Molecular Cell

CellPress

Technology review

The CRISPR-Cas toolbox and gene editing technologies

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https://doi.org/10.1016/j.molcel.2021.12.002

SUMMARY

The emergence of CRISPR-Cas systems has accelerated the development of gene editing technologies, which are widely used in the life sciences. To improve the performance of these systems, workers have engineered and developed a variety of CRISPR-Cas tools with a broader range of targets, higher efficiency and specificity, and greater precision. Moreover, CRISPR-Cas-related technologies have also been expanded beyond making cuts in DNA by introducing functional elements that permit precise gene modification, control gene expression, make epigenetic changes, and so on. In this review, we introduce and summarize the characteristics and applications of different types of CRISPR-Cas tools. We discuss certain limitations of current approaches and future prospects for optimizing CRISPR-Cas systems.

INTRODUCTION

The term ''gene editing'' refers to a set of procedures for introducing desired changes at specific genomic loci that hold great promise for the life sciences. The original strategy for gene editing was to use programmable sequence-specific nucleases (SSNs) to generate DNA double-strand breaks (DSBs) at predetermined genomic sites, the desired changes being produced by subsequent non-homologous end joining (NHEJ), microhomology-mediated end joining (MMEJ), or homology-directed repair (HDR) (Gao, 2021). CRISPR (clustered regularly interspaced short palindromic repeats)-Cas (CRISPR associated) systems are prokaryotic adaptive immune system that bind and cleave foreign nucleic acids (Marraffini and Sontheimer, 2008; Brouns et al., 2008; Barrangou et al., 2007). The most frequently used type II CRISPR system is composed of two components: Cas9 nuclease and an artificial single guide RNA (sgRNA), a fusion of a CRISPR RNA (crRNA) and a *trans*-activating crRNA (tracrRNA) (Jinek et al., 2012; Deltcheva et al., 2011). When the SpCas9-sgRNA complex recognizes an NGG (N = A, T, C, or G) protospacer-adjacent motif (PAM) sequence, the spacer of the sgRNA pairs with the target DNA strand to form an ''R-loop'' structure. Subsequently, the Cas9 nuclease cleaves the DNA strands and produces a blunt-end DSB 3 bp upstream of the PAM into the protospacer (Figure 1A; Table 1) (Cong et al., 2013; Mali et al., 2013; Jinek et al., 2012). CRISPR-Cas gene editing tools are flexible, highly efficient, and inexpensive and have been widely applied. Recently, various Cas orthologs and variants with useful additional properties have been identified and harnessed for use in gene editing. Moreover, novel tools for precise gene modification, such as base editors (BEs) and prime editors (PEs), have greatly expanded the applications of gene editing (Li et al., 2021a; Anzalone et al., 2020) and have been leveraged for use in a variety of fields of research.

In this review, we first describe the naturally occurring Cas nucleases used for gene editing and variants engineered to expand the range of potential targets, provide increased specificity, and improve efficiency. Then, we describe CRISPR-Cas-mediated DSB-dependent and DSB-independent gene editing tools, with an emphasis on precision tools, such as base editors and prime editors. These gene editing tools are generally able to generate site-specific DNA lesions that are resolved by cellular DNA repair pathways (Nambiar et al., 2022). Finally, we summarize existing CRISPR-Cas-related technologies such as CRISPR screens, CRISPR-associated transposase-mediated insertion, multiplex gene editing, encoding of memory in DNA, and other fields.

CRISPR-CAS NUCLEASE SYSTEMS AND THEIR ENGINEERING

CRISPR-Cas systems have been divided largely into two classes: class 1 systems, including types I, III, and IV, that use multiprotein complexes to destroy foreign nucleic acids, and class 2 systems, including types II, V, and VI, that use single proteins (Makarova et al., 2020). In this section, we provide an overview of natural Cas nucleases that have been adopted for use as gene editing tools and describe engineered variants with improved performance.

Various Cas9 nucleases and their engineering

Cas9 nucleases, which are components of type II CRISPR-Cas systems, are RNA-guided DNA endonucleases that induce DSBs at target sites (Gasiunas et al., 2012; Garneau et al., 2010). Cas9 has two distinct nuclease domains, HNH and

