Genetic Engineering Techniques in Fruit Science

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Abstract— *Today, fruit science have well been established in world trade networks and sophisticated cultural and postharvest technologies that allow fruits to be enjoyed throughout much of the year, instead of mere weeks per year like our ancestors experienced. Especially modern biotechnological methods including genetic engineering technologies have been taken part in breeding strategies of fruit crops. Several biotechnological methods can be applied to plant to have better ones in the process of fruit breeding. Genetic engineering is a powerful tool for plant improvement and has the potential to allow the integration of desirable characteristics into existing genomes. Transformation technology developed a path to transfer important genes into plant genome for enhancing resistance against fungal, viral pathogens, other pests, drought, and salinity as well as silencing undesirable genes and improvement in nutrient acquisition. Different gene transfer techniques could be employed for fruit species. As well as direct and indirect transformation, modern genome editing methods recently have been used in plant science. In this review, we illustrated how to use these technologies in fruit science.*

Keywords— Agrocbacterium, direct gene transfer, CRISPR-CAS9, TALENs, ZFN.

I. INTRODUCTION

Horticulture is a branch of agriculture concerned with the cultivation and breeding of fruits, vegetables, grapevine and ornamentals. The horticultural market is gradually increasing because they have many benefits human health and the environment. Among the horticultural plants; fruits and vegetables are the primary source of essential minerals and vitamins. Fruits will continue to expand due to demand of better tasting, more varied and nutritious. Breeding of new varieties becomes day after day even more importance especially for growing population. Many different breeding strategies and techniques have been available for fruit crops. In this article, we will address general breeding strategies and biotechnological methods in fruit breeding, general principles and methodologies of genetic engineering techniques in fruit crops and discuss some issues related to the procedures and applications of these methodologies in practical breeding.

II. FRUIT BREEDING

Plant breeding is concerned with the development of cultivars to fit specific environmental and production practices, and to fulfill requirements for food, feed and fibre. Techniques from genetics, mathematics, chemistry, pathology, cytology and more recently, bioinformatics, are routinely used [1]. Plant breeding in the modern sense can be defined as purposeful genetic improvement. Yet genetic improvement of crop plants has an ancient tradition and in fact the greatest feat of plant breeding dates to Neolithic Revolution when our crop plants were domesticated starting about 10,000 years ago. Progress was achieved by selection of elite clones or lines of useful plants, mass growing of elite clones, followed by selection from naturally occurring seedlings. Most varieties of fruit crops have been developed by plant breeding. The principles of traditional plant breeding, in which desirable traits are combined from different plants first established by Gregor Mendel in the 19th century. These techniques resulted in the introduction of hybrids that led the way to green revolution and improving the efficiency of agriculture production throughout the world [2]. But, according to Mendel's principles recombination of DNA in offspring is random. Traditional plant breeding often took decades and frequently yielded crop varieties with unforeseen and undesirable properties. Today, conventional plant breeding remains inherently random and slow, also constrained by the availability of desirable traits in closely related plant species [3].

Plant breeding has resulted in numerous improved food, feed, ornamental, and industrial crop varieties and traditional breeding based on crossing and selection remains an important activity for crop improvement. Although the efficiency of

crossing and selection has been improved by using marker-assisted selection, it faces limitations in crops with complex genetics (e.g., due to polyploidy, heterozygosity, or self-incompatibility) or a long generation time (e.g., fruit trees). In addition, the search for useful genetic variation is often laborious, and introgressing such variation from wild relatives into the cultivated germplasm through crossing can be tedious [4]. The requirement of fruits is increasing proportionally with the increasing population in the countries. How do we keep fruit production on par with the burgeoning population? Although conventional plant breeding techniques have made considerable progress in the development of improved varieties, they have not been able to keep pace with the increasing demand for vegetables and fruits in the developing countries. Therefore an immediate need is felt to integrate biotechnology to speed up the crop improvement programs.

