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Efficient and reproducible DNA delivery methods for trees genome editing

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Abstract: Genome editing aimed at manipulating and improving targeted genes is widely used for the study of basic biological processes and specific improvement of desirable and novel characters in commercially important tropical as well as subtropical fruit, nuts and forest trees. The technique involves precise and accurate changing and editing of the genome through DNA insertion, deletion, or replacement via multiple genome editing tools. Trees are considered an invaluable commodity that not only provides energy, fiber and materials but also safeguards global climate and such genome editing techniques are reliable and have great potential to further improve these imperative traits and allow us to boost productivity, enhance wood quality and improve resistance to several biotic and abiotic stresses. Tree breeding is considered a lengthy procedure that often requires a few to more than 10 years due to the tree's long juvenile phases, large size and asexual propagation nature. Traditional tree breeding strategies via conventional cross-breeding and induced mutations have led to the development of new fruit tree cultivars. However, precise tree genome editing techniques might play a valuable supplementary tool for their improvement. Over the last decade, numerous methods have been exploited for DNA delivery, such as the application of biotechnology in breeding via Agrobacterium-mediated transformation has been proven successful and possesses a huge potential with increased availability of sequenced genomes of Fruits and nuts that can be efficiently used for the improvement of the trait. Various other potential genome editing tools such as ZFNs, TALENs and most recently CRISPR/Cas9 have been effectively utilized for several fruit trees. Various improvements and alterations have been introduced worldwide to enhance the efficiency and reproducibility of the existing delivery protocols. In this review, various DNA delivery methods for genome editing together with their fundamental principles, procedures, efficacy and future prospects will be discussed.

Keywords: Genome editing, DNA, Agrobacterium, CRISPR/Cas9, Trees

1. Introduction

Trees have a unique impact on the well-being of this world and are now becoming increasingly imperative to restore forest stands globally and meet the world's increasing demands. Forest trees are the most significant element of the earth's biomass exhibiting vital ecological as well as economic roles (Endo et al. 2002). Various forest tree species produce raw materials, assist in maintaining biodiversity and lessen the effects of climate change. Furthermore, forest trees neutralize several toxic substances such as CO_2 and additional air pollutants that pose a dangerous risk to humans and the ecosystem. Also, they play a significant role in environmental protection (Endo et al. 2002). Some species of trees are also utilized as feedstocks for the production of bioenergy (Harfouche et al. 2011). Likewise, fruits and nuts are rich in nutrients that are essential for human health and growth (Song et al. 2019).

As a result of climate change, the emergence of new diseases, the increase in world population and the decrease the in production of cultural crops (Fiore et al. 2018), the demand for sustainable production of woody trees exhibiting improved traits is higher than before (Orbović 2019). Attaining all these goals may need the introduction of genes or their modified expression in order to improve biomass production in a sustainable and ecologically responsible way (Harfouche et al. 2011, Cao et al. 2022, Oliveira et al.2024).

Genomic diversity plays an important role in the production of novel varieties and cultivars. In this context, gene diversification aimed at the improved genetic architecture of various crops and trees has been performed for many years through conventional breeding techniques or via physicochemical and biological-induced random mutagenesis (Arora and Narula 2017). Improvement of trees encompasses not only dealing with genetics with resources but also conservation, breeding, selection and propagation of preferred genotypes.

Genome editing refers to a collection of various strategies and techniques established to create defined or tailored modifications in the genomic composition of an organism (Zaidi and Mansoor 2017). Genome editing permits targeting and altering specific DNA sequences (Wang et al. 2016, Jaganathan et al. 2018). It enables breeders to introduce single point mutations or novel DNA sequences at a specific locus in the genome thus permitting the specific modulation of desired traits with unparalleled control and efficiency for the first time (Schiemann et al. 2020). Genetic engineering of trees has progressed to a stage at which genes for traits of interest can now be introduced and expressed effectively such as tolerance to biotic and abiotic stresses, enhanced root formation, wood properties and phytoremediation (Harfouche et al. 2011).

This review will provide detailed knowledge about various DNA delivery methods for genome editing together with their fundamental principles, procedures, efficacy and future prospects.

2. Mechanisms of Genome Editing Systems

Numerous methods have been practiced by various scientists for successful gene delivery into the plant genome without critical limitations (Rashid and Lateef 2016, Tanuja and Kumar 2017, Keshavareddy et al. 2018). The choice for selecting a method for introducing transgenes into the plant system relies on the expression vector and the host (Chen and Lai 2015). Gene delivery to plant cells can be attained in two ways i.e. direct or indirect methods. The direct gene transformation does not require Agrobacterium-mediated transformation but the plasmids carrying desired DNA information will be delivered to the plant tissues through physical or chemical processes (Anami et al. 2013).

