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Review

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CRISPR/Cas: a Nobel Prize award-winning precise genome editing technology for gene therapy and crop improvement

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Abstract: Since it was first recognized in bacteria and archaea as a mechanism for innate viral immunity in the early 2010s, clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein (Cas) has rapidly been developed into a robust, multifunctional genome editing tool with many uses. Following the discovery of the initial CRISPR/Cas-based system, the technology has been advanced to facilitate a multitude of different functions. These include development as a base editor, prime editor, epigenetic editor, and CRISPR interference (CRISPRi) and CRISPR activator (CRISPRa) gene regulators. It can also be used for chromatin and RNA targeting and imaging. Its applications have proved revolutionary across numerous biological fields, especially in biomedical and agricultural improvement. As a diagnostic tool, CRISPR has been developed to aid the detection and screening of both human and plant diseases, and has even been applied during the current coronavirus disease 2019 (COVID-19) pandemic. CRISPR/Cas is also being trialed as a new form of gene therapy for treating various human diseases, including cancers, and has aided drug development. In terms of agricultural breeding, precise targeting of biological pathways via CRISPR/Cas has been key to regulating molecular biosynthesis and allowing modification of proteins, starch, oil, and other functional components for crop improvement. Adding to this, CRISPR/Cas has been shown capable of significantly enhancing both plant tolerance to environmental stresses and overall crop yield via the targeting of various agronomically important gene regulators. Looking to the future, increasing the efficiency and precision of CRISPR/Cas delivery systems and limiting off-target activity are two major challenges for wider application of the technology. This review provides an in-depth overview of current CRISPR development, including the advantages and disadvantages of the technology, recent applications, and future considerations.

Key words: Genome editing; Clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein (Cas); Coronavirus disease 2019 (COVID-19); Cancer; Precision breeding; Crop improvement; Gene knock-out/in; Gene repair/replacement

1 Introduction

Precision targeting of specific nucleotide sequences has been a long-standing dream in research and industrial fields, with potential applications in gene functional studies, gene therapies, and precision breeding of crops and domesticated animals. In the 1990s, discovery of genome editing meganucleases brought this dream into reality and initiated the beginning of a new era of targeted genome editing. Meganucleases are a class of endodeoxyribonucleases, which occur naturally in a variety of different organisms (Silva et al., 2011). They function through the recognition and cleavage of specific double-stranded DNA (dsDNA) sequences, which are typically of >14 bp in length, the sequence of which varies among different meganucleases. As the first class of molecular DNA "scissors" that were successfully used to edit genetic sequences precisely, meganucleases allowed previously unattainable targeted replacement, elimination, and modification of DNA. Also, the long

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length of the recognition site allowed meganucleases to have high target specificity and low off-target effects. However, this also created challenges, as there is a limited number of pre-defined targets and it is therefore hard to find a naturally occurring meganuclease that can target a chosen DNA sequence. Although scientists have attempted to modify meganucleases to broaden the editing site potential, construction is arduous and success has been limited.

Several other nucleases have since been identified, and subsequently modified, for genome editing purposes. These include zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs). ZFNs and TALENs have similar properties, with both being composed of non-specific DNA cutting domains linked to specific DNA recognition domains. However, ZFNs recognize C2−H2 domains, whereas TALENs recognize DNA through transcription activator-like effectors (TALEs) (Baker, 2012). Although both ZFNs and TALENs have been well used to target and edit specific genes, with some applications in clinical treatments (Ellis et al., 2013; Xu et al., 2014; Aravalli and Steer, 2016; Bañuls et al., 2020), they both have some shortcomings for genome editing. Like meganucleases, engineering ZFNs and TALENs to target desired sequences can be time-consuming, and often requires specialist knowledge (Table 1).

In comparison, the more recently discovered clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein (Cas) system uses RNA-guided nucleases to create double-stranded breaks (DSBs) in DNA, and can be rapidly engineered to target almost any sequence with high efficiency, specificity, and ease of use (Table 1). Consequently, since its adaptation as a genome editing tool in 2012 (Gasiunas et al., 2012; Jinek et al., 2012), CRISPR/Cas has quickly become the dominant genome and gene-editing tool for precision editing, with much research already underway in relation to its potentials for gene therapy and crop improvement.

All of the above-mentioned methods have played significant roles in the history of precision genome editing, with *Nature Methods* (2012) listing meganucleases, ZFNs, and TALENs under the umbrella of molecular "scissors" as the Method of the Year in 2011, highlighting the significance of their development. All the methods are capable of knocking out/in individual genes, creating allelic mutations, changing gene regulatory controls, and adding reporters and epitope tags. Recently, the focus has shifted from their ability to edit genomes, to their efficiency and range. Each method has both benefits and drawbacks in its use. However, the perceived challenges to engineering meganucleases, ZFNs, and TALENs put them at a disadvantage, as it is not easy to design constructs to target the desired sequences. In this sense, the flexibility of CRISPR/Cas has allowed it to take the lead, and it has subsequently been used widely in applied and practical research. In recognition of this, CRISPR/Cas was selected by *Science* as the 2015 Breakthrough of the Year (Travis,

Table 1 Comparison of major genome editing tools

Genome editing tool	Interaction	Recognition site	Required agents	Required PAM	Inducing DSB	Cell toxicity	Specificity
Meganucleases	Protein–DNA	Large (12–40 bp dsDNA)		No	Yes	Low	Very high
ZFN	Protein–DNA	Long	ZFN with a *Fok*I DNA cleaving domain and a DNA-binding domain	No	Yes	Low	Very high
TALEN	Protein–DNA	Long	TALEN with a *Fok*I DNA cleaving domain and a DNA-binding domain (TAL repeats)	No	Yes	Low	Very high
CRISPR/Cas	RNA–DNA or RNA–RNA	Short	Cas and sgRNA	Yes	Yes	High	High

Genome editing tool	Off-target	Multiplex	Editing efficiency	Single nucleic acid targeting	Cost	Easy for construction
Meganucleases	Low	Difficult	Low	Yes	High	No
ZFN	Low	Difficult	Relatively low	Yes	High	Relatively hard
TALEN	Low	Difficult	Relatively low	Yes	High	Relatively hard
CRISPR/Cas	Relatively high	Yes	High	Yes	Low	Simple, easy, and robust

ZFN: zinc finger nuclease; TALEN: transcription activator-like (TAL) effector nuclease; CRISPR: clustered regularly interspaced short palindromic repeats; Cas: CRISPR-associated protein; dsDNA: double-stranded DNA; sgRNA: single guide RNA; PAM: protospacer-adjacent motif; DSB: double-stranded break.

2015), and in 2020, two pioneers of the CRISPR/Cas system, Dr. Emmanuelle CHARPENTIER and Dr. Jennifer A. DOUDNA, won the Nobel Prize in Chemistry for their work related to developing the technology.

