*General Article*_____ *Chapter –13*

GENOME EDITING IN FRUIT CROPS

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Abstract

The conventional breeding of fruit crops can take more than two decades due to the long juvenile period of fruit species.Genetic engineering allows improved varieties to be developed more quickly and the vegetative propagation of fruit trees allows the engineered cultivars to achieve coverage of larger areas than crops that depend on sexual reproduction.All genetically engineered fruit crops have been produced either by *Agrobacterium*-mediated transformation or direct DNA transfer. 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 interests for fruit crops are traits in biotic or abiotic stress response, shelf life, flavor, nutritional value, plant architecture and flowering time. Genome editing is type of genetic engineering in which DNA is inserted, deleted, modified or replaced in the genome of a living organisms. Gene-editing approaches have been extensively documented in plants. Transcription activator-like effector nucleases (TALENs) and zinc-finger nucleases (ZFNs) were among the first editing technologies to be developed. In the last decade, clustered regularly interspaced short palindromic repeats (CRISPR) and associated proteins (CRISPR/Cas) have surpassed their predecessors to become the most reliable and cost-effective approach for gene-editing.

Keywords: Genome editing, TALENs, ZFNs, CRISPR/Cas

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Introduction

Plant breeding, one of the oldest agricultural activities, parallels human civilization. Many crops have been domesticated to satisfy human's food and aesthetical needs, including numerous specialty horticultural crops such as fruits, vegetables, ornamental flowers, shrubs, and trees. Crop varieties originated through selection during early human civilization. Other technologies, such as various forms of hybridization, mutation, and transgenics, have also been invented and applied to crop breeding over the past centuries. The progress made in these breeding technologies, especially the modern biotechnology-based breeding technologies, has had a great impact on crop breeding as well as on our lives.In each case, the efficiency of transformation is highly dependent on the species and even cultivar, requiring the development of special optimized methods consisting of efficient gene delivery, selection and regeneration from transformed explants.Most fruit tree species are highly heterozygous and to maintain the characteristics of the original variety the transgenic events should be derived from mature tissue (such as leaves) rather than embryogenic explants.

Horticultural crops provide humans with many valuable products. The improvement of the yield and quality of horticultural crops has been receiving increasing research attention. Given the development and advantages of genome-editing technologies, research that uses genome editing to improve horticultural crops has substantially increased in recent years. The first genetically engineered fruit product (Flavr Savr™ tomato) was deregulated in 1992 and introduced into the market in 1994. A gene that triggers pectin solubilization was downregulated in the transgenic fruits, resulting in delayed fruit softening and an extended shelf-life.Several additional fruit crops with traits improved by genetic engineering have received regulatory approval for commercialization in different parts of the world and are intended for cultivation either as human food or animal feed.Most of the transgenic fruits were developed to improve agronomic productivity by conferring pest or disease resistance or delayed ripening.Theseare tomato (*Solanum lycopersicum*), papaya (*Carica papaya* L.), pepper (*Capsicum annuum*), plum (*Prunus domestica*), eggplant (*Solanum melongena* L.), apple (*Malus domestica* Borkh.), melon (*Cucumis melo* L.) and pineapple (*Ananas comosus* L. Merr.).

Some engineered fruit crops have been withdrawn from the market because they were not commercially viable (Flavr Savr™ tomato) or were never commercialized (Melon A and B).The USA has issued the most approvals for transgenic fruit cultivation either for human consumption or as animal feed.Like other genetically engineered crops, three government agencies are responsible for the oversight of transgenic fruit cultivation and import: the US Department of Agriculture (USDA), Animal and Plant Health Inspection Service (APHIS), the US Environmental Protection Agency (EPA) and the US Food and Drug Administration (FDA)which is part of the Department of Health and Human Services. Depending on its characteristics, a genetically engineered fruit may fall under the jurisdiction of one or more of these agencies(Lobato-Gomez*et al.*, 2021).

What is Genome Editing?