On the other hand, indirect methods are achieved by Agrobacterium strains or plant viruses (Rivera et al. 2012). Currently, gene delivery in most of the laboratories is usually conducted via

biolistic and Agrobacterium-associated methods (Rashid and Lateef 2016, Keshavareddy et al. 2018). Though, in fruit trees, gene delivery studies are mostly based on Agrobacterium-mediated transformations compared to the biolistic approach with less usage (Litz and Padilla 2012).
More recently, numerous modern gene-editing techniques have been implemented to counteract the difficulties occurring in plants in order to compensate for the continuously increased food demand in the future (Zhang et al. 2017). Genome-editing tools, including engineered endonucleases/ meganucleases (EMNs), zinc-finger nucleases (ZFNs), TAL effector nucleases (TALENs) and clustered regularly interspaced short palindromic repeats (CRISPR) (Gaj et al. 2013) are essential in plant research, as they permit the remodeling of future crops. Some methods used in gene delivery to plant cells are given below (Fig 1).

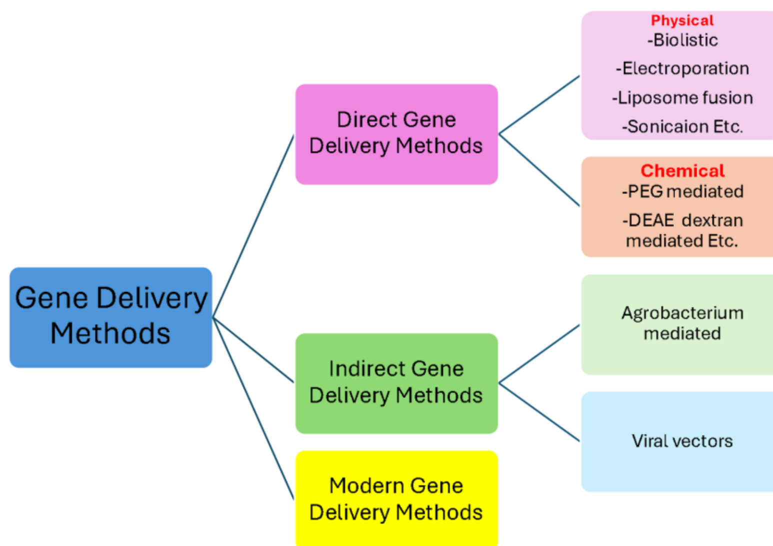

Fig 1. Some methods for gene delivery to plant cells

2.1. Direct or Vector-less Gene Delivery Methods

The term involves the direct introduction of foreign DNA into the plant genome and relies on the transfer of naked DNA into the plant cells. These methods are quite simple and much effective and several plants have been transformed using these approaches (Dönmez et al. 2016).

2.1.1 Particle Bombardment (Biolistic Method)

The particle bombardment method also termed a gene gun or biolistic is a unique high-performance technology that allows direct delivery of any foreign DNA, RNA, or protein into the plant genome. The method involves less physiological risk on the genome as there is no requirement for microbial intermediaries i.e. Agrobacterium and needs few additional DNA. Additionally, this method can adopt for both plant species i.e. monocotyledon and dicotyledonous (Mousavi and Fard 2019). One limitation of the biolistic method is the co-transfer of large-sized fragments of the vector backbone DNA, which is able to negatively affect the expression of the transgene (Tassy et al. 2014). In this technique, gold or tungsten microparticles are coated with DNA and bombarded at maximum velocity in a stream of helium into intact cells or tissues (Wu et al. 2016). The process is further grouped into 2 stages i.e. (a) nucleic acid is coated on metal particles (microprojectiles), and (b) the nucleic acid-coated microprojectiles are accelerated to

velocities suitable for invasion of target cells or tissues by avoiding unnecessary disruption of biological integrity (Sanford 1990).