In this review, we focus on the CRISPR/Cas system in regard to its applications in the precision genome editing and crop improvement of a variety of plant species, particularly agriculturally important crops. Major problems associated with advancing the biotechnology will also be discussed alongside its future potential.

2 CRISPR/Cas classification and protospacer-adjacent motif requirements

The CRISPR/Cas system was first discovered in bacteria and archaea, where it functions as a form of adaptive immunity against viruses (Ishino et al., 1987; Nakata et al., 1989; Hermans et al., 1991; Mojica et al., 1993; Jansen et al., 2002; Mojica et al., 2005). As an overview, Cas proteins typically recognize small motifs (about 3–6 bp) present in the invading DNA, known as the protospacer-adjacent motif (PAM) (Gleditzsch et al., 2019). The particular PAMs recognized differ among host species and are rarely present in the bacteria's own DNA to avoid self-cleavage (Gleditzsch et al., 2019). Following PAM recognition, a segment of downstream DNA (about 20 bp), known as a protospacer, is copied out of the foreign DNA and into a CRISPR array for transcription into short CRISPR RNAs (crRNAs) (Jinek et al., 2012). These then anneal to trans-activating crRNAs (tracrRNAs) already present in the cell, which form a stem loop structure to allow Cas enzymes to bind (Jinek et al., 2012). The RNA complex then acts as a guide for the Cas endonuclease to initiate sequence specific cleavage of the foreign DNA, creating a DSB and silencing the pathogen (Jinek et al., 2012). When the system was adapted as a molecular tool, the crRNA/tracrRNA complex was replaced with a single guide RNA (sgRNA), which can be designed to target any desired sequence, and is introduced alongside a Cas protein to cleave the target site.

Although the function of CRISPR/Cas has only recently been elucidated, scientists had been building up to the discovery for over 30 years. In 1987, whilst cloning an *iap* gene in *Escherichia coli*, Ishino et al. (1987) accidently cloned a sequence which contained uncommon, interspaced repeats. Two years later, similarly repeated sequences were found in two bacteria closely related to *E. coli*, *Shigella* and *Salmonella* (Nakata et al., 1989), followed by the less closely related species, *Mycobacterium tuberculosis*, in 1991 (Hermans et al., 1991; Mojica et al., 1993). Subsequently, scientists from research groups across the world began confirming that these sequences exist widely in both bacteria and archaea. In 2002, they were finally named as CRISPR (Jansen et al., 2002). However, CRISPRs were initially thought to function in genotyping (Mojica and Montoliu, 2016), and their true role was revealed only following the advancement of recombinant DNA technologies and the discovery of Cas proteins (Mojica et al., 1995, 2005; Jansen et al., 2002). Upon detection of its DNA cleaving capabilities, CRISPR/Cas was quickly modified for use as a genome editing tool in 2012 (Gasiunas et al., 2012; Jinek et al., 2012).

Among different species of bacteria and archaea, a wide range of CRISPR/Cas systems have now been classified. These typically make use of different Cas endonucleases. Among these systems, Cas endonucleases show significant differences not only in their organization, but also in their size and functional structures. By using this diversity as a base for classification, it was recently suggested that CRISPR/Cas systems collectively form 2 classes, 6 types, and 33 subtypes (Makarova et al., 2020). Class I represents systems which generally contain multiple Cas enzymes collaboratively functioning to target DNA, and can be segregated into 3 types (I, III, and IV) and 16 subtypes (I-A, I-B, I-C, I-D, I-E, I-F, I-G, III-A, III-B, III-C, III-D, III-E, III-F, IV-A, IV-B, and IV-C). Due to the complexity of engineering and introducing multiple Cas enzymes into a cell, Class I systems are rarely used as genome editing tools.

In comparison, Class II systems typically require only a single, large, multifunctional Cas enzyme, making them simpler for adaptation. As a result, much research has been expended on developing current Class II systems, and discovering more. Similar to Class I, in Class II there are currently 3 types (II, V, and IV) and 17 subtypes defined. Type II systems are the most well studied, following the early discovery of Cas9, which is currently the endonuclease most commonly used in CRISPR/Cas genome editing. However, research is ongoing, and the family is quickly expanding as more prokaryotes are explored. For example, in a recent study, Pausch et al. (2020) discovered a new CRISPR/Cas

system, termed CRISPR/CasΦ, in bacteriophages, which has a much smaller Cas endonuclease than previously observed (about 50% smaller than Cas9). In terms of genome editing, the smaller size may offer advantages, as it will be easier to deliver the molecule into cells. This highlights one way in which the inherent diversity is found in CRISPR/Cas, and allows the technology to be rapidly adapted for different purposes.

Cas9 endonucleases found in Type II systems contain both an RuvC and an HNH nuclease domain, each of which is responsible for cleaving one strand of a dsDNA sequence, allowing a blunt-ended DSB. As Type II systems are so well-studied, they have been widely applied for a variety of purposes, including knock-in/out, base editing, transcriptional regulation, and gene imaging. Each CRISPR/Cas subtype contains many Cas enzymes with high similarity, typically obtained from different species. Although they show high evolutionary conservation, slight differences in the sequences can affect their activity, and overall size can greatly impact success. A good example of this is a comparison between SaCas9, derived from *Staphylococcus aureus*, and SpCas9, derived from *Streptococcus pyogenes*. SaCas9 has 1053 amino acid residues, making it much smaller than the more commonly used SpCas9 with 1368 amino acid residues. This difference makes SaCas9 more suitable for delivery in vivo (Nishimasu et al., 2015), and it has recently been shown to have high editing efficiency in mammalian and plant cells (Ran et al., 2015; Steinert et al., 2015; Kaya et al., 2016; Xie HH et al., 2020).

Differences in the sequence of Cas variants can also lead to distinct PAM preferences. SpCas9 recognizes a 5'-NGG-3' PAM. However, several other Cas9 orthologues have been characterized which have distinct recognition and targeting requirements. For example, NmCas9, derived from *Neisseria meningitides*, recognizes a 5'-N4GATT-3' PAM (Esvelt et al., 2013; Hou et al., 2013), whereas St1Cas9 and St3Cas9, derived from *Streptococcus thermophilus*, both require either 5'-NNAGAAW-3' (where W represents A or T) or 5'-NGGNG-3' PAMs (Garneau et al., 2010; Magadán et al., 2012; Müller et al., 2016). CjCas9, derived from *Campylobacter jejuni*, recognizes 5'-NNNNACAC-3' or 5'-NNNNRYAC-3' (R and Y represent purines (A/G) and pyrimidines (C/T), respectively) (Kim E et al., 2017), and ScCas9, derived from *Streptococcus canis*, prefers 5'-NNG-3' PAM (Chatterjee et al., 2018). CasX, another Type II derived from Deltaproteobacteria and Planctomycetes, has been shown to recognize 5'-TTCN-3' (Burstein et al., 2017). Lastly, SaCas9 recognizes a 5'-NNGRRT-3' PAM (Nishimasu et al., 2015; Ran et al., 2015). This provides great flexibility when identifying target sites for a desired edit, as different Cas variants provide greater scope.