Genome editing is type of genetic engineering in which DNA is inserted, deleted, modified or replaced in the genome of a living organisms.To alter plant genomes and

produce desirable traits without introducing foreign genes.The nucleases create specific double-strand breaks (DSBs) at desired locations in the genome and harness the cell's endogenous mechanisms to repair the induced break by natural processes of homologous recombination (HR) and non-homologous end-joining (NHEJ).Sequence modifications then occur at the cleaved sites, which can include deletions or insertions that result in gene disruption in the case of NHEJ, or integration of exogenous sequences by HR (Osakabe and Osakabe, 2014).

Why Genome Editing?

To understand the function of a gene or a protein, one interferes with it in a sequence-specific way and monitors its effects on the organism.In some organisms, it is difficult or impossible to perform site-specific mutagenesis and therefore more indirect methods must be used, such as silencing the gene of interest by short RNA interference (siRNA).But sometime gene disruption by siRNA can be variable or incomplete.Nucleases such as ZFNs or TALENs can cut any targeted position in the genome and introduce a modification of the endogenous sequences for genes that are impossible to specifically target using conventional RNAi.

Scope of Genome Editing:

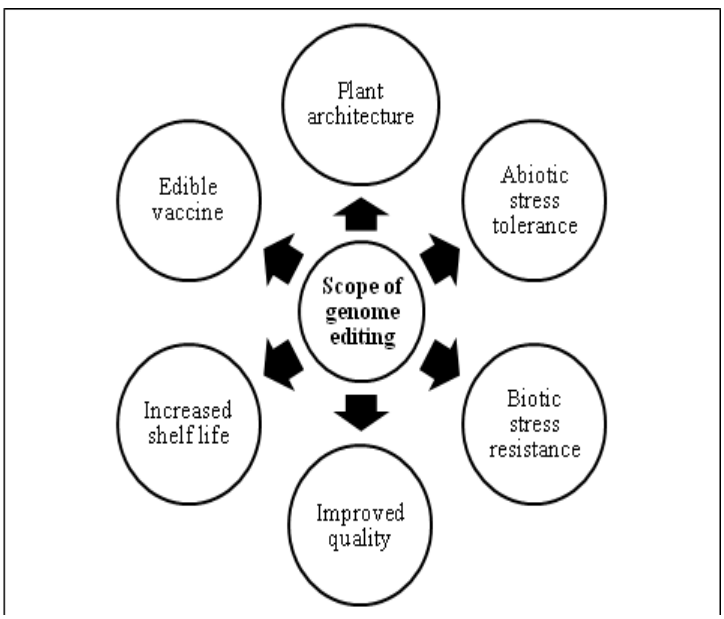


Table 1. Status of Improving Fruits Through Molecular Tools (Lobato-Gomez*et al.,* 2021)

Fruit crop	Trait	Modification strategy	G	F	Outcome
Apple	Flowering time	OE, GE	✓		Early flowering
	Fruit morphology	OE, GS	✓		Different color and shape
	Qualityimprovement	GS		✓	Increased firmness
	Plant morphology	OE	✓		Dwarf tree
	Tolerance to abiotic stress	OE	✓		Increased tolerance to salinity
Banana, Orange, Kiwi	Nutritional improvement	GE	✓		Increased carotenoid content
Grape	Fruit morphology	OE		✓	Reduce pathogen-induced mortality
Papaya	Quality improvement	GS		✓	Delayed fruit ripening and ethylene production
Pear	Nutritional improvement	OE	✓		Increased tocopherol content
Plum	Flowering time	OE		✓	Early flowering
Strawberry	Nutritional improvement	GS		✓	Decreased starch and increased soluble sugarand anthocyanin content

Stage of development: G: Greenhouse, F: Field trials

Modification strategy: OE: Overexpression, GS: Gene silencing, GE: Genome editing

Genetically Engineered Fruits Approved for Commercialization:

1. Papaya Resistant to Papaya Ringspot Virus:

In 1992, papaya ringspot virus (PRSV) was detected in Puna, the major papaya-producing district in Hawaii. PRSV resistance was not found in papaya germplasm or in wild *Carica* species suitable as candidates for interspecific hybridization. Furthermore, insecticides failed to control the aphid vectors responsible for virus transmissionand many orchards were therefore abandoned due to PRSV infestation. The widely cultivated 'Sunset' papaya was transformed with a gene derived from a Hawaiian strain to produce the transgenic papaya 'SunUp', which is completely resistant to PRSV in Hawaii. 'SunUp' papaya was crossed with 'Kapoho', a non-engineered cultivar, to obtain the yellow-flesh 'Rainbow' papaya, which is also resistant to PRSV.

