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REVIEW

Approach for in vivo delivery of CRISPR/Cas system: a recent update and future prospect

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Abstract

The clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) system provides a groundbreaking genetic technology that allows scientists to modify genes by targeting specific genomic sites. Due to the relative simplicity and versatility of the CRISPR/Cas system, it has been extensively applied in human genetic research as well as in agricultural applications, such as improving crops. Since the gene editing activity of the CRISPR/Cas system largely depends on the efficiency of introducing the system into cells or tissues, an efficient and specific delivery system is critical for applying CRISPR/Cas technology. However, there are still some hurdles remaining for the translatability of CRISPR/Cas system. In this review, we summarized the approaches used for the delivery of the CRISPR/Cas system in mammals, plants, and aquacultures. We further discussed the aspects of delivery that can be improved to elevate the potential for CRISPR/Cas translatability

Keywords CRISPR/Cas · Gene editing · Gene delivery · Gene therapy

Introduction

The innovation of gene editing has enabled the precise modification of specific genomic regions in a wide variety of organisms. Gene editing is mainly accomplished using programmable nucleases that are highly specific. These nucleases create double-strand breaks (DSBs) in regions of interest of the genome. These DSBs are then repaired by nonhomologous end-joining (NHEJ), which is error-prone, or homology-directed repair (HDR), which is error-free; specific changes, such as insertions or deletions (indels), are thus introduced into desired regions of the genome [1–3]. By introducing HDR repair template, the defects in genes

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may be corrected, thus providing hope for correcting inherent errors in DNA.

A recent new programmable nuclease technology; clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas)-type II RNA-guided nucleases system [4], has revolutionized the scientific field of gene editing. Although the versatility and ease of construction and target design make CRISPR/Cas extremely attractive for breakthrough gene therapy achievements and crop improvement, there are still important limitations to consider [5, 6]. One of the obstacles is the immune response in animal systems; since the components of the CRISPR/Cas system are bacterially derived, this system is expected to trigger host immune responses. Another obstacle is the size of the components in the system, which are all macromolecules; thus, they are unable to spontaneously enter the cytosol and then the nucleus [6], which are essential for successful gene modification [7]. In addition, the large size of the CRISPR/Cas system may also make it difficult to package into delivery vehicles such as viral vectors. Another aspect of the difficulty of the CRISPR/Cas system is its stability. The CRISPR/Cas system needs to be highly stable and functional; otherwise, it will be degraded or eliminated during circulation in the targeted organs or tissues. Efficient delivery is one of the last major hurdles to overcome in CRISPR/Cas-mediated gene editing. As such, developing stable and effective delivery approaches is critical for its application. In this review, we summarized the approaches used for the delivery of the CRISPR/Cas system in different biological systems, including mammals, aquacultures, and plants (Fig. 1). We also discussed the aspects of delivery that can be improved to elevate the potential translatability the CRISPR/Cas system.

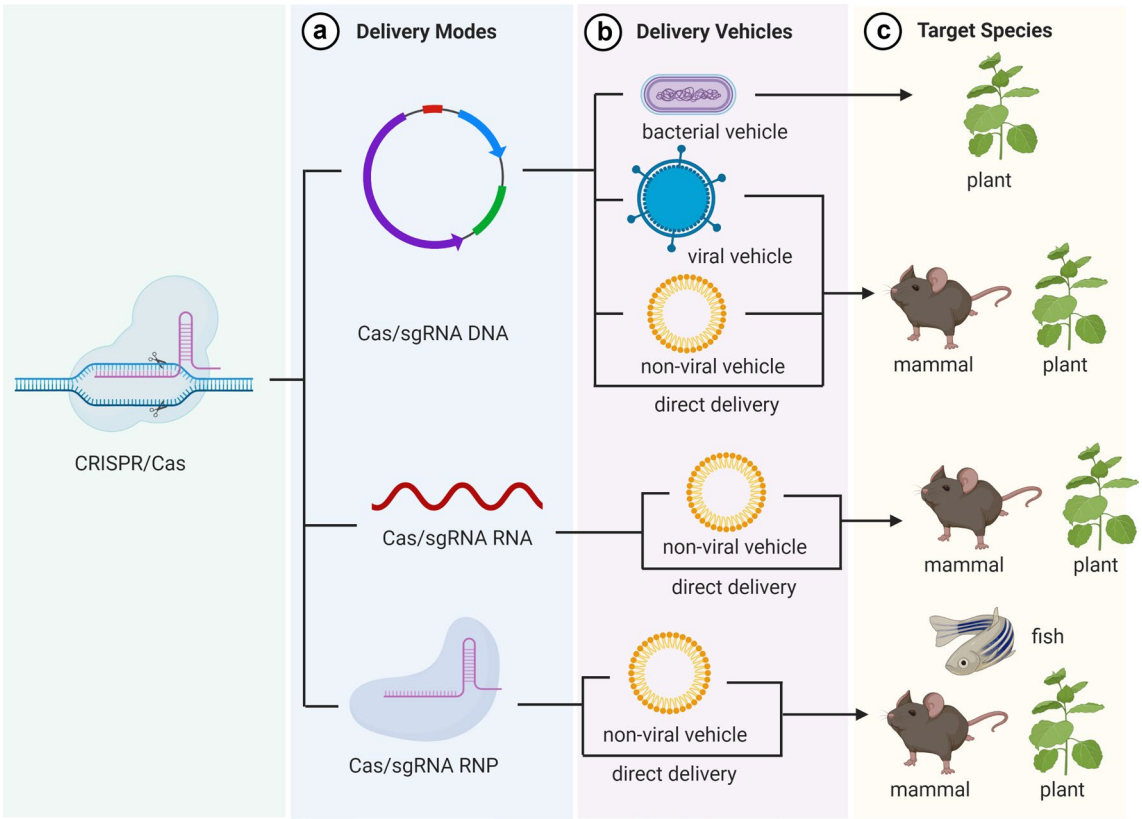

Fig. 1 Schematic diagrams of in vivo CRISPR/Cas delivery modes and vehicles in different biological systems**.** Systems used for delivery of CRISPR/Cas components (**a**) can be separated into two major categories, CRISPR/Cas delivery mode (**b**) and delivery vehicle (**c**). Three CRISPR/Cas delivery models including DNA (plasmid encoding both the Cas protein and the gRNA), RNA (mRNA for Cas protein translation and a separate gRNA) and protein (Cas protein with gRNA as a ribonucleoprotein complex, RNP) can be delivered in to mammalians, aquacultures or plants via bacterial or viral vectors, non-viral carriers and physically direct delivery (**d**). The figure was created with BioRender.com

CRISPR/Cas gene-editing system

The CRISPR/Cas system was first identified as a prokaryotic adaptive immune system. It was the first programmable nuclease system that was found to function as ribonucleoprotein particles that utilized base pairing to recognize its targets [8]. This system for gene editing has been widely adopted since it is relatively easy to redesign to produce target specificity. Scientists have engineered and modified this system to allow CRISPR/Cas to act as a successful gene-editing tool [4].

There are three key components in the CRISPR/Cas9 system: the tracrRNA, Cas9 protein, and pre-crRNA. The tracrRNA forms a complex with pre-crRNA after transcription. The Cas9 protein stabilizes the complex, and the pre-crRNA is then processed by RNase III to generate crRNA [9]. The Cas9/gRNA (made up of crRNA and tracrRNA) complex recognizes the protospacer adjacent motif (PAM), which is a short motif that is located adjacent to the target DNA sequence [10]. Then, the complex unwinds the target DNA beginning at the seed region (10–12 nucleotides) [11]. When the DNA sequence corresponds to the gRNA, two nuclease domains of Cas9 cleave the target strands [12–14]. The Cas9/gRNA complex can tolerate single or sometimes multiple mismatches, with mismatches downstream of the seed region typically being more frequently tolerated [13, 15].

There are six types of CRISPR/Cas systems (type I–VI) that are further classified into two classes: the class 1 CRISPR/Cas system and the class 2 CRISPR/Cas system [16, 17]. The main feature of the class 1 CRISPR/Cas system, which is subclassified into types I, III, and IV, is that they have multisubunits of effector nuclease complexes. The class 2 CRISPR/Cas system differs from class 1 because it requires only a single effector nuclease; class 2 is subclassified into types II, V, and VI. Their programmable single effector nucleases enable nucleic acid detection and genome engineering [3, 18–21]. Types II, V, and VI are based on Cas9, Cas12, and Cas13 effectors [22–24]. Among them, the CRISPR/Cas9 system is the most commonly used system to date.

Application of the CRISPR/Cas system for gene editing

Strategies based on Cas nuclease activity

Based on the nuclease activity of CRISPR/Cas, there are various gene editing strategies that have been developed for DNA (gene disruption, precise repair, targeted insertion, large-scale DNA editing) and RNA modification (Supplementary Table S1; Fig. 2a, b).

