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Review

Target-specific gene delivery in plant systems and their expression: Insights into recent developments

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In order to improve crop plants in terms of their yield, drought resistance, pest resistance, nutritional value, etc., modern agriculture has relied upon plant genetic engineering. Since the advent of recombinant DNA technology, several tools have been used for genetic transformations in plants such as *Agrobacterium tumefaciens*, virus-mediated gene transfer, direct gene transfer systems such as electroporation, particle gun, microinjection and chemical methods. All these traditional methods lack specificity and the transgenes are integrated at random sites in the plant DNA. Recently novel techniques for gene targeting have evolved such as engineered nucleases such as Zinc Finger Nucleases, Transcription Activator like effector nucleases, Clustered regular interspaced short palindromic repeats. Other advances include improvement in tools for delivery of gene editing components which include carrier proteins, and carbon nanotubes. The present review focuses on the latest techniques for target specific gene delivery in plants, their expression and future directions in plant biotechnology.

Keywords. Plant genetic engineering; targeted gene delivery; ZFN; TALEN; CRISPR; carrier protein; carbon nanotubes

1. Introduction

Genetic engineering refers to the direct manipulation of DNA to alter the organism's characteristics in a particular way. This process of genetic manipulation has radically transformed agriculture by empowering introduction of transgenes into plants. Transgenic plants are generated that exhibit novel traits. For example transgenic crop plants are often equipped with qualities such as pest resistance (Douglas 2018), drought and disease resistance, yield enhancement production of fortified food (Ye *et al.* 2000; Paine *et al.* 2005; Black *et al.* 2008) etc. But improvement of crop plants is not the sole achievement of transgenic plant development. Recombinant protein, vaccine productions are also achieved by genetic manipulation of plants and plant cells (Laere *et al.* 2016). Some transgenic plants are developed for purely ornamental purposes (Chandler and Sanchez 2012). Transgenic plants are also used to uncover and study the function of certain genes for research purpose. The first report on genetically engineered plant dates back to 1983, where an antibiotic resistant gene was cloned into the T1 plasmid of *Agrobacterium* and introduced into a *Nicotiana tabacum* plant (Bevan *et al.* 1992; Lemaux 2008). Following this, a number of techniques have been developed for introduction of transgenes into plants. Some techniques involve vectors such as *Agrobacterium tumefaciens* and certain viruses (Laere *et al.* 2016). Other vector-less methods, such as electroporation, microinjection, particle gun bombardment and chemical methods for DNA uptake into protoplasts have also been developed. Chloroplasts have been an important target for genetic engineering and in 1995, McBride et al. engineered the tobacco (*Nicotiana tabacum*) chloroplast genome to be herbicide and insect resistant (McBride *et al.* 1995). In several other reports, homologous recombination utilising transgenes

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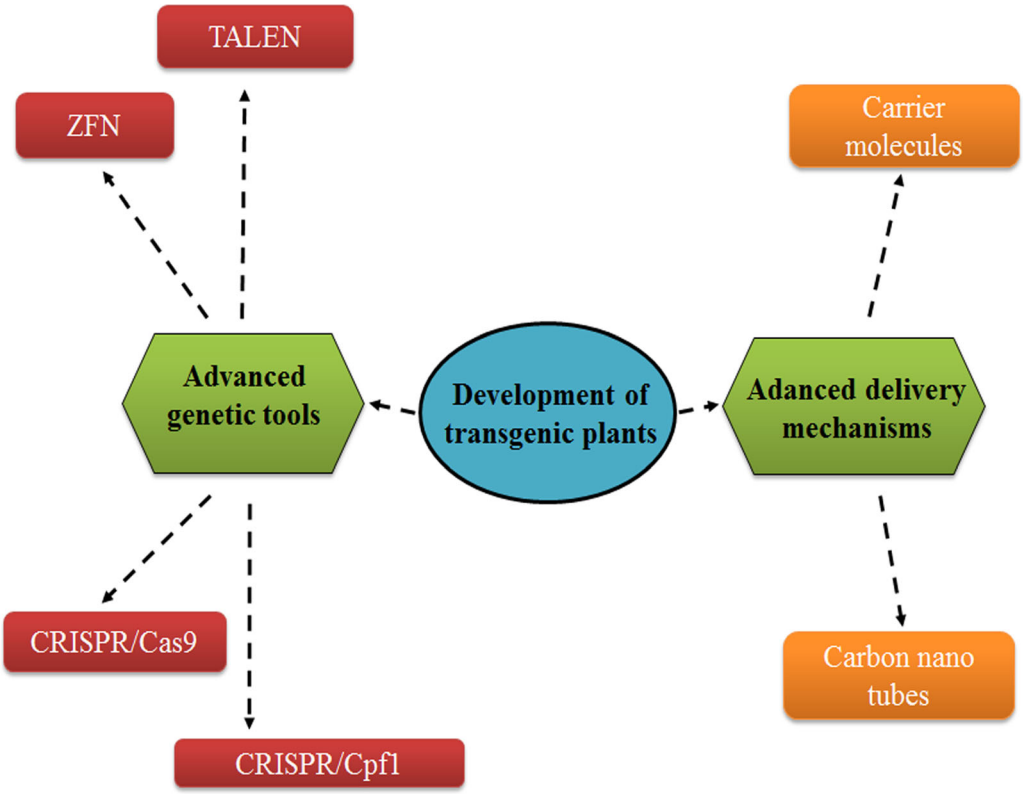