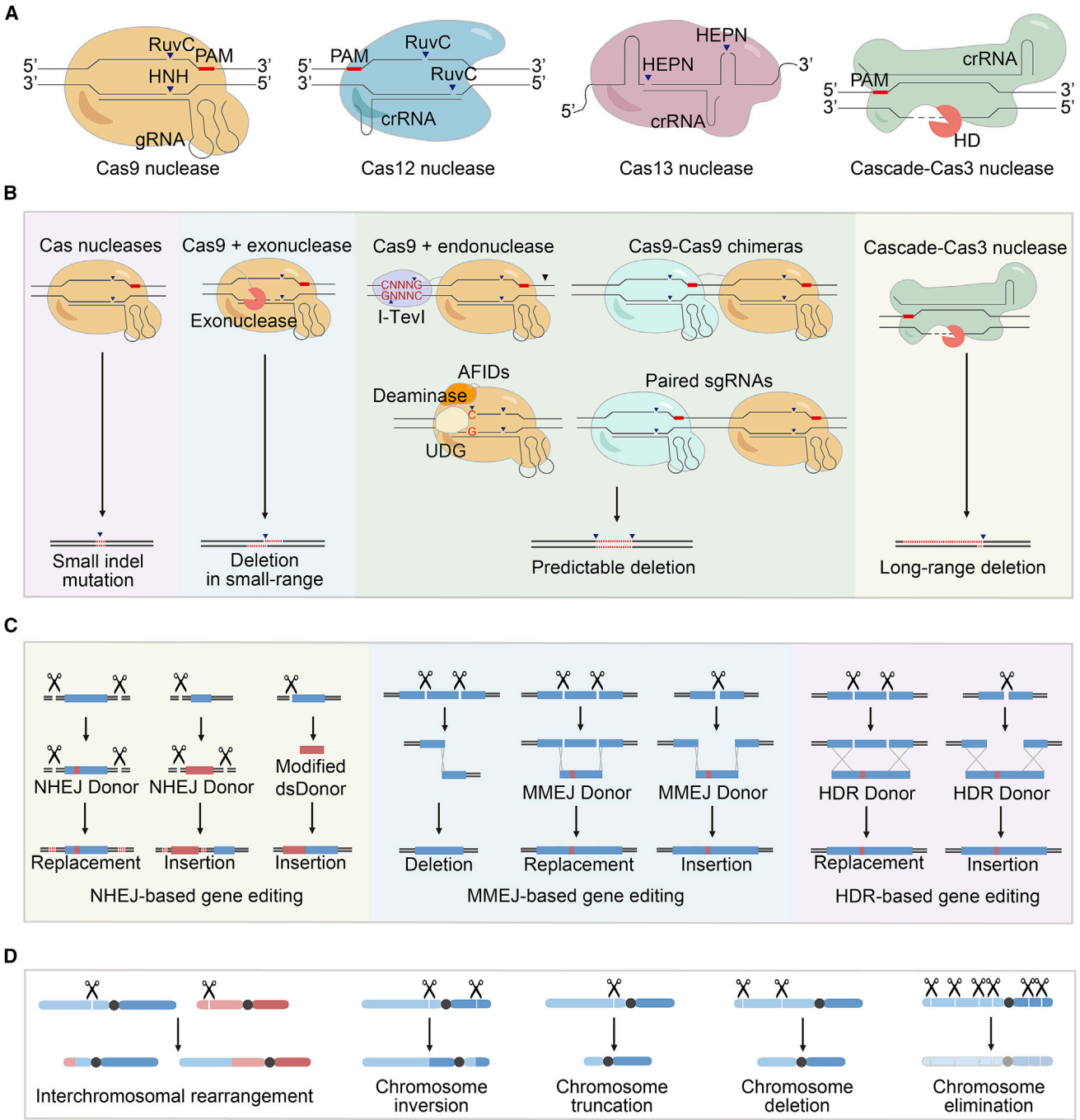

Figure 1. CRISPR-Cas tools for inducing DSBs
(A) Diagrams of gene editing by Cas9, Cas12, Cas13, and Cascade-Cas3 nucleases.
(B) Diagrams showing the production of various types of indel by CRISPR-Cas systems.
(C) Schematic diagrams of NHEJ-, MMEJ-, and HDR-based gene editing.
(D) Schematic diagrams of the production of chromosomal rearrangements by CRISPR-Cas.

RuvC, which cleave the target and non-target strand, respectively (Figure 1A; Table 1) (Gasiunas et al., 2012; Jinek et al., 2012). Inactivation of either nuclease domain creates a Cas9 nickase (nCas9), which cleaves only one DNA strand. Inactivation of both nuclease domains generates dead Cas9 (dCas9), which still binds to target DNA (Qi et al., 2013). nCas9 is useful in base editors and prime editors, which perform precise genome editing without requiring DSBs (Anzalone et al., 2020),

Table 1. Characteristics of CRISPR Cas nucleases

Nucleases	Cas9	Cas12	Cas13	Cascade-Cas3	Cas7-11
Type	type II	type V	type VI	type I	type III
Nuclease domain	RuvC and HNH	RuvC	HEPN	HD	RRM
Guide RNA	crRNA and tracrRNA	crRNA/crRNA and tracrRNA	crRNA	crRNA	crRNA
Substrate	dsDNA	mostly target dsDNA	RNA	dsDNA	RNA
PAM/PFS	generally G-rich	generally T-rich	no PFS bias[a]	variable[b]	no PFS bias
Cleavage pattern	mostly blunt end	staggered end	depends on local target sequence and secondary structure	nicks and degrades NTS DNA, then induces DSB	cleaves at two sites separated by 5–6 nt near the 3′ end of the binding site
Indel type	small indels	small indels	degraded RNA	long-range deletions	degraded RNA

[a]Although protospacer flanking sequence (PFS) bias has been identified *in vitro* and in *E. coli*, PFS bias probably does not affect interference in mammalian cells.
[b]GTH PAM for *M. aeruginosa* type I-D; AAG, AGG, ATG, GAG, TAG PAM for *E. coli* type I-E; AAG PAM for *T. fusca* type I-E.

and dCas9 serves as a scaffold for recruiting effectors proximal to specific genomic sites. dCas9 is widely used for regulating transcription, altering epigenetic controls, imaging living cells, and other purposes (Nakamura et al., 2021; Wang et al., 2016).

Although wild-type SpCas9 has robust activity in eukaryotic cells, its specific PAM requirement limits its applications, especially for carrying out precise genome editing, such as base editing and prime editing (Anzalone et al., 2020). To deal with this issue, many Cas9 orthologs and variants with a wide variety of PAM sequence preferences have been characterized and used in gene editing (Collias and Beisel, 2021). The properties of these Cas9 orthologs and variants are listed in Table S1. "Off-target" refers to nuclease cleavage at unintended genomic sites, caused by the nuclease tolerating mismatches between gRNA and off-target DNA. This is a major barrier to gene editing applications, especially to gene therapy. One strategy for improving Cas9 specificity is to use a pair of nCas9 nickases or a pair of fusions of dCas9 with the FokI DNA cleavage domain to generate a DSB at the on-target site (Tycko et al., 2016). Another strategy is to modify the gRNA. Truncated gRNAs (Tru-gRNAs) with spacer sequences of <20 nt, hp-gRNAs with hairpins at their 5′ ends (Kocak et al., 2019; Tycko et al., 2016), and CRISPR hybrid RNA-DNA (chRDNA) guides (Donohoue et al., 2021) have been reported to increase editing specificity. A third strategy is to develop high-fidelity Cas9 variants by rational design or directed evolution. The properties of these high-fidelity Cas9 variants are listed in Table S1. Two high-throughput screens have been performed to profile the specificity of such Cas9 variants (Kim et al., 2020; Schmid-Burgk et al., 2020), and these provide useful guides to application of these variants in different situations.

Efficient editing by Cas9 nuclease is also essential for its applications. There have already been reports of modifying CRISPR-Cas9 systems to improve their editing activities, for example, by modulating chromatin accessibility (Chen et al., 2017; Ding et al., 2019; Liu et al., 2019a), and recruiting end-processing enzymes (Čermák et al., 2017; Zhang et al., 2020a). In addition, optimizing gRNA structure by increasing the length of the duplex and disrupting potential terminator sequences increases the editing efficiency of Cas9 (Dang et al., 2015).