This approach has a great potential for utilization in the breeding of numerous tropical as well as subtropical fruit tree species allowing the transfer of different genes to these trees for diverse purposes. Among these genes, the majority were selectable and scorable marker genes employed for the development of the optimized transformation protocols and a few other desired genes encoding the economical characters. Such as, the method has been successfully utilized for the transformation of bananas for transferring numerous genes to different banana cells for diverse purposes. However, it is recommended to integrate this method with the Agrobacterium to increase the gene transformation efficiency (Mousavi and Fard 2019). Embryogenic cells originated from diverse tissues such as immature male flowers (Becker et al. 2000), immature embryos (Chee et al. 2005), male inflorescence, and buds (Houllou-Kido et al. 2005) have been described with greater potential to the transformation of the gene in banana. Similarly, the stable transformation of the Cavendish banana (*Musa* spp. AAA group) cv. Grand Nain has also been also reported using uidA and potential virus-resistance (BBTV) genes together with selectable marker gene nptII gene employing different plasmids (Becker et al., 2000). Likewise, the level of resistance was increased against Fusarium wilt race 1 caused by *Fusarium oxysporum* cubense f. sp. by gene transformation in banana with chitinase and β-1,3-glucanase genes utilizing a biolistic approach (Subramaniam et al. 2010). They further transferred reporter genes viz. gfp and uidA together with the chitinase and β-1,3-glucanase genes for transformation occurrence detection and later expression in Rastali cultivar (*Musa* spp. AAB group) buds. Also, increased tolerance to Black Leaf Streak Disease (BLSD) caused by *Mycosphaerella fijiensis* has been reported by Vishnevetsky et al. (2011) by transferring 2 genes viz. endochitinase (ThEn-42) and grape stilbene synthase (StSy) antifungal genes exhibiting with synergistic effect coupled with chloroplastic (chl) Cu, Zn superoxide dismutase gene (Cu, Zu-SOD) (scavenging of free radical) to embryogenic callus of Cavendish banana (*Musa* spp. AAA group) cv. Grand Nain.

In citrus breeding, the biolistic method has also shown several advantages like great transformation efficiency, simplicity of the plasmid constructs allowing for the integration of larger inserts, the co-transformation of more than one construct and reduced biological harm to the explant (Dönmez et al. 2016). Most studies on the biolistic transformation of citrus have been conducted on the transformation and expression detection of the selectable and scorable marker genes (Mousavi and Fard, 2019). In a study, Bespalhok et al. (2003) successfully delivered the uidA/nptII genes to thin epicotyl sections of the Carrizo citrange (*Citrus sinensis* (L.) Osbeck × Poncirus trifoliata (L.) Raf.) and sweet orange (*Citrus sinensis* (L.) Osbeck) cv. Pera. Later, the Carrizo immature epicotyl along with reporter gene gfp and selectable marker gene was transferred (Wu et al. 2016). In date palm, the first transformation was carried out using the biolistic procedure (Mousavi et al. 2007) in which embryogenic callus and somatic embryos were bombarded with gold particles coated with plasmid DNA construct carrying gus gene in different helium pressures (900, 1100, and 1350 psi) and target distances (6, 9, and 12 cm). As a result, the highest gus expression in embryogenic callus was attained when bombarded with 1100 psi/6 cm (helium pressures/target distance), whereas, in somatic embryos, it was obtained at 1350 psi/9 cm.

2.1.2 Electroporation

The electroporation technique involves the treatment of plant tissues or cells using short high voltage electric pulses that result in concise permeability of the plasmalemma for high molecular

particles, like DNA (Bates 1989). The movement of DNA occurs through pores in the cytoplasmic membrane created after applying electric pulses (Sowers 1992). These pores exhibit temporal features and are associated with the increased dipole moment of hydrophilic heads building cell membrane lipids. The dipole heads of phospholipids are dislocated in the direction of the applied electric field, which results in the formation of breaks in the continuity of the cell membrane (Wojcik and Rybczynski 2015). The particular impact of this electric field when applied on tissues grown *in vitro* has been assessed in a study by examining the isolated protoplasts' growth and growth with protoplast-derived calli of Colt cherry (*Prunus avium* × *Pseudocerasu*). The plant regeneration capacity of resulting electroporated tissues was also examined in the study. The callus acquired from the protoplasts and exposed to 3 successive exponential pulses at 250 V or 500 V depicted the maximum fresh weight increase between subcultures (Ochatt et al. 1988, Wojcik and Rybczynski 2015). Similarly, in the case of the coffee tree, electroporation of embryo and somatic embryos at the torpedo stage of the tree has been done with DNA containing gus and bar genes and resulted in plant regeneration via secondary somatic embryogenesis (Barton et al. 1991, Wojcik and Rybczynski 2015).

