J Agric. Res., 2026, Vol. 64(1):27-47
www.jar.com.pk
Agriculture Department
Government of Punjab

ISSN: 2076-7897 (Online) / ISSN: 0368-1157 (Print)
DOI: https://doi.org/10.58475/2026.64.1.1275
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BIOTECHNOLOGY

CRISPR/CAS DELIVERY STRATEGIES IN ANIMALS AND PLANTS: CURRENT APPLICATIONS, LIMITATIONS AND FUTURE DIRECTIONS

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Article received on: 19/11/2025
Accepted for publication: 24/03/2026

ABSTRACT

CRISPR-Cas systems revolutionize genome editing with high precision. New variants like base and prime editors enable targeted nucleotide changes without double-strand breaks. This technology facilitates therapeutic gene correction, functional genomics, and applications in agriculture and biotechnology. Despite advances, delivering these systems remains challenging due to the complexity of components like Cas proteins and guide RNAs, impacting stability and uptake. Additionally, CRISPR has expanded into transcriptional regulation and molecular diagnostics but continues to face hurdles in delivery efficiency, safety, and specificity. Various delivery strategies have been investigated for in vivo and in vitro incorporation of the CRISPR/Cas components such as viral vectors, non-viral vectors, and physical methods. Each delivery tool has its applications and limitations, and selection of appropriate methods is necessary for treating several diseases. In plants, the widely used approach for delivering the CRISPR/Cas components is Agrobacterium-mediated transformation, whereas animal systems primarily utilize viral vectors as well as emerging nanoparticles-based carriers. This review provides a comprehensive overview of the current delivery methods with minimal drawbacks which cause efficient genome editing in both plants and animals.

KEYWORDS: CRISPR/Cas9 System; genome editing; delivery tools; viral vectors; non-viral vectors; Pakistan

INTRODUCTION

CRISPR-Cas System: Prior to the CRISPR/Cas system (also referred to as a third-generation genome editing tool), ZFNs and TALENs were the most widely utilized gene-editing techniques. These two methods have some drawbacks, including high off-target rate, high cost, labour consumption, limited editability, high cytotoxicity, and time consumption. The CRISPR/Cas system is easier to design, less expensive, more effective at targeting, less expensive, less off-target, and less cytotoxic than TALENs and ZFNs. Additionally, this method may modify a wide variety of genes both in vitro and in vivo (Bharathkumar *et al*., 2022). CRISPR/Cas9 technology originated from the bacterial adaptive immune system and protects cells against invading genetic material. This technology involves the use of Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and CRISPR-associated protein 9 (Cas9). It has been considered a safe approach for editing the genome (Doudna and Charpentier, 2014; Hsu *et al.,* 2014; Tabebordbar *et al.,* 2016). CRISPR-Cas technology has two components: Cas9 nuclease and a gRNA (guide RNA). Cas9 requires a complementary RNA for site-specific editing (Sternberg and Doudna, 2015). The Cas9 protein from *Streptococcus pyogenes* is a DNA endonuclease that creates double-stranded breaks in target DNA and also named as genetic scissor. It comprises of the recognition (REC) lobe, which binds guide RNA, and the nuclease (NUC) lobe, containing RuvC and HNH domains for DNA cleavage and PAM (Protospacer Adjacent Motif) for target DNA binding (Nishimasu *et al.,* 2014). Guide RNA is composed of CRISPR RNA (crRNA) and trans-activating CRISPR RNA (tracrRNA). The crRNA, which is 18–20 base pairs long, recognizes the specific DNA sequence by matching with the target sequence, while tracrRNA is a series of loops that function as a binding platform for Cas9 nuclease. Prokaryotes utilize guide RNA to direct viral DNA targeting, while in gene editing technology, synthetic construction of single guide RNA (sgRNA) is possible by merging crRNA and tracrRNA to target various gene sequences for editing (Mei *et al.,* 2016).

Rapid advancements in CRISPR/Cas9 technology have led to its application in both animal and plant

systems (Cong *et al.,* 2013). In animals, over the past 10 years, research on the CRISPR/Cas system has demonstrated its enormous promise for treating conditions including Alzheimer's disease, Huntington's disease, non-small-cell lung cancer (NSCLC), and cardiovascular disorders that are linked to genetic mutation or alteration. The CRISPR/Cas system is more significant in the field of biomedicine due to its enormous capabilities and possibilities in gene therapy (Huang *et al.,* 2022). In plants, this technology has been effectively used to increase crop resilience against a variety of biotic stresses, such as disease resistance, and abiotic challenges, such as heat, salt, and drought (Chennakesavulu *et al.,* 2022).

For efficient genome editing, the CRISPR/Cas components (Cas9 and gRNA) must be directly delivered to the target cell. When CRISPR/Cas components are introduced into the human body, they travel through the bloodstream where they undergo nuclease degradation and are eventually removed. The CRISPR components need to be structured to pass through the interstitial space and reach the suitable tissues and target cells, ensuring accurate genome modification and minimizing off-target risks. Therefore, the delivery strategies are essential for optimizing success rates and achieving accurate genome editing (Wang *et al.,* 2017).

Classification of CRISPR delivery technologies: Efficient delivery of the CRISPR/Cas9 complex into target cell nuclei is critical for its efficacy and can be achieved via plasmid DNA, messenger RNA, or ribonucleoprotein complex (RNP, Cas9 protein complexed with sgRNA) (Wang *et al.,* 2017).

CRISPR/Cas cargo; DNA, mRNA or RNP: CRISPR/Cas9 and gRNA Ribonuleoprotein (RNP) complex, as a cargo molecule, should be present in cell nucleus to achieve CRISPR-mediated genome editing. But direct delivery of Cas9 across cell membrane can create problems because of Cas9 large size (about 160kDa) and highly negative charge on the phosphate backbone of gRNA (Jinek *et al.,* 2014). Besides this approach of delivering protein, research has focused to direct the specific target cell to generate its own Cas9 protein *in situ* by administrating genetic material such as pDNA or mRNA, resulting in CRISPR-mediated genome editing (Liang *et al.,* 2015). There are pros and cons of these delivery formats (Table 1).

Plasmid DNA: Plasmid DNA, being both stable and inexpensive, makes Cas9 encoded with plasmid a cost-effective and efficient option. To effectively transport DNA cargo into eukaryotic cells, the DNA must have the ability to penetrate nuclear and cellular membranes of the target cell to allow for DNA transcription into mRNA and subsequent translation into Cas9 protein. Two other delivery formats exhibit faster expression kinetics than this one (Xu *et al.,* 2019). Delivery of plasmid DNA leads to a longer period of increased expression as compared to other delivery methods, making it advantageous for situations where sustained expression is needed. Extending the duration of plasmid activity leads to a rise in off-target incidents. Moreover, the plasmid DNA size of 10 kb is equal to 6400 kDa, which could impede the efficacy of in-vivo delivery. Delivery of plasmid results in an immunogenic response (Hughes *et al.,* 2009).

mRNA: In mRNA delivery format, Cas9 is encoded with

Table 1: Summary of Delivery modes of CRISPR/Cas9.