In China, PRSV has threatened the papaya industry for 50 years. Similarly, to the 'SunUp' variety, transgenic Huanong No. 1 papaya is resistant to the four pre-dominant PRSV strains found in South China (Hainan, Guangdong, Guangxi and Yunnan provinces), namely Ys, Vb, Sm and Lc. Additionally, Huanong No. 1 produces bigger fruits with thicker flesh than the original cultivar. In 2012, some Huanong No. 1 papayas grown in Hainan exhibited PRSV-like symptoms, suggesting that resistance is beginning to break.

Phylogenetic analysis revealed the presence of a new virus lineage in Hainan and Guangdong papaya plantations, which may pose a threat to Huanong No. 1 papaya cultivation.

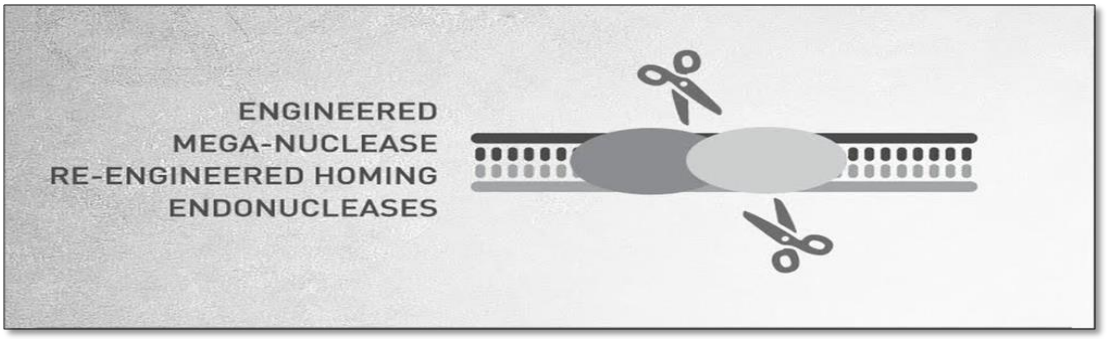
2. Pink-fleshed Pineapple:

Fruits with different skin and flesh colors have been developed by conventional breeding and in proof-ofconcept engineering experiments. In 2005, the Pinkglow™ transgenic pineapple was developed, in which the pink flesh accumulates lycopene due to the modification of the carotenoid pathway. The skin of the Pinkglow™ pineapple also has a combination of green, yellow, orange and red colors, whereas conventional pineapple is green and yellow. In addition to the modulation of carotenoid accumulation, an endogenous ethylene biosynthesis gene was suppressed to control flowering, but this trait has yet to be evaluated.

Genome Editors:

1) Meganucleases:

- It discovered in the late 1980s, are enzymes in the endonuclease family which are characterized by their capacity to recognize and cut large DNA sequences (from 14 to 40 base pairs).
- The most widespread and best known meganucleases are the proteins in the LAGLIDADG family, which owe their name to conserved amino acid sequences.
- It has benefit of causing less toxicity in the cell than methods such as ZFNs because of more stringent DNA sequence recognition.
- One major drawback is construction of sequence-specific enzymes for all possible sequences is costly and time consuming, as one is not benefiting from combinatorial possibilities that methods such as ZFNs and TALENs based fusions utilize.