Various Cas12 nucleases and their engineering

Cas12 type V nucleases possess a single RuvC-like domain that cleaves both target and non-target strands and generates staggered ends downstream of PAM sites (Figure 1A; Table 1) (Zetsche et al., 2015). Cas12a (formerly known as Cpf1) was the first Cas12 nuclease to be used as a gene editing tool (Zetsche et al., 2015). It only requires a crRNA and can self-process pre-crRNA into mature crRNA, which is advantageous for multiplex gene editing (Zetsche et al., 2017). Cas12b, which requires both a crRNA and tracrRNA, has achieved gene editing in human cells and plants (Ming et al., 2020; Strecker et al., 2019a). Cas12c, Cas12d (formerly CasY), Cas12h, and Cas12i have RNA-guided DNA interference activity in *E. coli*; Cas12g is an RNA-guided ribonuclease (RNase) with collateral RNase and single-strand DNase activities; Cas12e (formerly CasX; 986 amino acids) and Cas12j (formerly CasΦ; 700–800 amino acids) have been adopted as gene editing tools in eukaryotic cells; and Cas12f (formerly Cas14) nucleases, such as Un1Cas12f1 (529 amino acids) and AsCas12f1 (422 amino acids) have been shown to achieve robust gene modification and regulation in mammalian cells (Anzalone et al., 2020; Karvelis et al., 2020; Kim et al., 2021a; Pausch et al., 2020; Wu et al., 2021; Xu et al., 2021b; Yan et al., 2019).

Various identified natural Cas12 orthologs have broader PAM recognition sites, and several Cas12a variants with weakened PAM constraints have also been developed (Table S1). Early studies showed that Cas12a was a high-fidelity nuclease (Anzalone et al., 2020). However, enAsCas12a, a AsCas12a variant with enhanced activity, has higher off-target activity than wild-type AsCas12a, but a high-fidelity derivative, enAsCas12a-HF1, has been generated to improve its specificity (Kleinstiver et al., 2019). Moreover, modifying crRNA structure can also enhance Cas12a editing activity (Bin Moon et al., 2018; Park et al., 2018).

Various Cas13 nucleases

Cas13 nucleases belonging to type VI CRISPR-Cas systems and containing two HEPN domains are RNA-guided ribonucleases. They can process their pre-crRNA into mature crRNA, only

require this crRNA to cleave target RNA, and have collateral activity (Figure 1A; Table 1) (Özcan et al., 2021; Abudayyeh et al., 2016). Several Cas13 nucleases, including Cas13a (Abudayyeh et al., 2017), Cas13b (Cox et al., 2017), Cas13d (Konermann et al., 2018), Cas13X, Cas13Y (Xu et al., 2021a), and Cas13bt (Kannan et al., 2021), have stable and robust gene knockdown activity in mammalian cells, and LwaCas13a can generate knockdown of endogenous genes efficiently in rice protoplasts (Abudayyeh et al., 2017). Additionally, transcript tracking and nucleic acid detection can also be achieved using Cas13a (Abudayyeh et al., 2017; Gootenberg et al., 2017). RNA base editors that edit A-to-I and C-to-U can be created by fusing catalytically inactive Cas13b (dCas13b) to the adenosine deaminase domain of ADAR2 and by directed evolution of ADAR2, respectively (Abudayyeh et al., 2019a; Cox et al., 2017). Moreover, using an ADAR-recruiting RNA (arRNA), endogenous ADARs can be recruited to achieve targeted RNA base editing (Qu et al., 2019). The fusion of catalytically inactive CasRx with a splicing factor can be used to manipulate alternative splicing (Konermann et al., 2018). Recently, the Cas13 nucleases Cas13X, Cas13Y, and Cas13bt have been identified and engineered for RNA knockdown and base conversion in mammalian cells (Kannan et al., 2021; Xu et al., 2021a).

Type I CRISPR-Cas systems

Although Class 1 CRISPR systems are the most abundant in bacteria and archaea, gene editing applications of class 1 systems are limited by the fact that they have multiple-subunit effectors. Of the class 1 systems, type I is the most widely used for gene editing, especially in creating long deletions. During type I-mediated gene editing, the CRISPR-associated complex for antiviral defense (Cascade), composed of multiple-subunit effectors and a crRNA, binds target DNA and forms an R-loop structure (Figure 1A; Table 1). Then, Cas3 specifically is recruited and cleaves the target DNA (Figure 1A; Table 1) (Zhu and Huang, 2019). Previous studies in prokaryotes have shown that reprograming of endogenous type I CRISPR-Cas systems can achieve editing of endogenous genes and alter gene expression (Xu et al., 2021c). Type I-E systems have been produced by fusing transcriptional activators or repressors to Cas effectors and have been shown to alter gene expression in human (Pickar-Oliver et al., 2019) and plant cells (Young et al., 2019). The type I-E system is capable of cleaving genomic DNA in human cells and induces large unidirectional deletions upstream of target sites (Dolan et al., 2019; Morisaka et al., 2019). Fusion of type I-E Cascade with the FokI nuclease domain can achieve gene editing in human cells and generates insertion/deletions (indels) at target sites (Cameron et al., 2019). Recently a type I-D system has been used to produce both small indels and bidirectional long-range deletions in human cells (Osakabe et al., 2021).

Other types of CRISPR-Cas systems

Type III CRISPR-Cas systems generally use several Cas proteins to target RNA, but the recently identified subtype III-E uses a single-protein effector (a fusion of multiple Cas7 proteins and a putative Cas11-like protein) to recognize and cleave target RNA (van Beljouw et al., 2021). Cas7-11 from *Desulfonema ishimotonii* (DiCas7-11) has robust RNA knockdown activity without any collateral cleavage activity (Özcan et al., 2021). Recently, ancestors of CRISPR-Cas nucleases have been identified and applied in gene editing. The IscB and TnpB proteins encode members of the IS200/IS605 transposon family that may be ancestors of Cas9 and Cas12, respectively. Both are RNA-guided nucleases that can generate DSBs in target sites and can be harnessed for gene editing in human cells (Karvelis et al., 2021; Altae-Tran et al., 2021).

CRISPR-CAS GENE EDITING TOOLS

CRISPR-Cas tools can be reprogrammed to bind to a given target and provide a platform for tethering other effectors that manipulate DNA or RNA within a restricted range of the target. In this section, we provide an overview of the versatility of CRISPR-Cas tools and their optimization for cleaving, precise gene editing, manipulating gene regulation, and producing epigenetic changes.

CRISPR-Cas tools for inducing double-strand breaks

NHEJ-based gene editing tools

NHEJ is the predominant pathway for repairing DSBs. It is considered an error-prone pathway that always results in indels at target sites (Figure 1B). To produce larger and predictable deletion, the cleavage motif of a homing endonucleases (such as I-TevI) or another Cas nuclease (such as SaCas9 or NmCas9) can be fused to Cas9 (Bolukbasi et al., 2018; Wolfs et al., 2016). Additionally, fusions with cytosine deaminase and uracil DNA glycosylase can induce targeted cytidine deamination and excision, respectively, thus generating predictable deletions extending from the deaminated cytidine to the DSB (Lin et al., 2021b; Wang et al., 2020). Paired sgRNAs are generally used to delete larger segments (Cong et al., 2013). Recently, type I systems have also been exploited to create large deletions in mammalian cells (Dolan et al., 2019; Morisaka et al., 2019; Osakabe et al., 2021).