DNA editing

For gene disruption, NHEJ ligates DSBs introduced by Cas endonuclease, and this break and repair pattern takes place repeatedly until the target sequence is altered and an indel occurs [25]. An indel can cause frameshifting or exon skipping and subsequent gene disruption. Gene disruption can also silence dominant negative mutations by disrupting the mutant allele while preserving the normal allele. In addition, HDR inserts a donor template, which has homology arms to match the target locus to the genome and cause a deletion [26].

For precise repair, HDR uses a donor template that has the desired insertion or modification. This donor template has homology arms that enable it to match the target locus and insert the desired genetic material or modify the genome with high precision [25].

For targeted insertion, HDR allows precise insertion of exogenous DNA sequences into the genomes of dividing cells, while homology-independent targeted integration (HITI) allows insertion of exogenous DNA sequences into the genomes of nondividing cells using an NHEJ-based homology-independent strategy [27].

For large-scale DNA editing (editing a size of up to several megabase pairs [Mbp]), DNA fragments can be deleted by introducing the CRISPR/Cas system with two guide RNAs that target different sites. In addition, allelic exchange can correct recessive compound heterozygous mutations. This is achieved by generating homologous DNA breaks in both chromosomes, and the allelic exchange between mutated alleles can rescue the disease phenotype [28].

RNA editing

The CRISPR/Cas system acts not only on DNA but also RNA. Previous studies have identified an RNA-targeting CRISPR/Cas effector complex, termed the psiRNA-Cmr protein complex, which comprise prokaryotic silencing (psi) RNAs and Cmr Cas proteins. This complex cleaves target RNAs at a predetermined site, indicating that prokaryotes have their own unique RNA silencing system [29]. Cas endonuclease has also been shown to bind to and cleave ssRNA targets [30]. Strutt et al. showed that type II-A and II-C Cas9 endonucleases are capable of recognizing and cleaving ssRNA without a PAM. [31]. Recently, scientists have discovered the RNA-editing Cas13 family. The Cas13 family has been shown to be a programmable RNA-editing CRISPR/Cas system. Compared to other RNA targeting approaches, this system is more specific and efficient [32–34]. Recently, Konermann et al. discovered a Cas13d

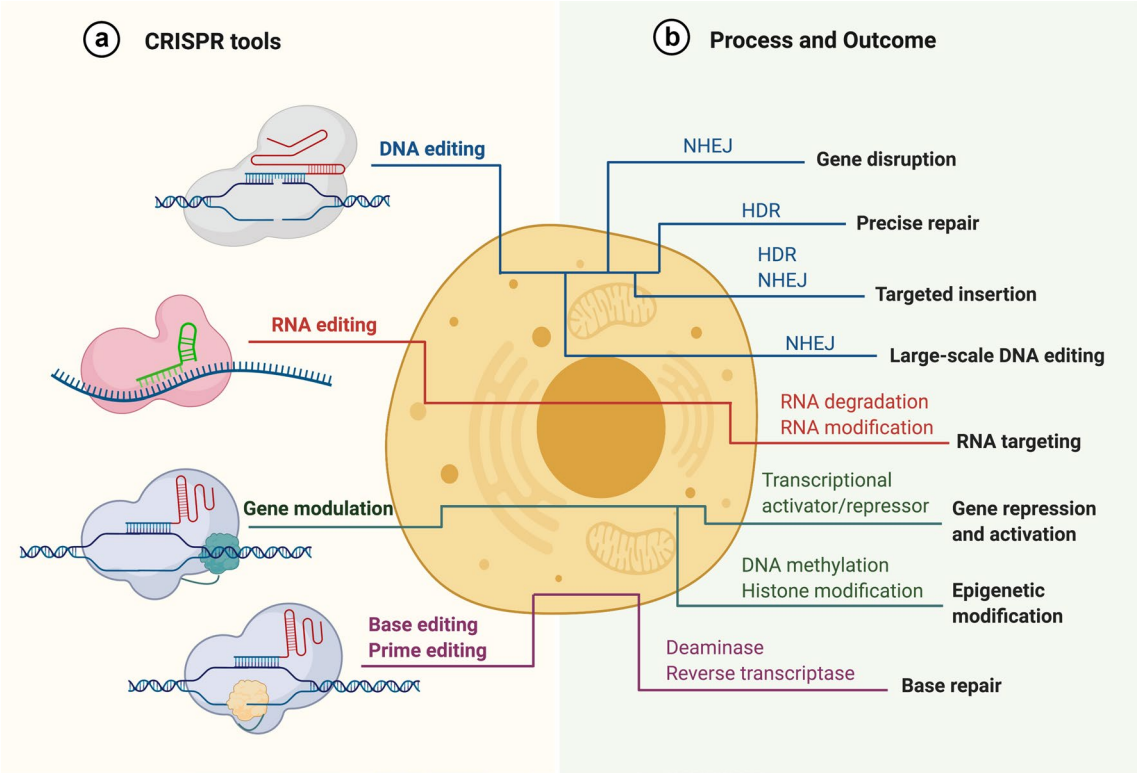

Fig. 2 CRISPR/Cas-mediated gene-editing strategies. The versatile CRISPR/Cas system is a powerful tool for DNA, RNA editing, gene modulation and base, prime editing by leveraging different approaches (**a**) to achieve numerous gene-editing outcomes (**b**). The figure was created with BioRender.com

in *Ruminococcus flavefaciens* XPD3002 (CasRx), and it possesses high activity in human cells. CasRx is small, consisting of 930 amino acids, and it can be flexibly packaged into an adeno-associated virus (AAV), making it suitable for delivery by AAV vectors. In addition to the knockdown activity, catalytically inactivated CasRx can be utilized to regulate pre-mRNA splicing by acting as a splice effector [35].

Strategies based on Cas-effector fusion protein activity

Since CRISPR/Cas possesses DNA-binding properties, it may play a crucial role in important applications other than site-specific gene editing. A catalytically dead Cas9 enzyme (dCas9) has been developed to control gene expression [36]. dCas9, like Cas9, is capable of recognizing and binding to a target DNA sequence. However, instead of cleaving the target DNA sequence, dCas9 has been used for transcriptional repression, transcriptional activation, introducing epigenetic modifications, and base editing. These functions are achieved by fusing dCas9 to gene activators, repressors, acetyltransferases or adenosine deaminases. Since dCas9 is in a catalytically inactive form, it is used here for precise targeting instead of its catalytic activity (Supplementary Table S2; Fig. 2c, d).

Transcriptional regulation

dCas9 can be fused with transcriptional repressors or activators to target the promoter region of the gene of interest and result in transcriptional repression (CRISPR interference), or activation (CRISPR activation) without changing the genome; this activity has been demonstrated in *Escherichia coli* as well as in plant and mammalian cells [36–38]. In addition, studies have shown that a modifying sgRNA can also enhance the specificity of transcriptional regulation. For example, using the Cas9-VP64 transcriptional activator together with an sgRNA that has two MS2 RNA aptamer hairpin sequences added to it can successfully induce sequence-specific transcriptional activation [39].

The CRISPR/Cas system can also be utilized for epigenetic modification. Hilton et al. fused dCas9 with acetyltransferase that catalyze the acetylation of histone H3 at lysine 27. This modulation has been shown to strongly activate specific gene expression. Not only acetylation but

also methylation may be accomplished using this approach [40].

Base and prime editing

CRISPR/dCas has been utilized for precise DNA base editing. The CRISPR/nickase Cas9 (nCas9)-based base editor was first developed by Komor et al. and was used to convert a targeted C–G base pair to T–A by a DNA cytosine deaminase [41]. Gaudelli et al. subsequently developed a transfer RNA adenosine deaminase that, when fused to nCas9, can convert A–T base pairs to G–C base pairs [42]. This kind of CRISPR/Cas-mediated editing is powerful since single point mutations account for a large category of genetic diseases.

Recently, a more powerful and versatile gene-editing method, prime editing, was discovered as a way to introduce indels and enable base conversions in both transitions and transversions [43]. The editor used in prime editing is termed prime editor. The prime editor is composed of nCas9 fused with reverse transcriptase. The prime editor is guided by a prime editing gRNA (pegRNA). After nCas9 nicks the target site, the pegRNA binds to a single strand DNA (ssDNA) and initiates reverse transcription. The reverse transcribed pegRNA is then incorporated into the target site.

Current approaches for delivering the CRISPR/Cas system in mammals

CRISPR/Cas can be delivered using different modalities, including DNA, RNA and protein. When it is delivered in a DNA mode, Cas and gRNA are delivered as a single plasmid. For the RNA mode, Cas mRNA is delivered with a separate gRNA. For the protein mode, Cas protein is delivered with gRNA as a ribonucleoprotein complex (RNP). Each mode exhibits overall effectiveness but also includes some limitations. Packaging Cas9 and gRNA in the same plasmid makes the delivered cargo more stable than that of other methods; however, the large size of the plasmid increases the difficulty of delivery, and the integration of plasmids into the host genome and prolonged expression are potential limitations of this delivery method. Delivery of Cas mRNA enables faster gene editing; however, RNA is fragile, and the degradation of gRNA may initiate before Cas9 mRNA is successfully translated. The RNP is the most direct and fastest mode for gene editing. However, compared to plasmids or mRNAs, it is much more challenging to obtain a pure protein. In addition, the sudden introduction of bacterial proteins may induce an immune response in the host.