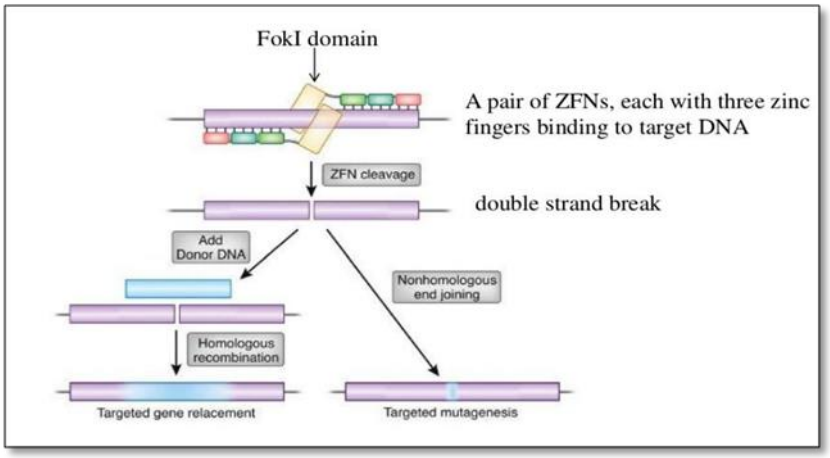


2) Zinc Finger Nucleases (ZFNs):

- It first discovered in the African clawed toad in 1985.
- A class of DNA-binding proteins.

- Facilitate targeted editing of the genome by creating double strand breaks in the DNA at specified locations which is important for site-specific mutagenesis.
- Stimulate the cell's natural DNA repair processes *i.e.* Homology directed repair (HDR) and Non-homology end joining (NHEJ).
- Generate precisely targeted genomic editing resulting in cell lines with targeted gene deletions, integrations or modifications.
- Highly specific genomic scissor.
- Consist of two functional domains:
  - DNA - binding domain
  - DNA - cleaving domain comprises of nuclease domain of FoK I

Diagrammatic Representation of ZFN Technology:



Applications of ZFNs:

- Repairing mutations
- Insertion of gene or DNA fragment at specific site
- Repair or replace aberrant genes
- Disabling an allele
- Allele editing
- Application in medical sector

a) Gene therapy
b) Treatment of HIV

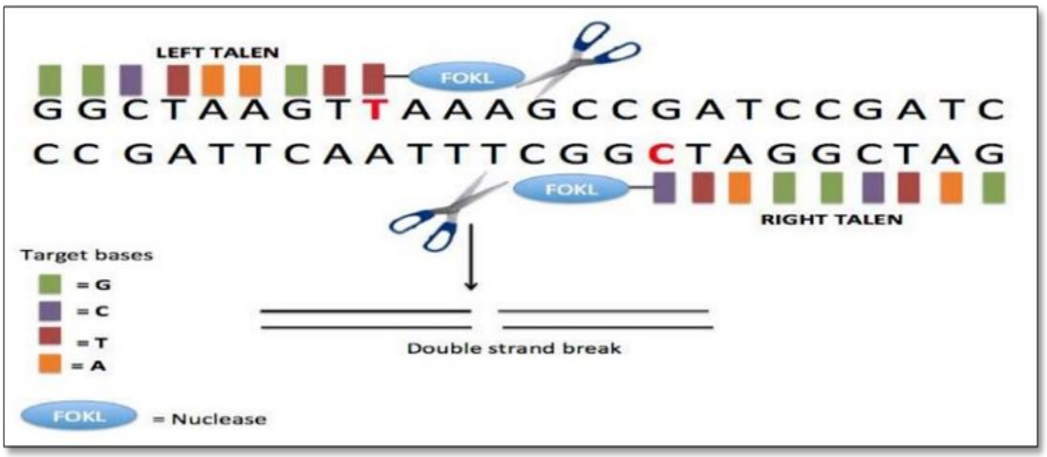
3) Transcription Activator-like Effector-Based Nucleases (TALENs):

- TALENs are the restriction enzymes engineered to cut specific sequences of DNA.
- They are made by fusing:
  - DNA-binding domain (TAL effector)
  - DNA-cleavage domain (Catalytic domain of RE FoK I)
- TALENs engineered to bind any desired DNA sequence to cut at specific locations in DNA.