NHEJ-based CRISPR tools can also be used to insert or replace large segments of DNA (Figure 1C) (Li et al., 2016; Suzuki et al., 2016). Chemically stabilized double-stranded oligodeoxynucleotides (dsODNs) can also increase the precision and efficiency of NHEJ-based insertion events. Furthermore, NHEJ can insert tandem-repeat structures to trigger MMEJ or HDR for accurately replacing small fragments (Lu et al., 2020). These findings indicate that NHEJ-based editing can be used for efficient seamless editing.

MMEJ- and HDR-based gene editing tools

Delivering a donor fragment with homologous sequencies along with a Cas nuclease should allow DNA strand breaks to be repaired by HDR, thus permitting precision editing (Figure 1C). However, the use of HDR-based gene modification is limited by its low efficiency (especially in plants) (Gao, 2021). Many strategies have been developed to improve the efficiency of gene targeting. For example, using donors with improved stability or higher copy numbers, adopting novel types of donor to facilitate HDR repair, recruiting donors proximal to the Cas nuclease cleavage site, manipulating DNA repair pathways, taking advantage of specific cell-cycle phases and cell types, and increasing the activity of Cas nuclease (Gao, 2021).

In plants, using geminivirus-based replicons as donors (which can be thousand-fold amplified) (Čermák et al., 2015), or nucleases driven by egg-cell promoters, greatly increases gene targeting efficiency (Miki et al., 2018; Wolter et al., 2018). However, there remains a need to identify geminiviruses and egg-cell promoters suitable for additional species.

MMEJ-mediated repair is capable of generating precise deletions between two micro-homologous sequences at target loci (Figure 1C) (Gao, 2021). MMEJ-based deletions could be predicted and the deletion fragments could be designed, providing a powerful way to generated targeted and predictable deletion (Ata et al., 2018; Shen et al., 2018; Xie et al., 2021). Seamless DNA fragment insertion and replacement can also be achieved via MMEJ by co-delivery of a donor with micro-homologous arms (Nakade et al., 2014), and these MMEJ-mediated modifications can be improved by overexpressing CtIP or exonucleases (Nakade et al., 2018; Weiss et al., 2020). In summary, approaches using MMEJ repair provide useful alternatives to HDR-mediated gene targeting.

Inducing chromosomal rearrangements via DSBs

Using CRISPR-Cas to introduce pairs of DSBs simultaneously in the same chromosome can generate targeted chromosomal deletions and inversions (Figure 1D) (Gao, 2021). Interestingly, entire chromosomes can also be eliminated by introducing multiple DNA cleavages (Adikusuma et al., 2017; Zuo et al., 2017), or they can be shortened by introducing a single DNA cleavage (Cullot et al., 2019; Figure 1D). Introducing two or more DSBs in different chromosomes can induce interchromosomal rearrangements including crossovers, translocations, and exchanges (Gao, 2021; Figure 1D).

For now, the frequency of targeted chromosome engineering remains low. Egg-cell promoters and SaCas9 nuclease can be exploited for chromosome engineering in plants (Rönspies et al., 2021). Moreover, because NHEJ inhibits the formation of chromosomal rearrangements by holding broken DNA ends together to promote their re-ligation, chromosomal rearrangements can be stimulated by disrupting NHEJ (Rönspies et al., 2021). These findings also hint that bringing the ends of different chromosome breaks close together might enhance chromosomal rearrangements.

Tools for CRISPR-Cas-mediated activities not requiring double-strand breaks

Base editors and their optimization

Base editors generate targeted base conversions without requiring DSBs (Figure 2A). Cytosine base editors (CBEs) (Hess et al., 2016; Komor et al., 2016; Ma et al., 2016; Nishida et al., 2016) and adenine base editors (ABEs) (Gaudelli et al., 2017), which combine deaminases with CRISPR systems, have been developed to produce C:G-to-T:A and A:T-to-G:C base transitions, respectively. In the case of CBEs, cytosine deaminase converts cytosines (C) to uridines (U) in the released single-stranded DNA (ssDNA), and the U:G mismatch can be repaired to U:A, finally resulting in a T:A base pair. Furthermore, fusions with uracil glycosylase inhibitor (UGI) are able to prevent the excision of U's and significantly increase the editing efficiency of CBEs (Anzalone et al., 2020). For ABEs, adenine deaminase converts adenines (A) to inosines (I), which may be recognized as guanines (G). Because there is no natural ssDNA adenine deaminase, the currently used DNA adenine deaminases have been evolved from *E. coli* tRNA-specific adenine deaminases (Gaudelli et al., 2017). Moreover, recently developed base editors can achieve C-to-G base conversions. However, the efficiency and product purity of these base editors remains low (Koblan et al., 2021; Zhao et al., 2021).

Several strategies have been used to improve the efficiency, precision, and specificity of base editors (Figure 2A). These include (1) increasing the efficiency of base editors by engineering the deaminase, using a deaminase of higher activity (Anzalone et al., 2020), or prolonging the exposure of the ssDNA region by, for example, using a single-stranded DNA-binding protein domain to facilitate the formation of an R-loop (Zhang et al., 2020b) (such optimizations also generally expand the editing window simultaneously); (2) increasing the precision of editing by fusing with natural or engineered deaminases with sequence context preferences to decrease bystander edits (Anzalone et al., 2020); (3) altering the editing window by altering the spatial location of the deaminase (Anzalone et al., 2020; Li et al., 2020b; Nguyen Tran et al., 2020) or recruiting multiple copies of deaminase (Anzalone et al., 2020); (4) reducing indels of CBEs by increasing the copy number of the UGI or using Gam to prevent degradation of DSBs (Anzalone et al., 2020); (5) using an engineered deaminase with lower affinity for ssDNA or RNA to reduce genome-wide and transcriptome-wide off-target effects induced by the ectopic expression of the deaminase (Anzalone et al., 2020; Jin et al., 2020a) (using domain-inlaid Cas9 base editors also reduces transcriptome-wide off-target effects; Li et al., 2020b; Nguyen Tran et al., 2020); and (6) using alternative or engineered deaminases to prevent suppression of the activities of certain APOBEC and AID deaminases by some epigenetic modifications (Anzalone et al., 2020).

Although these improvements significantly increase the performance of BEs, they will need to be further optimized to generate base transversions and targeted single-base transitions efficiently without bystander editing.