The delivery vehicles can be separated into two groups: viral and nonviral vectors. For in vivo delivery of CRISPR/Cas, viral vectors are the preferred vehicle. To date, nonviral vector delivery has not been as commonly used as viral-based delivery. However, nonviral vectors are comparable to viral vectors and are a topic of intense research. The delivery vehicles for the in vivo CRISPR/Cas system discussed below are summarized in Fig. 3 and Table 1.

Viral-based CRISPR/Cas gene editing and delivery

Viral vectors are commonly used vehicles for introducing gene-editing materials such as DNA. Lentivirus, adenovirus, and AAV are three major types of viral vectors widely used for the gene delivery of CRISPR/Cas system. Though viral delivery has high efficiency in vivo, there are some disadvantages, including safety issues. These viruses work by releasing the viral genome into host cells after infection. This means that the interactions between the virus and host cells must be strong; thus, viral delivery methods are more complicated than most of the nonviral methods under in vivo conditions.

Lentivirus

Lentiviruses are RNA viruses with the capability to integrate into dividing and nondividing cells. Lentiviruses are an excellent delivery vehicle for cells that are hard to transfect by chemical methods. Furthermore, it has a large packaging capacity of ~ 10.7 kb [44]. This property allows it to carry multiple sgRNA sequences that can induce multiple gene edit at once [45]. Due to these advantages, lentiviruses have been used in many initial gene-editing studies. Mouse models of myeloid malignancy [46] and lung cancer [47] have been generated using lentivirus delivery. However, there are some disadvantages of using lentivirus, including the integration of the viral genome, which may be carcinogenic [48].

To overcome these issues, lentiviral vectors have been further developed into integration-deficient lentiviral vectors (IDLVs) to reduce the undesired integration of the viral genome into the host cell genome [49, 50]. IDLVs retain the property of being able to edit genes in hard-to-transfect cells [51, 52]. Although IDLVs have been found to cause unwanted gene modifications, the study also showed that IDLVs have effective site-specific gene repair activity due to their active recruitment of host HDR proteins [53]. Therefore, pairing IDLVs with safer endonucleases such as SpCas9-HF or eSpCas9 may improve its application [54, 55].

Adenovirus

Adenoviruses are double-stranded DNA (dsDNA) viruses. Similar to a lentivirus, an adenovirus can infect both dividing and nondividing cells. However, since they do not generally induce genome integration in the host DNA, adenoviruses do not cause potential off-target effects the way a

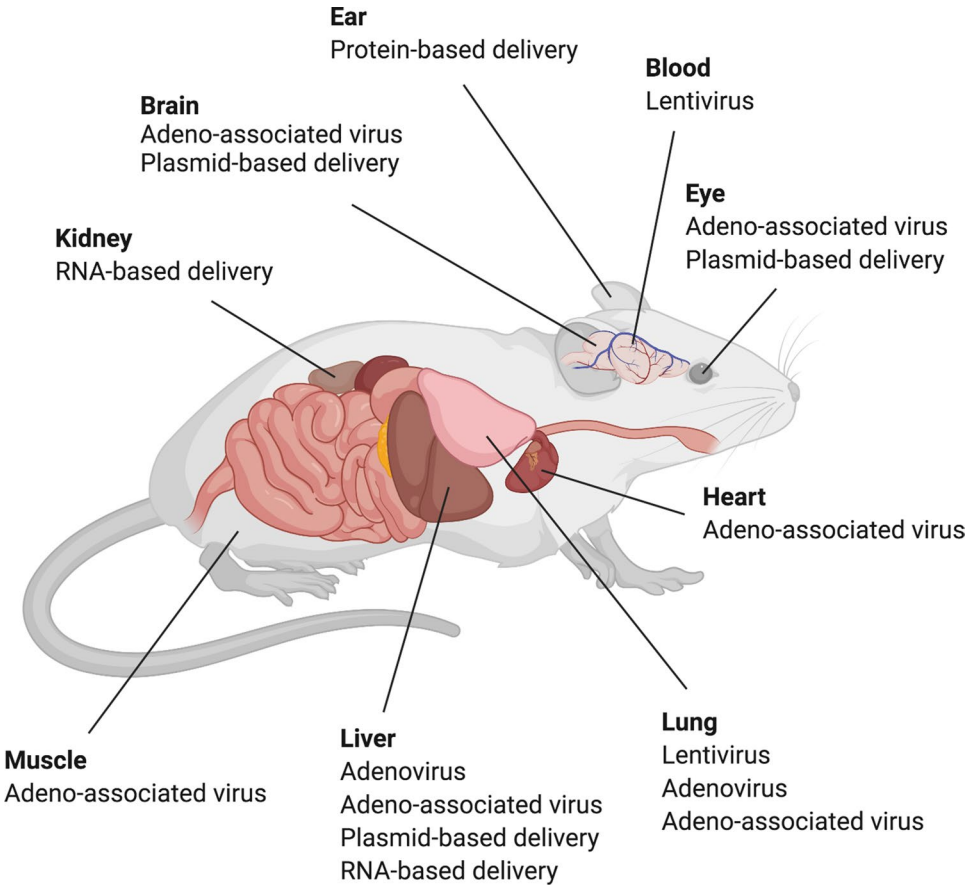

Fig. 3 Representation of different delivery methods of the CRISPR/Cas system to target organs in the rodent. Delivery methods including virus-based (lentivirus, adenovirus and adeno-associated virus) and non-virus-based (plasmid-, RNA- or protein-based) delivery have been used to deliver CRISPR/Cas system to different organs in the rodent. The figure was created with BioRender.com

lentivirus does. It has been shown that adenovirus-based delivery of the CRISPR/Cas system can result in the efficient editing of the *Pcsk9 (proprotein convertase subtilisin/kexin type 9)* and *Pten (phosphatase and tensin homolog)* genes in adult mouse liver [56, 57]. Moreover, adenovirus-based delivery also has been successfully used to induce specific chromosomal rearrangements to generate echinoderm microtubule-associated protein like 4-anaplastic lymphoma kinase (EML4-ALK)-driven lung cancer in vivo [58]. However, adenoviruses can elicit a significant immune response. Adenoviruses are also costly and difficult to produce in high volumes. These shortcomings set a limit for the applications of adenovirus-mediated delivery in clinical gene therapy [56].

Adeno-associated virus

AAVs are small ssDNA viruses. Compared to lentivirus- and adenovirus-based delivery, AAV-based delivery is safe and efficient since it results in only minor cytotoxicity and immune responses [59, 60]. AAVs have a wide range of serotypes, which helps to achieve a broad range of tissue tropisms and are used for efficient gene editing [61]. For example, Swiech et al. reported a first successful AAV-based CRISPR/Cas9 gene editing in the mouse brain [62]. A similar approach was used by Hung et al. for retinal gene editing and achieved high editing effects in the adult mouse retina [63]. Studies have also demonstrated successful AAV-based CRISPR/Cas9 gene editing in the retina of retinal degeneration mouse models [64, 65]. AAV-based delivery of CRISPR/Cas components have also been used to knockdown IGF in the central nervous system [66]. In addition, studies have also demonstrated that muscle tissue-specific delivery of CRISPR/Cas components using AAV vectors can correct the mutated dystrophin gene in Duchenne muscular dystrophy (DMD), and functional recovery was observed in vivo [61, 67–69]. Zhang et al. recently demonstrated improved CRISPR-Cas9-mediated gene-editing efficiency in DMD mouse model using self-complementary AAV (scAAV) system [70]. AAV-based delivery of CRISPR/Cas9 has also been used to achieve effective gene correction in metabolic liver disease in newborn mice [71, 72]. Moreover, delivery of sgRNAs using AAVs in a tissue-specific SpCas9 transgenic mouse can be employed to generate the disease animal model such as cardiomyopathy [73] and lung adenocarcinoma [74]. Also, Murlidharan et al. used chimeric AAV (AAV2g9) to deliver gRNAs targeting the schizophrenia risk gene MIR137 into the brain of a CRISPR/Cas9 knock-in mouse model, to achieve brain-specific gene deletion of the gene [75]. Furthermore, delivery of sgRNAs using AAVs