Delivery modes	Expression kinetics	Delivery tools	Stability rate	Advantages	Disadvantages	References
Plasmid DNA	slower kinetics of expression	Lipid nanoparticles, AAV, lentivirus, hydrodynamic injection, electroporation, and microinjection	Higher stability	Simple and easy to understand	immunogenic reaction, unintended consequences, Postponed Onset	Liu *et al.,* 2017; Liang *et al.,* 2015
mRNA	Transient expression, faster than plasmid DNA	Lipid nanoparticles, microinjection, and electroporation	Lower stability	Fast start, minimal chance of off-target effects, and no insertional mutagenesis	Quick Deterioration	Liu *et al.,* 2017; Liang *et al.,* 2015
RNP	More rapidly than other modes	DNA nanostructure, CPP delivery, polymer nanoparticles, lipid nanoparticles, iTOP, electroporation, and gold nanoparticles.	Moderate Stability	Minimal immunogenicity, barely off-target. No mutagenesis due to insertions. Partial genome editing	Expensive and structurally complex	Liu *et al.,* 2017; Liang *et al.,* 2015

mRNA instead of plasmid DNA. This Cas9-encoded mRNA is then translated directly into Cas9 protein in the cytoplasm instead of transcribing first as in DNA-encoded Cas9. Thus, this delivery format has quicker expression kinetics than that of DNA delivery mode (Liang *et al.,* 2015). The quicker onset of gene editing in mRNA leads to temporary expression of protein. This transience may assist in reducing off-target editing by tightly controlling the dose of Cas9 in cells, but it also suggests that long-term expression of Cas9 will cause certain problems. The stability of mRNA is lower than DNA which makes it more prone to deterioration by RNases. Thus choosing chemically modified RNA molecules to protect mRNA from degradation is useful as it helps in improving stability and expression inside the cell and genome editing efficiency (Pardi *et al.,* 2015; Wang *et al.,* 2013; Williams *et al.,* 2010; Zohra *et al.,* 2007; Li *et al.,* 2017).

RNP: The third delivery mode is RNP form, in which Cas9 protein (160kDa) and gRNA (34kDa) are combined to create a complex of RNP before entering the host cell (Kagita *et al.,* 2021). Once this complex enters the cell, it performs genome editing and a nuclear localization signal (NLS) delivers this complex to the nucleus. This form has the fastest kinetic expression than the other two delivery formats. However, caution is necessary when using this method, as the existence of gRNA in RNP could lead to deterioration, just as with mRNA delivery (Liang *et al.,* 2015).

Classification of delivery tools: For the transportation of cargo molecules to the desired target, we should choose a suitable delivery vehicle (carrier). It is very difficult to precisely and efficiently deliver CRISPR/Cas system to target's cell. To overcome the challenges mentioned above, delivery tools are classified into viral, non-viral, and physical delivery tools and these methods are used to introduce CRISPR components into animal and plant cells for modifying critical genes. Viral delivery tools include AAV vectors, AV vectors and LV vectors. Physical delivery tools include electroporation, sonoporation, microinjection and hydrodynamic injection. Non-viral delivery tools are liposomes, nanoparticles, cell-penetrating peptides and virus-like particles (Huang *et al*., 2022; Kazemian *et al*., 2022). CRISPR delivery strategies used in animals are summarized in Table 2 & 3 below.

In this review, we are going to highlight the uses and challenges of each delivery tools described above in animals and plants individually. This study will describe about the most suitable delivery tool in animals for therapeutic applications and in plants for crop improvement.

Delivery tools in animals: Viral Delivery Tools

Viruses have been proved as the most efficient vector for expression of Cas9 because viruses have evolved to effectively getting into cells inside host and create expression of foreign protein inside the host. The cargo molecule is protected from degradation by host enzymes by virion structure inside the host. For safety reasons, rep genes responsible for replication or other disease-causing genes have been removed from the vector. Therapeutic transgenes of interest are substituted for rep genes. Thus, viruses can still induce the expression of transgene by entering into host cell but cannot replicate itself (Naso *et al*., 2017; Ludwig, *et al*. 2004). Commonly used viral vectors are described below:

Adeno-associated viral vectors (AAV): Adeno-associated viruses are members of the Parvoviridae family. Their potential use as vector has attracted the attention of various researchers to use this tool as gene delivery systems (Samulski and Muzyczka, 2014; Naso *et al*., 2017). Currently, this in vivo delivery vehicle for CRISPR is effective due of its minimal cytotoxicity and limited integration (Verdera *et al*., 2020; Asmamaw and Zawdie, 2021).

A limitation of AAVs is its capability to integrate genes into host genome and it has been overcome by the development of recombinant AAV (rAAV) (Bijlani *et al*., 2022). Although AAV vectors have significant achievement in this field, their application is impeded by the limited cloning capacity of virus and its small size (5 kb) (Le Rhun *et al*., 2019). Scientists have created many approaches to overcome the cargo limit of AAV. First approach involves delivery of the sgRNA using AAV into cells that already express Cas9 protein. Other way is introducing the sgRNA and Cas9 packaged into distinct AAV with different tags into cells, followed by screening for effective co-transfection. However, high viral dose is required for this dual AAV delivery method which results in limiting therapeutic editing potential and raising safety concerns (Hayashi *et al*., 2020; Fang *et al*., 2021). Triple AAV system has been developed by scientists to deliver larger genes of 10 kb and 11.1 kb such as CDH23 (Usher syndrome) and DMD (muscular dystrophy) (Koo *et al*., 2014; Maddalena *et al*., 2018). It increases the cargo size capacity of AAV to 14 kb. Dual and triple AAV vectors have efficiency limitations (Trapani *et al*., 2021).

Another technique to overcome challenges of cargo size is to utilize smaller variants of Cas9 and different effector proteins instead of the Sp Cas9 (Uddin *et al*., 2020). Cholesterol regulatory gene Pcsk9 have been targeted by using Sa Cas9 in AAV vectors (Ran *et al*., 2015). Additionally, Cpf1 has gained attention as a substitute to Cas9 because of its versatile characteristics (Safari *et al*., 2019). In addition Cas13 proteins are being employed alongside viral vector

delivery methods (Gupta *et al*., 2022).

Intein-mediated trans-splicing is another method that increases the carrying capacity of AAV (Tornabene *et al*., 2019). Split inteins, which function similarly to introns in pre-mRNA splicing, facilitate protein trans-splicing (Wang *et al*., 2022). A concern regarding the application of AAV vectors is the pre-existing immunity that many people have developed against the bacterial Cas9 protein, which is derived from bacteria (Ibraheim *et al*., 2021). To resolve this issue, optimization and engineering of the AAV capsid protein coating have been explored (Ronzitti *et al*., 2020).