Figure 1. The different aspects of development of transgenic plants. Both genetic tools and delivery machinery require advancement for successful site specific genetic manipulation. Recent advancements in gene targeting include development of specific nucleases like ZFNs, TALENs, CRISPR /Cas9 and CRISPR/Cpf1 systems. Gene delivery into plant systems have improved with the development of carrier molecules and carbon nanotubes.

flanking a small region of chloroplast genome was used and was widely successful (Daniell *et al.* 1998; Day and Madesis 2007; Won *et al.* 2011) in comparison to nuclear transgene expression. The common disadvantage of these techniques is that the transgene is inserted at random sites in the host genome which is manifested in its level of expression (Peach and Velten 1991; Kohli *et al.* 2003; Francis and Spiker 2005) as well as in redundant mutations that occur because of disruption of active plant genes (Kim *et al.* 2007).

To circumvent these problems, it was imperative to develop tools for targeted gene delivery, which aims at altering a specific DNA sequence in an endogenous gene at its original locus in the genome leading to permanent site specific modification of the genome. Gene targeting depends upon homologous recombination (HR) and not only ensures accurate gene integration but also single copy insertion and increased expression of transgene. In 1988, Paszkowski performed the first target specific gene delivery into protoplast of tobacco (*Nicotiana tabacum*) (Paszkowski *et al.* 1988) where an antibiotic resistance gene was integrated into the plant genome. Subsequently, numerous approaches have been made towards gene targeting in plants (Iida and Terada 2005; Tzfira and White 2005; Voytas 2013).

In the following review, recent tools available for target specific gene delivery in plants have been elucidated with respect to their mechanism as well as expression (figure 1). Utilisation of Site-specific homologous recombination, Zinc Finger Nucleases (ZFNs), Transcription Activator-Like Effector Nucleases (TALENs) and Clustered Regularly Interspaced Short Palindrome Repeats (CRISPR) have been discussed. Moreover, the methods of delivery of these genome altering components into plant cells have also been discussed with special mention to carrier proteins and carbon nanotubes.

2. Tools for gene targeting

2.1 *Homologous recombination*

Natural homologous recombination is a DNA maintenance mechanism that repairs DNA damage such as

double stranded breaks (DSBs) and inter-strand cross-links. Repair of DSBs are imperative to ensure accurate replication of the genome. Generally, DSBs are repaired by two well-known mechanisms: Non-homologous end joining (NHEJ) and Homologous recombination (HR). NHEJ can not only cause deletions but also insertions that occur by random integration of sequences copied elsewhere from the genome into these breaks. DSB repair using homologous recombination involves exchange of genetic information between genomic and exogenous DNA molecules via crossover events, the exchange being guided by homologous sequences acted upon by enzymatic machinery of the cell. Whereas homology present in allelic and ectopic positions is rarely used for repair, homologous sequences in close proximity to breaks are frequently accessed by the repair machinery. Single strand annealing (SSA) is also a process that is initiated when a DSB occurs between two repeated sequences oriented in the same direction. Single stranded regions are created adjacent to the break extending to the repeated sequences such that the complementary strands can anneal to each other. This annealed intermediate can be processed by digesting away the single stranded tails and filling in the gaps, therefore leading to sequence deletions between direct repeats. According to Puchta *et al.* (2005), induction of DSBs may be effectively used for guided manipulation of plant genome for gene excision and site specific integration. Accordingly, the frequency of homologous recombination can be improved and the repair machinery of the cell following a DSB can be efficiently utilized for gene targeting.

HR has been effectively used for gene targeting in yeast and mice whereas in plants the earlier attempts had been inadequate (Salomon and Puchta 1998; Hanin and Paszkowski 2003). In an early report by Puchta (1998), *Nicotiana plumbaginifolla* protoplasts were cotransfected with a plasmid carrying a synthetic I-Scel gene, encoding a highly sequence-specific endonuclease, and recombination substrates carrying an I-Scel-site adjacent to their homologous sequences. The drawback of this strategy was that target sites were randomly inserted into the genome, and thus, it would not be likely to target endogenous genes. Improvement of HR frequency was the prime focus when reports suggested that rad9 and rad17 mutants improve HR frequency (Puchta *et al.* 1996; Chen *et al.* 2008). For this purpose bacterial RecA protein was expressed in Tobacco which increased the HR frequency but not gene targeting (Reiss *et al.* 2000). These attempts lead to discovery of intriguing facts and contributed towards the understanding of the pathways involved in DSB repair and homologous recombination machinery in plants.