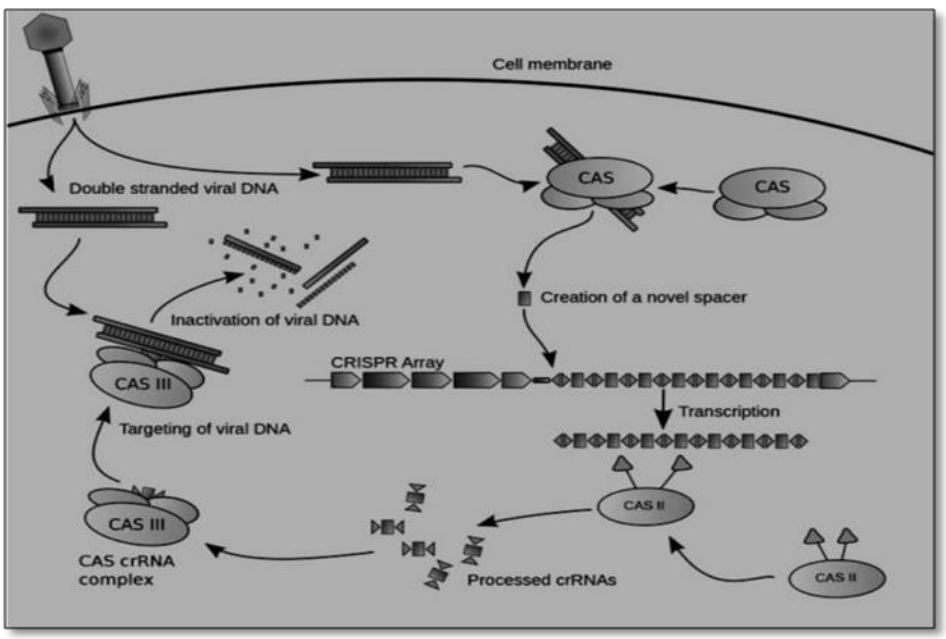
- TALEN constructs are used in a similar way to designed zinc finger nucleases and have advantages in targeted mutagenesis:

1. DNA binding specificity is higher
2. Off-target effects are lower
3. Construction of DNA-binding domains is easier

Based on the maximum theoretical distance between DNA binding and nuclease activity, TALEN approaches result in the greatest precision.



Diagrammatic representation CRISPR/Cas9:



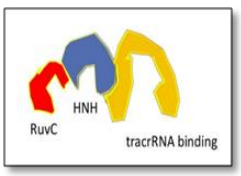
4) CRISPR/Cas9:

- CRISPRs (Clustered Regularly Interspaced Short Palindromic Repeats) are genetic elements that bacteria used as a kind of acquired immunity to protect against viruses.
- They consist of short sequences that originate from viral genome and have been incorporated into the bacterial genome.
- Cas (CRISPR associated proteins) process these sequences and cut matching viral DNA sequences.
- By introducing plasmids containing Cas gene and specifically constructed CRISPRs into eukaryotic cells, the eukaryotic genome can be cut at any desired position.
- Several companies including Cellectis and Editas have been working to monetize the CRISPR method while developing gene-specific therapies.

Components of CRISPR:

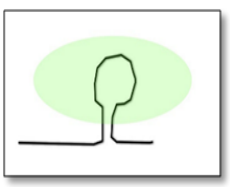
1. Enzyme Cas9:

The Cas9 nuclease has two functional domains: RuvC and HNM, each cutting a different DNA strand. When both of these domains are active, the Cas9 cause double strand breaks (DSBs) in the genomic DNA.



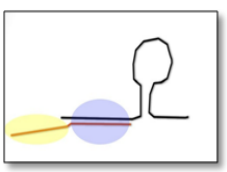
2. tracrRNA (Trans-activating CRISPR RNA):

The tracrRNA is a component of host immune system. Each species has unique tracrRNA which will only bind to the host specific Cas9. It means that tracrRNA will remain inactive until it encounters it matching Cas9.



3. crRNA:

The crRNA consist of two domains. The first domain located at the 3′ end combines with the 5′ terminal region of tracrRNA. The second domain which is located at the 5′ end is target specific and can be engineered to base pair with the target DNA region.