Recently, Tabebordbar and his colleagues have described about "myotube AAV or MyoAAV" which is a modified AAV formed by introducing a random peptide in AAV nucleus (Tabebordbar *et al*., 2021). Immunogenicity, cytotoxicity, long-term expression of Cas9/gRNA and incorporation of viral genome sequence are some limitations of AAV vector. AAV has a packing capacity of ~4.7 kb which is not suitable for larger Cas9 variants (Long *et al*., 2016).

Adenoviral vectors: Adenoviridae belongs to family of adenovirus. AdV-mediated delivery enables in both in vivo and in vitro genome editing (Ramos-Kuri *et al*., 2015). One benefit of adenoviral vectors is that rather than integrating into host genome, they produce an episomal DNA next to the target DNA after transduction and consequently this AdVs episomal gene expression lower the risk of off-target effects and insertional mutagenesis (Danthinne and Imperiale, 2000; Tatsis and Ertl, 2004; Li and Lieber, 2019). Successful targeted knock-in approach has been made possible through AdVs. The recombinant AdV5 vector, for example, successfully delivered the CRISPR/Cas9 system in a pre-clinical trial, leading knock-in of human AAT (alpha-1-antitrypsin) genes under in vivo conditions in mice (Stephens *et al*., 2018).

Adenovirus (AdVs) vectors can carry larger CRISPR cargo due to their lack of size limitations. Researchers have developed high capacity adenoviral (HC-AdVs) vectors, or gutless vectors, by deleting all viral coding genes (Tasca *et al*., 2020). AdVs are an attractive in vivo delivery system for CRISPR due to clinical safety records (Boucher *et al*., 2020).

The transient expression in AdV is considered beneficial for cargo delivery because it lowers the risk of off-target mutagenics. The versatile characteristics of AdVs like their safety in clinical trials, large scales manufacturing, and cost-effectiveness, make them invaluable for the delivery of CRISPR/Cas (Boucher *et al*., 2020; Srivastava *et al*., 2021). The immunological response elicited by AdVs vectors for delivering CRISPR components, however, is a significant problem. This immune reaction can result in inflammation in the tissues. High immunogenicity made repeated administration of AdV ineffective (Lee *et al*., 2017).

Lentiviral vectors: Lentivirus possess single-stranded genome and an enveloped RNA structure. Lentiviral vectors are enclosed within a capsid enriched with lipids and packaging capacity is around 9 kb. Most used lentivirus is HIV variant which considered as the standard vector for delivering target genome into dividing and non-dividing cells (Milone and O'Doherty, 2018). Unlike AAV vectors, lentiviral vectors provide greater cloning capacity and effectively deliver CRISPR/Cas components like Cas9 and sgRNA in one transfection. (Yip, 2020). Advantages of LVs include low intrinsic immunogenicity, cost-effective, safe for clinical applications. However, a major challenge is the random integration into the genome of the target cell, which can enhance proto-oncogene expression and lead to the transformation of cells (Behr *et al*., 2021). Lentiviral vectors enable efficient gene delivery, making them a perfect choice for expressing the entire CRISPR/Cas system in human cells (Shalem *et al*., 2014).

The LV-delivered CRISPR/Cas9 system has many applications. It targets HIV-1, HBV, genetically inherited diseases like cystic fibrosis, and neurological disorders (Choi *et al*., 2016). However, concerns exist regarding off-target effects from LV integration of CRISPR/Cas transgene, potentially leading to long-term gene expression. To reduce this risk, scientists created integration-deficient lentiviral vectors (IDLV). These vectors offer a temporary expression of the CRISPR/Cas system, minimizing the risk of off-target effects and providing a safer method of genome editing (Ortinski *et al*., 2017).

Researchers recently created non-integrating lentivirus vectors (NILVs) for introduction of the CRISPR components to reduce the possibility of integration. Additionally, NILVs have diverse applications in clinical settings, while also addressing infectious and genetic disorders (Rilo-Alvarez *et al*., 2021; Gurumoorthy *et al*., 2022).

The creation of replication-competent lentiviruses (RCLs) as a result of recombination with wild-type HIV in the case of HIV-infected individuals is another limitation linked with LV or IDLV. Different plasmids code limited LV vector-producing genes separately, reducing homologous sequences to lower RCL generation risk. Therefore, to further improve safety, it is beneficial to exclude viral genes from the LV vector (Segall and Sutton *et al*., 2003).

Physical delivery tools

Electroporation: The most common physical method for delivery of proteins and nucleic acid inside host cell is electroporation. This method utilizes high-

voltage electric current which temporarily made plasma membrane permeable permitting the entry of all cargo molecules of CRISPR/Cas9 into the cell (Rols, 2008). Electroporation has been utilized for in-vitro genome editing. For instance, when Cas9 and two different plasmid-encoded sgRNA are delivered via electroporation, it significantly results in gene deletion in Hematopoietic stem and Progenitor cells (HSPCs) and in primary T-cells (Mandal *et al*., 2014).

Technological solutions have been developed by several groups to enhance the electroporation prominence in delivering CRISPR/Cas9 reagents. For ex-vivo genome editing therapy, ablation of CCR5 (C--motif chemokine receptor 5) gene via electroporation of Cas9 RNP was reported by Xu *et al*. (2019), and then this gene was transplanted for treatment of HIV infection (Xu *et al*., 2019). Uses of electroporation for in-vivo genome editing has been reported. In in-vivo genome editing therapy, electroporation is done on smaller organs and at fetus stage which might have some ethical issues, so electroporation methods for in-vivo genome editing require optimization and safety assessment (Taha *et al*., 2022).

Microinjection: Microinjection involves directly injecting plasmid DNA, mRNA, or Cas9 protein with sgRNA into specific cells by breaching the plasma membrane using a microscope. Microinjection has its applications only in in-vitro and ex vivo editing of genome. The most widely used cargo delivered by microinjection is nucleic acid. There are pros and cons of these methods. Chuang *et al*. (2017) and Nakagawa *et al*. (2015) used this method to prevent time-consuming transcription reactions in vitro. However, ssDNA results in off-target effects. In case of delivering mRNA, it is better to deliver sgRNA and Cas9-encoding mRNA separately into the nucleus and cytoplasm to make it easier for Cas9 to translate and go to the nucleus. This method is difficult and time-consuming as it is impractical to inject two distinct microinjections into a single cell (Yang *et al*., 2013). Thus, CRISPR/Cas9-encoding mRNA components are delivered into the cytoplasm via microinjection. Ma *et al*. utilized microinjection for knocking out four genes simultaneously in rat zygotes from a single injection (Ma *et al*., 2014). As mRNA is prone to degradation in eukaryotic cells, so CRISPR/Cas9 components delivered via microinjection has limited duration of activity in eukaryotic cell (Ross, 1995). This is often useful as it lowers the potential of off-target events. Large size of cargo can be delivered through microinjection. Animal models can be generated by using microinjection. Delivery of gene editing cargo into zygote via microinjection permits germline editing (Horii *et al*., 2014). Microinjection is not useful for processing of large number of cells because it can target only one cell per injection (Ross, 1995).