Initially, the frequency of HR for random combination of vector was found to be inadequate and gradually improved with induction of DSBs. Furthermore, engineered nucleases were introduced for gene targeting in mammals (Joung and Sander 2013), which showed a new direction towards improving gene targeting in plants.

In recent times three types of engineered nucleases are used for gene targeting: Zinc Finger Nucleases (ZFNs), Transcription Activator-Like Effector Nucleases (TALENs), Clustered Regularly Interspaced Short Palindrome Repeats (CRISPR) (figure 2).

2.2 *Zinc finger nucleases (ZFNs)*

Zinc finger domains (ZFNs) are artificial restriction enzymes that are developed by fusion of zinc-finger DNA binding domain recognizing a particular DNA sequence, to DNA cleavage or nuclease domain of restriction enzyme FokI (Kim *et al.* 1996). This fusion is facilitated by the use of a peptide linker. By engineering the Zinc-Finger domain at the N-terminal, Cys2-His2 (C2H2), to recognise novel DNA sequences in a complex genome, it was possible to manipulate ZFNs to precisely alter endogenous gene loci in eukaryotic organisms (Carroll 2008). In a single ZFN, the DNA binding apparatus consists of 3–6 individual ZF repeats, each of which recognize 9–18 bp of DNA sequences. Therefore, a single ZF domain may contain almost 30 amino acids and if the ZFs identify a 3bp sequence, they can form a 3-finger array recognizing a 9bp target and so on. This procedure however has significant downside in overlapping of the specificities of the individual ZFs which is influenced by surrounding ZFs and DNA. The DNA cleavage domain, i.e. the non-specific nuclease domain from FokI at the C-terminal cleaves DNA after dimerization, hence requiring a pair of ZFNs for gene targeting. To deliver ZFNs into cells, an efficient transient transfection agent is required. In most cases, electroporation has been used (Liu *et al.* 1997). Other less frequently used delivery mechanisms include adenoviruses, adeno-associated viruses, lentiviruses (IDLV), and lipofectamine 2000 (Wang *et al.* 2016b).

ZFNs are utilized to cleave the DNA in a site specific manner creating DSBs in the absence of pre-engineered target sites in the genome. ZFN mediated gene targeting provides the ability to modify the plant genomes in