2.1.3. Liposome-mediated transformation

Liposomes are lipid vesicles developed artificially and contain a phospholipid membrane. Transformation using liposomes involves the attachment of liposomes to the surface of the protoplast, followed by its fusion at the attachment site and then the release of plasmids carrying the genes inside the host cell. This transformation thus allows the delivery of functional DNA into the host cell using the more natural processes via endocytosis and lipid-plasmalemma fusions. Previously, it was presented to be comparatively non-toxic, to be simple to execute with readily available chemical reagents, to need no sophisticated lab equipment and to be extremely reproducible and efficient (Sawahel 2002). Regarding the application of Liposome-based transformation in fruit crops, this method was found limited (Donmez et al. 2016).

2.1.4. Sonication

Sonication or ultrasound (ultrasonication) based direct DNA delivery method can modify the transient permeability of plasma membrane to assist in uptake (Tachibana et al. 1999). This delivery system proved simpler to perform comparably to other direct delivery procedures, such as biolistic or electroporation. Though, this ultrasound treatment could result in cell damage or sometimes even rupture (Liu et al. 2006). It has been reported that ultrasound mediates the uptake of the gene in protoplast, suspension cells and intact tissues pieces. The technique employs the same simple process for gene transfer regardless of the nature of the planting material to be used for transformation (Liu et al. 2006).

2.2. Indirect or Vector-mediated Gene Delivery Methods (*Agrobacterium-mediated*)

Indirect gene delivery methods exploit the potential of plant viruses or certain pathogenic bacterial species like *Agrobacterium tumefaciens* that have the ability to naturally transfer their genome (plant virus) or section of their tumor-inducing plasmid (Ti plasmid) DNA (T-DNA) into plant cells (Agrobacterium). A transgene has the ability to enter cells of the plants as a by-product of virus infection if cloned into the full viral genome. Plant infection can be assisted by rubbing tissues of plants with infection-causing viral particles or nucleic acids carrying a transgene (Davies 2010). Yet, this procedure is only pertinent to plant hosts or viruses showing susceptibility to mechanical inoculation rather than insect vectors for transmission of viral particles.

The members of the genus Agrobacterium are gram-negative, mostly soil-inhabiting and plant pathogenic bacteria occurring on many plant species. The phytopathogenic strains often cause crown gall disease primarily associated with the presence of tumor-inducing (Ti) plasmid in their genome, while other strains cause hairy root disease and harbor root-inducing (Ri) (Kuzmanović et al. 2015). The Ti (tumor-inducing) plasmid contains the T-DNA region which is bordered by the left and right repeats. This T-DNA region assists in the DNA transfer enclosed by these borders (Gelvin 2003) and can be substituted by any desired gene(s), which is then transferred to plant cells during Agrobacterium infection. In a few Agrobacterium species, more than one T-DNA is present on their Ti plasmids that leading to more than two T-DNA borders from which T-DNA can be processed. In this binary vector system, the functions of virulence (vir) genes vital for transformation are usually provided in trans on a second plasmid in order to secure their transfer to the plant cell (Gelvin 2003). Transformation efficiency is assessed via a type of gene (Dhekney et al. 2009), type of tissue (Franks et al. 1998), conditions for co-cultivation (Li et al. 2008), Agrobacterium strain (Torregrosa et al. 2002) and selection regime (Zhou et al. 2014).
Genetic engineering via Agrobacterium offers possibilities of developing novel genotypes at a higher rate in various forest tree species. Various studies have been conducted in the past that showed the transfer of a number of alien genes to forest tree species via Agrobacterium-mediated transfer system (Fillatti et al. 1987, Klopfenstein et al. 1993, Nilsson et al. 1996, Tzfira et al. 1997, Fladung et al. 1997a, Holland et al. 1997). In 1987, the first hardwood species that was transformed using Agrobacterium was poplar for the herbicide resistance gene (Fillatti et al. 1987). It has been demonstrated that conifer species are usually difficult to transform using Agrobacterium technology, although various successful transformations have also been documented (Henderson and Walter 2006) such as larch (*Larix decidua*) (Huang et al. 1991), pine (*Pinus radiata*) (Charity et al. 2005) and spruce (*Picea* spp.) (Klimaszewska et al. 2001; Le et al. 2001).