III. BIOTECHNOLOGY IN FRUIT CROPS

Many different biotechnological methods can be applied to plant to have better ones in the process of plant breeding. One of the widest applications of biotechnology has been in the area of plant tissue culture in fruit crops. Today plant tissue culture applications encompass much more than clonal propagation and micropropagation. The range of routine technologies has expanded to include somatic embryogenesis, somatic hybridization, and virus elimination as well as the application of bioreactors to mass propagation [5].

Molecular marker technology is another area of the plant biotechnology. Molecular markers are known to be important tools for plant breeding and have developed into many systems based on different polymorphism-detecting techniques or methods, such as RFLP, AFLP, RAPD, SRAP SSR, ISSR SNP, SSCP etc [6].

Transformation which is main subject of this review is one of the interesting areas of biotechnology because characteristics can be added with minimal alteration of the target plants genome, and gene transfer technology need to be utilized in this important crop species [7, 8, 9, 10]. In order to obtain genetic transformation with a reproducible methodology several requirements should be considered [11, 12, 13, 14, 15]:

- Low costs and easy procedures that lead to large numbers of transformations per event, operator safety avoiding dangerous procedures or substances,
- Technical simplicity involving the minimum manipulations,
- Capability to introduce in a stable way the desired DNA without vector sequences which are not required for gene
- integration or expression,
- Small number of genetic copies introduced into each cell, and facility to regenerate transgenic plants from single transformed cells.

Common methods for genetic transformation are usually divided into indirect or direct transformation. Biological methods using bacteria are referred to as indirect, while direct methods are physical; that is, based on the penetration of the cellular wall [15]. On the other hand recently modern techniques were developed. In the present article, we summarized genetic engineering techniques and some examples were given from fruit crops.

IV. GENETIC ENGINEERING TECHNIQUES

Genetic engineering is a powerful tool for plant improvement and has the potential to allow the integration of desirable characteristics into existing genomes [16]. Transformation technology developed a path to transfer important genes into plant genome for enhancing resistance against fungal, viral pathogens, other pests, drought, and salinity as well as silencing undesirable genes and improvement in nutrient acquisition [17].

GM technology has some advantages and disadvantages. GM technology used the less time it will take to clean up the genetic background, i.e., reduce the number of backcross generations; the ideal scenario being targeted gene editing with no alteration of the genetic background (no off-target effects). Thus GM breeding has the potential to be very fast. A major disadvantage of GM breeding is that the target gene must be known and sequenced. Other disadvantages of GM breeding are: it requires specialized laboratories and is expensive, though cheaper, easier options are being developed [18].

Genetic transformation methods are usually divided into two groups (1) indirect transformation, (2) direct transformation methods.

4.1 Direct Gene Transformation Methods

The term direct transfer of DNA is used when the foreign DNA is directly introduced into the plant genome. Direct DNA transfer methods rely on the delivery of naked DNA into the plant cells. Majority of the direct DNA transfer methods are known to be simple and more effective. Several transgenic plants have been developed by these approaches. Direct gene transfer technologies have an important disadvantage; because of the frequency of transgene rearrangements is high, higher transgene copy number, and high frequencies of gene silencing could be observed.

4.1.1 Particle Bombardment (Biolistic)

In biolistics technology, DNA is coated onto gold or tungsten micro-particles and bombarded at high velocity in a stream of helium into intact cells or tissues [19, 20, 21, 22]. Biolistic process is subdivided into two stages: (i) coating metal particles (microprojectiles) with nucleic acid, and (ii) accelerating the coated microprojectiles to velocities appropriate for penetration of target cells or tissues without excessive disruption of biological integrity [20]. Biolistic transformation is simple and safe since it does not require the manipulation of genetically modified organisms such as *Agrobacterium* [23, 24]. Moreover, it allows for the integration of larger transgenes [25], the co-transformation of multiple constructs [26, 27]. One disadvantage of biolistics is the cotransfer of large fragments of the vector backbone DNA, which can negatively affect transgene expression [28, 29].