Prime editors and their optimization

Prime editors are powerful tools for installing base substitutions and precise DNA insertions and deletions. They are composed of two components: an engineered Cas9 nickase (H840A)-reverse transcriptase (RT) fusion protein and a prime editing guide RNA (pegRNA) (Figure 2B) (Anzalone et al., 2019). The pegRNA contains an RT template (RTT) encoding the desired edits and a primer binding site (PBS) for hybridization of the 3′ end of the nicked DNA strand to initiate reverse transcription. After reverse transcription, the RT template is reverse transcribed, forming a 3′ DNA flap followed by a 5′ DNA flap, and this enables the desired edit to be integrated into the target site (Anzalone et al., 2019). Recent research indicates that PEs do not induce detectable pegRNA-independent off-target effects and do not affect endogenous reverse transcription mechanisms (Jin et al., 2021). However, the efficiency of current PEs remains low, and several strategies have been used to increase it (Figure 2B): (1) using PE3(b) to create an additional nick in the non-edited strand (Anzalone et al., 2019), using engineered RTs or fusions with functional elements or peptides (Song et al., 2021; Velimirovic et al., 2021), or increasing the binding ability of nCas9 (Park et al., 2021); (2)

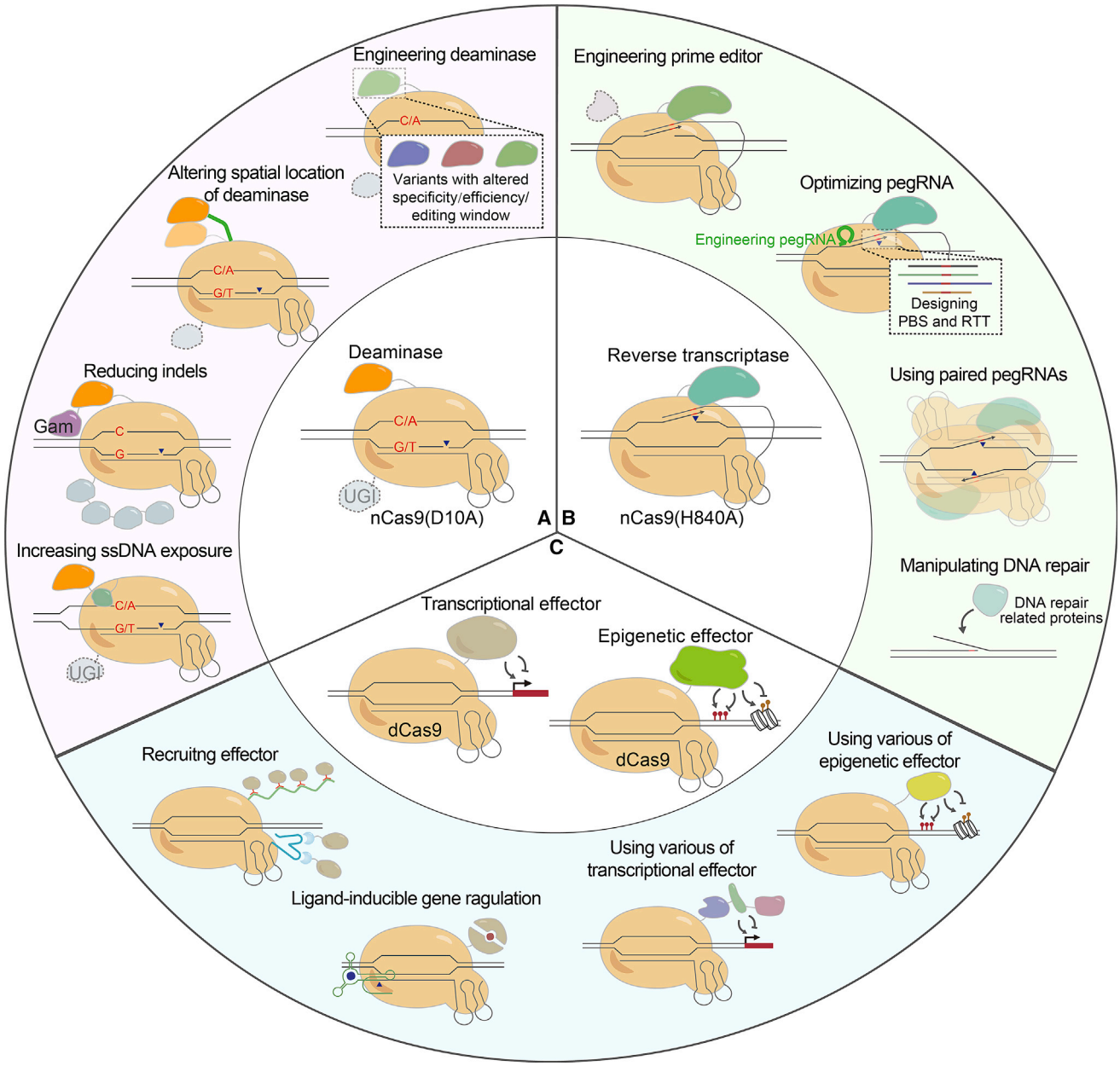

Figure 2. Systems for generating CRISPR-Cas-mediated effects without double-strand breaks
(A–C) Schematic diagram of base editing (A), prime editing (B), CRISPRa/CRISPRi and epigenetic modification (C), and optimization strategies.

using an optimized pegRNA with a higher level of transcription (Jiang et al., 2020) or with additional RNA aptamers to recruit RTs (Chai et al., 2021), or preventing pegRNA circularization or degradation (Liu et al., 2021; Nelson et al., 2021); (3) designing pegRNAs with appropriate PBS or RT templates, for example, by optimizing the melting temperature of the PBS of pegRNA (Lin et al., 2021a), or evaluating the efficiency of the pegRNA by deep learning (Kim et al., 2021b; Koeppel et al., 2021; Li et al., 2021b); (4) manipulating repair pathways, for example by downregulating the mismatch repair pathway (Chen et al., 2021; Ferreira da Silva et al., 2021); (5) using paired pegRNAs so that the reverse-transcribed RT templates can anneal and facilitate insertion of the desired edits (Anzalone et al., 2021; Choi et al., 2021; Jiang et al., 2021; Lin et al., 2021a; Zhuang et al., 2021); and (6) co-selecting prime edited events with selective markers to enrich for them (Levesque et al., 2021; Xu et al., 2020a; Xu et al., 2020b). However, PEs are still substantially less efficient than BEs in general and should be further optimized. Apart from improving their efficiency, there is a need to optimize the efficiency of their delivery, such as using RNP, split PE systems, or all-in-one vectors (Adikusuma et al., 2021; Aird et al., 2021; Petri et al., 2021; Zheng et al., 2021; Zhi et al., 2021).