Sonoporation: Sonoporation involves combining appropriate amount of cargo molecule and microbubbles then add mixture to target cells after target cell is exposed to ultrasound. Ultrasound waves causes the rupturing of microbubbles and formation of transient pores in the plasma membrane which permits the cargo molecule to enter into target cell (Lentacker *et al*., 2014). For example, Ryu *et al*. delivered Cas9 DNA plasmid by applying the ultrasound-activated particles for hair growth recovery in mouse by destroying the (SRD5A2) gene (Ryu *et al*., 2020). In another example, gene encoding the epidermal growth factor receptor 2 (C-erbB-2) was knocked out in (HEC)-1A cells by utilizing ultrasound microbubble to deliver CRISPR-Cas 9 plasmid (Cai *et al*., 2019).

Hydrodynamic delivery: Hydrodynamic delivery is utilized for in vivo genome editing for large macromolecules to deliver into target cell with the use of hydrodynamic injection. This sudden increase in volume creates transient pores, permitting the entry of cargo molecule into target cell. Delivery of plasmids encoding Cas 9 and gRNA into the lungs, liver, heart, and kidney tissue has been successfully done by hydrodynamic injection via the tail vein in mice (Yin *et al*., 2014; Bonamassa *et al*., 2011; Kamimura *et al*., 2015). Yin *et al*. (2014) employed hydrodynamic delivery to correct Fah mutation in mouse hepatocytes using Cas9 and sgRNA plasmid. The liver's ability to regenerate itself approved for the proliferation of the engineered cells and phenotypic rescue (Yin *et al*., 2014). Despite the fact that hydrodynamic delivery is feasible for genome editing of cells in adult animals, there are presently no clinical uses for this approach. Hydrodynamic delivery can cause certain physiological complications (Suda *et al*., 2007; Bonamassa *et al*., 2011). Low transfection rates and successful delivery in only certain cell types are other limitations of hydrodynamic delivery (Lino *et al*., 2018).

Non-viral vectors

Virus-like particles (VLP): Designer nucleases like ZFN, TALEN, and CRISPR/Cas are utilized in mechanistic research, animal model creation, and gene therapy. Challenges remain with off-target effects and immune responses, making transient expression and advanced delivery methods critical for clinical applications. Virus-like particles have garnered significant interest as delivery vehicles for the transient expression of designer nucleases (Lyu and Lu, 2022). Initially, VLPs were utilized for protein delivery in viral-particle tracking and cell elimination (Kaczmarczyk *et al*., 2011; Peretti *et al*., 2005; Joo and Wang, 2008). Their application for transient expression of genome

editing effectors led to using VLPs for transporting designer nucleases proteins or ribonulceoproteins (RNPs). Two strategies emerged to package cargoes into the particles: the fusion strategy and the aptamer/ABP interaction strategy (Lyu and Lu, 2022).

The absence of viral genomic nucleic acid and non-replication property of VLP classify it as a non-viral delivery technology. Expression of CRISPR/Cas9 system is transient in VLP for genome editing therapy and gained attention because of its potential to develop vaccine. Off-target effects caused by prolonged Cas9 expression can be avoided. Pre-packaging of Cas9 protein in lentiviral particles by Cas9P LV, passive incorporation of SpCas9 by VEsiCas system, Nano-Blades system, or Gesicle system are some of the example of VLP system developed recently. Another VLP method has been developed called NanoMEDIC (Lyu *et al*., 2020).

Polymer-based nanoparticles: Commonly used non-viral method for transporting of the CRISPR/Cas 9 components is polymeric vector. Polyethylenimine (PEI), poly (L-lysine), poly [2-(dimethylamino) ethyl methacrylate] (PDMAEMA), polyamidoamine (PAMAM) dendrimers are cationic polymers and are efficient than viral vector because of its strong biocompatibility, biodegradability, minimal immunogenicity, and gene therapy uses (Chen *et al*., 2020). PEI of high molecular weight has high cytotoxicity but are efficient while PEI of low mass is less efficient but also rarely cytotoxic. Thus, during the formation of PEI-based vector, the major concern is to balance strong transfection capacity with minimal cell toxicity of PEI (Kunath *et al*., 2003; Liechty *et al*., 2010; Petersen *et al*., 2002). It was proved that by modifying PEI, transfection efficiency can be improved and cytotoxicity can be minimized. One of the most commonly used method was PEGylation (Neu *et al*., 2007).

Cationic polymeric vectors can cause higher toxic effects (Yin *et al*., 2016). According to researchers, this limitation can be resolved by modifying cationic polymeric vectors (Kaczmarek *et al*., 2016; Miller *et al*., 2017; Sun *et al*., 2015; Zhang *et al*., 2019). Previous studies have demonstrated that the CRISPR-mediated genome editing material for a target-specific antimicrobial can be delivered with higher efficiency by using polymer-based nanoparticles. Recently, a CRISPR nanoparticles was produced by covalently conjugating cationic polymer bPEI to Cas9-sgRNA complex. This polymer-conjugated Cas9 cause its target gene to undergo DSB by maintaining its nuclease activity. Delivery of CRISPR/Cas9 system to MRSA for effective editing of target genome was done by utilizing polymer-based nanoparticle (Kang *et al*., 2017).

Lipid-based nanoparticles: Liposomes and lipid-based nanoparticles are the mostly used cationic lipid-based vectors. Lipid NPs are the most efficient delivery approach for nucleic acids and consist of hydrophilic head and hydrophobic tail domain. Some of the lipid-nanoparticles have their application in clinical trials for RNAi therapy (Fitzgerald *et al*., 2014; Coelho *et al*., 2013). Lipid NPs are formed when conjugation of nucleic acids that are negatively charged and lipids that are positively charged have done through electrostatic interaction, which prevent nucleic acid degradation by nucleases. Lipid nanoparticles have its application in delivering CRISPR-Cas9 system to various target cells (Cong *et al*., 2013; Mali *et al*., 2013) and also utilized for clinical therapeutic purposes or to establish knockout animal models (Raghavan *et al*., 2016; Platt *et al*., 2014).

To go beyond the limitations of lipid nanoparticles, such as risk of toxicity, lower stability, lower gene editing efficiency, and delivery of commercially cationic lipids at non-specific sites, considerable modifications have been investigated. Many studies have reported the betterment in the efficiency of transfection by chemical alteration of lipids and lowering the risk of toxicity of cationic lipids by the structural alteration of ammonium groups, such as DOTAP (Chen *et al*., 2020; Wang *et al*., 2017), and DOPE (Li *et al*., 2015; Miller *et al*., 2017; Wang *et al*., 2017), etc. Additionally, gene editing efficiency of RNPs is higher and potential of off-target effects is reduced as compared to CRISPR/Cas9 that is encoded by plasmid. Thus, for developing new lipid nanoparticles for RNPs, considerable efforts have been devoted (Liu *et al*., 2018; Wang *et al*., 2017; Wang *et al*., 2017).