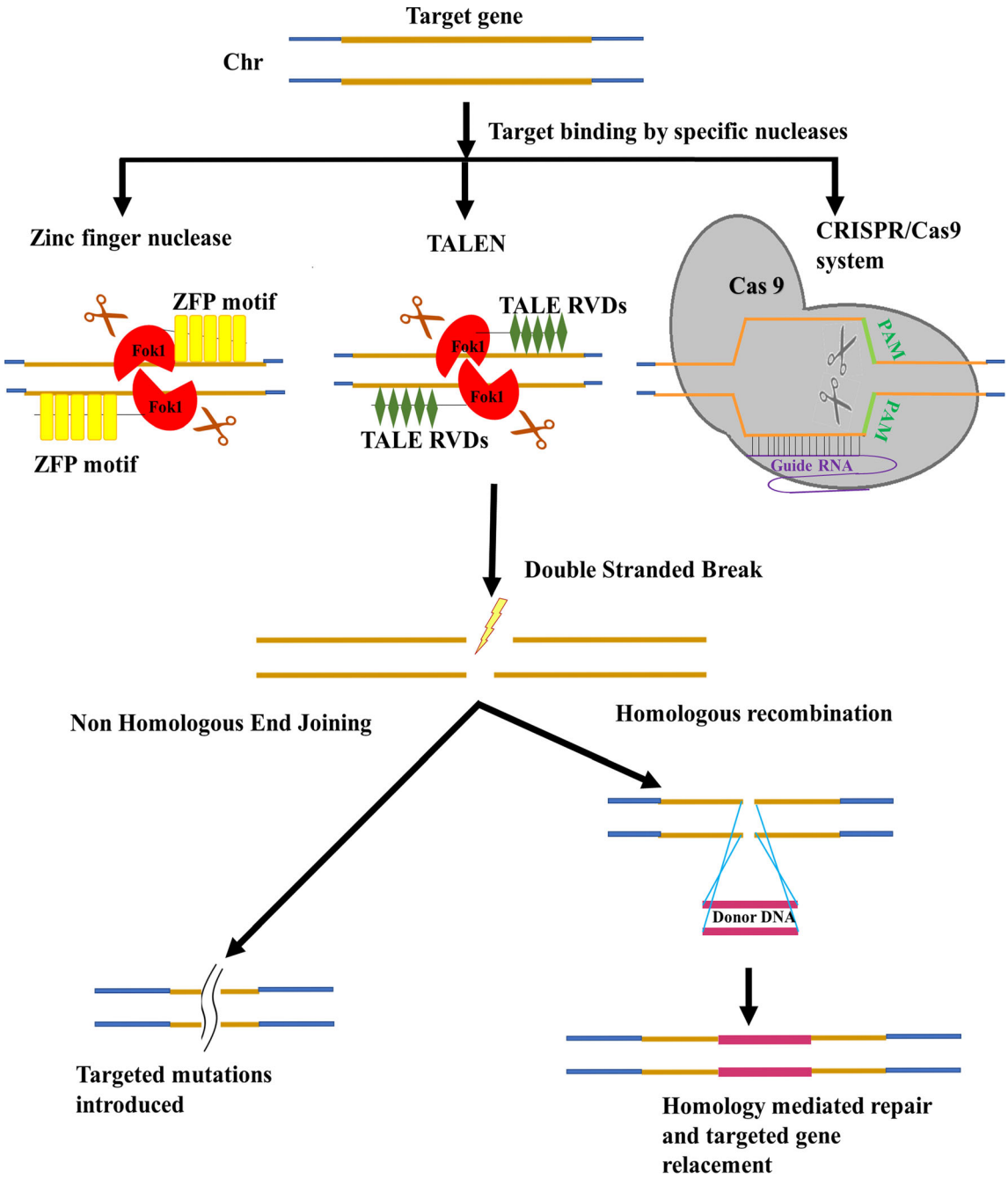

Figure 2. Tools for gene targeting: ZFNs, TALENs, CRISPR. First, the target sequence is recognized by these specific nucleases which then give rise to double stranded breaks (DSBs). DSBs can undergo either homology directed repair leading to target gene replacement or introduction of gene of interest via non-homologous end joining (NHEJ) that lead to targeted mutagenesis.

a site-specific manner using homology-directed repair of a targeted genomic DSB. In an attempt to assess the activity of ZFNs with respect to induction of mutations at specific sites in Arabidopsis (Lloyd *et al.* 2005), inserted a ZFN gene driven by a heat- shock promoter and its target into the plant genome. On inducing heat shock, ZFN expression occurred and resulted in high frequency mutations at ZFN recognition sites demonstrating the utility of ZFNs for targeted plant mutagenesis. ZFNs have also successfully induced mutagenesis in Arabidopsis at numerous gene loci such as the stress response regulator, ABA INSENSITIVE4 (ABI4) (Osakabe *et al.* 2010), ADH1 and TT4 genes (Zhang *et al.* 2010). Subsequently, ZFNs have been

used for targeted gene delivery in a number of other plant species. The notable among them being the use of ZFN to target IPK1 locus in maize (Shukla *et al.* 2009), which not only alters the inositol phosphate profile of developing seeds but can also be used to induce herbicide tolerance. ZFNs have also been used in soybean (Curtin *et al.* 2011; Bonawitz *et al.* 2019), tobacco (Townsend *et al.* 2009) and bread wheat (*Triticum aestivum*) (Ran *et al.* 2018) for manipulation of endogenous genes.

Therefore, from its development, ZFNs have served as effective tools for removal of endogenous gene function, targeted mutagenesis demonstrated by creating numerous gene knockouts, and gene deletion, with removal of gene segments both native and transgenic of up to 9 Mb size (Novak 2019). In addition to loss of gene function, but ZFNs have also demonstrated gain of function in some cases.

Although the ZFNs present numerous advantages, these engineered nucleases are prone to off target cleavage if the ZF domain is not accurately specific to the target site or if the target is not unique in the eukaryotic genome. Off-target nuclease activity may result in induction of DSBs at a frequency that can no longer be successfully repaired by the cells repair machinery leading to chromosome rearrangement or cell death or random transgene integration. Production of exogenous proteins may also lead to immunogenicity due to introduction of ZFNs (Durai *et al.* 2005).

An important advancement in ZFN technology is the development of Zinc-finger Nickases (ZFNickases), tailored by rendering inactive, the catalytic activity of one ZFN monomer in the ZFN dimer required for double-strand cleavage (Ramirez *et al.* 2012), thus inducing highly specific single stranded breaks (SSBs). These nucleases demonstrate a preference towards the HR mediated gene repair in contrast to the ZFNs that induce DSBs at a comparatively higher frequency but do not distinguish between HR and the error prone NHEJ. Therefore, ZFNickases can prevent off target effects displayed by ZFNs. Nevertheless, the fidelity of ZFNs and its easy availability and use makes it an important tool for gene targeting in plants.

2.3 *TALENs*

Transcription Effector like Nucleases (TALENs) are developed by fusing transcription activator-like effectors (TALEs) to the catalytic domain of the FokI endonuclease, that induce double stranded breaks (DSBs) at specific targeted sites in the genome. One of the challenges towards using ZFNs has been towards engineering new DNA binding specific ZF domains. TALENs were hence developed as an alternative to ZFNs (Moscou and Bogdanove 2009). TALENs are similar to ZFNs in containing the non-specific nuclease domain of *Fok1*and also a customizable DNA binding domain (Boch *et al.* 2009). The DNA-binding domain consist of highly conserved repeats acquired from transcription activator-like effectors (TALEs), which are proteins secreted by the plant pathogen *Xanthomonas* bacteria (Bogdanove *et al.* 2010). TALEs are the proteins introduced by *Xanthomonas* into the plant cells during infection through type III secretion pathway. The TALEs enter the plant cell nucleus and on binding effector-specific DNA sequences, transcriptionally activate expression of target genes which normally increase plant susceptibility to infection or may elicit plant defence.