Tools regulating gene expression

Combining transcriptional regulators that play roles in gene activation and repression with deactivated CRISPR-Cas systems can be used to regulate the expression of target genes (Figure 2C) (Gilbert et al., 2013, 2014). The first generation of CRISPR interference (CRISPRi) systems uses deactivated Cas9 itself or dCas9-repressive effector domains (such as KRAB) (Qi et al., 2013; Gilbert et al., 2013), whereas the first generation of CRISPR activation (CRISPRa) systems fuses deactivated Cas9 with single transactivator domains, such as VP64 (four copies of VP16) (Gilbert et al., 2013). Various transcription effectors can be used to improve the performance of both CRISPRa and CRISPRi, and RNA aptamers and multimeric peptide arrays can be incorporated to stimulate the recruitment of such transcription effectors (Xu and Qi, 2019). Moreover, regulating gene expression with CRISPR-Cas tools is becoming more controllable. The expression of a target gene can be tuned by recruiting different numbers and types of effectors to the target region, or using multiple or mismatched gRNAs for targeting (Bikard et al., 2013; Xu and Qi, 2019). It can also be controlled by incorporating ligands that respond to particular input signals (Xu and Qi, 2019) or incorporating ligand-responsive self-cleaving catalytic RNAs (aptazymes) into the guide RNA (Tang et al., 2017).

Epigenetic engineering technology

Epigenetic modifications regulate the expression of many genes and play essential roles in many cellular processes. A variety of CRISPR-based tools have been developed for investigating epigenetic control and generating targeted epigenetic modifications (Figure 2C). In fact, many effectors of CRISPRa/CRISPRi regulate the expression of epigenetic modifications (Nakamura et al., 2021). These CRISPR epigenetic engineering tools generally induce lower fold changes than standard CRISPRa/CRISPRi, but the changes are longer lasting (Chavez et al., 2016; Nakamura et al., 2021). The newly developed CRISPRoff could now initiate long-lasting DNA methylation and gene repression in more than hundreds of cell divisions and also persist through differentiation (Nuñez et al., 2021). By tethering the appropriate epigenetic effectors, CRISPR epigenetic engineering tools can achieve a variety of desired epigenetic modifications in the vicinity of the target, for example, DNA methylation/demethylation, histone methylation/demethylation, and histone acetylation/deacetylation. RNA epigenetic engineering tools to achieve targeted RNA epigenetic modifications have also been developed (Liu et al., 2019b; Wilson et al., 2020). Given the large number of different DNA and RNA modifications, many new types of epigenetic engineering tools could theoretically be developed by incorporating the appropriate epigenetic modification "writers" and "erasers" (Chen et al., 2020).

Similar strategies to those used for CRISPRa/CRISPRi could be exploited to improve the performance of CRISPR epigenetic engineering tools. Their specificity and stability could likewise be optimized (Hofacker et al., 2020; Nuñez et al., 2021).

CRISPR-CAS-RELATED GENE EDITING TECHNOLOGIES

CRISPR screens

CRISPR screens are used to dissect direct phenotype-to-genotype relationships in a high-throughput manner. CRISPR-Cas systems in combination with an sgRNA library can induce a large number of changes and integrate a unique sgRNA sequence for barcoding purposes into each cell (Figure 3A). In this way, NGS analysis of sgRNA abundance in response to different treatment can identify related genes.

CRISPR-based knockout usually produces random indels at target sites and has been extensively used in loss-of-function screens for identifying essential gene functional elements, long non-coding RNAs (lncRNAs), enhancers, microRNAs (miRNAs) and important components of cellular pathways (Doench, 2018; Ford et al., 2019). CRISPRa and CRISPRi are powerful tools for modulating gene expression and are extensively used for pooled gain-of-function and loss-of-function screens, respectively (Doench, 2018). It is worth mentioning that CRISPRa/CRISPRi screens have also facilitated the discovery of DNA repair factors that affect CRISPR-Cas tools, and knowledge of these factors can be helpful for understanding how these tools function, and for optimizing the tools (Koblan et al., 2021). CRISPR-dependent base editing screens can identify functional elements at single-base resolution (Cuella-Martin et al., 2021; Hanna et al., 2021). Furthermore, combining single-cell RNA sequencing and CRISPR screening technology has permitted the creation of new methods of profiling transcriptome perturbations, such as CROP-seq, CRISP-seq, and Pertub-seq (Doench, 2018; Ford et al., 2019; Jin et al., 2020b; Replogle et al., 2020).

Compared with animal cells, CRISPR screening in plants is still at an early stage. The studies reported in plants focus mainly on generating genome-wide mutant libraries or on specific gene loci in rice, tomato, maize, and soybean (Gaillochet et al., 2021). Unlike cell culture-based CRISPR screening, plant CRISPR screening studies only survey phenotypes at the level of the individual. Individual-level CRISPR screening has the advantage of permitting a comprehensive survey of traits but also requires more time and labor. Plant suspension cell cultures and protoplasts are two well-established systems that may facilitate the development of pooled CRISPR screening in plants.

Multiplex gene editing technology

Multiplex gene editing CRISPR-Cas systems have advantages over TALENs and ZFNs. Several strategies have been used to express multiple gRNAs in the same cell (Figure 3B). First, multiple gRNAs can be produced by tandem expression of Pol III promoter-driven expression cassettes (Cong et al., 2013). Second, multiple gRNAs can be transcribed into a single transcript from a Pol II or Pol III promoter, and mature gRNAs can then be released by transfer RNA processing, ribozyme self-cleavage, or Csy4 ribonuclease cleavage (Figure 3B) (Gao, 2021). Finally, multiple gRNAs can also be matured on the basis of the natural self-processing activity of CRISPR systems (Figure 3B). For type II CRISPR arrays, a tracrRNA needs also to be expressed (Cong et al., 2013). The diversity of Cas12 proteins further simplifies multiplexed genome editing because of the shorter length of the crRNA sequences and their efficient processing (Campa et al., 2019; Yan et al., 2019; Zetsche et al., 2017)

Beyond producing multiple sgRNAs to induce DSBs, several strategies have been used to implement multiplexed orthogonal editing. The first application established was using fully orthogonal Cas proteins fused to different effectors to mediate gene