Gold nanoparticles: RNP is delivered by a unique delivery technique known as gold nanoparticles (Wang *et al*., 2017). Transient gene editing in vitro is achieved by this method. However, the effectiveness of this system in primary cells of humans have not been observed by researchers till now. Research on mice has demonstrated that cells in which complex of CRISPR and RNP and donor DNA delivered in vivo, both CRISPR technology and HDR editing with higher efficiency and lower off-target effects is caused by AuNP (Lee *et al*., 2017).

Researchers have also showed that gene editing in brains of different mice can be done having low toxicity by using CRISPR-Au RNP delivery vectors (Mout and Rotello, 2017). Application of AuNPs in biomedical science is very vast. Gold nanoparticles can be utilized in in-vitro, ex vivo and in-vivo gene editing system. Targeted gene editing and efficient CRISPR/Cas9 delivery is achieved by using this method of delivery. Mout *et al*. delivered Cas9 protein which is modified chemically and sgRNA by designing ArgNPs (cationic

arginine-functionalized gold nanoparticles). High cytoplasmic/nuclear delivery efficiency approximately ninety percent and efficiency of editing gene ranging from 23-30% was achieved by this Cas9 protein delivery system (Yang *et al*., 2013; Mout *et al*., 2017; Mout and Rotello, 2017).

AuNP-CRISPR technology can efficiently and precisely edit specific brain cells. Not only diseases in which only single gene is mutated but also polygenic diseases can be treated like Huntington's disease with AuNP-CRISPR technology by using dual sgRNA. Additionally, AuNPs exert no immunogenic response (Lee *et al*., 2017). Biological effects can be monitored in real-time while editing genes by using AuNCs. CRISPR technology are broadly utilized in diagnostics and therapy by developing more AuNC-based nanocomposites (Tao *et al*., 2021).

Chitosan: Chitosan has many uses in industries of food and bioengineering, in agriculture and as a carrier for controlled drug delivery. Biodegrability, non-toxicity, bioactivity, polycationic nature and biocompatibility are characteristics of chitosan (Divya and Jisha, 2018). The property that makes chitosan suitable for drug delivery uses is its capability to produce gel when contact with negatively charged particles and then form beads. However, the limitation of chitosan is the enormous size of these beads typically ranging from 1-2mm (Shiraishi *et al*., 1993). Ohya and his colleagues firstly discovered chitosan nanoparticles (ChNP) in 1994. They prepared chitosan nanoparticles by emulsification and cross-linking of chitosan which further utilized to deliver 5-fluorouracil, an anticancer drug through the bloodstream (Grenha, 2012).

For delivering RNP and donor DNA complex into cells for HDR (Homology-directed Repair), Chitosan had been suggested as a polymeric carrier. Free CS polymer has proved unsuccessful in efficiently encapsulating and delivering the Cas9 RNP complexes (Qiao *et al*., 2019). To overcome this problem, RFP@CS nanocomplexes were created by combining a negatively charged red fluorescent protein with CS polymer. Donor DNA and glutamate-modified Cas9 protein were then complexed with RFP@CS, enabling gene editing in multiple cell types, achieving HDR efficiency similar to CRISPRMAX and targeting gene PRDX4. Furthermore, RFP provides a fluorescent marker in nanoassemblies to track the delivery of RNP inside the cells (Zhang *et al*., 2021).

These nanoparticles are utilized in effective drug delivery and improve the therapeutic efficiency of the drugs. Chitosan nanoparticles are utilized in ocular, oral, pulmonary, and nasal drug delivery due to their mucoadhesive and in-situ gelling properties. But a thorough investigation of the toxicity and safety concerns surrounding chitosan nanoparticles and their production process is necessary (Garg *et al*., 2019).

Cell-penetrating peptides: CPPs (Cell penetrating peptides) are also referred as PTDs (protein transduction domains), and have emerged as versatile tools for transporting a variety of cargo across cellular membranes. These cargoes include full-length proteins, tiny peptides, full-length proteins, liposomes, viral particles, nucleic acids such as RNA and DNA, nanoparticles, and even radioisotopes and fluorescent probes used for imaging purposes (Taylor and Zahid, 2020).

CPPs has been used in in vitro and ex vivo genome editing. A novel approach by Ramakrishna *et al*. 2014 involves the treatment of human cell lines with CPP-complexed guide RNAs and CPP-conjugated recombinant Cas9 protein, resulting in successful disruption of endogenous genes (Ramakrishna *et al*., 2014). Previous researches indicate that in order to achieve gene-editing effect, conjugation of both Cas 9 protein and sgRNA is necessary (Suresh *et al*., 2017; Worthen *et al*., 2017). Certain limitations of CPP hinder their clinical application. First, CPP has a brief plasma half-life and is unstable by proteolytic enzymes. This causes CPP to deteriorate before reaching the target tissue. Endosomal escape is another limitation even after cellular entry. Second limitation is decreased specificity of cell of CPP that may cause systemic toxicity and efficiency of therapeutic is limited. (Taha *et al*., 2022).

DNA nanostructure: In cells, DNA nanostructure shows potential as a means of delivery tool for molecular payloads. DNA origami structures, compact nanostructures formed by folding long single-stranded DNA, are attractive for gene packaging. However, delivering genetic material into cell nuclei remains a challenge. To address this, DNA nanostructures with CRISPR-Cas9 binding sites were designed for enhanced nuclear shuttling and successful integration through homology-directed repair. Efficient shuttling and integration were demonstrated using transfection, electroporation, and virus-like particles. As compared to unstructured DNA templates, these nanostructures demonstrated greater insertion efficiency and reduced toxicity, making them valuable for gene delivery and potential applications in biosensing within cell nuclei (Lin-Shiao *et al*., 2022).

The potential of rolling circle amplification (RCA)-synthesized nucleic acid nanostructures, such as DNA nanoclews, in medication (Sun *et al*., 2014) and gene delivery (Ruan *et al*., 2018) has drawn interest. The great biocompatibility, predictability, programmability, and ease of functionalization of these nanoclews are among its benefits. All things considered, this

method offers a viable way to deliver therapeutic payloads with DNA nanoclews in an effective manner (Sun *et al*., 2015).

DNA nanoclews are that nanoparticles which are created by rolling circle amplification and composed of DNA cage. In U2OS.EGFP cells, mutation frequency of approximately 28% was achieved by utilizing this technology. Moreover, after intra-tumor injection in vivo destruction of 25% of the EGFP is due to this technology (Liu *et al*., 2017). In particular, more research is necessary to determine potential immunogenicity of DNA nanoclews. However, primary outcomes for this innovative delivery method are encouraging (Lino *et al*., 2018).