TALEs bind DNA by their central regions consisting of 30 tandem repeats of a 33- to 35-amino-acid-sequence motif, the amino acid sequence of each repeat being largely invariant, with the exception of two adjacent amino acids (the repeat variable di-residue or RVD). Different DNA base pairs are recognized by repeats with different RVDs, there being one-to-one correspondence between the RVDs in the repeat domain and the nucleotides in the target DNA sequence, constituting a cipher using which targets of new TALEs can be correctly predicted (Romer *et al.* 2010). Functional targets of TALEs composed of randomly assembled repeats have been generated using this cipher. The capability of prediction of their DNA binding specificity allows rapid construction and utility of TALENs in targeting DSBs at premeditated locations within the complex plant genome.

TALENs have been used regularly as a choice of engineered nucleases for DSB induction in human cell lines and animal species but due to the considerable generation time in plants, few reports are available for successful use of these enzymes in plants. In 2012, Li *et al* demonstrated the use of TALEN for introduction of a mutation into the promoter of the OsSWEET14 gene, leading to enhanced disease resistance in rice. In another report TALENs were employed for targeted mutations within the ALS gene of transformed tobacco protoplasts (Li *et al.* 2012). Furthermore, using donor template an in-frame gene fusion between ALS and a YFP marker gene was created and on measuring the GT efficiency by quantifying YFP fluorescence, almost 14% of protoplasts showed fluorescence, therefore indicating a very high frequency of gene targeting. In the same study, TALEN and a 322-bp donor molecule differing by 6-bp from the ALS coding sequence was

introduced yielding even higher evidence of targeted gene transfer (Zhang *et al.* 2013). Recent reports of TALEN technology include the development of fragrant rice by targeted knockout of the OsBADH2 - gene (Shan *et al.* 2015), introduction of transgenes into potato plants (Forsyth *et al.* 2016) and glyco-engineering and monoclonal antibody production in *Nicotiana benthamiana* (Li *et al.* 2016). Therefore, TALENs can indeed be regarded as very convenient and effective tools to perform DSB-induced targeted gene transfer in plants.

2.4 *CRISPR*

The latest tool that has emerged for gene targeting is the RNA guided double strand break induction. This new technique is based on efficiency enhancement of Clustered Regularly Interspaced Short palindromic Repeats (CRISPR)/CRISPR-associated (Cas) type II prokaryotic system. CRISPR forms a part of bacterial and archaeal adaptive immunity against viruses and plasmids in the form of immunological memory of a previous infection (Barrangou 2015a; Barrangou 2015b).Expression of these sequences result in small non-coding RNAs or interfering CRISPR RNAs (crRNAs) which in turn guide the Cas endonucleases towards targeted sequences thus cleaving them. This seemingly simple prokaryotic defence system has been used for gene targeting and transcriptional manipulation in eukaryotes by customization of the short guide RNA and repurposing the Cas endonuclease (Jinek *et al.* 2012). At present two different endonucleases: Cas 9 and Cpf1 are used for CRISPR targeted genome modification.

2.4.1 *CRISPR/Cas9:* In recent years, the CRISPR/Cas9 system has emerged as the most widely used technique for accurate gene editing in eukaryotes. Cas 9 is a type II endonuclease from *Streptococcus pyogenes* originally utilizing a crRNA and a transactivating CRISPR RNA (tracrRNA) which has been bioengineered by fusion of the RNA molecules into a single guide RNA for efficient manipulation of the target gene of interest. It is now possible to target the recombinant Cas9 to any DNA region of interest by synthetic single-guide (sg) RNAs (Jinek *et al.* 2012) or crRNA (Mojica and Montoliu 2016).

CRISPR/Cas9 has been used to manipulate eukaryotic genome such as human cells, zebra-fish (Blackburn *et al.* 2013) and mice. This revolutionary technique has also been used in plants to generate a wide variety of benefits ranging from pest resistance, drought resistance to productivity improvement.