In some crops biolistic bombardment assays were carried out. Embryogenic suspensions of ‘Chancellor’ (*Vitis* L. complex interspecific hybrid) were bombarded with tungsten particle coated with plasmid pBI426 encoding GUS and NVFII. Hebert et al. [30] obtained up to 850 transformed callus colonies per plate, 23 days after bombardment. These results demonstrated the biolistic process has a potential tool for achieving stable transformation of grapevines.

4.1.2 Electroporation

Electroporation consists of the treatment of plant cells with short high voltage electric pulses. The electric pulse shock causes brief permeability of the plasmalemma for high molecular particles, such as DNA [31]. The DNA movement is via pores formed after electric pulses in the cytoplasmic membrane [32]. The pores are of temporal character and they are related to the increased dipole moment of hydrophilic heads building cell membrane lipids. The dipole heads of phospholipids dislocate in the direction of the electric field, which causes breaks in the continuity of the cell membrane [33, 34, 35, 36]. The specific effect of the electric field on tissues cultured *in vitro* was determined by analyzing the growth of isolated protoplasts as well as with protoplast-derived calli of Colt cherry (*Prunus avium* × *pseudocerasu*). The plant regeneration capacity of electroporated tissues was also investigated. The callus obtained from the protoplasts and subjected to three successive exponential pulses at 250 V or 500 V showed the largest fresh weight increases between subcultures [36, 37]. Embryo and somatic embryos at the torpedo stage of coffee have been electroporated with DNA containing gus and bar genes, and plant regeneration through secondary somatic embryogenesis has been obtained [36, 38].

4.1.3 Pollen Tube Pathway

The transformation method via the pollen tube pathway, introduces exogenous DNA into plants through pollination as first reported by Zhou et al. [16]. Although the underlying transforming mechanism is still unclear, this tissue culture independent procedure makes the technique simple and convenient. It has been applied to introduce exogenous genomic DNA or plasmid DNA or minimal linear gene cassettes into plants [39]. Thus, it would allow us to generate vector- and selectable marker-free transgenic plants [40]. A marker-free and vector-free antisense 1-aminocyclopropane-1-carboxylic acid oxidase construct was transformed into melon via the pollen-tube pathway by Hao et al. [40]. Based on phenotype analysis together with RT-PCR data, a transformation frequency of 0.7% was achieved. Established an *aiiA* gene transformation system of soft rot resistant. In another study, the aiiA gene was introduced into Chinese cabbage through the pollen-tube pathway using the plasmid vector pBBBast-*aiiA* (7, 315 kb), which included *aiiA* gene, expression regulatory sequence (CaMV 35S promoter, OCS terminator), the herbicide resistant Bar gene and other elements by Han et al. [41]. Five out of 20632 (0.02%) of T_0

plants was positive for *aiiA* gene through PCR. Further PCR Southern blot analysis displayed that the gene *aiiA* segments were transformed into T_0 plants.

4.1.4 Liposome-mediated transformation

Liposome-mediated transformation, which facilitates the delivery of functional DNA into the cell by the more natural processes of endocytosis and lipid-plasmalemma fusions, was previously shown to be relatively non-toxic [42] to be simply to perform with readily available chemical reagents, to require no sophisticated equipment, and to be highly reproducible and efficient [42, 43, 44]. Application of Liposome-mediated transformation method was limited in fruit crops.

4.1.5 Sonication

Sonication (ultrasound) can alter the transient permeability of plasma membrane to facilitate uptake [45]. Compared to other direct DNA delivery methods, such as particle gun bombardment, electroporation and microinjection, the ultrasound treatment may be simpler to carry out. Sonication, however, could cause cell damage or ever rupture [46]. Ultrasound has been reported to mediate gene uptake in plant protoplast, suspension cells and intact pieces of tissues. Gene transfer by ultrasonication employs the same simple procedure irrespective of the nature of the plant material to be transformed [46].