Table 1 Delivery methods for CRISPR/Cas system in mammals

Model-target tissue (disease)	Delivery methods		Editing gene	References
	Viral delivery system	No viral delivery system		
Mouse blood (myeloid malignancy)	Lentivirus		*Tet2, Runx1, Dnmt3a, Ezh2, Nf1, Smc3, p53 and Asxl1*	[46]
Mouse lung (lung cancer)	Lentivirus		*Pten and Nkx2-1*	[47]
Mouse liver (NASH)	Adenovirus		*Pten*	[56]
Mouse liver (cardiovascular disease)	Adenovirus		*Pcsk9*	[57]
Mouse lung (lung cancer)	Adenovirus		*Eml4 and Alk*	[58]
Mouse brain	AAV		*Mecp2, Dnmt1, Dnmt3a and Dnmt3b*	[62]
Mouse eye (LCA)	AAV		*Yfp*	[63]
Mouse muscle (DMD)	AAV		*Dmd*	[68, 69]
Mouse lung (lung cancer)	AAV		*Kras, p53 and Lkb1*	[74]
Mouse liver (OTC deficiency)	AAV		*Otc*	[72]
Mouse liver (cardiovascular disease)	AAV		*Pcsk9*	[78]
Mouse brain (Huntington disease)	AAV		*Htt*	[192]
Mouse brain (GBM)	AAV		*Trp53, Nf1 and Rb1*	[76]
Mouse brain	AAV		*Camk2a, Erk2 and Actb*	[193]
Mouse muscle (DMD)	AAV		*Dmd*	[67]
Mouse muscle (MDC1A)	AAV		*Lama2*	[194]
Mouse eye (retinal degeneration)	AAV		*Nrl*	[64]
Mouse spleen, lungs, heart, colon, and brain (HIV/AIDS)	AAV		*HIV-1 provial DNA*	[195]
Mouse liver (Hemophilia B)	AAV		*F9*	[196]
Mouse liver (cancer)	AAV		*Tsgs*	[77]
Mouse liver	AAV		*HBV cccDNA*	[197]
Mouse eye (X-linked retinitis pigmentosa)	AAV		*Rpgr*	[65]
Mouse liver (tyrosinemia)	AAV		*Fah*	[198]
Mouse liver (OTC deficiency)	AAV		*Otc*	[71]
Mouse muscle (DMD)	AAV		*Dmd*	[70]
Mouse brain (schizophrenia)	AAV		*Mir137*	[75]
Mouse muscle (ALS)	AAV		*Igf1*	[66]
Mouse liver (tyrosinemia)		Plasmid	*Fah*	[84]
Mouse liver		Plasmid	*HBsAg*	[85]
Rat eye (retinitis pigmentosa)		Plasmid	*(Rho(S334))*	[86]
Mouse eye (retinitis pigmentosa)		Plasmid	*Rho*	[87]
Mouse brain (MB and GBM)		Plasmid	*Trp53, Pten and Nf1*	[199]
Mouse brain		Plasmid	*Satbs*	[89]
Mouse eye (IRDs)		Plasmid	*Rho-P23H*	[88]
Mouse liver (tyrosinemia)		mRNA	*Fah*	[90]
Mouse liver (cardiovascular disease)		mRNA	*Pcsk9*	[91, 93]
Mouse liver (tyrosinemia)		mRNA	*Ttr*	[92]
Mouse liver, kidney, and lung		mRNA	*floxed tdTomato*	[200]
Mouse ear		Protein	*Egfp*	[94]
Mouse muscle (DMD)		Protein	*Dmd*	[100]
Mouse brain (FXS)		Protein	*Grm5*	[101]
Mouse brain (Alzheimer's disease)		Protein	*Th, Bace1*	[96]
Mouse liver and spleen		Protein	*Pten*	[99]
Mouse liver and spleen (HT1)		Protein	*Hpd*	[95]

AAV adeno-associated virus, *ALS* amyotrophic lateral sclerosis, *DMD* Duchenne muscular dystrophy, *FXS* fragile X syndrome, *GBM* glioblastoma, *HIV/AIDS* human immunodeficiency virus/acquired immunodeficiency syndrome, *HT1* hereditary tyrosinemia type I, *IRDs* inherited retinal degenerations, *LCA* Leber congenital amaurosis, *MB* medulloblastoma, *MDC1A* congenital muscular dystrophy type 1A, *NASH* non-alcoholic steatohepatitis, *OTC* ornithine transcarbamylase

into CRISPR/Cas9 knock-in mice can be used to perform high-throughput mutagenesis to generate autochthonous mouse models of cancer [76, 77]. Despite progress in using AAVs for CRISPR/Cas-based gene editing, the small cargo capacity (< 4.7 kb) of AAVs can limit its application. Thus, when combining conventional SpCas9, which has a size of 4.2 kb, with the addition of sgRNA, another vector system is usually required. Later on, several smaller Cas9 orthologs (such as *Staphylococcus aureus* (SaCas9) [78], *Campylobacter jejuni* (CjCas9) [79], *Streptococcus thermophilus* (StCas9) [80] and *Neisseria meningitidis* (NmCas9) [79]) were developed by scientists to enable the in vivo gene editing by a single AAV vector.

Nonviral-based CRISPR/Cas gene editing and delivery

DNA-based delivery

DNA-based delivery is commonly used for introducing the CRISPR/Cas system into cells because it is more stable than RNA. CRISPR/Cas-encoding DNA facilitates greater gene-editing efficiency than other methods [81–83]. For example, the CRISPR/Cas9 components were delivered in the form of DNA by tail-vein hydrodynamic injection to a mouse model of tyrosinemia and achieved > 6% gene correction in the liver cells after a single application [84]. Furthermore, Zhen et al. also reported that hydrodynamic injection of CRISPR/Cas9-encoding DNA can effectively disable the hepatitis B virus replication by creating mutations in virus DNA [85]. Apart from systemic administration, subretinal injection of CRISPR/Cas components in a plasmid form in combination with electroporation has also been reported to enable an allele-specific gene editing in the retina of a rat model of retinitis pigmentosa [86]. A similar effect also found by Latella et al. in a mouse model of retinitis pigmentosa, which significantly reduced mutated protein levels and prevented major visual dysfunction [87]. In addition, Li et al. demonstrated an allele-specific gene editing in the retinas of *Rho*-P23H knock-in mice which selectively targeting the P23H allele that has a single-nucleotide mutation [88]. Moreover, Shinmyo et al. introduced a plasmid containing CRISPR/Cas components into the mouse brain using in utero electroporation for effective brain-specific gene editing in vivo [89]. These works demonstrated the applicability of DNA-based delivery of CRISPR/Cas9 in vivo.

RNA-based delivery

RNA-based delivery methods largely decrease the risk of host genome integration. However, the effective time of RNA-based delivery methods is relatively fast, and there are some additional shortcomings of such delivery methods. For example, the stability of RNA, and the need to deliver the components (Cas mRNA and sgRNA) separately are the two main concerns of this method. Yin et al. demonstrated a delivery method that utilized different vehicles for introduction of the CRISPR/Cas9 components, lipid nanoparticles delivered the Cas9 mRNA, and an AAV delivered the sgRNA/HDR template. By utilizing this strategy, they showed an efficient correction of *Fah* (*fumarylacetoacetate hydrolase*) gene in a mouse model of hereditary tyrosinemia [90]. However, it is important to note that this combination approach still requires viral codelivery to achieve certain efficacy, and compared to DNA and protein, RNA is unstable. Moreover, the degradation of sgRNA may significantly affect editing efficiency. Future research into increasing sgRNA stability is required to improve the efficiency of these methods. Studies have showed that modifying sgRNA has beneficial effects on the stability of sgRNA. Yin et al. modified sgRNA by switching the 2′OH group of RNA to 2′OMe and 2′F and added phosphorothioate bonds [91]. This study reported that a single injection induced more than 80% efficiency in editing *Pcsk9* in the livers of mice, demonstrating a potential modified method for improving the stability of RNA to overcome the obstacles of RNA-based delivery. In addition, other researchers reported a similar study in which modified sgRNA and Cas9-encoding mRNA were packaged into a lipid nanoparticle vehicle. With a single administration, a more than 97% reduction in the mouse *Ttr* (*transthyretin)* gene was shown in the serum protein levels of the liver. This study demonstrated efficient gene editing that could persist for at least 1 year [92]. Another study has also demonstrated a high editing efficacy (~ 80%) by unitizing a lipid nanoparticle with disulfide bonds (BAMEA-O16B) to deliver Cas9 mRNA and sgRNA in vivo [93].