2.3. Modern Gene Delivery Methods

In recent decades, natural variations as well mutations carrying germplasm produced by exploiting exogenous mutagens have been utilized for breeding purposes to enhance quality as well as yield. To distinguish individual plants carrying the desired phenotype via mutant population screening is required to detect desired mutant lines. This process is relatively laborious and genetic engineering has been widely employed to minimize the time needed for molecular breeding. Though, conventional genetic engineering technologies are based on the mechanism of transferring random genes. Novel methods focused on modification of targeted genes are consequently are essential for improvement and gene function analysis of woody plants (Osakabe et al. 2016).
Genome editing via modern approaches is now becoming the most practiced and versatile tool for the improvement of plants as well as functional genomics (Ahmar et al. 2020). The advancement in terms of engineered nucleases has permitted targeted gene engineering with more accuracy i.e. genome editing. At present, four systems of engineered nucleases are in practice viz. ZFNs, TALENs, EMNs and the CRISPR/Cas9 system (Fig 2). All these four systems are based on the generation of site-directed double-stranded breaks (DSBs) in the target genomic DNA (Urnov et al. 2010, Osakabe and Osakabe 2015). This results in efficient site-specific gene modifications via a non-homologous end joining (NHEJ) repair pathway or homologous recombination (HR) repair gene targeting (Osakabe et al. 2016).

Limited studies regarding genome editing in tree species have been documented compared to higher plants. Several difficulties are subjected to trees in applying genome editing techniques such as low efficiencies regarding transformation, deficiency of information and data for optimal expression cassettes for expressing engineered nucleases and trouble in isolation of clonal engineered plants. This is coupled with the slower speed of growth in perennial trees in comparison to grass species. Consequently, the advancement of genome editing techniques for tree species needs to be done in a controlled figure of trial-and-error cycles (Osakabe et al. 2016).

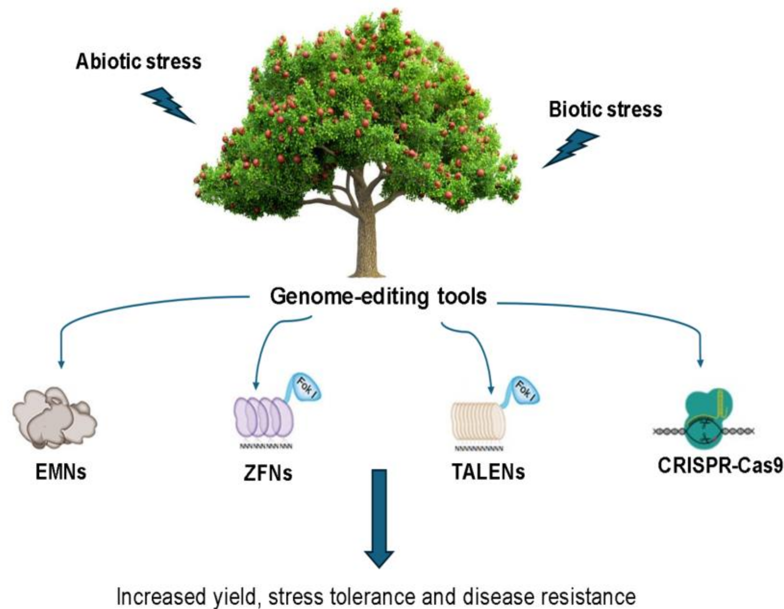

Fig 2. Modern gene delivery methods

2.3.1. Zinc Finger Nucleases (ZFNs)

Zinc-finger nucleases (ZFNs) are among the most significant tools that allow effective and efficient genome editing through targeting DSBs (Durai et al. 2005). Initially, genome editing using ZFNs was developed by employing chimerically engineered nucleases and it was facilitated by the discovery of the functional Cys2-His2 zinc-finger domain (Papworth et al. 2006, Gaj et al. 2013). ZFNs are structurally composed of two domains i.e. (a) the DNA-binding domain consisting of 30 to 600 zinc-finger repeats (Carlson et al. 2012) and each of these repeats can observe and read between 9 and 18 bp; and (b) the DNA cleavage domain or the non-specific cleavage domain of the type II restriction endonuclease Fok1 performs as the DNA cleavage domain (Carroll et al. 2006). ZFNs consist of two monomers assigned to their particular target sequences reversely flanking in between 5 and 6 bp of the DNA target (Carroll et al. 2006; Minczuk et al. 2008). Dimers having Fok1 domains slice DNA within its flanking sequence. The monitoring of a specific 24–30 bp sequence is done by a zinc-finger domain having specific or rare targeting sites in the genome (Gaj et al. 2013).
In a study, Peer et al. (2015) employed ZFN targeting of the uidA transgene involved in expressing β-glucuronidase (GUS) and demonstrated ZFN-based site-directed mutagenesis in apple (*Malus domestica*) and fig (*Ficus carica*). Similar to Arabidopsis (Osakabe and Osakabe 2015), a heat-