4.1.6 Silicon Carbide Whiskers

The exact mechanism for whisker-mediated transformation is not known. Silicon carbide has great intrinsic hardness and fractures readily to give sharp cutting edges [47]. Scanning electron microscopy work on whisker-treated BMS ceils described by Kaeppler et al. [48] suggested that a SiC whisker may have penetrated the wall of a maize cell. Unlike asbestos fibers [49], the surface of SiC whiskers is negatively charged. This negative surface charge probably results in there being little affinity between DNA molecules (which are also negatively charged) and whiskers in neutrat pH medium [50].

4.1.7 PEG (PolyEthylene Glycol)

In protoplast transformation using PEG transgenes can be integrated in single copies but also in multiple copies linked together or at separate loci, rearrangement of transgene sequences has been observed too. Protoplasts are beneficial as a starting material because they are totipotent, which allows transgenic plants to be regenerated from single cells thus avoiding the issue of chimeras [51]. The cell cycle stage of the protoplasts seems to play an important role with regard to the integration pattern, with protoplasts in M phase (mitotic phase) giving rise to transgenic plants containing more copies of the transforming plasmid, usually at separate loci. Protoplasts in S phase (DNA synthesis phase) result in high copy numbers and frequent rearrangement of plasmid sequences [52]. PEG has generally led to relatively low frequencies of transformation (less than 1% of treated cells). Nevertheless, due to the availability of a large number of cells in such systems, a number of transgenic plants can be produced by using effective selection systems [53, 54].

4.2 Indirect Gene Transformation Methods

Members of the genus *Agrobacterium* are predominantly soil-inhabiting and plant-associated bacteria. They are primarily recognized as plant pathogens occurring on numerous plant species, including many agricultural crops. Among the phytopathogenic strains may cause crown gall disease which is mainly determined by the presence of tumor-inducing (Ti) plasmid in their genome, while strains harboring root-inducing (Ri) plasmid are the causal agents of hairy root disease [55, 56, 57].

The T-DNA region on the Ti (tumor-inducing) plasmid, bordered by left and right repeats, facilitates the transfer of DNA enclosed by these border regions [58].

Some *Agrobacterium* species carry more than one T-DNA on their Ti plasmids, leading to more than two T-DNA borders from which T-DNA can be processed. In the binary vector system, the virulence (*vir*) gene functions necessary for transformation are usually provided in trans on a second plasmid to secure that they are not transferred to the plant cell [58].

Transformation efficiency is determined by gene type [59], tissue type [60], co-cultivation conditions [61, 62], *Agrobacterium* strain [63] and selection regime [64, 65].

Zhou et al. [65] performed transformation assays via *Agrobacterium tumefaciens* in grape genotype. In that study an ubiquitin ligase gene (*VpPUB23*) from Chinese wild *Vitis pseudoreticulata* was transferred into Thompson Seedless for functional evaluation. Of the 351 transgenic plants obtained, those overexpressing *VpPUB23* exhibited decreased resistance to powdery mildew compared with non-transgenic plants. In another study a highly important grape vine rootstock, 110 Richter (*V. rupestris* X *V. berlandieri*), was a target cultivar for several transformation experiments aiming to introduce agronomically important genes (mainly virus resistance). Transgenic plants of 110 Richter were regenerated from embryogenic cultures co-cultivated with a disarmed LBA4404 strain of *A.tumefaciens*, harboring a binary vector contained chimeric genes for hygromycin resistance (*hpt*), Icanamycin resistance (*npt11*), the *uidA* gene and the CP-GCMV [66].

In most transformation systems, selectable marker genes are used to select regenerated transgenic shoots. However, the presence of these marker genes in the transgenic plants becomes undesirable after transformation. Theoretically, the use of a selectable marker gene can be avoided and transgenic plants can be directly selected by molecular analyses [67].