To further expand the scope of genome editing, several Cas9 nuclease variants have been engineered to introduce new PAM recognition sites. Cas9 VQR variant (D1135V/R1335Q/T1337R) recognizes 5'-NGA-3' PAM (Kleinstiver et al., 2015b; Hirano et al., 2016; Hu XX et al., 2016, 2018). Cas9 EQR variant (D1135E/R1335Q/T1337R) recognizes 5'-NGAG-3' PAM (Kleinstiver et al., 2015b; Hirano et al., 2016). Cas9 VRER variant (D1135V/G1218R/R1335E/T1337R) recognizes 5'-NGCG-3' PAM (Kleinstiver et al., 2015b; Hirano et al., 2016). The xCas9 variant (A262T/R324L/S409I/E480K/E543D/M694I/E1219V) recognizes NG, GAA, and GTA PAMs (Hu JH et al., 2018; Wang JJ et al., 2019; Zhong et al., 2019). SpCas9-NG variant (R1335V/L1111R/D1135V/G1218R/E1219F/A1322R/T1337R) recognizes 5'-NG-3' PAM (Nishimasu et al., 2018; Ge et al., 2019; Zhong et al., 2019), and the SaCas9-KKH variant (E782K/N968K/R1015H) recognizes 5'-NNNRRT-3' PAM (Kleinstiver et al., 2015a).

Two advances have recently been reported which further eliminate the constraint of PAM sequences in Cas9. Walton et al. (2020) developed two SpCas9 variants, named SpG and SpRY, that are able to recognize 5'-NGN-3' and 5'-NRN-3'/5'-NYN-3' (Y represents C or T) PAMs, respectively. Using phage-assisted non-continuous evolution, David LIU's group has also characterized three new SpCas9 variants, SpCas9 NRRH, SpCas9 NRCH, and SpCas9 NRTH, which collectively show preference for the 5'-NRNH-3' (H represents A, C, or T) PAM (Miller et al., 2020).

Class II, Type V CRISPR/Cas is the second most researched group and has proven effective in both animal and plant species. It is composed mostly of Cas12a (Cpf1) endonucleases (Zetsche et al., 2015; Kim H et al., 2017; Tang et al., 2017; Xu et al., 2017). These systems have been discovered in *Acidaminococcus* sp., *Francisella tularensis* subsp. *novicida* and *Lachnospiraceae bacterium* (Zetsche et al., 2015; Tang et al., 2017). Although Cas12a is analogous to Cas9, it has unique features that differ from Cas9 and its orthologs. Firstly, cleavage via a Cas12a protein relies on a single RuvC-like endonuclease domain, not the RuvC

and HNH combination found in Type II (Zetsche et al., 2015). Secondly, Cas12a cleaves target DNA using only crRNAs, rather than a crRNA/tracrRNA complex (Zetsche et al., 2015). Thirdly, CRISPR/Cas12a genome editing depends on recognition of a T-rich PAM sequence (5'-TTTV-3' PAM) (Zetsche et al., 2015). Together, this creates a 4- or 5-nt 5'-staggered DSB at targeted sites, which facilitates more precise gene replacement mediated by the non-homologous end joining (NHEJ) DNA repair pathway (Zetsche et al., 2015).

As with Cas9, several Cas12a variants have been identified for engineered genome editing. The AsCas12a RR variant (S542R/K607R) and AsCas12a RVR variant (S542R/K548V/N552R) recognize 5'-TYCV-3' and 5'-TATV-3' PAMs, respectively (Gao et al., 2017). In comparison, the LbCas12a RR variant (G532R/K595R) recognizes 5'-CCCC-3' and 5'-TYCV-3' PAMs and the LbCas12a RVR variant (G532R/K538V/Y542R) recognizes 5'-TATV-3' PAM (Li SY et al., 2018; Zhong et al., 2018).

Among the Class II systems, Type VI is the most distinct. Cas13 dominates Type VI and contains neither an RuvC nor an HNH nuclease domain. Instead, Cas13 contains two HEPN domains which target RNA instead of DNA, opening up a novel avenue for epigenetic editing. In addition, although PAM recognition is a requirement for most CRISPR/Cas systems, the ability to target RNA mitigates this requirement in proteins such as Cas13. However, to date, Cas13 is the only Cas found that can directly target RNA sequences (Makarova et al., 2020).

3 CRISPR/Cas precision genome editing

3.1 Repair pathways

Once DSBs have been formed by CRISPR/Cas in DNA, cells typically undergo one of two main repair mechanisms. The most commonly used pathway is NHEJ, in which a DNA ligase links the two broken DNA ends together to reconnect the sequence. During this process, it is common for one or more nucleotides to be inserted and/or deleted at the DSB site. Due to this, NHEJ often results in either a frameshift in the DNA that changes the amino acid sequence or a nonsense mutation. Thus, CRISPR/Cas is efficient at inducing gene mutations and is widely used for functional studies of individual genes. DNA can also be repaired via homology-directed repair (HDR). HDR is an error-free repair pathway in which a homologous DNA template, typically obtained from a gene copy or homologous gene, is inserted to fill the DSB and reform the original DNA sequence. As understanding of these repair mechanisms has increased, researchers have been able to add artificial DNA templates into cells, such as whole genes with arms homologous to the DSB (Heyer et al., 2010), allowing the insertion of specific DNA sequences. This facilitates overexpression studies, as it allows targeted gene insertion at precise DNA locations, a feat which cannot be achieved with traditional transgene technologies.

Although both NHEJ and HDR can be used to repair DSBs generated by CRISPR/Cas, NHEJ is the most common (Feng et al., 2021). The HDR pathway is used much less often because of its lower frequency and efficiency, even when a donor DNA template is present. Many factors can affect this efficiency, including cell type, cell cycle stage, concentration of the donor DNA template (Dickinson et al., 2013), length of the homologous arms, the CRISPR/Cas system (as discussed above), and the delivery system (Lin et al., 2014). HDR efficiency was dramatically increased in HEK293T human primary neonatal fibroblast and human embryonic stem cells relative to experiments with unsynchronized cells, with rates of HDR of up to 38% observed (Lin et al., 2014), and dividing rather than nondividing cells. In addition, controlled cell cycling provided a 6-fold increase in the ratio of HDR to NHEJ repair pathways in quiescent stem cells ex vivo and in vivo (Shin et al., 2020). Co-transfection of a CRISPR/Cas system with RAD51, a key molecule during the initial step of HDR, also increased HDR efficiency 2.5-fold in layers 2/3 of pyramidal neurons in the somatosensory cortex of mouse brains (Kurihara et al., 2020). Inhibition of DNA ligase IV (LIG4), an essential molecule for NHEJ, increased efficiency of HDR-mediated knock-in (Cao et al., 2020). Overall, inhibition of LIG4 appeared more effective than RAD51 overexpression for induced HDR-mediated knock-in (Cao et al., 2020). HDR-mediated genome editing has been reported in both plants and animals. Generally, HDR-mediated genome editing in plants has been achieved with only up to 10% success, although there are few reports of high efficiency (Li, 2009; Li et al., 2013; Sauer et al., 2016). Efficiency of 30% or even higher can be achieved after certain modifications, such as using single-stranded oligodeoxynucleotides (ssODNs) instead of a plasmid donor

in animals (Yang et al., 2014). Thus, it is much easier to obtain HDR-mediated genome editing in animal cells than in plant cells.