shock promoter was employed to express ZFN cassettes in order to escape the toxicity of ZFN (Peer et al. 2015). Individual plants were generated in this study and cloned via tissue culture after one year period. As the transformation of engineered nucleases via Agrobacterium generates NHEJ-based mutagenesis in transfected cells only, isolation of these transfected cells along with their regeneration is an inevitable stage in the cloning of engineered plants (Peer et al. 2015). In another approach i.e. systemic infection caused by a virus carrying genome editing vectors, that does not need regeneration from mutated cells if genome editing took place in apical meristems of the shoot, has been given by Peer et al. (Peer et al., 2015). It is therefore proposed that a combination of a virus causing systemic infection and an engineered nuclease is one possible approach toward a fast-breeding program in woody plants.

2.3.2. Transcription Activator-Like Effector Nucleases (TALENs)

Transcription activator-like effector nucleases (TALENs) recently appeared to be an alternative tool to ZFNs for genome editing and inducing targeted double-strand breaks (DSBs) into particular DNA sites (Joung and Sander 2013). These nucleases proficiently identify and cleave DNA in a sequence-dependent approach (Wang et al., 2021). Similar to ZFNs, TALENs are comprised of a non-specific FokI nuclease domain which is attached to a customized DNA-binding domain carrying highly conserved repeats originating from proteins called transcription activator-like effectors (TALEs). These functional proteins are 33 to 35 amino acids in length and have been derived from plant pathogenic bacteria Xanthomonas in order to modify the transcription of genes in host cells (Joung and Sander 2013, Ede et al. 2017). Compared to ZFNs, TALENs express higher target binding specificity due to their length (Jaganathan et al. 2018) and also the assembly of TALENs is easier, resulting in their wider acceptance as editing methods (Zhang et al. 2017).

TALEN can also be employed for the specific removal of mitochondrial mutations (Reddy et al. 2015). It has an unlimited targeting range and it eases the engineering of novel proteins, this makes it an appealing platform for targeted genome modification (Maeder and Gersbach 2016).

TALENs can be efficiently practiced to protect plants from the adverse effects of climate change. Genes involved in resistance to plant pests and diseases or protection from severe environmental conditions, like drought and salinity, can be effectively edited to improve resilience. TALENS have also been used to improve the quality of products derived from crops. Their unique function to rapidly and efficiently modify any gene sequence assures them to have a profound influence on biological research (Joung and Sander 2013).

2.3.3. Oligonucleotide-Directed Mutagenesis (ODM)

Oligonucleotide-directed mutagenesis (ODM) is also a novel gene-editing tool developed for plants (Sauer et al., 2016; Abdurakhmonov, 2016). ODM performs targeted mutagenesis and uses a specific 20-100 base long oligonucleotide, whose sequence is similar to the target genome sequence excluding that it comprises a single base pair change towards attaining site-directed editing of gene/ sequence of interest (Kamburova et al. 2017). When these synthetic oligonucleotides or repair templates with homology to a specific region of the target gene are transiently exposed to the plant cells through a range of specific DNA delivery tools, they attach to the targets and trigger the natural repair machinery of cells which then identifies the single mismatch in the template and then copies that mismatch or mutation into the target sequence using repair process (Sauer et al. 2016). This creates the desired targeted single nucleotide or base editing in the genome of the plant that allows unique function or character whereas the plant cell degrades the repair template oligonucleotide. By means of tissue culture procedures, cells with

modified sequences are then regenerated and novel varieties exhibiting edited genomes carrying improved traits are produced via traditional breeding (Abdurakhmonov 2016).

2.3.4. Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)

A new genome-editing tool, CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats), has been created in recent years that enable accurate genome engineering for gene silencing and activation, transcriptional regulation and genetic base edits (Doudna and Charpentier 2014, Noman et al. 2016). The discovery of this system has greatly changed our plant genome understanding (Cardi et al. 2023). This editing system includes two components including a Cas9 endonuclease isolated from bacterium *Streptococcus pyrogenes* and a single guide RNA (gRNA) that has the ability to target a 20 nt DNA sequence before every NGG (protospacer-adjacent motif (PAM) site). This system has been discovered earlier in bacteria that utilize it naturally for defense against bacterial virus attacks (Doudna and Charpentier 2014). Later, this discovery was exploited to develop a novel biotechnological tool to be used for the improvement of plant genetics.