A. rhizogenes mediated transformation was applied to *V. rupestris*, *V. riparia*, *V. vinifera* and several *Vitis* hybrids infected by different phloem-limited viruses suchas grape vine fleck virus, grapevine virus A and grape vine virus B [68]. All viruses multiplied and persisted in these proliferating root cultures, which were successfully utilized for viral particle purification [69].

4.2.1 Sonication-Assisted *Agrobacterium*-mediated transformation (SAAT)

An important modification in *Agrobacterium*-mediated transformation involves subjecting the plant tissue to brief periods of ultrasound in the presence of *Agrobacterium*. SAAT treatment produces a large number of small and uniform wounds throughout the tissue, allowing easy access to the *Agrobacterium*, resulting in improved transformation efficiency in several different plant tissues including immature cotyledons, leaf tissue, suspension cultures, somatic and zygotic embryos [70]. Recently, many experiments have been demonstrated that SAAT tremendously improved the efficiency of *Agrobacterium* infection by introducing large numbers of micro-wounds into the target plant cells or tissues [71, 72, 73]. SAAT increased the transformation efficiency in the *Agrobacterium*-mediated transformation of some fruit crops. For example; *Vitis vinifera* [74], *Prunus mume* [75].

4.2.2 Agrolistics

The agrolistics approach combines the advantages of efficient biolistic delivery and the precision of the *Agrobacterium* T-DNA insertion mechanism, minimizing the regions of homology contributing to genetic and/or epigenetic instability [76]. Biolistic transformation is the method of choice for some plant species but many of the integration events resulting from these transformations are not desirable. By combining features of *Agrobacterium*-mediated transformation it is possible to achieve relatively predictable inserts in plants that are not normally transformable using *Agrobacterium* [70].

4.3 Modern Genetic Engineering Techniques

Over the past 15 years, several new techniques have been developed and are being implemented to facilitate breeding of improved crop varieties. Compared with traditional breeding, these techniques increase the precision of making changes in the genomes and thereby reduce the time and effort that is needed to produce varieties that meet new requirements. A common denominator of these techniques is that they make use of a GM step, but result in products in which no foreign genes (i.e., genes other than from the species itself or from cross-compatible species) are present [77].

GM is normally defined as an alteration of the genotype by the insertion or alteration of a specific DNA sequence using ‘recombinant DNA technologies’ involving artificial delivery systems. Early GM technology focused on the insertion of DNA from a foreign species, but there has been a trend away from transgenics (foreign DNA insertion) to cisgenics (same species DNA insertion) and most recently to targeted mutagenesis (genome editing) of a favoured genotype [18].

The exception is the use of genome editing to insert transgenes using sequence-specific nuclease technology (SSN-3; see Glossary), where the innovation is that genes can be inserted at a precise, predefined location, without the need for T-DNA border sequences, occurrence of small deletions, and so on [77].

Conventional genetic engineering is labor-intensive and requires time-consuming screens to identify the desired plant mutants. Genome-editing is an advanced genetic engineering tool that can more directly modify a gene within a plant genome [78, 79]. 'Genome editing' includes a set of techniques that allow to edit, delete, replace or insert, in specific genomic sequences of interest [80].

The desired genetic modification is initiated by inducing double stranded breaks (DSBs) into a target sequence by using nucleases, and is subsequently attained by DNA repair through NHEJ or homology-directed repair (HDR) [81]. The NHEJ pathway efficiently yields a small insertionor deletion (referred to as indel) in a specific locus withoutthe use of exogenous DNA. By contrast, the HDR pathwaycan introduce as a desired DNA sequence or gene into atargeted site, depending on the length of the exogenous DNA that is delivered to the plant cells together with the nucleases [82].

The breaks in the double-strand DNA can be induced by four systems based on specific enzymes. These; meganucleases, zinc finger nucleases (ZFN), transcription activator-like effector nucleases (TALEN) and clustered regular interspaced short palindromic repeats/CRISPR-associated nucleases (CRISPR/Cas9) [80].