Induced transduction by osmocytosis and propanebetaine (iTOP): D'Astolfo *et al*. (2015) reported a CPP-independent delivery method for gene editing system called iTOP (D'Astolfo *et al*., 2015). For delivering endogenous proteins and additional substances into cells, iTOP is considered a novel method. The iTOP method, which stands for "induced transduction by osmocytosis and propanebetaine," makes use of propanebetaine, a transduction agent, and hyperosmolality generated by NaCl to promote macropinocytosis, the process by which extracellular macromolecules are taken up by cells. Despite being a relatively novel delivery method, iTOP's uses have thus far mostly been restricted to in vitro environments. In a research, CRISPR/Cas9 ribonucleoprotein (RNP) was introduced into primary human KBM7 and H1 cells by iTOP. This led to a gene knockout efficiency of about 70% and the acquisition of resistance to diphtheria toxin. For direct delivery of RNPs or individual delivery of the Cas 9 protein and sgRNA's, iTOP transduction is considered effective (Lino *et al*., 2018). iTOP causes reduced effectiveness of editing of gene in primary cells (Zuris *et al*., 2015; Kim *et al*., 2014; D'Astolfo *et al*., 2015; Ramakrishna *et al*., 2014; Suresh *et al*., 2017) . Additionally, the Cas9 protein is not appropriate for in vivo applications (Liu *et al*., 2017). CRISPR delivery strategies in animals are illustrated in figure 1 below.

Various delivery tools are used to introduce CRISPR components into animal cells including, viral vectors (AAV, AdV, and lentivirus), non-viral nanoparticles (polymer nanoparticles, lipid nanoparticles, and gold nanoparticles), physical delivery tools (electroporation, microinjection, sonoporation, and hydrodynamic delivery), chemical delivery (iTOP, cell-penetrating peptides) and DNA nanostructure. These delivery tools allow the transport of Cas nucleases and gRNA into host cells for efficient gene editing.

Delivery tools in plants: The effective delivery of the CRISPR/Cas system into plants is critical for efficient genome editing. As advancements continue, thorough consideration of the delivery and expression of the CRISPR/Cas components within plant cells is crucial for achieving efficient and safe genome editing outcomes (Kuluev *et al*., 2019). Several methods of delivering CRISPR/Cas components into plants have utilized for effective delivery of CRISPR/Cas9-mediated genome editing. These include direct methods like, physical (protoplast-mediated transfection and microprojectile bombardment) and chemical vectors (liposomes, exosomes) and indirect methods like, agrobacterium-mediated transformation and viral vectors. These methods are dependent on plasmids, RNP or viruses to deliver the target sequences into the plant cell. Viral and non-viral delivery vectors induce the expression of nuclease in target cells. In contrast, physical delivery tools can also be utilized for transferring nuclease into target cells. Delivery of CRISPR can be in vivo and in vitro. Viral vectors have limitations like carcinogenesis and immunogenicity, unlike non-viral vectors. Plants have unique characteristics that limit applying animal cell methods, necessitating the creation of plant-specific approaches (Prestwich *et al*., 2023). CRISPR delivery tools in plants are illustrated in Figure 2 below.

Direct methods: Most widely used methods for transient expression of genes are direct gene delivery methods. Direct methods may also be used for stable transformation. Many scientists opt for direct method as compared to indirect gene transfer method because of its higher transformation efficiencies (Klein, 2011).

Protoplast-mediated transformation: Cell wall of plants digested with enzymes and editing reagents to obtain plant protoplast which can be delivered via polyethylene glycol (PEG) treatment. With editing efficiency ranging from 3% to 46%, transfection of CRISPR/Cas components into protoplasts and consequent plant regeneration enabled the successful introduction of mutations in a variety of crops, including potato, wheat, rice, soybean, A. thaliana, etc. (Xie and Yang *et al*., 2013; Feng *et al*., 2014; Butt *et al*., 2017; Malnoy *et al*., 2016; Andersson *et al*., 2018; Woo *et al*., 2015; Kim *et al*., 2017; Gil-Humanes *et al*., 2017). Delivering several cargo molecules to a huge population of transfectable cells and producing DNA-free plants have done through this method (Cho *et al*., 2013). Regulation may not be prevented despite using exogenous DNA templates for knocking in. Further protoplast transfection is limited by plant regeneration and potential soma clonal mutations (Cho *et al*., 2013).

Biolistic bombardment: Biolistic bombardment is another technique for delivering components of CRISPR/Cas into plants. It is composed of micro projectiles generally coated with tungsten, silver, or gold particles which penetrate the cell wall by firing with high pressure into plant cells. Biolistic bombardment

Table 2: Summary of CRISPR delivery tools in animals

Delivery	Delivery vehicle	Composition	Delivery cargo	Packaging capacity	Immuno-genicity	Advantages	Dis-advantages	References
Viral vectors	Adeno-associated virus(ADV)	Non-enveloped, ssDNA	Plasmid DNA	4.7kb nucleic acid	Low	Very little immunogenicity High efficacy of transduction	Low capacity for packaging	Yang *et al.,* 2013; Long *et al.,* 2016; Carroll *et al.,* 2016; Platt *et al.,* 2014
	Adenovirus	Non-enveloped, dsDNA	Plasmid DNA	8kb nucleic acid	High	High-efficiency delivery	Inflammatory reaction, challenging production scaling	Voets *et al.,* 2017; Maddalo *et al.,* 2014; Wang *et al.,* 2015; Ding *et al.,* 2014
	Lentivirus	Enveloped, ssRNA	Plasmid DNA	~10kb	Moderate-high	Gene transfer that is persistent	Gene rearrangement susceptibility and transgene silencing	Shalem *et al.,* 2014; Platt *et al.,* 2014; Heckl *et al.,* 2014
Physical Vectors	Electro-poration	Electric current	Plasmid DNA; mRNA	nm levels of sgRNA and Cas9	No	Delivery to the population of cells	Only in vitro, certain cells are resistant to Cytotoxic	Hashimoto and Takemoto, 2015; Chen *et al.,* 2016; Qin *et al.,* 2015
	Micro-injection	Needle	Plasmid DNA; mRNA Protein (RNP)	Cas9 and sgRNA levels in nm	No	Delivery into the desired cell is guaranteed.	Inject just one cell, which takes time.	Yang *et al.,* 2013; Horii *et al.,* 2014; Chuang *et al.,* 2017
	Sono-poration	Ultrasound	Plasmid DNA	50 micrograms of plasmid DNA	No	Safe, simple, and less hazardous approach	Low transmission of genes efficiency and target cell damage	Tomizawa *et al.,* 2013
	Hydro-dynamic delivery	High pressure injection	Plasmid DNA; Protein (RNP)	nm levels of Cas9 and sgRNA	No	Low-cost, virus-free	Not utilized in clinical studies, non-specific	Yin *et al.,* 2014; Guan *et al.,* 2016; Xue *et al.,* 2014
Non-viral vectors	Virus-like particles	Viral structural proteins (Vpr)	mRNA; Protein (RNP)	nm levels of Cas9 and sgRNA	No	Decreased impacts off-target, Temporary mode of distribution	In vivo efficiency is lower.	Lyu *et al.,* 2020
	Cell-penetrating peptides	Short amino acid sequences	Plasmid DNA, Full length gene, Protein (RNP)	nm levels of Cas9 and sgRNA	No	liable for delivering intact RNP and free of viruses	Variable penetration effectiveness	Rama-Krishna *et al.,* 2014
	DNA nano-structure	DNA spheroid	Protein (RNP)	nm levels of Cas9 and sgRNA	No	Free of viruses	DNA template modification is necessary.	Sun *et al.,* 2015
	iTOP	Hyper-osmlality+ transduction compound	Protein (RNP)	nm levels of Cas9 and sgRNA	No	Highly successful and virus-free	Reduced gene editing effectiveness and no in vivo use	D'Astolfo *et al.*, 2015