Protein-based delivery

Delivering Cas protein with gRNA as a Cas9 RNP is the fastest and most direct pathway for gene editing, and it is suitable for in vivo therapeutic applications. To facilitate the delivery of Cas9 RNPs into target cells, a fusion protein of Cas9 and negatively supercharged proteins was created to enable the delivery by cationic lipid formulated transfection reagents such as RNAiMAX [94]. Delivery of the Cas9 RNP/RNAiMAX complex via injection into the cochlea of transgenic *Atoh1* (*atonal bHLH transcription factor 1*)-*GFP* mice caused a 13% reduction in GFP in the ears of the transgenic mice. Mangeot et al. designed a vector based on murine leukemia virus (MLV), termed nanoblades, to deliver Cas9 RNPs for in vivo gene editing [95]. Moreover, an amphiphilic nanocomplex has also been developed to deliver Cas9 RNPs in vivo and showed effective gene editing in the brain of mouse model of Alzheimer's disease [96]. Furthermore, to enhance endosomal escape, PEI polymers or

combined PEI polymers with liposomes were used for Cas9 RNP delivery in vivo. Sun et al. coated a DNA nanoclew with PEI polymers to deliver Cas9 RNPs into the nuclei of human cells. Using this vehicle, target gene disruption can be achieved with negatively impacting cell viability [97]. The study also noted that the modification of DNA nanoclew to partially complementary with the sgRNA can further enhance the editing efficacy. In addition, the modification of Cas9 protein can also improve the efficacy of direct cytoplasmic/nuclear delivery of Cas9 RNP. Mout et al. developed the Cas9En protein, in which the N-terminus of Cas9 protein has an attached oligo glutamic acid tag that is negatively charged [98]. Cas9En RNPs were delivered using arginine-functionalized gold nanoparticles (Arg-AuNPs), which are positively charged. With the NLS attached, Cas9 RNPs were delivered directly to the cytosol, accumulated in the nucleus, and provided ~ 30% editing efficiency. Recently, this nano-assembled platform has been used for Cas9 RNP delivery in vivo and achieved > 8% gene editing efficiency [99].

AuNPs have also been used to deliver Cas9 RNPs in vivo for gene editing and correction in the disease models. AuNPs can be conjugated with donor DNA, Cas9 RNPs and the endosomal disruptive polymer poly[N-[N-(2-aminoethyl)-2-aminoethyl]aspartamide] (PAsp(DET) to form a vehicle termed CRISPR-Gold. Lee et al. reported that CRISPR-Gold-based Cas9 RNPs delivery can achieve 5.4% correction of the dystrophin gene in the muscle tissue of DMD mice [100]. Another study also showed that intracranial injection of CRISPR-Gold in the brain rescued mice from abnormal behaviors caused by fragile X syndrome [101]. CRISPR-Gold may offer the opportunity in the development of therapeutic approaches targeting the muscle and brain diseases, while effective endosomal escape is still required for higher delivery efficiency.

Overall, protein-based delivery offers reduced off-target effects and a low immune response compared to DNA and RNA-based delivery [102]. Cas9 RNPs increase efficacy by avoiding the degradation of sgRNA. However, transport of Cas9 RNPs into the cytosol or the nucleus is critical for therapeutic effects. Thus, endosomal entrapment is still a crucial obstacle to overcome [103].

Table 2 Delivery methods for CRISPR/Cas system in aquacultures

Species	Applications	References
Zebrafish (*Danio rerio*)	Gene editing of multiple genes	[104]
Rohu carp (*Labeo rohita*)	Gene editing of *tlr22* gene	[106]
Atlantic salmon (*Salmo salar L.*)	Gene editing of *dnd* gene	[107]
	Gene editing of *tyr* and *slc45a2* genes	[201]
	Gene editing of *elov12* gene	[202]
Channel catfish (*Ictalurus punctatus*)	Gene editing of *mstn* gene	[108]
	Gene editing of *ticam* and *rb1* gene	[110]
Common carp (*Cyprinus carpio*)	Gene editing of *sp7* and *mstn* genes	[109]
Grass carp (*Ctenopharyngodon idella*)	Gene editing of *gcjam-a* gene	[111]
Nile tilapia (*Oreochromis niloticus*)	Gene editing of *nanos2, nanos3, dmrt1* and *fox12* genes	[203]
	Gene editing of *gsdf* gene	[204]
	Gene editing of *aldh1a2* and *cyp26a1* genes	[205]
	Gene editing of *sf-1* gene	[206]
	Gene editing of *dmrt6* gene	[207]
	Gene editing of *amhy* gene	[208]
	Gene editing of *wt1a* and *wt1b*genes	[209]
Southern catfish (*Silurus meridionalis*)	Gene editing of *aldh1a* gene	[210]
Sea bream (*Pagrus major*)	Gene editing of *mstn* gene	[211]
Rainbow trout (*Oncorhynchus mykiss*)	Gene editing of *igfbp2b1* and *igfbp2b2* genes	[212]
Pacific oyster (*Crassostrea gigas*)	Gene editing of *mstn* and *twist* genes	[213]
Northern Chinese lamprey (*Lethenteron morii*)	Gene editing of multiple gene	[214]

Current approach of delivering the CRISPR/Cas system in aquaculture

Genomes of several aquaculture species, including zebrafish, Atlantic salmon, Nile tilapia, sea bream, catfish, carp, rainbow trout, Northern Chinese lamprey and Pacific oyster, have been successfully modified with the CRISPR/Cas system (Table 2). CRISPR/Cas protocols developed in model species such as zebrafish have been followed for gene editing in aquaculture species [104]. The standard gene transfer method used in aquaculture species is microinjection. Microinjection is performed using special equipment to inject the CRISPR/Cas complex into newly fertilized eggs; this method has high gene-editing efficiency [105]. In most cases, NHEJ was used to induce mutations, while HDR has been successfully used in rohu carp [106]. However, if gene editing continues at different stages of embryonic development, mosaicism could occur. These concerns are the focus of current research, which aim to enable more widespread adoption of CRISPR/Cas techniques in aquaculture. CRISPR/Cas techniques have been used to address characteristics such as sterility, growth, and disease resistance of aquaculture species. The reason for inducing sterility in fish is to preserve the domesticated strains by preventing gene flow. For example, CRISPR/Cas techniques have been used to induce sterility in Atlantic salmon [107]. Several papers have demonstrated gene editing of the myostatin gene using the CRISPR/Cas approach to enhance the growth of fish, including channel catfish and common carp [108, 109]. The CRISPR/Cas approach has also been used to investigate immunity and disease resistance in channel catfish, rohu carp, and grass carp [106, 110, 111]. Disruption of the *tlr22* gene in rohu carp resulted in a model for studying immunology, demonstrating the capability of CRISPR/Cas to aid in the development of effective treatments for aquaculture. By understanding the underlying pathways of transcription and translation through CRISPR/Cas-based mechanisms, it is possible to strengthen disease resistance, decrease disease incidence, and improve species resilience in aquaculture. Aquaculture is highly suited for the application of CRISPR/Cas gene editing for numerous reasons. Sample sizes can be large without generating cumbersome costs; thousands of externally fertilized embryos enable microinjection by hand. The large sample size is impartial and useful for comparisons of successfully edited samples with controls and for the assessment of pathogen resistance. Furthermore, a large sample size enables the development of well-developed disease challenge models since extensive phenotypes are practical. With the technology becoming mature in aquaculture species, it is becoming easier to study gene function, improve disease resistance, and generate new strains with selected characteristics that can improve economic value.

Current approach of delivering the CRISPR/Cas system in plants

As shown above, the CRISPR/Cas system is highly adept at modifying animal genomes. Studies have also demonstrated its ability to modify plant genomes. Conventionally, a mixed dual promoter system is used to express CRISPR/Cas system in plants. In mixed dual promoter systems, RNA polymerase II promoters are used to express Cas protein and RNA polymerase III promoters specifically expressed in plants, such as AtU6 for Arabidopsis or tomato, TaU6 for wheat, and OsU6 or OsU3 for rice, are used to express gRNA [112–115]. However, to utilize CRISPR/Cas9 technology in creating new traits in plants, efficient delivery of the CRISPR/Cas system into cells is essential. The two delivery methods utilized in plants are indirect and direct methods. Indirect methods (such as agroinfiltration, agroinfection, and viral infection/agroinfection) use plant bacteria or viruses to mediate the introduction of DNA constructs into target plant cells. By contrast, no biological organisms are used as mediators for direct delivery. Protoplast transfection and biolistic particle delivery are the most commonly used direct methods. Agroinfiltration is usually used as a transient assay and has been widely used for its versatility and simplicity [116–120]. Agroinfection, biolistic particle delivery, and viral infection are usually used for stable editing. Protoplast transfection can be used for both transient and stable editing. The delivery methods used in plant gene editing (Fig. 4 and Table 3) will be summarized in the following sections.