In 2013, Shan et al. demonstrated the use of customized single-guide RNA (sg-RNA) to direct Cas9 for inducing sequence-specific genome manipulations in two common crop plants rice (*Oryza sativa*) and wheat (*Triticunaestivum*)(Shan *et al.* 2015). Similar efforts by other molecular biologists resulted in development of *Nicotiana benthamiana*, Arabidopsis thaliana resistant to Beet severe curly top virus (Ji *et al.* 2015), *Solanum lycopersicum* resistant to tomato yellow leaf curl virus (Tashkandi *et al.* 2018), *Citrus sinensis* resistant to the bacterium *Xanthomonas citri subsp*. *Citri* (Peng *et al.* 2017) and *Oryza sativa* resistant to the fungus *Magnaportheoryzae* (Wang *et al.* 2016a PMID 27116122). CRISPR/Cas9 has also been utilized to increase the productivity of rice by increasing grain weight (Xu *et al.* 2016; Xu *et al.* 2017) and increasing blast resistance by targeting OsERF922 gene (Wang *et al.* 2016a, b). Likewise, Maize and soybean genomes have also been successfully modified using CRISPR/Cas9, for not only increasing agricultural efficiency (Chilcoat *et al.* 2017) but also for understanding the functions played by certain genes (Li *et al.* 2015; Svitashev *et al.* 2015; Svitashev *et al.* 2016; Char *et al.* 2017).

2.4.2 *CRISPR/Cpf1:* CRISPR/Cpf1 has been reported currently as the CRISPR nuclease-based technique that can enable more precise gene editing compared to previous Cas9 based methods. In this case, the CRISPR sequences are derived from the *Prevotella* and *Francisella* bacteria and Cpf1 is a RNA-guided type II endonuclease and a type V CRISPR effector. Processing, target site recognition as well as DNA cleavage is performed by a single Cpf1 molecule guided by a single crRNA, unlike Cas9 that requires a trans-acting crRNA. The Cpf1 protein being a ribonuclease, also processes precursor crRNAs. Another classical difference between Cas9 and Cpf1 is that Cpf1 recognizes T-rich PAM sequences located at 5' end of target DNA, upstream of protospacer sequence whereas Cas9 has no such preference (Kim *et al.* 2017).

Though CRISPR/Cpf1 is a very recent addition to gene targeting tools, it has been widely experimented with in plants. CRISPR/Cpf1 systems have been used for targeted genome editing in rice and tobacco plants (Endo *et al.* 2016; Xu *et al.* 2017). It has also been found as an efficient tool towards transcriptional repression in plants (Tang *et al.* 2017). Latest publications include use of CRISPR/Cpf1 for accurate genome editing in rice, maize, and Arabidopsis (Malzahn *et al.* 2019) and citrus (Jia *et al.* 2019). These

reports suggest that the accuracy of gene targeting can be increased by using this system.

3. Advanced tools for delivery of gene editing components into plant cell

In the last decade a considerable progress has been made in terms of development of techniques for gene targeting. But, the delivery of these gene editing components into the plant cells and their assessment for gene targeting continues to be a challenge (Baltes *et al.* 2014). Most common gene transfer methods for transient expression in plants include biolistics, Agrobacterium mediated transformation, infection with genetically engineered plant viruses and protoplast transformation. Biolistic method is a vector independent method and hence aids co-transformation. However, the particle gun device is very costly and this method can cause severe damage to the plant tissues. This method also has a low transformation efficiency which further add up to its demerits. On the other hand, Agrobacterium mediated gene transfer is a simple and cost effective method but is considered as a slow process and produces low yield. Genetically engineered plant viruses are widely used for the production of plant-based vaccines. Plant virus mediated infection produces high yield within a short period of time but once the vaccine is harvested, another plant needs to be infected and this reinfection procedure has to be continued for vaccine production in plants which turns out to be a disadvantage of this method of gene delivery. Protoplast transformation involves isolation of protoplast from mesophylls which is a difficult procedure (Laere *et al.* 2016). Protoplasts can respond differently from intact cells and hence make this method unsuitable for certain types of gene expression (Liu *et al.* 2018). Agroinfiltration is a modified technique of gene transfer which involves the infiltration of exogenous DNA-carrying Agrobacteria suspension into the intracellular spaces of plants. It is a rapid and cost effective method of transformation. Liu et al. highlighted the concept of stamping antigens onto leaves to generate vaccines using agroinfiltration technique. They developed SEB vaccines by agroinfiltrating SEB genes into the leaves of radish (Liu *et al.* 2018). Chemical additives, heat shock and co-expression of genes known for suppression of stress and gene silencing improves agroinfiltration-based transient gene expression (Norkunas *et al.* 2018). Recently certain other methods have also come to light allowing effective delivery of genes for targeted gene editing. Among these techniques are use of carrier proteins and nanotechnology that have revolutionized targeted gene delivery mechanisms (figure 3).

3.1 *Carrier proteins*

Plasmid DNA is a negatively charged biomolecule. They require masking of their negative charges and condensation into small particles for their efficient uptake into eukaryotic cells. This can be achieved by the use of polycationic reagents like poly L-Lysine and polyethylenimine. For efficient delivery of DNA to a specific target inside the cells, proteins can be coupled to these cationic carriers. These cationic carriers establish a contact between DNA and cell membrane which results in their uptake into the cell. Carrier proteins are well known for their potential as gene carriers for animal cells but their use for plant cells still remain the topic of profound research.