4.3.1 RNAi and micro RNA technology

RNA silencing is a novel gene regulatory mechanism that limits the transcript level by either suppressing transcription (transcriptional gene silencing [TGS]) or by activating a sequence- specific RNA degradation process (posttranscriptional gene silencing [PTGS]/RNA interference [RNAi]). Although there is a mechanistic connection between TGS and PTGS, TGS is an emerging field while PTGS is undergoing an explosion in its information content. Pioneering observations on PTGS/RNAi were reported in plants, but later on RNAi-related events were described in almost all eukaryotic organisms, including protozoa, flies, nematodes, insects, parasites, and mouse and human cell lines. The critical common components of the paradigm are that (i) the inducer is the dsRNA, (ii) the target RNA is degraded in a homology dependent fashion, and, as we will see later, (iii) the degradative machinery requires a set of proteins which are similar in structure and function across most organisms. In most of these processes, certain invariant features are observed, including the formation of small interfering RNA (siRNA) and the organism-specific systemic transmission of silencing from its site of initiation [83].

Plant regulatory small RNAs, which are usually 20–24 nt in length, are classified into different sub-categories based on their biogenesis and function [84]. Small RNA genes can be empirically annotated by the pattern of alignments and size of predominant small RNAs [85, 86] Plant microRNAs (miRNAs), usually 21 nt in length, and heterochromatic small interfering RNAs (het-siRNAs), usually 24 nt in length, are the two major types of plant small RNAs. Plant miRNAs are processed from single stranded hairpin-forming primary RNAs and mediate post-transcriptional silencing by triggering target mRNA slicing and/or translational repression [87]. Plant het-siRNAs are processed from double stranded RNA and mediate silencing by RNA-directed DNA methylation (RdDM) [88, 89].

4.3.2 Zinc finger nucleases (ZFNs)

One well-characterized engineered nuclease is the zinc finger nuclease (ZFN), in which the nonspecific DNA cleavage domain of the FokI enzyme is fused with the DNA-binding motif of the zinc finger protein. The ZFN is composed of two monomers, each containing three or four zinc fingers. As each finger typically recognizes three nucleotides, a total of 18 nucleotides are recognized at a given target site, resulting in high DNA specificity [90, 91]. Over the last decade, ZFNs have been successfully used for genome editing in a wide range of eukaryotic species, including Drosophila [92], zebrafish [93, 94], rats [95], mammalian cells [96], and human cells [97, 98]. In addition, ZFNs have been successfully used for targeted mutagenesis by NHEJ in *Arabidopsis* [99, 100] and soybean [101] as well as for gene targeting by HR in *Arabidopsis* [100], tobacco [102] and maize [103]. Peer et al. [91] were first demonstrated of efficient and precise genome editing, using ZFN at a specific genomic locus, in two different perennial fruit trees—apple and fig. They concluded that targeting a gene in apple

or fig with a ZFN introduced by transient or stable transformation should allow knockout of a gene of interest. Using this technology for genome editing allows for marker gene-independent and antibiotic selection-free genome engineering with high precision in fruit trees to advance basic science as well as nontransgenic breeding programs.

4.3.3 TALENs

TALEs are naturally occurring proteins from the plant pathogenic bacteria genus *Xanthomonas*, and contain DNA-binding domains composed of a series of 33–35-amino-acid repeat domains that each recognizes a single base pair. TALE specificity is determined by two hypervariable amino acids that are known as the repeat-variable di-residues (RVDs) [104, 105]. A TALE protein has led to the explosive expansion of an alternative platform for engineering programmable DNA-binding proteins [79]. Like zinc fingers, modular TALE repeats are linked together to recognize contiguous DNA sequences. However, in contrast to zinc-finger proteins, there was no re-engineering of the linkage between repeats necessary to construct long arrays of TALEs with the ability of targeting single sites in a genome [79].