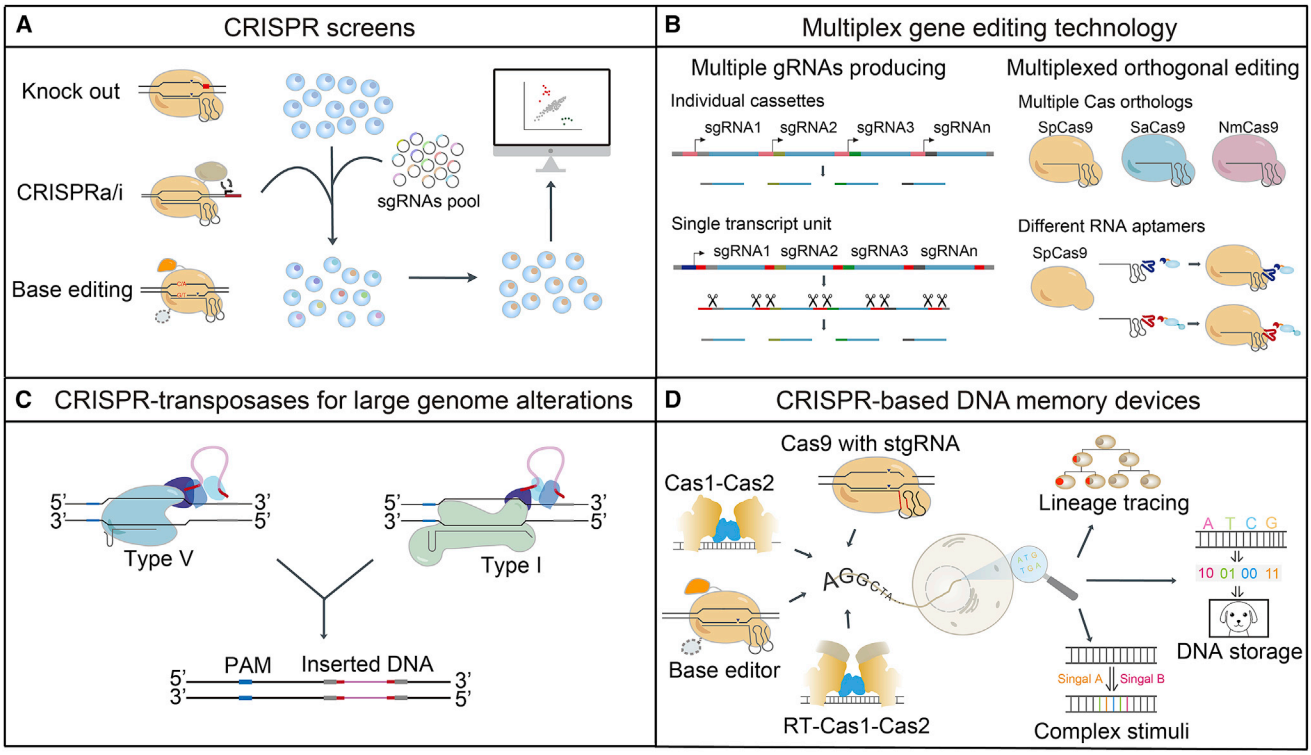

Figure 3. CRISPR-Cas-related gene editing technologies
(A) Pooled genetics screening using various CRISPR-related gene editing tools.
(B) Strategies for producing multiple sgRNAs and implementing multiplexed orthogonal editing.
(C) Site-specific DNA integration by type I and type V transposon-associated CRISPR systems.
(D) Recording multiple intracellular and extracellular events by various CRISPR-based DNA memory devices.

editing and simultaneously alter gene regulation within the same cell (Esvelt et al., 2013; Lian et al., 2017). Additionally, by modifying gRNAs, it was also possible to achieve multiplexed orthogonal editing (Fan et al., 2020). For example, aptamer-modified gRNAs can recruit various effectors and implement gene regulation and base editing simultaneously (Li et al., 2020a; Zalatan et al., 2015).

There is an urgent need for the application of multiplex gene editing technologies in plants. Polyploidization of many genes occurred during the evolution of plants, which suggests that it was necessary to mutate several copies of various genes to obtain the desired traits (Doust et al., 2014). The use of ribozymes, Csy4 and tRNA-mediated gRNA expression methods for multiplex editing in plants has been comprehensively surveyed (Tang et al., 2019), and multiplex gene editing technologies have been applied to *de novo* crop domestication (Gao, 2021). These technologies should further facilitate plant breeding.

CRISPR-transposases for large genome insertions

Transposon-associated CRISPR-Cas systems are paving the way for targeted, precise, and efficient insertion by a wide variety of mechanisms (Ma et al., 2021). Following on from earlier bioinformatics studies (Peters et al., 2017), two groups have shown that type I-B, I-F, and V-K CRISPR-Cas systems can direct a Tn7-like transposase to integrate donor DNA into target sites downstream of targeted DNA sequences in *E. coil* (Figure 3C) (Saito et al., 2021; Klompe et al., 2019; Strecker et al., 2019b). The type V-K transposon-associated CRISPR system, named CAST, consists of Cas12k and a Tn7-like transposase and can catalyze unidirectional DNA integration with up to 80% efficiency without selection in bacterial cells (Strecker et al., 2019b). The type I-F transposon-associated CRISPR system, named INTEGRATE, uses the TniQ-Cascade complex, which has high on-target non-directional integration activity and low off-target activity (<5%) for DNA integration (Klompe et al., 2019). Subsequently, a single-plasmid INTEGRATE system has achieved high-efficiency and multiplexed bacterial DNA integration (Vo et al., 2021). A DNA-editing all-in-one RNA-guided CRISPR-Cas transposase (DART) process has also been developed that is capable of generating organism- and location-specific insertions within natural communities (Rubin et al., 2020).

One of the major advantages of transposon-associated CRISPR systems is that they can achieve targeted DNA insertion directly without having to rely on endogenous repair machinery (Ma et al., 2021), and this has great potential in applications to organisms with low HDR repair efficiencies, especially plants. However, up to now there has been no report of the application of transposon-associated CRISPR systems in eukaryotes, perhaps because of their complex chromatin structure,

repression of transposases, or differences in post-transcriptional modifications.