Small arginine rich intracellular peptides were used to deliver plasmid DNA into root tissue cells of mung bean (Chen *et al.* 2007). According to Chugh *et al.* (Chugh and Eudes 2008), treatment of wheat immature embryos with toluene/ethanol (1:20, v/v with permeabilization buffer) showed enhanced uptake of cell penetrating peptides (CPPs). Fluorometric analysis has revealed high penetration ability of Tat monomer and dimer. Addition of lipofectamine with Tat_2-plasmid DNA complex and permeabilizing agent has showed even better results. CPP can mediate delivery of T-DNA complex into the single locus of *Triticale* genome resulting in integration of intact transgene (Ziemienowicz *et al.* 2012). Peptides with higher pI value have stronger ionic interactions which stabilise p-DNA and polycation sequences of fusion peptides are required for condensation of p-DNA. Transfection efficiencies of R9-Bp 100, $(KH)_9$-Bp 100 and R9-Tat_2 for both *Arabidopsis thaliana* and *Nicotiana benthamiana* were equally high (Lakshmanan *et al.* 2013). A fusion peptide consisting of histidine and lysine with a CPP Bp 100 has been proved to be a potential carrier of ds RNA for plant cells by Keiji Numata *et al.* (Numata *et al.* 2014). This ds RNA complex successfully infiltrated plant leaves to induce gene silencing. For delivery of exogenous DNA to mitochondria of *A. thaliana*, Jo Ann Chuah used a combination of mitochondrial targeting peptide and CPP (Chuah *et al.* 2015). A combination of Cytcox-(KH_9) and BP 100 was able to increase the gene expression exclusively in the mitochondrial compartment of *A. thaliana* cells (Chuah *et al.* 2016). J A Chuah used BPCH7, an

