*) gene (Uluisik et al., 2016).

Many natural compounds from fresh fruits such as lycopene, carotenoids, anthocyanins, and gamma-aminobutyric acid (GABA) are biologically active, having antiinflammatory, anticancer, antioxidation, and other physiological effects. Therefore, the accumulation of bioactive substances has been the main focus of numerous studies (Amish et al., 2015). As lycopene synthesis decrease during the fruit ripening, due to the conversion to

Table. Improvement in fruit crops by CRIPSR-Cas9 technology.

Technology	Fruit crop	Target gene	Trait Improvement	References
CRISPR/Cas9	Apple	*PDS*; *TFL1*; *DIPM-1*, *DIPM-2*, *DIPM-4*; *IdnDH*	Albino phenotype; early flowering; fire blight disease resistance; biosynthesis of tartaric acid	Nishitani et al., 2016; Malnoy et al., 2016; Charrier et al., 2019; Osakabe et al., 2018
CRISPR/Cas9	Banana	*PDS*; *MaGA20ox2*; *eBSV*	Albino and variegated phenotype; semi-dwarfing size; control of virus pathogenesis	Kaur et al., 2018; Shao et al., 2019; Tripathi et al., 2019
CRISPR/Cas9	Cacao	*TcNPR3*	*Phytophthora tropicalis* resistance	Fister et al., 2018
CRISPR/Cas9	Citrus (Carrizo Citrange)	*PDS*	Albino phenotypes	Zhang et al., 2017
CRISPR/Cas9	Citrus (Grapefruit)	*CsLOB1*; *PDS*	Canker disease resistance; albino phenotype	Jia et al., 2017a, b
CRISPR/Cas9	Citrus (Kumquat)	*PDS*	Albino phenotypes	Zhu et al., 2019
CRISPR/Cas9	Citrus (Sweet Orange)	*CsLOB1*; *CsWRKY22*; *DMR6*	Canker disease Resistance; canker disease resistance; Huanglongbing resistance	Peng et al., 2017; Wang et al., 2019b; Zhang et al., 2018
CRISPR/Cas9	Grape	*VvPDS*, *MLO-7*; *PDS*; *IdnDH*; L-idonate dehydrogenase gene (*IdnDH*); *VvWRKY52*	Albino phenotype; powdery mildew resistance; albino phenotype; biosynthesis of tartaric acid; tartaric acid content; *Botrytis cinerea* resistance	Malnoy et al., 2016; Nakajima et al., 2017; Ren et al., 2019; Osakabe et al., 2018; Ren et al., 2018; Wang et al., 2018a
CRISPR/Cas9	Groundcherry	*ClV1*	Fruit size	Lemmon et al., (2018)
CRISPR/Cas9	Kiwifruit	*PDS*	Albino phenotype	Wang et al., 2018b
CRISPR/Cas9	Pear	*PDS*; *TFL1*	Albino phenotype; early flowering	Nishitani et al., 2016; Charrier et al., 2019
CRISPR/Cas9	Strawberry	Apetala3, *FaTM6;* (*AP3*); Auxin Response Factor 8 (*FvARF8*) and Auxin biosynthesis gene (*FveTAA1,* FveARF8); *PDS; MLO; FvMYB10, FvCHS*	Flowering control and anther development; auxin biosynthesis; albino phenotypes; resistance to powdery mildew; anthocyanin biosynthesis; anther development	Martín-Pizarro et al., 2019; Zhou et al., 2018; Wilson et al., 2019; Jiwan et al., 2013; Xing et al., 2018; Martin-Pizzaro et al., 2019
CRISPR/Cas9	Watermelon	*ClPDS, PDS*	Albino phenotype, carotenoid biosynthesis	Tian et al., 2017; Wang et al., (2019c)

β-carotene and α-carotene, the conversion of lycopene was reduced by knocking out the *SGR1*, *LCY-E*, *BLC*, *LCY-B1*, and *LCY-B2* genes. As a consequence of CRISPR/Cas9 editing, the lycopene content in tomato fruits increased about 5.1 times (Li et al., 2018d). GABA content in fruits was also significantly enhanced by editing five genes (*GABA-TP1*, *GABA-TP2*, *GABA-TP3*, *SSADH*, and *CAT9*) in the tomato genome (Li et al., 2018a).

The CRISPR/Cas9 technology also has a great potential to change the fruit coloration. Editing the genes involved in pigment synthesis may also affect the production of bioactive compounds. The mutation of the *SlMYB12* gene has produced pink tomato fruits (Ballester et al., 2010), while the mutation of the *ant1* gene enhanced the accumulation of anthocyanins and produced purple tomatoes (Čermák et al., 2015). Several silent mutations of polygalacturonase 2a (*PG2a*) and β-galactanase (*TBG4*) genes encoding pectin degrading enzymes that usually affect fruit ripening were associated with changes in the fruit color (Wang et al., 2019a).