One route which is being explored to increase efficiency and specificity is modifying the Cas endonucleases. To date, much of this research has been focused on modifying the cutting domains in Cas9. Loss function of a single cutting domain, known as nick Cas (nCas), or both domains, known as dead Cas (dCas), has been shown not to affect Cas-binding activity. Also, loss of function of one domain does not affect the cutting activity of the other. These modified Cas proteins can then be fused with other functional proteins, which has paved the way towards new CRISPR/Cas genome editors with expanded purposes, including recently developed base editors and prime editors (Table 2).

3.2 Base editors

Base editors are capable of creating specific base changes. This is achieved by fusing a dCas9 nuclease with an engineered base converter enzyme, which mediates the conversion of one base to another under the guidance of a sgRNA. Currently, three kinds of base editors have been generated. The first, cytosine base editors (CBEs), catalyze the conversion of a single C to a T, which in turn initiates a change from a C/G pair to a T/A pair. This is accomplished by fusing dCas9 with cytidine deaminase and uracil glycosylase inhibitor (UGI) domains (Komor et al., 2016; Li XS et al., 2018; Zong et al., 2018). The next category is adenine base editors (ABEs), which consists of dCas9 fused with an evolved transfer RNA adenosine deaminase (TadA*). This converts an A to a G, initiating an A/T to G/C base pair switch (Gaudelli et al., 2017; Kang et al., 2018; Hua et al., 2020). Lastly, and most recently, two independent research groups have established two new base editors, known as glycosylase base editors (GBEs) and C-to-G base editors (CGBEs), which convert C-to-A and C-to-G, respectively (Kurt et al., 2020; Zhao et al., 2020).

Base editor tools are rapidly being developed to achieve even higher base conversion efficiencies and broader activity window spans. To date, there have been four generations of CBE base editors developed, each with optimized sequence composition and cytidine deaminases or derivatives, including BE1, BE2, BE3, BE3-YE1/BE3-YE2/BE3-YEE/BE3-EE, and BE4 (Komor et al., 2016, 2017; Kim YB et al., 2017; Gehrke et al., 2018; Endo et al., 2019; Wu Y et al., 2019; Doman et al., 2020; Tan JJ et al., 2020). In addition, for ABE base editors, ABE7.10, ABE8, and ABE8e have been reported to have high catalytic activity for installing A/T to G/C point mutations (Gaudelli et al., 2017, 2020; Ren et al., 2019; Li C et al., 2020; Richter et al., 2020).

Although base editors have been developed to accomplish conversion of C/G to T/A, A/T to G/C, C-to-A, and C-to-G, they are still constrained by their ability to insert, delete, or create other transversion mutations,

Table 2 Comparison of major CRISPR/Cas genome/gene editors

CRISPR/Cas genome/ gene editors	Cas enzyme	Additional enzyme	Induced DSB or SSB	Require DNA template	gRNA	Major application
Regular CRISPR/Cas editor without DNA template	Cas		DSB	No	sgRNA	Gene knock-out, inducing silence mutations
Regular CRISPR/Cas editor with DNA template	Cas		DSB	Yes	sgRNA	Gene knock-in, DNA replacement
CRISPR/Cas epigenetic editor	dCas	Epigenetic modifier, including DNA methyltransferase	No	No	sgRNA	Regulation of epigenome and gene expression
CRISPRi editor	dCas	dCas alone or fusing with a repressor	No	No	sgRNA	Knock-down
CRISPRa editor	dCas	Fusing an effector with dCas9	No	No	sgRNA	Activation of gene expression
Base editor	dCas	Nucleobase deaminase enzyme	No	No	sgRNA	Base change
Prime editor	nCas	Reverse transcriptase	SSB	No	pegRNA	Sequence repair

CRISPR: clustered regularly interspaced short palindromic repeats; Cas: CRISPR-associated protein; CRISPRi: CRISPR interference; CRISPRa: CRISPR activator; dCas: dead Cas; nCas: nick Cas; DSB: double-stranded break; SSB: single-stranded break; gRNA: guide RNA; sgRNA: single guide RNA; pegRNA: prime editing guide RNA.

such as G to C and G to T (Komor et al., 2016; Nishida et al., 2016; Gaudelli et al., 2017; Kim YB et al., 2017; Anzalone et al., 2019; Chen et al., 2019; Lin et al., 2020).

3.3 Prime editors

In 2019, David LIU's group at Harvard University developed a new CRISPR/Cas genome editing system known as a prime editor, by fusing a reverse transcriptase (RT) to a catalytically impaired Cas9 endonuclease (CRISPR/dCas9 H840A) (Anzalone et al., 2019). Prime editing is a search-and-replace genome editing technology that directly inputs a new genome sequence without a DNA template in the targeted genome site, and is revolutionizing this field. Prime editing systems harbor a programmable prime editing guide RNA (pegRNA) that functions to produce sgRNAs and RT templates of desired edits (Anzalone et al., 2019). These systems have been demonstrated to work in both human and plant cells, including wheat and rice (Anzalone et al., 2019; Li HY et al., 2020; Lin et al., 2020; Tang et al., 2020; Xu et al., 2020). Compared to classic CRISPR/Cas systems, prime editing systems have the capability to accomplish targeted DNA insertions, deletions, and 12 types of base conversions, without generating DSBs or needing donor DNA templates (Anzalone et al., 2019). These hold great potential for the future of genome editing, but the editing efficiency of prime editors is closely determined by the design of an appropriate pegRNA, and so requires specific knowledge to obtain success (Anzalone et al., 2019).

Prime editors are a new type of genome editor, many details still need to be modified, and more studies are required to realize their full potential. This new technology was first developed in animal models and can achieve a reasonable genome editing efficiency of up to 50% in human cells (Anzalone et al., 2019). Although it has been used successfully to edit plant genes, the editing efficiency is much lower in plants than in animals (Anzalone et al., 2019; Li HY et al., 2020; Lin et al., 2020; Tang et al., 2020; Xu et al., 2020). Also, more targeted genes should be tested in both plants and animals in the future.

3.4 Epigenome editors

As understanding of the factors regulating genetic processes has improved, epigenetics has been revealed as an extremely important factor for controlling biological pathways. This has led to it becoming a novel target for clinical therapy and crop improvement. DNA methylation, histone modification, and non-coding RNAs are all primary epigenetic factors that aid in controlling gene expression. The extent of their control is being explored via CRISPR/Cas editing.