Both ZFNs and TALENs have tandem repeats in their DNA-binding domains that can be engineered to recognize specific DNA sequences; the resulting chimeric nucleases can thus be guided to the desired target sequences in the genome to generate DSBs. For each target site, a new ZFN or TALEN chimeric protein needs to be engineered to recognize the target [106].

4.3.4 CRISPR/Cas9

Until 2013, the dominant genome editing tools were zinc finger nucleases (ZFNs) [107] and transcription activator-like effector nucleases (TALENs) [108]. Both are artificial fusion proteins comprising an engineered DNA-binding domain fused to the nonspecific nuclease domain of the restriction enzyme FokI, and they have been used successfully in many organisms including plants [109, 110, 111]. The latest ground-breaking technology for genome editing is based on RNA-guided engineered nucleases, which already hold great promise due to their simplicity, efficiency and versatility. The most widely used system is the type II clustered regularly interspaced short palindromic repeat (CRISPR)/Cas9 (CRISPR-associated) system from *Streptococcus pyogenes* [112]. Among the various site-specific nuclease-based technologies available for genome editing, the clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein 9 (Cas9) systems have shown the greatest potential for rapid and efficient editing of genomes in plant species. CRISPR/Cas9 can target a specific genomic sequence, guided by an engineered 20-nt RNA sequence that binds to its DNA target by base-pairing rules pyogenes [112] and holds great potential for analysis of loss-of-function, gain-of-function and gene expression. CRISPR/Cas9 has been applied to the functional characterization of individual genes through loss-of-function, gain-of-function and perturbation of gene expression [113].

In comparison with ZFNs and TALENs, the CRISPR/Cas9 approach looks more attractive based on higher simplicity, accessibility, cost, and versatility, possibility of multiplexing and other aspects, including an easier open access to resources. Although contrasting results have been reported, the off-target effects remain the main concern for a wider application of genome editing to functional genomics and plant breeding [80]. Unlike ZFN and TALEN, in CRISPR/Cas9 technology, recognition of the DNA sequence to be modified is determined not by proteins, but by a chimeric sequence of RNA (single guide RNA – sgRNA), which results from the fusion of the two sequences (crRNA and tracrRNA) present in the natural system (the CRISPR/Cas system is used by bacteria to defend themselves against phages), while the cutting of DNA sequence is performed by the associated monomeric enzyme Cas9 [80, 107, 108, 112, 114,]. The preceding technologies, zinc finger and TALEN nucleases, were based on specific polypeptide-to-DNA binding which istedious to optimize; CRISPR-Cas9 is based on DNA-RNA hybridiza-tion which is well mastered. CRISPR-Cas9 nowadays appears as the most efficient system to achieve site-specific genome editing—easiest, more reliable and cheapest as well [115].

The first report of targeted genome modification in citrus using the Cas9/sgRNA system—a system that holds significant promise for the study of citrus gene function and for targeted genetic modification- carried out by Jia and Wang [116]. In that study successfully employed *CsPDS* gene, into sweet orange. DNA sequencing confirmed that the *CsPDS* gene was mutated

at the target site in treated sweet orange leaves. The mutation rate using the Cas9/sgRNA system was approximately 3.2 to 3.9%. Off-target mutagenesis was not detected for *CsPDS*-related DNA sequences in this study.

Some recent examples by using genetic engineering methods and transferring genes for fruits were presented Table 1.