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Table 3. Nanoparticles-based delivery tools in animals

Nano-particles	Composition	Delivery Cargo	Packaging Capacity	Immuno-genicity	Advantages	Dis-advantages	References
Polymeric nano-Particles	Polymers	Protein (RNP)	nm levels of Cas9 and sgRNA	Low	Safe and virus-free	Low effectiveness	Liu *et al.,* 2017
Lipid nano-Particles	Natural or Synthetic lipids	mRNA, Protein (RNP)	nm levels of Cas9 and sgRNA	No	Low-cost, virus-free	Cargo endosomal degradation and particular cell tropism	Yin *et al.,* 2016; Wang *et al.,* 2017; Zuris *et al.,* 2015
Gold nano-particles	Cationic arginine-coated AuNP	Protein (RNP)	nm levels of Cas9 and sgRNA	No	Membrane-fusion-like, inert delivery	Non-specific inflammatory reaction	Mout *et al.,* 2017, Lee *et al.,* 2017
Chitosan	Chitin, (1-4)-2-amino-2-deoxy-β-D-glucan	Proteins, Plasmid DNA and oligo-nucleotides.	nm levels of Cas9 and sgRNA	Low	Good bio-tolerability, low level of toxicity, durability, high specificity, ease of storage and transportation, biocompatibility, and biodegradability.	Expensive and poorly soluble.	Sharifi-Rad *et al.,* 2021

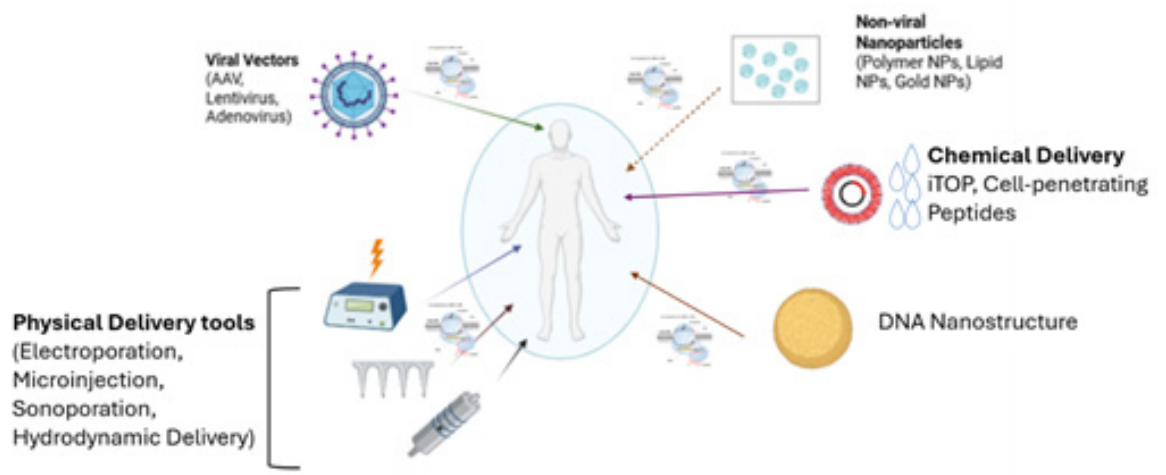


Fig 1. CRISPR delivery tools in animals

cause stable integration in rice, wheat, and soybean genomes, introducing targeted mutation in plants by carrying and delivering CRISPR/Cas9 reagents in plasmids by using gold particles (Shan *et al*., 2014; Li *et al*., 2015; Wang *et al*., 2014). Other studies showed transient expression of CRISPR/Cas9 DNA (TECCDNA) in wheat (Zhang *et al*., 2016). Biolistic bombardment, an efficient method, deliver several constructs simultaneously and used for various plant species. Major limitation includes random integration of several copies of the inserted genes within genomes. Also, it is expensive (Liang *et al*., 2017).

Electroporation: Electroporation uses high-field electrical pulses to create temporary pores in cell membranes, increasing their permeability (Saeed and Shahzad, 2015; Ozyigit, 2020). Protoplasts and DNA are incubated together, followed by the application of these pulses to generate pores in the target cell's membrane (Kotnik *et al*., 2019). A study conducted by Zhang *et al*. 2013 transformed

pollens of common wheat (Triticum aestivum L.) by electroporation and showed stable expression of transformants in transgenic progeny (Zhang *et al*., 2013). Electroporation-mediated transformation offers rapid, low-cost, stable transformation rate as compared to other transformation methods (Kar *et al*., 2018). Electroporation faces challenges in transforming thick-walled plant cells, limited receptor species, and intense electric pulses that can damage naked genes, resulting in inaccurate product translation (Saeed and Shahzad, 2015; Yan *et al*., 2022).

Nanoparticle-based carriers in CRISPR/CAS

exosome-like nanoparticle: Exosomes transmit signaling molecules, such as nucleic acids (mainly mRNA and microRNA), functional proteins, and lipids (Valadi *et al*., 2007; Li *et al*., 2016; Liu *et al*., 2015). Composition of exosome vesicles include proteins, lipids and nuclear components (Dad *et al*., 2021). It might be possible that within animal and plant tissues, these vesicles function as nanocarriers (Cai *et al*., 2018). Exosomes offer advantages over synthetic carriers due to their small size, low toxicity, natural targeting, and encapsulation of bioactive molecules while crossing physical barriers. They present high potential for delivering drugs, DNA, oligonucleotides, proteins, and peptides. Additionally, naturally and synthetically produced exosome-like nanoparticles show promise as biocompatible systems for drug delivery (Fernandes *et al*., 2020). The main disadvantages of the exosomes are its ineffective large nucleic acid encapsulation and loading capacity (Lin *et al*., 2018).