It is well known that DNA methylation is a major epigenetic modifier in plants and animals (Bogdanović and Lister, 2017). Methylation plays a role in controlling many biological and metabolic processes, including cell identity (Bogdanović and Lister, 2017), tissue differentiation and development (Zhang HM et al., 2018), response to environmental stresses (Zhang HM et al., 2018), fruit ripening (Huang et al., 2019), and cell wall biosynthesis and formation (Haas et al., 2020; Zhang and Zhang, 2020a). The term DNA methylation encompasses the process whereby specific DNA sequences become methylated or demethylated, either inhibiting or promoting gene expression, respectively. With this in mind, regulating DNA methylation represents a novel pathway for controlling gene expression and subsequent gene function.

DNA methylation is initiated by DNA methyltransferase (DNMT), so controlling DNMT activity should in turn control DNA methylation. By fusing dCas9 to a catalytic domain of DNMT, such as DNMT3 or MQ3, CRISPR/Cas systems have successfully targeted methylation to specific sites in both plants and animals (Liu et al., 2016; Vojta et al., 2016; Lau and Suh, 2018). By fusing dCas to a demethylation enzyme, such as a ten-eleven translocation (TET) family member, the CRISPR/dCas system may also be used to remove methyl groups from specific DNA sequences, initiating increased expression of the target gene. Lau and Suh (2018) successfully demonstrated this by targeting a CGG expansion mutation in the 5'-untranslated region (5'-UTR) of the fragile X mental retardation 1 (*FMR1*) gene in Fragile X syndrome (FXS) patients, which is the most common form of genetic intellectual disability in males. Several studies have shown CRISPR/dCas9-TET1 capable of removing up to 90% of targeted methylations, significantly increasing gene expression at the target sites (Liu et al., 2016; Morita et al., 2016; Hanzawa et al., 2020; Horii et al., 2020). Horii et al. (2020) also used this technology successfully to generate an epigenetic disease model in mice via targeted demethylation of the epigenome.

DNA in cells is wrapped in chromatin fibers which are held together with histone proteins that keep the DNA

condensed. Thus, histones play an important role in controlling gene expression. Many studies have shown that both histone methylation and acetylation affect chromatin and histone remodeling, which in turn affects gene expression (Strahl and Allis, 2000; Adli, 2018). Several of these studies have focused on the enzymes associated with histone methylation and acetylation, to further elucidate gene regulation. One such enzyme, lysine-specific demethylase 1 (LSD1), is a histone demethylase that selectively removes one or two methyl groups from histone H3 at the Lys4 position (Hayward and Cole, 2016). By fusing dCas9 with LSD1, Kearns et al. (2015) were able to successfully target active enhancer markers, H3K4me2 and H3K27ac, and alter the expression profiles of target genes (Kearns et al., 2015). In addition, by fusing a dCas9 with a transcriptional repressor histone deacetylase 1 (HDAC1), Liu et al. (2020) successfully silenced the *KRAS* gene, one of the most frequently mutated oncogenes in cancer patients. With the *KRAS* mutation, cell growth was significantly inhibited and the cell death was increased in cancer cells (Liu et al., 2021).

Non-coding RNAs have recently been revealed as important epigenetic gene regulators. In particular, small non-coding RNAs such as microRNAs (miRNAs), have been highlighted to play diverse roles in development and stress responses in plants and animals (Zhang et al., 2007b; Gebert and MacRae, 2019), including serving as oncogenes and tumor suppressors (Zhang et al., 2007a). Due to their new-found significance, a lot of attention has been placed on developing methods to edit individual miRNA genes, as the function of many miRNAs is still unclear. Using CRISPR/Cas9, Huo et al. (2017) were able to successfully knock out the miRNA-21 (miR21) gene in ovarian cancer SKOV3 and OVCAR3 cell lines. The edited cell lines revealed that the miR21 knock-out significantly inhibited cancer cell proliferation, migration, and invasion, highlighting a prime target for cancer gene therapies (Huo et al., 2017). To add to this, again using CRISPR/Cas9, Jiang et al. (2020) were able to knock out several miRNA genes, including miR30c, miR205, and miR663a, in human LNCaP cell lines, and were able to determine that miR-1225-5p and miR-663a knock-outs decreased lactate production in LNCaP cells. However, although miRNA-related research is a quickly developing field with thousands of miRNAs already identified, most research has been focused on humans, and there is still much to learn. With the application of CRISPR/Cas genome editing, information can be obtained in a specific, targeted manner, accelerating progress towards miRNAs as targets for clinical treatments and crop improvement.

3.5 CRISPRi and CRISPRa editors

There are many factors that affect gene expression, including binding of transcription factors and associated proteins to promotor regions during transcription/translation. By targeting Cas enzymes to such regions, either alone or with fused elements such as trans-effectors, gene expression may be repressed or enhanced. This represents a new role for CRISPR/Cas, termed CRISPRa for enhancing gene expression and CRISPRi for inhibiting gene expression. An early study showed that catalytically dCas9 alone could strongly bind to target DNA and interfere with the binding activity of other proteins, such as RNA polymerase and transcription factors, blocking transcription initiation and elongation in *E. coli* and mammalian cells (Qi et al., 2013). The efficiency of CRISPRi strongly depended on sgRNA quality and target site efficiency, although it was shown that targeting any part of a gene, including the promoter, transcript sequence, and even UTR regions, could provide up to 86% repression with dCas9 alone (Lawhorn et al., 2014). The most active sgRNAs used were shown to repress gene expression by up to 99% (Gilbert et al., 2014). Thus, screening and modifying sgRNAs is very important for achieving high repression efficiency using CRISPRi technology. CRISPRi-based inhibition can be significantly enhanced by fusing dCas9 with a gene expression repressor, such as the Krüppel-associated box (KRAB) domain of Kox1, the chromoshadow (CS) domain of HP1α, the WPRW domain of Hes1, or four concatenated copies of the mSin3 interaction domain (SID4X), in a variety cells, including human cells (Gilbert et al., 2013; Konermann et al., 2013). Among all tested gene expression repressors, dCas9-KRAB was the most effective at repressing expression of a target gene (Gilbert et al., 2013; Konermann et al., 2013).

In comparison, CRISPRa is usually composed of dCas9 fused with a transcriptional activator, allowing targeted enhancement of gene expression. This was demonstrated by Maeder et al. (2013), who fused a dCas9 with a transcriptional activation domain from the herpes virus activator VP64 to successfully increase gene expression levels in human cells. This technology has also been used to activate multiple endogenous genes (*IL1RN*,

SOX2, and *OCT4*) in both human and mouse cells. To date, the highest efficiency for CRISPRa activation was obtained by using multiple sgRNAs, which suggests a synergistic relationship during gene regulation (Cheng et al., 2013). CRISPRa-targeting sites may also affect CRISPRa efficiency. Cheng et al. (2013) demonstrated that CRISPRa binding to the proximal region of a promoter had a particularly high activation efficiency for the target gene. Binding to tandem arrays, such as SunTag, can also significantly enhance CRISPRa-based gene expression through recruiting multiple activator copies (Tanenbaum et al., 2014). Fusing multiple gene expression activators with dCas9 also increased CRISPRa activity and significantly enhanced gene expression (Chavez et al., 2015; Konermann et al., 2015). In addition, Joung et al. (2017) were able to use the technology to screen and identify a long noncoding RNA (lncRNA) locus that regulates a gene neighborhood, highlighting a novel use for CRISPRa in identifying epigenetic relationships.