CRISPR-based DNA memory devices

CRISPR-Cas-induced targeted mutagenesis has been linked to events of interest in living cells and repurposed as DNA memory devices to record multiple intracellular and extracellular events (Farzadfard and Lu, 2018; Kim and Lu, 2019) (Figure 3D). A CRISPR-Cas system that induces and accumulates mutations progressively in arrayed target sites during cell division has been used to measure the lineage relationships between different cell types (McKenna et al., 2016). A self-targeting guide RNA (stgRNA; also called a homing sgRNA) carries out continuous, localized mutagenesis and greatly expands the capabilities of CRISPR lineage tracing systems (Kalhor et al., 2018; Perli et al., 2016). Furthermore, combining single-cell sequencing and CRISPR lineage tracing systems enables simultaneous cell-type identification and lineage tracing (Spanjaard et al., 2018). The CAMERA and DOMINO systems use base editing for analog recording of multiple events at single-nucleotide resolution (Farzadfard et al., 2019; Tang and Liu, 2018).

CRISPR spacer acquisition systems, which consist of proteins Cas1 and Cas2 coupled with a CRISPR array, can store foreign DNA sequences within genomic arrays and have been used to record biological events (Shipman et al., 2016). The type I-E CRISPR system was the first to be harnessed for recording specific DNA information by electroporating sets of oligonucleotides into *E. coli* (Shipman et al., 2016), and it has also been used to encode images and short movies (Shipman et al., 2017). More recently, the reverse transcriptase-Cas1-Cas2 complex from *F. saccharivorans* has been leveraged to record transcriptional events in *E. coli* (Schmidt et al., 2018). This system can record transcriptional information concerning responses to RNA viruses, arbitrary sequences, and complex stimuli (Schmidt et al., 2018). Although these DNA memory devices have not yet been applied in plant research, they will no doubt have powerful applications in plant synthetic biology.

Other CRISPR-Cas-related technologies

The CRISPR-Cas system has also been applied in a broader manner. In genetic engineering applications, it has been used to generate gene drives, anti-virus, *in vivo* directed evolution, modulation of chromatin structure, and other applications. Generally, the term ''gene drive'' refers to systems in which one of two alleles is inherited more than 50% of the time (Bier, 2021). The efficient and flexible targeted DNA cleavage that can be achieved with CRISPR-Cas allows the gene drive allele to replace its homolog through HDR, thus creating super-Mendelian inheritance. CRISPR-based gene drive technology has been used in animals and plants (Bier, 2021; Gantz and Bier, 2015). CRISPR-Cas9 has also been harnessed to generate virus resistance in eukaryotic cells (Chen, 2021; Zaidi et al., 2020) and for directed evolution, by fusing DNA polymerases to Cas9 nickase to create the EvolvR system, which continuously diversifies all base pairs within a tunable window in a targeted locus (Halperin et al., 2018). Also, by coupling dCas9 with a compartment-specific protein, the so-called CRISPR-GO system can control the spatial position of genomic loci (Wang et al., 2018).

Apart from its use for genetic engineering, the CRISPR-Cas system is also used in nucleic acid detection, live cell imaging, and other fields. CRISPR-based nucleic acid detection technology offers a promising approach to the diagnosis of disease, because of its sensitivity, simplicity, and low cost (Kaminski et al., 2021; Nouri et al., 2021). Thus far, a series of ultrasensitive nucleic acid detection methods, such as SHERLOCK, HOLMES, DETECTR and others, have been developed (Chen et al., 2018; Chen, 2021; Gootenberg et al., 2017; Li et al., 2018), and CRISPR-Cas9-based diagnostics have also been created (Kaminski et al., 2021; Khodakov et al., 2016). In the agricultural field, Cas9 and Cas12a systems have been developed to detect DNA and RNA viruses (Chang et al., 2019; Jiao et al., 2021; Mahas et al., 2021), and SHERLOCK has been used for genotyping and quantifying traits using crude soybean extracts (Abudayyeh et al., 2019b). CRISPR-mediated live cell imaging technology provides a method for imaging specific genomic and transcriptomic loci in a dynamic fashion (Chen et al., 2016; Pickar-Oliver and Gersbach, 2019). The original version of the CRISPR-based imaging tool consisted of a fusion of dCas9 with enhanced green fluorescent protein (EGFP) and a structurally optimized gRNA; this system was able to display visually the location of specific genomic regions in living cells (Chen et al., 2013). Subsequently, CRISPR-based imaging was improved so as to display the locations of multiple loci simultaneously by labeling them with different fluorescent proteins, orthogonal Cas proteins, and RNA aptamers (Batool et al., 2021; Khosravi et al., 2020a). Researchers have used transiently transformed CRISPR imaging systems in *Nicotiana benthamiana* to visualize telomere regions (Khosravi et al., 2020a). Unfortunately, CRISPR imaging systems cannot label telomere regions in stably transformed *A. thaliana* or *D. carota*, possibly because binding of multiple Cas-GFP protein-induced R-loop regions leads to genome instability (Khosravi et al., 2020b).

CONCLUSIONS

Biological research has been revolutionized by harnessing CRISPR-Cas systems as genome manipulation tools. Various types of CRISPR-Cas nucleases and their engineered variants have been developed for multiple aspects of biological research, and the CRISPR-based gene editing toolbox has been greatly expanded beyond simple cutting of two strands of DNA. At the same time, the presently available CRISPR-Cas systems will no doubt be combined in future with novel functional elements to create an ever expanding toolbox for genome engineering research.

The precision of gene editing-based modification strategies needs to be further optimized to obtain higher efficiencies and avoid undesired byproducts. Moreover, strategies to tune the activity of CRISPR-Cas or CRISPR-derived editors in a more precise manner should be further developed to reduce the negative effects of ectopic expression of the effectors. We note that each expansion of the targeting scope or specificity and precision of engineered Cas nucleases tends to be accompanied by a reduction in editing efficiency. Thus, it is important to create robust and controllable CRISPR-Cas systems and Cas variants without reducing their editing efficiency.

Delivery of CRISPR-Cas reagents to specific organs or tissues remains a challenge in therapeutic applications as well as in agriculture applications. Thus, other types of optimization, especially of delivery strategies, will also be important for improving gene editing in addition to improving the performance of the CRISPR-Cas toolbox itself. In future, rapidly evolving and expanding CRISPR-Cas systems will become more efficient and safe and will be further leveraged for applications in multiple fields.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.molcel.2021.12.002.

ACKNOWLEDGMENTS

We thank Kevin Tianmeng Zhao for his insightful comments on the manuscript. We apologize to our colleagues whose work could not be included because of space constraints. This work was supported by grants from the Strategic Priority Research Program of the Chinese Academy of Sciences (XDA24020102 to C.G.), the Postdoctoral Innovative Talent Support Program of China (BX2021353 to Q.L., BX2021358 to G.L.), and the Young Elite Scientists Sponsorship Program of the China Association for Science and Technology (2020QNRC001 to S.J.).

DECLARATION OF INTERESTS

The authors declare no competing interests.

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