Liposomes nanoparticles: Liposomes are types of nanoparticles for delivering CRISPR constructs. Liposomes are spherical vesicles with phospholipid bilayers carrying compounds (Kwon *et al*., 2020; Qamar *et al*., 2019). Liposomes act as a vector for delivery of genetic material into cells of plant and have several advantages such as prevent nucleic acid degradation by nucleases, targeted delivery to specific cells, and delivery via entry through plasmodesmata (Wiesman *et al*., 2007; Gad *et al*., 1990). Liposome-based nano-biosensors monitor the low level of organo-phosphorus pesticides dichlorvos and paraoxon (Vamvakaki and Chaniotakis, 2007). Several kinds of materials can be delivered to plant cells by transfection agent such as liposomes (Felgner *et al*., 1987; Eoh and Gu, 2019). Nitric oxide can be delivered intracellularly by incorporation of cationic liposomes into membranes (Huang *et al*., 2009). Liposomes are restricted by their low-solubility, short half-life, expensive, and leakage and fusion of encapsulated drug/molecules (Himanshu *et al*., 2011).

Indirect methods: Many indirect carrier methods for stable and for transient transformation has application in plants. These methods include Agrobacterium-mediated transformation and viral vectors (Rao *et al*., 2009).

Agrobacterium-mediated transformation: Agrobacterium tumefaciens is a frequently utilized technique to produce transgenic plants and for delivering components of CRISPR/Cas into cells of plant. Agrobacterium transfer T-DNA to the nucleus of target cell. In Ti plasmid of Agrobacterium tumefacians, Cas9 and sgRNA expression cassettes are cloned, transformed into Agrobacterium and then introduced into plants. Viral transient expression with efficiency of gene editing 85% has been achieved in Nicotiana benthamiana, rice and Arabidopsis thaliana. Agrobacterium rhizogenes is useful in achieving stable integration in soybeans when components of CRISPR/Cas are delivered into cells of plants (Gelvin, 2017). Several advantages of Agrobacterium-mediated delivery includes, easy availability of technology in most laboratories required for Agrobacterium-mediated delivery, cheap method, delivery of multiple binary vectors into Agrobacterium and co-transformation into cells of plant thus, permits multiplex genome editing. The main drawback of this method is the utilization of binary vector. Small DNA fragments, RNA or proteins cannot be delivered by this method. Furthermore, Agrobacterium faces host range issues, requires large plasmid cloning, and is optimized for low-copy transgene integration, hindering transgene-free gene editing (Kocsisova and Coneva, 2023).

Virus-mediated delivery: In plants, viruses facilitate virus-induced gene silencing (VIGS) by delivering small molecules like siRNAs, viruses have been used to deliver mega nucleases and zinc finger nuclease for knocking out an individual gene (Laforest and Nadakuduti, 2022). This method delivers the viral genome via a T-DNA vector through Agrobacterium infiltration of leaves on intact plants. The T-DNA can integrate locally, allowing the expressed viral genome with CRISPR reagents to form viral particles that can spread systemically. This enables editing across multiple plant cells lacking the editing reagents' DNA. If meristematic cells are affected, editing can pass to embryos, yielding transgene-free edited plants without tissue culture (Kocsisova and Coneva, 2023).

The delivery of the CRISPR/Cas system into plant cells via viral vectors is hindered by capacity limitations of DNA and positive-strand RNA viruses. Due to the large size of CRISPR (typically < 4.5kb), negative-strand RNA viruses, such as Barley yellow striate mosaic virus (BYSMV) and Sonchus yellow net rhabdovirus (SYNV), have been employed to effectively deliver reagents of CRISPR/Cas and sgRNA in cells of plant (Ma *et al*., 2023). The primary benefit of viral vectors

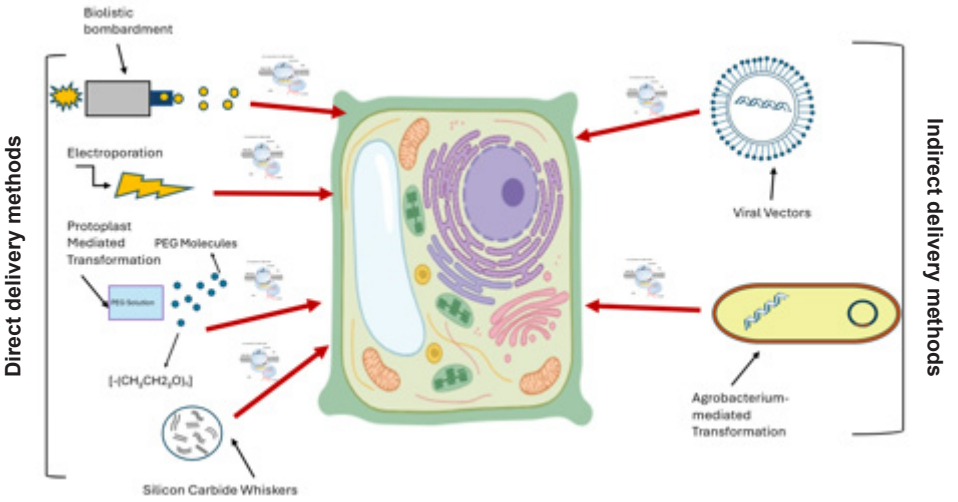


Fig 2. CRISPR delivery tools in plants.

is that they offer transgene-free and potentially tissue-culture free delivery of gene editing reagents. However, this technique faces many limitations, including a narrow host range and insufficient cargo capacity for entire expression cassettes for gRNAs and nucleases (Kocsisova and Coneva, 2023).

CRISPR components can be introduced into plant cell via two methods, direct and indirect method. Direct methods include biolistic bombardment, electroporation, protoplast mediated transformation, and silicon carbide whiskers, whereas indirect delivery methods involve viral vectors and agrobacterium-mediated transformation.

CONCLUSION

In this review, we highlighted the uses and drawbacks of several delivery tools used in animals and plants. In animals, viral vectors are most widely used but also have disadvantages like immunogenicity, toxicity, packaging capacity which favors off-target events. For this, use of nanoparticles is considered safer and more efficient for therapeutic purposes. On the other hand, plants use protoplast-mediated transformation, biolistic bombardment, nanoparticles viral vectors and Agrobacterium-mediated transformation for efficient genome editing in plants. The drawbacks of each delivery tools must be considered before use and should opt for delivery tool with minimal disadvantages.

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1.	Farwa Yaqub	Collected and organized the research material, and wrote the main content of the paper	
2.	Mahnoor Fatima	Reviewed the paper carefully and assisted with proofreading to ensure accuracy	
3.	Ayesha Waheed	Contributed by editing the paper and providing additional proofreading support.	
4.	Ahmad Munir	Guided the selection of the research topic and advised on the structure and writing of the paper	