TABLE 1
LIST OF FRUIT SPECIES, TARGET GENES AND TRANSFORMATION METHODS

Species	Target genes	Transformation method	References
Carrizo (*Citrus sinensis* Osb. X *Poncirus trifoliata* L. Raf.)	*gfp* and *nptII*	Biolistic	[22]
'Pineapple', 'Hamlin', 'Sucarri' and 'Valencia' sweet orange [*Citrus sinensis* (L.) Osbeck], 'Carrizo' Citrange [*Citrus sinensis* (L.) Osbeck X *Poncirus trifoliate* (L.) Raf.] and Eureka lemon [*Citrus. limon* (L.) Burm.f.]	knotted1 (*kn1*)	*Agrobacterium tumefaciens*	[117]
Fortunella crassifolia Swingle	*nptII-nos* and *pab5-flp-nos*	*Agrobacterium tumefaciens*	[118]
Citrus paradisi Macf.	*CsLOB1*	Cas9/sgRNA	[119]
Vitis vinifera cv Monastrell SCC	*nptII* and *eyfp/IV2*	SAAT	[74]
Malus domestica and *Ficus carica*	*uidA*	ZFN	[91]
Diospyros kaki Thunb	*gfp*	*Agrobacterium tumefaciens*	[120]
Citrus sinensis	*CsPDS*	Cas9/sgRNA	[116]
Prunus mume	*sGFP(S65T)*	SAAT	[75]
Vitis vinifera	*VpPUB23*	*Agrobacterium tumefaciens*	[65]
Pyrus pyrifolia Nakai	*gfp*	SAAT	[121]
C. paradisi Macf.	*RdRp*, *gfp* and *gus*	*A.tumefaciens*	[122]
C. sinensis L. Osb.	*ctv-cp*	*A.tumefaciens*	[123]
C. aurantifolia Swingle	*AtSUC*2, *RSs*1, *RTBV*, *gus* and *rol*C	*A.tumefaciens*	[124]
C. aurantifolia	*p25, p20,* and *p23*	RNAi	[125]
C. paradisi	*att*E	*A.tumefaciens*	[126]
C. unshiu Marc	*miraculin*	*A.tumefaciens*	[127]
C. sinensis	*CPsV cp (ihpCP), 54K (ihp54K),* and *24K (ihp24K)*	*A. tumefaciens* RNA silencing	[128]
Olea europaea L.	*nos-nptII* and *uidA*	*Agrobacterium tumefaciens*	[129]
Olea europaea	*gus* and *nptII*	Biolistic	[130]
Malus domestica Borkh.	*MdTFL1*	RNAi	[131]
Vitis amurensis	*rol*B and *npt*II	*Agrobcterium tumefaciens*	[132]
Malus micromalus	*rol*C, *gus*	*Agrobacterium tumefaciens*	[133]
Coffea canephora	*rol* genes, *uid*A, *hpt*II	*Agrobacterium rhizogenes*	[134]
Fragaria × *ananassa* cv. Elsanta	*chs*	RNAi	[135]
C. sinensis L. Osb	*gfp* and *pme*	PEG	[136]
Carrizo (*Citrus sinensis* Osb. X *Poncirus trifoliata* L. Raf.)	*uidA* and *nptII*	Biolistic	[137]
C. sinensis L. Osb	*gus*	Electroporation	[138]
C. sinensis L. Osb	*gfp*	PEG	[139]
Citrus reticulata Blanco × *C. paradisi* Macf	*gus* and *nptII*	Biolistic	[140]
Vitis L. complex interspecific hybrid	*gus* and *nvfII*	Biolistic	[30]

V. CONCLUSION

Many fruit and vegetable crops are generated by hybridization and selection, such as garden strawberry (*Fragaria X ananassa*), apple (*Malus domestica*), sweet orange (*Citrus sinensis*), tomato (*Solanum lycopersicum*), and squash (*Cucurbita maxima*). Another application of hybridization breeding is the generation of seedless horticultural crops, such as watermelon, by employing diploid and tetraploid parents. However, crop hybridization breeding has limitations that are difficult to overcome [141]. This requires enormous amounts of labor and land resources, although fast track breeding techniques and genetic engineering methods may accelerate breeding and selection processes. Recently, in addition to classical gene transfer technology, modern genetic engineering methods also have been started to apply for many plant species. It seems that the techniques illustrated in the present review will be more important with combination of classical plant breeding.

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