CRISPR-Cas9 has been broadly utilized in a number of plant species for attaining targeted gene editing (Chen et al. 2019, Zhang et al. 2019). Its application for disease resistance is among the most applicable genetic engineering approaches in agricultural research (Borrelli et al. 2018). It is now also rapidly becoming the tool of choice regarding targeted mutagenesis in a number of woody species, including forest trees. Trees including forest, fruit, nuts are considered an invaluable commodity that not only provides energy, fiber, food products and materials but also safeguards global climate and CRISPR technology has the great potential to further improve all these essential traits (Bewg et al. 2018, Cao et al. 2022).

Few studies have applied CRISPR/Cas9 for gene editing in woody tree species including some fruit trees (Breitler et al. 2018) and forest tree species such as poplar (Fan et al. 2015, Wang et al. 2017, Jiang et al. 2017, Elorriaga et al. 2018). To date, the huge development regarding woody species has been achieved with poplar i.e. the first firmly transformed and genome-edited tree by CRISPR with greater efficiency (Zhou et al. 2015). CRISPR/Cas9 has been demonstrated as the most efficient system at producing targeted mutations in transgenic plants of poplar and homozygous mutations can be generated at the desirable sites in the first generation (Fan et al. 2015). The likelihood of obtaining null mutations in the T0 generation is specifically essential for trees having prolonged reproductive cycles (Fan et al. 2015, Tsai and Xue 2015) and like poplar or eucalyptus trees are vegetatively propagated (Dai et al. 2020). As poplar is important in industries for its wood properties, most gene editing studies based on CRISPR have specifically targeted SCW composition as well as phenylpropanoid metabolism including lignin (Wang et al. 2017, Wan et al. 2017, Yang et al. 2017, Shen et al. 2018). Similarly, in walnuts, the availability of sequence of its complete genome now permits selection of a precise guide RNA and prevention of off-target sequences (Walawage et al. 2019).

The CRISPR/Cas9 technology has also been successfully employed for genome editing in numerous fruit trees, such as apple, banana, citrus, cacao, grape, pear and kiwifruit. Several methods have been assessed to optimize this technique for its utilization within a fruit cultivar (Corte et al. 2019). The majority of these studies targeted the phytoene desaturase (PDS) gene that encodes an enzyme involved in the carotenoid biosynthesis pathway. When disrupted, this gene leads to the impairment of chlorophyll and carotenoid production that results in an albino phenotype (Qin et al. 2007) and serves as a simple manipulation target to verify the effectiveness of the genome modification system. Such as, transgenic expression of Cas9 guided by 19-bp

sgRNA designed to target the conserved region of 2 PDS genes in the banana genome generated complete albino and variegated phenotype among regenerated banana plantlets exhibiting 59% mutation efficiency (Kaur et al. 2018). Maximum editing efficiency of 100% targeting the same gene through polycistronic gRNAs has also been reported in bananas (Naim et al. 2018). Similarly, the same results were observed in Carrizo citrange regarding the editing of the PDS gene, where the Cas9 gene was driven by the Arabidopsis YAO gene promoter rather than cauliflower mosaic virus 35S promoter and the mutation efficiency increased to 100% (Zhang et al. 2017). Mutations in the PDS gene generated by CRISPR/Cas9 system have also been presented to confer an albino phenotype in several trees including apple (Nishitani et al. 2016, Charrier et al. 2019), kiwifruit (Wang et al. 2018), pear (Charrier et al. 2019) and kumquat (Zhu et al. 2019). Few more effective applications of the CRISPR/Cas9 system have also been documented in citrus trees (Peng et al. 2017, Zhang et al. 2017). Likewise, in sweet orange (*Citrus sinensis*) Jia and Wang (2014a) have documented genome editing using the CRISPR/Cas9 system. In this report, transient expression of plant-codon-optimized Cas9 and CsPDS-targeted gRNA disrupted the endogenous CsPDS locus. Remarkably, this low transformation efficiency in sweet orange was overcome by employing Agrobacterium infection facilitated by pre-infection with *Xanthomonas citri* subsp. *citri* (Xcc). Xcc enhances efficiencies regarding infection of Agrobacterium to citrus (Jia and Wang 2014b). Fister et al. (2018) have documented the use of transient leaf transformation targeting the non-expression of pathogenesis-related 3 (NPR3) gene, which is a suppressor of the immune system in Theobroma cacao, has resulted in enhanced resistance to pathogen *Phytophthora tropicalis* in tree crops. Furthermore, the cauliflower mosaic virus (CaMV) 35S promoter, transcribed by RNA polymerase II, has been utilized for the expression of gRNA in sweet oranges (Jia and Wang 2014a). RNA polymerase III-transcribed promoters, like U3 and U6, have more commonly been utilized to express gRNA. Though, in tree species, information regarding U3/U6-snRNA expression is lacking to some extent. Thus, RNA polymerase II-based gRNA expression appears to be one of the possible procedures currently being adopted to design predictable CRISPR/Cas9-based expression in trees.