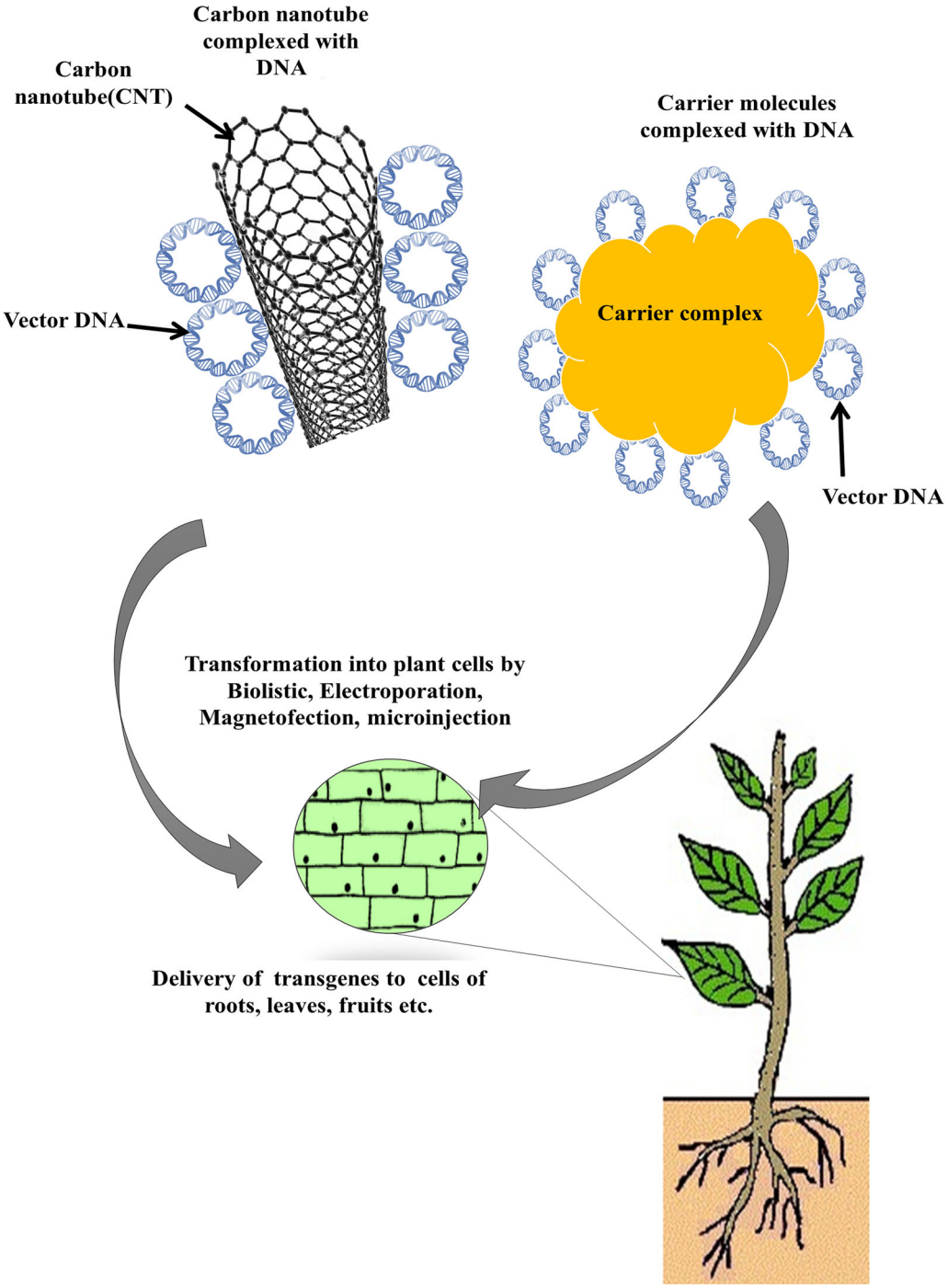

Figure 3. Advanced techniques for gene delivery into plant system. Carbon nanotubes and carrier molecules are the most recent developments. CNTs and Carriers complexed with DNA or RNA are delivered into plant roots, leaves or fruit cells via biolistics, electroporation, magnetofection, microinjection, etc., for highly efficient and accurate gene delivery.

efficient bioresponsive CPP for delivery of gene into plants. BPCH7 consists of a cyclic structure which binds to DNA with avidity. It is capable of efficient release of pDNA in both cytosol and nucleus of plant cell (Chuah and Numata 2018). These reports assure that proficiency of carrier protein complexes to deliver gene editing components into plant cells.

3.2 *Carbon nanotube (CNT)*

Synthesis and identification of carbon nanotubes were reported by Iijima (Iijima 1991) in early 1990s. CNTs are classified as single walled or multi-walled depending on the number of graphene sheets used. These are made by moulding graphene sheets in

specific angles, creating hollow structures of nanometre size.

For the first time in 2009, Liu et al. showed that single walled CNTs (SWCNTs) are capable of travelling across both plant cell wall and cell membrane (Liu *et al.* 2009). They can deliver pDNA inside plant cells and hence can be a potential nano-transporter for walled plant cells. Water soluble carbon nanotube treated gram has been shown to have better water absorption capacity and enhanced growth parameters (Tripathi *et al.* 2011). CNTs were found to do no harm to alfalfa and wheat plants rather their catalytic impurities like Fe species and Al_2O_3 promoted their growth. Plants were able to tolerate high amount of CNTs. Their germination and root development got enhanced in the presence of CNTs (Miralles *et al.* 2012). Chitosan wrapped SWCNTs can traverse plant cell walls, plasma membrane and double lipid bilayers of chloroplasts due to their high surface charge (Giraldo *et al.* 2014). In 2016, Ochoa-Olmos *et al.* made an attempt to deliver DNA in protoplast as well as walled plant cells of *Nicotiana tabacum* via single walled CNTs and multi-walled CNTs (MWCNTs) (Ochoa-olmos *et al.* 2016). SWCNTs were able to transform protoplasts as well as walled plant cells; on the other hand, MWCNTs were less efficient in gene transfer. CNT mediated gene silencing was shown to be transient in *Nicotiana benthamiana* plants (Demirer *et al.* 2019). When compared, Arg-SWCNTs were able to deliver pDNA successfully in tobacco root cells according to fluorescence microscopy images and western blot analysis whereas in case of chimeric peptides despite of positive results in fluorescence microscopy, gene expression in cells were not confirmed (Golestanipour *et al.* 2018).

Recently, in 2019, two individual research groups have used CNTs to deliver fluorescent protein coded pDNA in plant cells. A group from MIT, California has used chitosan complexed SWCNTs for delivery of pDNA to chloroplast. pDNA encoding YFP reporter gene was used to demonstrate chloroplast targeted gene delivery in arugula, spinach, watercress, tobacco plant as well as in *Arabidopdis thaliana* (Kwak *et al.* 2019). Another group from University of California, Berkeley demonstrated that CNTs can mediate species independent pDNA delivery. pDNA and polyethylenimine coated CNT treated leaves showed increase in GFP fluorescence intensity for three consecutive days which disappeared by day 10 (Demirer *et al.* 2019). Therefore, carbon nanotubes, with a scope of advancement are a strong contender for plant gene delivery.

4. Conclusion

This report explores the advancements in gene transfer as well as their delivery mechanisms in plants. Here, we have critically analysed the recently developed plant gene therapy techniques which have evolved owing to the increasing need for achievement of stably expressing plants. Among the tools of gene editing, CRISPR has outshone all others. Compared to other tools it is much efficient, specific and less time consuming. CRISPR-Cas9 technology is currently the simplest, most precise and versatile mode of gene editing.

Alongside these advancements in gene editing tools, there has been considerable progress in development of delivery machinery such as novel carrier molecules and carbon nanotubes. Carbon nanotubes have gained attention of many researchers not only because of its efficiency in gene delivery but also due to its ability to promote growth in plants with no potential toxicity to them.

The next big step would be using these CNTs to carry gene editing tools like CRISPR-Cas9 which can set a milestone in the course of development in gene delivery systems.

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