Both CRISPRi and CRISPRa are highly specific and can be used to repress or activate multiple target genes simultaneously. In addition, the effects are reversible and have no detectable off-target effects (Gilbert et al., 2013; Qi et al., 2013). CRISPR/Cas technology has also been adapted for use in live cell chromatin imaging (Ma et al., 2015; Fu et al., 2016; Wu XT et al., 2019; Khosravi et al., 2020) and manipulation of chromatin topology (Adli, 2018; Yim et al., 2020).

4 Wide use of CRISPR/Cas in clinical and preclinical research as well as disease treatment

Since it was considered a genome editing tool, CRISPR/Cas has attracted huge attention from scientific communities and industries for treating human genetic diseases. In the past ten years, great progress has been achieved in using CRISPR/Cas genome editing technology clinically and preclinically to treat human genetic disorders and screen and diagnose human diseases, and for fundamental studies in biomedicine.

4.1 Using CRISPR/Cas to screen and detect diseases

Early screening and detection of different diseases, particularly deadly diseases, is very important for disease treatment. In recent decades, many different technologies have been developed to screen, detect, and diagnose various diseases, including cancers. Due to its unique characteristics, CRISPR has been quickly developed and adopted as a diagnostic tool for different human diseases.

Many human diseases are genetic disorders or are associated with a specific gene mutation in the human genome. All bacterial and viral diseases, such as the current coronavirus disease 2019 (COVID-19), are associated with specific nucleic acid sequences from the pathogen. These sequences provide perfect targets for the CRISPR/Cas system to produce a readable signal, termed a biomarker, for screening, detecting, and diagnosing various diseases. COVID-19 is currently the most infectious disease, and also is one of the deadliest infectious diseases in human history (Shi et al., 2020). It is caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). By December 2020, SARS-CoV-2 had infected more than 70 million people and was responsible for more than 1.5 million deaths. Currently, the most reliable method for diagnosing COVID-19 is by using polymerase chain reaction (PCR) to detect SARS-CoV-2 sequences. However, CRISPR/Cas-based diagnostic tools may have advantages for detecting different pathogens, including the SARS-CoV-2 virus, due to their higher sensitivity and specificity compared to traditional PCR and real-time quantitative reverse transcription-PCR (qRT-PCR) (Kumar et al., 2020). CRISPR/Cas-based technology can be used to distinguish a single nucleotide difference and thus has high specificity for detecting different genetic variations, even with a single nucleotide polymorphism (SNP). CRISPR/Cas-based disease diagnostics may reshape the profiles of global diagnostic and health care systems (Gootenberg et al., 2017; Chertow, 2018; Kumar et al., 2020). Recently, the CRISPR/Cas12a system was also developed as a CRISPR/Cas-mediated lateral flow nucleic acid assay (CASLFA) for rapidly and sensitively detecting different pathogens, including COVID-19 (Wang XJ et al., 2020b). A CRISPR/Cas12a fluorescent cleavage assay coupled with recombinase polymerase amplification was also developed for sensitive and specific detection of pathogens (Kanitchinda et al., 2020). Other CRISPR/Cas-based diagnostic tools, such as SHERLOCH (specific high-sensitivity enzymatic reporter unlocking) and DETECTR (DNA endonuclease-targeted CRISPR trans reporter), have also been developed for detecting emerging infectious diseases, including COVID-19 (Gootenberg et al., 2017; Chen et al., 2018; Kellner et al., 2019; Li ZJ et al., 2020;

Brandsma et al., 2021; Mustafa and Makhawi, 2021). This novel technology can detect as few as ten copies of virus genes in less than one hour. The principle of CRISPR/Cas-based diagnostics is based on collateral cleavage activity, in which Cas12a/Cas13 nucleases become activated after the crRNA-targeted cleavage. After they are activated, Cas cleaves the nearby single-stranded DNA (ssDNA)/RNA molecules non-specifically, a feature called collateral cleavage or trans-cleavage (Kumar et al., 2020; Wang M et al., 2020). There have been several reports on diagnostics and detection of SARS-CoV-2 using CRISPR/Cas-based therapeutic tools (Chen et al., 2018; Ali et al., 2020; Broughton et al., 2020; Ding et al., 2020; Islam and Iqbal, 2020; Javalkote et al., 2020; Wang M et al., 2020; Wang XJ et al., 2020b).

Apart from COVID-19, CRISPR/Cas-based therapeutic tools have also been used to detect and diagnose other human disease-causing pathogens, including the Zika, West Nile, and yellow fever viruses (Gootenberg et al., 2018; Myhrvold et al., 2018; Quan et al., 2019), human papillomavirus (HPV), *M. tuberculosis*, and *Salmonella* (Ai et al., 2019; Wang XJ et al., 2020a). CRISPR/Cas-based therapeutic tools can also be used to diagnose non-infectious diseases, including cancers and genetic disorders (Tian et al., 2019).

4.2 Using CRISPR/Cas to study pathogenesis and the related mechanisms as well as disease treatment

Many human diseases are associated with genetic information. Some are associated with endogenous genetic disorders, whereas others are associated with exogenous genetic infection, such as virus-infected diseases. Because CRISPR/Cas specifically targets a DNA/RNA sequence it can be used to monitor, break down, replace, or regulate target sequences for various purposes. CRISPR/Cas technology has attracted huge attention from both academic communities and biotechnological industries to treat various human diseases since it was recognized as a natural genome editing tool in 2012. Currently, CRISPR/Cas has huge potential applications for studying and treating human diseases, particularly genetic disorders.