Carbohydrate polymers present in lignocellulosic biomass are useful in paper and pulp industry. Owing to the association of cellulose micro-fibrils with the condensed lignin coverage, delignification of wood using chemicals is crucial to remove lignin and thereby producing high-quality paper with improved brightness and whiteness (Chutyser et al. 2018). However, this Chemical delignification needs expensive chemicals that are extremely harmful to wood polysaccharide components and also for the environment because of toxic pollutants (Wang et al. 2018). To escape such problems, genetic engineering could successfully be utilized to minimize lignin content and modify its composition in woody tree plants so as to improve pulping quality, escalate wood extractability and decrease mill effluents (Verma and Dwivedi 2014). In this context, gene knockout and silencing using the CRISPR approach were effectively used in poplar tree species to strongly down-regulate genes involved in the lignin biosynthetic pathway. For the first time, genome-edited poplar using CRISPR with greater efficiency was described by Fan et al. (2015). In a study, CRISPR-Cas9 mutational efficiency was also tested on the biosynthesis of lignin and flavonoid in the woody perennial *P. tremula* × alba by disrupting three 4-coumarate: CoA ligase genes viz. 4CL1, 4CL2 and 4CL5 (Zhou et al. 2015). The results showed that genes 4CL1 and 4CL2 play a key role in lignin and flavonoid biosynthesis. Mutations in gene 4CL1 identified a decrease in lignification, while gene 4CL2 was confirmed to be involved in the production of chlorogenic acid in leaves. The same study also revealed a 20% decrease in lignin content and a 30% reduction in S/G ratio through CRISPR/Cas9-based mutagenesis in gene 4CL.

Notably, each independent 4CL1 line established identical reddish-brown wood, which is a phenotype linked with lignin deficiency. In another study of poplar, brassinosteroid biosynthetic gene PtoDWF4 knockout plants created by CRISPR considerably reduced biomass production depicting the significant role of this gene in secondary cell wall synthesis as well as wood formation (Shen et al. 2018). Few current studies have also documented the successful mutational efficiency of CRISPR/Cas9 for poplar flowering genes and a large mutation dataset (Elorriaga et al. 2018, Bruegmann et al. 2019). These reports demonstrated promising approaches for lignocellulosic biomass production consuming less land and assisting the conservation of natural forests and minimizing the environmental issue of paper and pulp processing.
This technology is a tool with significant potential for solving numerous biological problems and coping with the pressure of anthropogenic impacts and climate change (Thapliyal et al. 2023).

4. Conclusion

Various conventional and unconventional methods have been practiced for efficient gene delivery in plants. Most of the tree species are generated by hybridization and selection procedure that has certain limitations hard to overcome. This requires huge labor and a land resource, the procedure is lengthy and impractical in long-duration tree species. Employing unconventional methods encompassing Agrobacterium-mediated gene delivery or direct gene transfer, a number of transgenic plants as well as tree species like Populus have been produced. Dominant selectable markers viz. kanamycin resistance, tolerance to herbicides and insecticidal genes, originally cloned from bacteria, have been transferred and expressed in various plants. In a few cases, the transmission of the foreign genes to the progeny has been validated. In plants, following foreign gene transfer may result in unexpected genetic variability which may be a cause of concern in the case of long-lived tree species. More recently, in addition to these, modern gene delivery systems have also been initiated for application in many tree species that allow editing, deletion, replacement, or insertion, in desired specific genome sequences. Genome editing via modern approaches is now becoming the most practiced and versatile tool for the improvement of various crop plants and trees as well as functional genomics. Additional efforts are in progress to create genome-based genetic markers linked to desired phenotypic characters to improve early selection and reduce the excessive period of time needed to develop superior cultivars (Aradhya et al. 2019). As novel plant breeding systems advance, all these efforts along with a detailed understanding of the structure as well as the function of the whole genome of a vast variety of woody tree plants, will permit the progression of future breeding technologies in novel and imperative characters in woody plants and also various fungal and bacterial species for effective use of woody plants biomass.

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