Transient events

Indirect method

Agroinfiltration. Agrobacterium spp. are plant pathogens. When infecting plants, *Agrobacterium tumefaciens* causes tumor-like growth on aerial parts of the plant (crown gall), while *Agrobacterium rhizogenes* induces root tumors. Agrobacteria contain a large plasmid (exceeding 200 kb), which is named Ti in the case of *A. tumefaciens* or Ri in the case of *A. rhizogenes*, and it can transfer a specific DNA segment (transfer DNA or T-DNA) into the infected plant cells, enabling the T-DNA to integrate into the host genome. These two strains of agrobacterium have been modified to contain a disarmed Ti/Ri plasmid where tumor-inducing genes have been deleted. The essential parts of the T-DNA, border repeats (25 bp), are needed for plant transformation

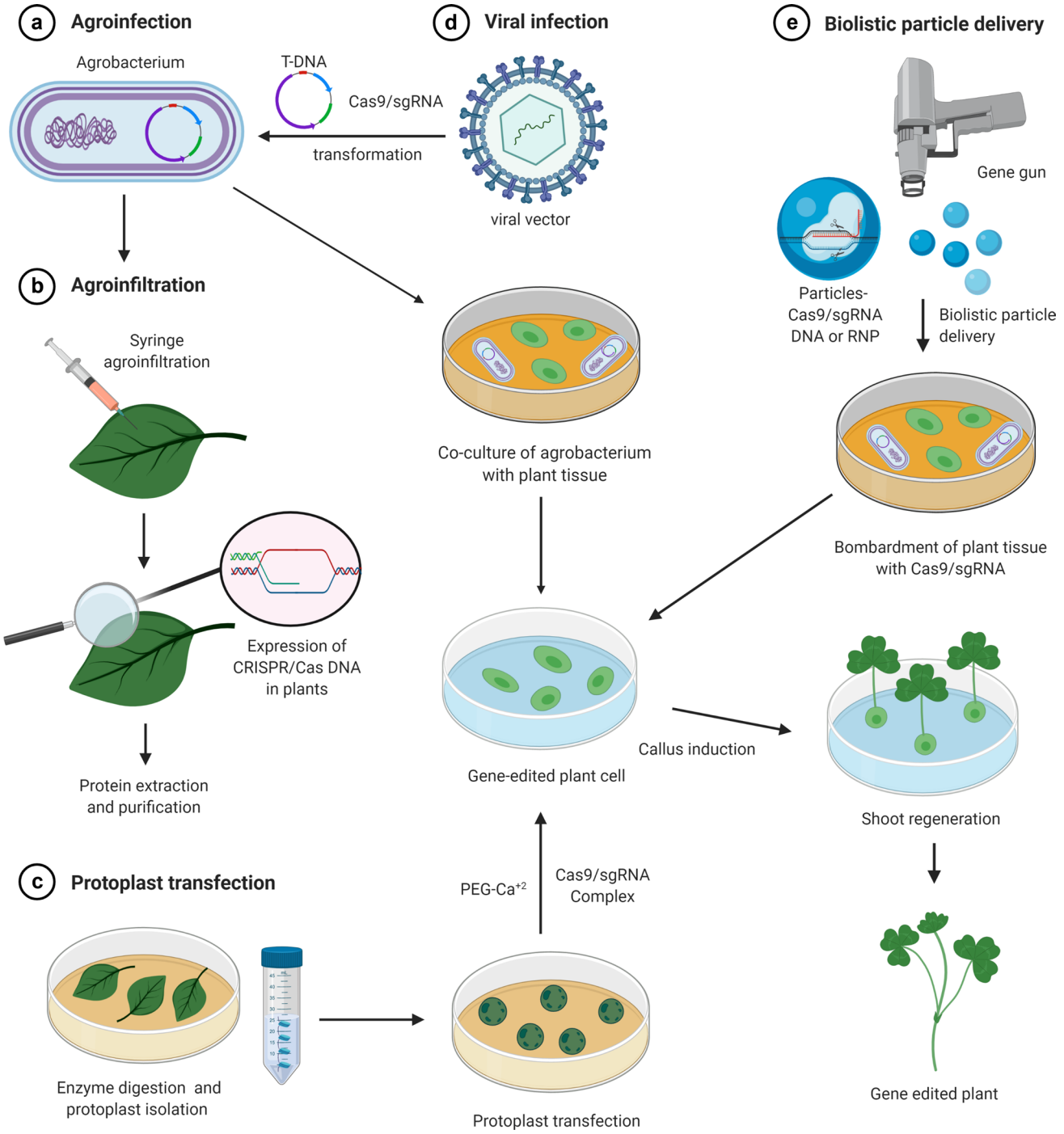

Fig. 4 Schematic representation of main methods used to modify plant genome by CRISPR/Cas system. A schematic diagram showing major steps involved in the generation of gene-edited plants using direct and indirect methods including agroinfiltration (**a**), protoplast transfection (**b**), agroinfection (**c**), and virus infection (**d**) and biolistic particle delivery (**e**)

and are used to generate transgenic plants. Agroinfiltration is a transient assay in which an *A. tumefaciens* culture containing modified T-DNA is directly injected into plant leaves (Fig. 4a) [121–123]. For root hair transformation, *A. rhizogenes* is specifically used to evaluate editing efficiency in plant root hairs, and this method has mainly been used in legume species such as Medicago and soybean [124–126].

Table 3 Delivery methods for CRISPR/Cas system in plants

Species	Delivery methods		Edited gene	References
	Stable	Transient		
Arabidopsis thaliana		Protoplast, Agroinfiltration	*PDS3, FLS2, RACK1b* and *RACK1c*	[122]
Arabidopsis thaliana		Agroinfiltration	*GFP*	[215]
Arabidopsis thaliana	Agrobacterium		*GFP*	[216]
Arabidopsis thaliana	Agrobacterium	Protoplast	*BRI1, JAZ1, GAI,* and *YFP*	[122]
Arabidopsis thaliana	Agrobacterium		*BRI1, JAZ1, GAI, CHLI1/2, AP1, TT4* and *GUUS*	[217]
Arabidopsis thaliana	Agrobacterium	Protoplast	*CHLI1, CHLI2* and *TT4*	[218]
Arabidopsis thaliana	Agrobacterium		*ADH1*	[219, 220]
Arabidopsis thaliana	Agrobacterium		*TRY, CPC* and *ETC2*	[221]
Arabidopsis thaliana	Agrobacterium		*At5g55580*	[145]
Arabidopsis thaliana	Agrobacterium		*ADH1* and *TT4*	[222]
Arabidopsis thaliana	Agrobacterium		*BRI1*	[224]
Arabidopsis thaliana	Agrobacterium		*ALS*	[225]
Arabidopsis thaliana	Agrobacterium		*ETC2, TRY, CPC* and *CHLI1/2*	[226]
Arabidopsis thaliana	Agrobacterium		*FT* and *SPL4*	[227]
Arabidopsis thaliana	Agrobacterium		*AP1, TT4* and *GL2*	[228]
Arabidopsis thaliana	Agrobacterium		*PDS3, AG, DUO1* and *ADH1*	[229]
Arabidopsis thaliana	Agrobacterium		*At3g04220*	[230]
Nicotiana benthamiana	Agrobacterium	Agroinfiltration	*PDS*	[121]
Nicotiana benthamiana		Agroinfiltration	*PDS*	[123, 262, 231]
Nicotiana benthamiana	Virus		*PCNA* and *PDS*	[136]
Nicotiana benthamiana		Agroinfiltration	*GFP*	[215]
Nicotiana benthamiana	Virus		*PDS, IspH* and *fsGUS*	[140]
Nicotiana tabacum		Protoplast	*PDS* and *PDR6*	[232]
Nicotiana tabacum	Virus		*SurA* and *SurB*	[138]
Nicotiana tabacum		Protoplast	*PDS*	[132]
Nicotiana tabacum	Agrobacterium		*PDS* and *STF1*	[233]
Nicotiana tabacum		Protoplast	*AOC*	[234]
Populus trichocarpa	Agrobacterium		*PDS*	[235]
Oryza sativa	Agrobacterium		*ROC5, SPP* and *YSA*	[122]
Oryza sativa		Protoplast	*SWEET11* and *SWEET14*	[215]
Oryza sativa		Agroinfiltration	*PDS*	[121]
Oryza sativa	Biolistic	Protoplast	*PDS-SP1, BADH2, Os02g23823* and *MPK2*	[144]
Oryza sativa	Agrobacterium	Protoplast	*MYB1*	[218]
Oryza sativa		Protoplast	*MPK5*	[236]
Oryza sativa	Agrobacterium		*CAO1* and *LAZY1*	[237]
Oryza sativa	Agrobacterium		*PTG1, PTG2, PTG3, PTG4, PTG5, PTG6, PTG7, PTG8* and *PTG9*	[236]
Oryza sativa	Agrobacterium		*OsBEL*	[238]
Oryza sativa	Agrobacterium		*FTL, GSTU, MRP15* and *Waxy*	[145]
Oryza sativa	Agrobacterium	Protoplast	*SWEET1a, SWEET1b, SWEET11, SWEET13* and *CYP76*	[239]
Oryza sativa	Agrobacterium		*PDS, PMS3, EPSPS, DERF1, MSH1, MYB5, MYB1, ROC5, SPP* and *YSA*	[240]
Oryza sativa	Agrobacterium		*DMC1A*	[241]
Oryza sativa		Protoplast	*PDS, DEP1, ROC5* and *miR159b*	[242]
Oryza sativa	Agrobacterium		*DL* and *ALS*	[233]