4.2.1 Creating animal genetic models for treating human genetic diseases

A good animal model will allow us to better understand targeted human diseases not only in relation to their pathogenesis and mechanisms, but also for screening drugs and studying their potential side effects. Since it can precisely target an individual gene for editing with high efficiency and fewer side effects, CRISPR/Cas genome editing, including base editing and prime editing, has been quickly adopted to generate a range of animal models for studying various human genetic diseases. In a recent literature survey, Zhang (2021) found that at least a dozen animal models have been generated using CRISPR/Cas genome editing technology. These include mouse, rat, pig, and rabbit models for studying various human diseases, including human non-small-cell lung cancers (NSCLCs) (Maddalo et al., 2014), hepatocellular carcinoma (HCC) (Liu et al., 2017), Hutchinson-Gilford progeria syndrome (HGPS) (Liu ZQ et al., 2018), corneal dystrophy (Kitamoto et al., 2020), and X-linked dilated cardiomyopathy (XLCM) (Liu ZQ et al., 2018). Fujihara et al. (2020) successfully created a rat model by using CRISPR/Cas9 genome editing for studying complex behavioral changes during schizophrenia. Schizophrenia is a serious mental disease that affects how a person thinks, feels, and behaves, and causes lots of problems for patients and their families. However, no effective treatments are available for schizophrenia. Genetic evidence has shown that GABAergic dysfunction is associated with the pathogenesis of schizophrenia, and the γ-aminobutyric acid (GABA) synthetic enzyme glutamate decarboxylase 67-kDa isoform (GAD67) is downregulated in the brains of patients with the disease (Fujihara et al., 2020). GAD67 is encoded by the *GAD1* gene. Some SNPs surrounding the *GAD1* gene were found to be associated with schizophrenia in North America and China (Addington et al., 2005; Du et al., 2008). Rats with a CRISPR knock-out of *Gad1* produced about 48% less GABA than wild-type rats, and showed a wide range of behavioral changes, including high sensitivity to an *N*-methyl-D-aspartate (NMDA) receptor antagonist, hypoactivity in a novel environment, and decreased preference for social novelty, which are similar to those exhibited by human patients with schizophrenia. Thus, the CRISPR knock-out of the *Gad1* rat could serve as a novel model for human schizophrenia.

Cardiovascular disease is the leading cause of global death and threatens millions of people in their daily lives. Recently, Lu SX et al. (2020) knocked out *heg1*, a gene encoding a heart development protein with epidermal growth factor (EGF) like domains 1, in

zebrafish. The *heg1* CRISPR zebrafish mutant developed severe cardiovascular malformations, including an abnormal heart rate, atrial ventricular enlargement, venous thrombosis, and slow blood flow. All these symptoms are similar to those associated with human heart failure and thrombosis (Lu SX et al., 2020). Thus, the zebrafish *heg1* CRISPR mutant has potential to serve as an animal model for analyzing the pathogenesis of cardiovascular diseases, and screening and testing new drugs and their potential therapeutic targets.

As research progresses, more animal models will be generated using CRISPR/Cas technology, particularly newly developed prime editors and base editors that can easily target a single-base change.

4.2.2 Studying the pathogenesis and mechanisms associated with human diseases

Many genes are aberrantly expressed during the pathogenesis of human diseases and they are directly or indirectly associated with these diseases. However, the roles of many these genes during these processes are unclear. CRISPR/Cas genome editing provides a robust way to study the molecular mechanisms associated with pathogenesis, and can be used to obtain new therapeutic targets for disease treatment.

In recent decades, cancers have been become the leading cause of death among all human diseases. Carcinogenesis is very complicated and usually involves genetic and epigenetic alterations in many genes and gene networks. Precise and efficient correction of these genes holds huge promise for cancer treatment. In the past eight years, CRISPR/Cas technology has been deeply explored to understand carcinogenesis and for cancer treatment, including impairing carcinogenesis-associated genes, exploring anticancer drugs, engineering immune cells and oncolytic viruses for enhancing cancer immunotherapeutics, and fighting oncogenic infections. By using an all-in-one lentiviral and retroviral delivery vectors hosting both Cas9 and sgRNAs, Malina et al. (2013) showed robust selection for the CRISPR-modified Trp53 locus following drug treatment. By linking Cas9 protein with green fluorescent protein (GFP), they also tracked disrupted Trp53 in chemoresistant lymphomas in the Eμ-*myc* mouse model. Chen et al. (2014) used a similar strategy to demonstrate that mixed lineage leukemia 3 (*MLL3*) is a haploinsufficient 7q tumor suppressor in acute myeloid leukemia (AML) in *shNf1; Trp53*$^{-/-}$ primary mouse haematopoietic stem and progenitor cells (HSPCs). Xue et al. (2014) used CRISPR/Cas9 technology to directly target *Pten* and *Trp53* tumor-suppressor genes in the mouse liver. They found that their CRISPR-generated *Pten* and *p53* mutant induced liver tumors. The CRISPR/Cas-created mutation can be used to mimic the model phenotype caused by Cre–*loxP*-mediated deletion of *Pten* and *p53*. These examples were among the first to show that CRISPR/Cas-based genome editing can be used to treat cancers and to study cancer pathogenesis. Since then, CRISPR/Cas genome editing has been quickly deployed to study mechanisms of disease occurrence, development, diagnosis, and therapeutic treatment.

CRISPR/Cas-based genome and epigenome editing has been widely used to study the role of different genes, including oncogenes and tumor-suppressor genes, in various diseases, particularly in cancers. CRISPR/Cas was also developed as a new gene therapy tool to study and treat various human diseases, such as cancers. Programmed death-ligand 1 (PD-L1) is a regulatory molecule expressed in T cells and plays an important function in T cell-mediated immunotherapy. The PD-1/PD-L1 pathway serves as a critical immune checkpoint for many different cancers. CRISPR/Cas9 knock-out of the *cdk5* gene blocked the expression of the *PD-L1* gene and enhanced cell antitumor immunity (Yahata et al., 2019; Deng et al., 2020). CRISPR/Cas9 knock-out of *cdk5* inhibited tumor cell growth in murine melanoma and lung metastasis suppression in triple-negative breast cancer (Deng et al., 2020). CRISPR/Cas9 disruption of the expression of *PD-L1*/*PD-1* may also be beneficial for treating other cancers, including Epstein-Barr virus (EBV)-positive gastric cancer (Su et al., 2017), melanoma (Mahoney et al., 2015), glioblastoma (Choi et al., 2019), colorectal cancer (Liao et al., 2020), and ovarian cancers (Yahata et al., 2019). Gao et al. (2021) used CRISPR/Cas genome editing to study the biological role of the AKT1 E17K mutation in a TP53-null background. Their results showed that AKT1 E17K inhibited cancer cell migration by abrogating β-catenin signaling. Using CRISPR/Cas9 genome editing, Bungsy et al. (2021) showed that the expression of *RBX1* was associated with chromosome instability (CIN). A knock-out of the *RBX1* gene significantly increased CIN phenotypes and increased Cyclin E1 levels and anchorage-independent growth in fallopian tube secretory epithelial cells, a cellular precursor of high-grade serous ovarian cancer (HGSOC) (Bungsy et al., 2021).

CRISPR/Cas can also be used as a screening tool for identifying genes associated with certain diseases, as well as a robust tool for drug screening. Soares et al. (2020) used CRISPR/Cas technology to screen and identify genes that confer susceptibility of AML to double negative T cell therapy. Their results suggested that CD64 is a predictive biomarker in AML patients. Takahashi et al. (2020) used a focused CRISPR/Cas9 screen with 3D culture models to target *NRF2* and other redox regulatory genes, and revealed that hyperactive *NRF2* serves as a prerequisite for spheroid cancer cell formation by regulating their proliferation and ferroptosis. By using targeted CRISPR/Cas9 dropout screening, Chen HR et al. (2021) identified methyltransferase-like 3 (METTL3) as the top essential m^6A regulatory enzyme in colorectal cancer.