4. PAM (Protospacer Adjacent Motif) Sequences:

For Cas9 to successfully bind to DNA, the target sequence in the genomic DNA must be complementary to the gRNA sequence and must be immediately followed by the correct PAM sequence. The PAM sequence is present in the DNA target sequence but not in the crRNA sequence. Any DNA sequence with the correct target sequence is followed by the PAM sequence will be bound by Cas9.

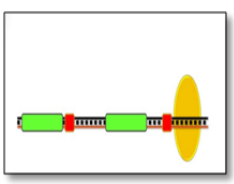


Table 2. Recent Studies of CRISPR Induced Gene Mutations In Fruit Crops

Fruit crop	Target genes	Target pathway or trait	Phenotype	Genome editing efficiency (%)	Transformation method	References
Apple	Kinase receptor MdDIPM 4	Plant immunity	Partial resistance to fire blight	73.3–77.8 %	*Agrobacterium*-mediated transformation	Pompili *et al.* (2020)
Banana	eBSV	Endogenous virus	Inactivation of eBSV into infectious viral particles	70–95 %		Tripathi *et al.* (2019)
Strawberry	FaTM6	Anther development and petal formation in strawberry	lack of flowers	50 %		Pizarro *et al.* (2019)
Kiwi	Phytoene desaturase (PDS)	Carotenoid biosynthesis	Albino	7.14–91.67 %		Wang *et al.* (2018)
Grapefruit	Lateral Organ Boundaries promoter (LOB1)	Plant immunity	Partial resistance to citrus canker	88.79–89.36 %		Peng *et al.* (2017)
Grape	IdnDH	Regulates the biosynthesis of tartaric	Stable accumulation of tartaric acid	~ 100 %	Protoplast-PEG-mediated transformation	Ren *et al.* (2016)

Challenges of Genome Editing in Fruit Crops:

- In fruit crops, molecular and genetic studies are difficult, which hinders the identification of genes responsible for desirable traits.

- The elimination of foreign DNA fragments (transferred T-DNAs) to obtain transgene-free edited plants remains difficult in some highly heterozygous and clonally propagated fruit species such as pineapple and banana.
- Polyploidization is found in many fruit species 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.
- Many fruit crops lack efficient transformation methods. Low editing efficiency may pose difficulty in identifying heritable mutations.
- Long juvenile stage for many fruit trees makes it difficult to experiment and optimize genome editing. Further, it takes a long time to obtain germline-transmitted homozygous mutations or next generation segregants.

Comparison of ZFN, TALEN and CRISPR/Cas9 Technologies

Nuclease platform	**ZFN**	**TALEN**	**CRISPR/Cas 9**
Source	Bacteria, Eukaryotes	Eukaryotes	Bacteria (*Streptococcus sp.*)
Binding specificity	3 Nucleotides	1 Nucleotide	1:1 Nucleotides pairing
Mutation rate (%)	10	20	20
Target site length (bp)	18-36	24-40	22
Endonuclease	FoK I	FoK I	Cas9
Off-target effects	High	Low	Variable
Ease of design	Difficult	Moderate	Easy
Dimerization required	Yes	Yes	No
Methylation sensitive	Yes	Yes	No
Best suited for	Gene knockout, Transcriptional regulation	Gene knockout, Transcriptional regulation	Gene knockout, Transcriptional regulation, Base editing

Conclusion

Genome editing applied successfully to a number of fruit crops to enhance fruit ripening, increase stress tolerance, modify plant architecture, control the timing of flower development and enhance the accumulation of desired metabolites and other commercially-important traits that are difficult or laborious to achieve by conventional breeding, either due to the lack of suitable germplasm or the long breeding cycles and need for multiple rounds of back-crossing. In some crops, direct transgene-free edits have already been achieved, while in others, T-DNAs have successfully been segregated out through crossing. In addition to the potential to produce non-transgenic edited cropsand thereby circumvent regulatory impediments to the release of new, improved crop varieties,

targeted gene editing can speed up trait improvement in crops with long juvenile phases, reducing inputs resulting in faster market introduction to the market. The development of transgene-free genome editing methods based on CRISPR/Cas9 promise to be more efficient and precise to edit genes when the genome sequences for target genes are known.

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