Table 3 (continued)

Species	Delivery methods		Edited gene	References
	Stable	Transient		
Oryza sativa		Protoplast	*PDS*	[132]
Triticum aestivum		Protoplast	*Mlo*	[144]
Triticum aestivum	Biolistic	Protoplast	*Gw2*	[147]
Triticum aestivum		Agroinfiltration	*Inox* and *Pds*	[231]
Zea mays		Protoplast	*zm ipk*	[128]
Zea mays	Agrobacterium	Protoplast	*zm hkt1*	[221]
Zea mays	Biolistic		*lig1, ms26, ms45, als1* and *als2*	[146]
Zea mays	Biolistic		*lig, ms26, ms45* and *als2*	[148]
Zea mays	Biolistic		*argos8*	[243]
Glycine max	Agrobacterium		*GFP, Glyma07g14530, 01gDDM1, 11gDDM1, Met1-04g, Met1-06g, miR1514* and *miR1509*	[244]
Glycine max	Biolistic		*DD20, DD43* and *ALS1*	[261]
Glycine max	Agrobacterium		*Glyma06g14180, Glyma08g02290* and *Glyma12g37050*	[246]
Glycine max	Agrobacterium		*bar, FEI1, FEI2* and *SHR*	[245]
Glycine max	Agrobacterium		*PDS11* and *PDS18*	[247]
Glycine max var		Protoplast	*FAD2-1A* and *FAD2-1B*	[234]
Solanum tuberosum	Agrobacterium		*StIAA2*	[223]
Solanum tuberosum	Agrobacterium		*StALS1*	[248]
Solanum tuberosum		Protoplast	*GBSS*	[142]
Hordeum vulgare	Agrobacterium		*HvPM19*	[249]
Marchantia polymorpha	Agrobacterium		*ARF1*	[250]
Solanum lycopersicum	Agrobacterium		*SlAGO7*	[251]
Solanum lycopersicum	Virus		*ANT1*	[127]
Solanum lycopersicum	Agrobacterium		*RIN*	[252]
Brassica oleracea	Agrobacterium		*BolC.GA4.a*	[249]
Papaver somniferum		Agroinfiltration	*4'OMT2*	[253]
Papaver somniferum	Agrobacterium		*eIF4E*	[254]
Xanthomonas citri subsp. citri	Agrobacterium	Agroinfiltration	*PDS*	[255]
Xanthomonas citri subsp. citri	Agrobacterium	Agroinfiltration	*LOB1*	[256]
Vitis vinifera	Agrobacterium		*IdnDH*	[257]
Chardonnay		Protoplast	*MLO-7*	[258]
Golden delicious		Protoplast	*DIPM-1, DIPM-2* and *DIPM-4*	[258]

Direct method

Protoplast transfection. A method for transfection and transient assays is protoplast transfection. This method enzymatically digests the cell walls of plant tissues and uses polyethyleneglycol (PEG) for transfection or electroporation for delivery (Fig. 4b). The same protoplasts can deliver several DNA constructs. Protoplast transfection has been proven to successfully deliver the CRISPR/Cas system and result in gene editing in *Arabidopsis thaliana, Nicotiana benthamiana*, rice, wheat, and maize, among others [113, 127–132].

Stable events

Indirect method

Agroinfection. Agrobacterium-mediated DNA delivery is the most commonly used method for almost all model plant species, main crop species, vegetable and fruit crops and forest crops. Similar to agroinfiltration, Agrobacterium can also create transgenic plants by genome integration in the plant nuclear DNA [133] (Fig. 4c).

Viral infection. The first viral vector used in plants was tobacco mosaic virus (TMV). Researchers used TMV to

silence a gene in *N. benthamiana* [134]. The majority of plant viruses are RNA viruses whose genomes are ssRNAs, as such they can be synthesized in vitro and used to inoculate plants, or they can be synthesized in vivo as DNA viruses from a plasmid introduced directly to plants by mechanical means for gene delivery [135]. To accelerate the delivery process, the viral genome can be inserted as a cDNA fragment into a binary vector and then can be used for agroinfection-mediated delivery into a plant cell (Fig. 4d).

Tobacco rattle virus (TRV) is an ssRNA virus that has two genome components, TRV1 (or RNA1) and TRV2 (or RNA2). Both genome components are required for inoculation. Plants edited using RNA viruses do not exhibit germline transmission of edits. For instance, Ali et al. used agroinfection to deliver the RNA1 genomic component of TRV and a vector derived from TRV RNA2 containing targeting gRNA into the leaves of *N. benthamiana* overexpressing Cas9 for gene editing in plant cells [136].

Geminiviruses, unlike TRV, do not require in vitro transcription prior to inoculation. Geminiviruses have a circular ssDNA genome [137]. Geminiviruses do not have a gene encoding DNA polymerase; therefore, their ssDNA genomes are converted into dsDNA genomes by host DNA polymerases in the nucleus. The dsDNA genome is then used as a template for virus transcription and rolling circle replication. Replication initiator protein (Rep) is essential for the initiation of rolling-circle replication. Rolling circle replication can either convert ssDNA genomes into dsDNA genomes or package ssDNA genomes into virions. Plant plasmodesmata pathways facilitate the transport of virions to adjacent cells [138, 139]. Bean yellow dwarf virus (BeYDV), which is a geminivirus, has been used to deliver the CRISPR/Cas system [138]. Studies have demonstrated gene editing using BeYDV in tomato (*anthocyanin mutant 1* gene, *ANT1*), and a modified cabbage leaf curl virus (CaLCuV) has been used in tobacco [127, 140]. Such approaches have also been applied in wheat, and researchers have enhanced the efficiency of this method by developing an optimized wheat dwarf virus (WDV) system [141].

Direct method

Protoplast transfection. Unlike the transient method of protoplast transfection, the stable transformation method generated targeted genome modifications in whole plants that were regenerated from gene-edited protoplasts [130, 131]. Two advantages of protoplast transfection are the ability to deliver multiple components and to do so at a high quantity. This method is highly suitable for gene editing using donor template repair. A high quantity of transfected cells can promote the recovery of gene editing via donor template repair. However, a disadvantage of protoplast transfection is the rate of plant regeneration in monocot plants. Protoplast transfection has been used for gene editing in potato [142], tobacco, and lettuce [131].

Biolistic particle delivery. Biolistic particle delivery is accomplished by transfecting cells via bombardment. Gene guns can penetrate the cell wall of plant cells with physical force to deliver DNA (Fig. 4e). This method is common in transforming plants due to its efficiency and its ability to deliver multiple DNA constructs simultaneously [143]. Most importantly, there is no plant species restriction to biolistic particle-based delivery. The main disadvantage of this method is that by introducing multiple copies of the DNA in the target plants, undesired effects such as gene suppression might occur in the recovered transgenic plants. Biolistic particle delivery has been used for gene editing in rice and wheat, soybean and maize using the CRISPR/Cas system [144–146]. In addition, this method is also used to deliver CRISPR/Cas9 RNPs for gene editing in crops, such as hexaploid wheat and maize [147, 148].

Future prospects in CRISPR/Cas delivery

The CRISPR/Cas system is simple but versatile. The CRISPR/Cas system has great potential for gene editing, but the delivery of CRISPR/Cas into cells dramatically impacts editing efficiency. There are still some aspects of delivery that can be improved to elevate the potential for translatability.

Immunity to the CRISPR/Cas system and its delivery vehicle

It is known that the Cas gene must be delivered into cells to express the Cas protein, and the long-term and robust expression of bacterially derived protein is expected to activate the host immune system. One solution to this problem is to use a protein-based delivery of the CRISPR/Cas system, which may have less immunogenicity, as the Cas protein would only be present in the target cell for a short period of time [98]. When combined with immunogenic effects caused by certain delivery vehicles, the level of immunogenicity might make negligible the efficiency of the CRISPR/Cas system. It has been reported that exogenous RNA delivered by lipid nanoparticles might activate Toll-like receptors and subsequent immune responses [149]. Therefore, the type of delivery vector should be carefully chosen. Moreover, it is especially important to consider the side effects of viral vectors. When compared to lentiviruses, AAVs and adenoviruses can avoid the risk of undesired DNA integration into the host genome. Producing viral DNA or protein within the cells of host can generate a risk for clinical applications [150, 151].