CRISPR/Cas9 system was also used to induce parthenocarpy in fruits by editing the genes involved in seed formation. The parthenocarpy is a high demand in fruits such as citrus cultivars, custard apple, grapes, peach, watermelon, bitter gourd (Ueta et al., 2017; Ahmar et al., 2020).

5.4. Resistance to pests and diseases

Numerous pests and diseases are widely present in fruit crops, affecting their growth and development and being responsible for economic loss. Thus, the development of resistant cultivars could be the alternative to solve these problems. Genome editing by CRISPR/Cas9 system could induce resistance to biotic stresses that greatly impact their production. *Xanthomonas citri* ssp. *Citri* (Xcc) produces citrus canker, and the key gene involved in this disease is *Citrus sinensis* Lateral Organ Boundaries (*CsLOB1*) (Hu et al., 2014). *CsLOB1* induction is promoted by Xcc pathogenicity factor PthA4, which binds to a specific element in the promoter region. The infection on the edited plants was reduced by using the CRISPR/Cas9 system to modify the PthA4 binding element in the promoter of the *CsLOB1* gene (Jia et al., 2016). Several mutations were observed in the promoter of both *CsLOB1* alleles generated plants, which were resistant to this disease. Similar experiments have been performed using five CRISPR/Cas9 constructs to modify the PthA4 binding element in the *CsLOB1* gene promoter of Wanjinchen orange. Different mutated lines with enhanced resistance to citrus canker were obtained, but deletion of the PthA4 binding element from both *CsLOB1* alleles was followed by a significant tolerance to infection (Peng et al., 2017).

In grapes, the knockout of the *WRKY52* gene by mutations in the first exon of the gene enhanced the resistance to *Botrytis cinerea* (Wang et al., 2018a). It was observed that the biallelic mutants were more resistant than the monoallelic ones.

Strawberry resistance to powdery mildew was obtained by editing the mildew-resistance locus (MLO) characterized in detail in barley. Due to the phylogenetically conservative nature of this locus, successful results have been obtained in strawberries as well (Jiwan et al., 2013).

In cacao, the CRISPR/Cas9 system was used to target the Non-Expressor of Pathogenesis-Related Genes3 (*NPR3*), which encodes a repressor protein involved in the defense mechanisms (Dorantes-Acosta et al., 2012). Consequently, 27% of the *NPR3* copies were deleted, and the resistance to *Phytophthora tropicalis* was achieved in the edited tissues. Future genome editing events of somatic embryos were performed in *Theobroma cacao* (Fister et al., 2018) and *Citrus* (Dutt et al., 2020) to test the effectiveness of the CRISPR/Cas9 system.

Banana streak virus (BSV) massively affects banana cultures and production. Several mutations in the BSV sequences integrated into the genome of *Gonja manjaya* cultivar were performed using the CRISPR/Cas9 system. It was observed that 75% of edited plants remained asymptomatic under water stress conditions (Tripathi et al., 2019).

5.4. Gibberellin biosynthesis and generation of dwarf cultivars

Dwarf cultivars with high productivity are preferable for many fruit crops, due to dense planting and low water and fertilizer requirements. Thus, desired mutations induced in the *MaGA20ox2* gene were correlated with dwarfism in banana (Chen et al., 2016). After genome editing, seven mutant lines with semi-dwarf phenotype were obtained, all of them having significant changes in gibberellin levels in leaves and roots as well (Shao et al., 2019). The CRISPR/Cas9 technology was also used in tomatoes to target mutations of the *PROCERA* gene encoding a DELLA protein, in order to select several loss-of-function mutations and a dominant dwarf mutation that carries a deletion of one amino acid in the DELLA domain. Heterozygotes display an intermediate phenotype at the seedling stage, but, regarding the dimorphism, they are the same as the homozygotes (Tomlinson et al., 2019).

6. Conclusions and prospects

Recent development of genome-editing technologies has greatly revolutionized the plant biotechnology. Even if ZFN and TALEN nucleases have been successfully used in various plant species, they were less applied for genome editing of fruit crops. The simpler and more efficient CRISPR/Cas9 system is the most powerful genome editing approach ever created for improving important breeding targets, such as the yield, quality, herbicide resistance, and biotic/abiotic stress tolerance. Its flexibility for targeting practically any DNA sequence with the utmost accuracy and mutation efficiency was already proven. Given its multiplexing capacity, the CRISPR/Cas9 system is a valuable tool for understanding and improving the function of the target genes. Moreover, genome editing does not involve transgenesis; thus, the resulting plants are not considered GMOs and are not subject to legal restrictions. Genome editing primarily by CRISPR/Cas9 and CRISPR/Cpf1 systems would be the most promising technology for developing new smart fruit crops with improved quality and yield.

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