Engineered biomaterials in improving the delivery efficiency

Among the delivery vectors, the most suitable vectors for in vivo delivery may be nonviral vectors rather than viral vectors. Nonviral delivery, compared with viral delivery, exhibits potential advantages. It reduces the risk of off-target effects by decreasing the expression period of nuclease and enables better control of dosing duration [90]. The emergence and development of nanotechnology and material sciences have produced versatile applications in gene editing. It has been shown that gold-based nanoparticles enable effective delivery of RNP both in vitro and in vivo [100]. In addition, polymeric-based and lipid-based nanoparticles exhibit low immunogenicity, especially in their ability to encapsulate large cargos [152]. Additionally, it has been demonstrated recently that PEI-magnetic nanoparticles can improve the delivery of CRISPR/Cas9 constructs in vitro with low cell toxicity and have been shown to be a promising delivery system that can improve the safety and utility of gene editing [153, 154]. Moreover, researchers have demonstrated the delivery of the Cas9 RNP complex directly into cells using the nanoneedle array system and showed approximately 32% and 16% gene disruption efficiencies in HeLa cells and mouse breast cancer cells, respectively. Although the efficiency needs to be improved, researchers were able to successfully demonstrate gene editing by the direct delivery of Cas9/sgRNA using a nanoneedle array, and this method of delivery may be applied to gene knock-in via HDR [155]. Recently, Chen et al. demonstrated a platform comprised of vertically aligned silicon nanotube (VA-SiNT) arrays for gene editing. They successfully delivered Cas9 RNP to the target gene and demonstrated more than 80% efficiency of SiNT-facilitated biocargo internalization. This indicated that the nanotube-facilitated molecular delivery platform has great potential to propel gene-editing technologies [156]. However, nanoparticle-mediated protein delivery still has challenges, including the difficult process of packaging into designed materials and the prevention of RNP degradation before it enters the nucleus. Therefore, biocompatible, well-tolerated, high capability, and nonimmunogenic delivery vehicles are required to deliver cargos to the nucleus for effective gene editing, and these characteristics are essential when designing any nonviral delivery material.

Spatial and temporal regulation of Cas9 activity

As previously discussed, the unintended off-target effect of the CRISPR/Cas system is a major concern. Regulating delivery of the components of the CRISPR/Cas system to specific target sites before Cas9 is turned on and delivery of certain factors that switch on this machinery at a specific time point is critical. A number of teams have identified Cas9 endonuclease inhibitors. These anti-CRISPR (Acr) proteins, such as AcrIIA4, can shut off Cas9 activity [157, 158]. Moreover, anti-CRISPRs could be used to limit editing activity to particular cells and tissues in the body. Researchers designed miRNA-responsive Acr switches, and delivery of this machinery with Cas9 or dCas9 enabled tissue-specific editing [159]. In a recent study, researchers generated Cas9 variants called ProCas9s that enabled the CRISPR/Cas9 system to be turned on only in target cells [160]. ProCas9 senses the type of cell it is in based on proteases. This machinery enables the safer translational application of CRISPR/Cas9 gene editing, and this technology could be used to help plants defend against viral pathogens.

Several strategies to control the activity or expression of Cas9 have also been demonstrated (Table 4). It has been reported that Cas9 can be expressed in a split [161–164] or inactive form [165, 166]. In addition, an inducible system enabled Cas9 to be activated only when stimulated by a chemical inducer [167–171] or by exposure to certain types of light [164]. Studies have engineered a split-Cas9 system in which the activity of Cas9 is induced only when the two domains, recognition domain and nuclease domain, are assembled [172]. This split-Cas9 system is also utilized for gene editing using inteins. Inteins are protein introns that excise themselves out of host polypeptides to generate a functional protein [173]. The intein-based split-Cas9 system is composed of the split Cas9 domains, each of which is fused to intein sequences. Upon dimerization, these intein sequences will be spliced out, and fully active Cas9 can be generated [161]. Truong et al. demonstrated that Cas9 domains can be delivered by AAV vectors separately and

Table 4 Summary of regulatory CRISPR/Cas systems

Type of system	Split-Cas9			Light-inducible	Destabilizing domain	NS3 domain
	Intein-inducible	Rapamycin-inducible	Photoactivatable			
In vivo studies	[259]	n/a	n/a	[178]	[260]	n/a
Delivery vehicle	Viral-based delivery: AAV	n/a	n/a	DNA-based delivery: electroporation	DNA-based delivery: tail vein hydrodynamic injection	n/a

retain comparable editing efficiencies as full-length Cas9 [161]. Cas9 can also be chemically inducible by exposure to rapamycin, which induces FK506-binding protein (FKBP)-FKBP rapamycin binding (FRB) dimerization [174]. Rapamycin-inducible split-Cas9 is composed of split Cas9 fragments each fused with FRB and FKBP fragments. In the presence of rapamycin, a fully active Cas9 is formed. Researchers have also demonstrated a photoactivatable Cas9 (paCas9) system that utilized photoinducible dimerizing protein domains termed Magnets [164]. This optically controlled split-Cas9 system was generated by fusing each Cas9 fragment with magnet fragments (pMagnet and nMagnet) and triggering magnet dimerization upon blue light treatment [175]. Several other optically controlled systems have also been reported to enable CRISPR/Cas-based transcriptional activation and gene editing [175–178]. Nihongaki et al. developed a light-inducible system. They fused integrin binding protein 1 (CIB1) with dCas9 and fused cryptochrome 2 (CRY2) with a transcriptional activator domain, and then they used blue light to trigger dimerization of CIB1 and CRY2, resulting in subsequent expression of downstream targets [175]. Shao et al. developed a optogenetic far-red light (FRL)-activated CRISPR/dCas9 effector (FACE) system based on dCas9 [179–181] and the bacterial phytochrome BphS [182] that induced transcription of target genes in the presence of FRL [178].

Other strategies can also enable tunable regulation of CRISPR/Cas9 systems. Wandless and colleagues used small cell-permeable molecules to regulate protein stability. This chemical-genetic approach allowed rapid and tunable expression of a specific protein by fusing the molecules to a destabilizing domain [183]. The destabilizing domain acts as a degron that directs the fusion protein to proteasome-dependent degradation without the presence of a small molecule ligand, which allows tunable control of protein function. Ligand binding to the destabilizing domain protects the fusion protein from degradation and allows the protein of interest to function normally. Thus far, several ligand-destabilizing domain pairs have been discovered, including Shield-1 with mutant K506-binding protein (FKBP) 12 destabilized domain (FKBP[DD]), trimethoprim with mutant dihydrofolate reductase (DHFR) destabilized domain (DHFR[DD]), and CMP8 with the 4-OHT-estrogen receptor destabilized domain (ER50[DD]) [183–185]. This concept can be utilized for switchable gene editing and activation [186–188]. FKBP[DD], DHFR[DD], and ER50[DD] were fused to Cas9 for drug inducible gene editing [187, 188]. DHFR[DD] or ER50[DD] were fused to PP7-activation domain [179], and DHFR[DD] can be fused directly to dCas9 activator [186] for drug inducible gene activation. Multidimensional control can be achieved by pairing different ligand-destabilizing domain pairs with different aptamers [187]. Another platform utilizes the hepatitis C virus (HCV) nonstructural protein 3 (NS3) protease domain and its various inhibitors and has also been used to regulate CRISPR/Cas activity [189]. Tague et al. integrated the NS3 protease domain into dCas9–VPR to form a ligand-inducible CRISPR activation platform [189, 190]. The NS3 protease domain was inserted between the DNA binding scaffold and the C-terminal region, which is where NLS and VPR are located, to form a dCas9–NS3–NLS–VPR complex. NS3 protease can separate VPR from dCas9 and subsequently inhibit transcriptional activation, while in the presence of protease inhibitor, transcriptional activation is achieved. Recently, Cas9 has been fused with small molecule-assisted shut-off tag (SMASh), which consists of the HCV NS3 and nonstructural protein 4a (NS4A, acting as a degron). Cas9 stability can be controlled by SMASh via asunaprevir, an HCV protease inhibitor. Cas9 protein is degraded when NS3–NS4A is inhibited in the presence of asunaprevir, while in the absence of asunaprevir, the gene editing activity of Cas9 was restored [191].

Unfortunately, there are still some obstacles to progressing with the application of the regulatory approach to the CRISPR/Cas system. Chemical inducers may elicit cytotoxicity, which would make application of this approach in vivo more difficult. Additionally, light-induced systems may be limited to in vitro studies since activating such a system with light in vivo would be invasive, and penetration of light into tissue may cause other problems. Further investigation, optimization, and development are needed to overcome these challenges to advance the clinical translation of the CRISPR/Cas system.

Conclusion

The discovery and application of the CRISPR/Cas system offers great hope for the human disease treatment as well as revolutionize plant breeding. Although research on the CRISPR/Cas system in the life sciences community is well underway, there are still substantial barriers to efficient delivery that need to be overcome to achieve effective gene editing. Factors related to specificity, efficacy and regulatable expression are important to consider when selecting an approach. The development of new delivery methods has overcome many disadvantages that severely impede the translatability of the CRISPR/Cas system. With the rapid development of delivery methods, the successful translation of CRISPR/Cas technology into medical and agricultural applications is imperative and major improvements can be anticipated.

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