Regulating gene expression (e.g., knock-out, knock-in, or gene regulation) by CRISPR/Cas can be used to increase target cell sensitivity to certain drugs, and can increase the efficiency of chemotherapy. CRISPR/Cas9 knock-out of the *rev7* gene showed significant synergy with 5-fluorouracil (5-FU) and oxaliplatin in colorectal cancer in cell culture and in a murine xenograft model (Gao et al., 2021). Sun et al. (2019) developed a drug-inducible CRISPR/Cas9 system that can be used both in vitro and in vivo for large-scale functional screening of human diseases.

4.2.3 Treating human genetic diseases including cancers and COVID-19

CRISPR/Cas has been developed as a powerful and promising tool for gene therapy (Lotfi and Rezaei, 2020; Zeballos and Gaj, 2020; Ferrari et al., 2021; Zhang, 2021). There are many CRISPR/Cas studies on preclinical and clinical treatments of various diseases, including cancers and infectious diseases.

In 2016, scientists at Sichuan University (Chengdu, China) and the Chengdu MedGenCell Co., Ltd., China, performed the first CRISPR/Cas gene therapy Phase 1 clinical trial, in which they knocked out the *PD-1* gene in T cells for treating metastatic NSCLC using CRISPR/Cas9 genome editing technology. Since then, more CRISPR/Cas gene therapy clinical trials have been approved by different countries, especially in the USA, China, Germany, UK, Canada, Italy, France, Spain, and Australia. The clinically treated diseases varied widely, from different cancers to eye diseases and Rubinstein-Taybi syndrome, as well as human immunodeficiency virus (HIV) infection.

Xu et al. (2019) reported their clinical trial (No. NCT03164135) involving treating HIV-infected patients with hematological malignances. They first used CRISPR/Cas genome editing technology to edit the *CCR5* gene in HSPCs, and then transplanted the CRISPR-edited CCR5-ablated HSPCs into patients with HIV-1 infection and acute lymphoblastic leukemia. Their results showed that the CRISPR/Cas-edited donor cells persisted for more than 19 months after transplantation without causing gene-editing-related adverse events (Xu et al., 2019; He, 2020). Stadtmauer et al. (2020) reported their results from a CRISPR/Cas9 gene therapy Phase 1 clinical trial on cancer treatment. First, they used CRISPR/Cas9 genome editing technology to knock-out *TRAC*, *TRBC*, and *PDCD1* genes in T cells isolated from three individual patients. Then, they transferred the CRISPR/Cas-engineered T cells with a cancer-targeting gene, *NY-ESO-1*, back into the patients. After nine months of treatment, all three patients showed promising results, demonstrating the high efficiency, technical safety, and feasibility of CRISPR/Cas genome editing technology for the treated cancers (Stadtmauer et al., 2020). In another clinical trial, Lu Y et al. (2020) also demonstrated the safety and feasibility of using CRISPR/Cas9-engineered T cells to treat human cancers at advanced stages (Lu Y et al., 2020). In their Phase 1 clinical trial (No. NCT02793856), they edited the *PD-1* gene in T cells obtained from patients with advanced NSCLC. With a total of 22 patients enrolled, 12 received CRISPR gene therapy treatment with CRISPR-edited T cells for up to 96 weeks for treatment-related adverse events. Based on their study, after CRISPR gene therapy treatment of NSCLC, the median progression-free survival of 12 patients was 7.7 weeks, and the median overall survival was 42.6 weeks. No treatment-related deaths were observed in their clinical trials (Lu Y et al., 2020). All these Phase 1 clinical trials demonstrated the safety and feasibility of CRISPR/Cas-based gene therapy using engineered immune T cells with a low off-target impact.

Many other CRISPR/Cas gene therapy trials are underway, especially Phase 1 clinical trials (Zhang, 2021). As current CRISPR/Cas genome editing technology improves, a new generation of CRISPR/Cas genome editors, such as base editors and prime editors, will emerge, as well as new methods for delivering

CRISPR/Cas reagents. Thus, the safety (e.g., lack of off-target impacts and low toxicity) and feasibility of the technology will be further improved.

5 CRISPR/Cas is a robust and powerful tool for precision breeding and crop improvement

Another major application of CRISPR/Cas genome editing technology is to improve crop yield and quality, as well as tolerance to various environmental stresses (Tan YY et al., 2020; Zhang et al., 2021). Since it was adopted in plants for genome editing, this technology has revolutionized studies in the field of plant molecular biology and is switching traditional plant breeding to precision breeding.

5.1 CRISPR/Cas systems have been quickly developed in a diversity of plant species

Since it was recognized as a naturally occurring genome editing tool, the CRISPR/Cas system has attracted increasing attention from the plant science community and was quickly applied to plant gene function studies and crop improvement. Shan et al. (2013) reported for the first time the establishment of a CRISPR/Cas system in two of the most widely cultivated food crops, wheat and rice. Since then, CRISPR/Cas genome editing technology has been widely established in almost all agriculturally important crops, including cotton, maize, soybean, and potato, as well as biofuel crops, such as switchgrass (Table 3).

5.2 CRISPR/Cas systems have been widely used for improving crop resistance to biotic and abiotic stresses

Abiotic and biotic stresses are two major factors limiting crop growth and development as well as yield and quality. Particularly during climate change and industrialization, both stresses threaten natural plant development and agricultural practices. Although plants have evolved certain mechanisms to handle these stresses, and people have made significant progress on improving crop tolerance using both traditional breeding and transgenic technologies, there are still a big gap and a challenge for agricultural practices. Since it was adopted in plants, CRISPR/Cas-based genome editing technology has opened a new era for precision breeding for improved plant tolerance to abiotic and biotic stresses.

With the increasing development and application of new technologies, such as next generation high-throughput deep sequencing technology, more and more genes, including both protein-coding and non-coding genes, have been identified which respond to different environmental stresses. Among these genes, some offer plant tolerance to these stresses, but there are many genes that make plants more sensitive to these stresses. For example, MILDEW-RESISTANCE LOCUS O (*MLO*) genes are widely considered to be plant susceptibility genes that are highly expressed in plants sensitive to powdery mildew, a widespread fungal disease in plants. MLOs play a negative role in the plant's response to powdery mildew infection. Loss-of-function of MLOs allows plants to gain resistance to powdery mildew disease in several important crops, including barley (Büschges et al., 1997). Thus, the manipulation of susceptibility genes (also called S-genes) has high potential in agricultural practices to increase crop tolerance to various diseases, reduce chemical pesticide usage, and protect the environment (Filiz and Vatansever, 2018). Inhibiting the expression of susceptibility genes is a great strategy for breeding new cultivars with high tolerance or even resistance to certain diseases. However, using traditional breeding technology and even transgenic technology to remove these genes is very hard, inefficient, and time- and lab-consuming. Although virus-induced gene silencing can be used to inhibit gene expression, it is difficult to completely inhibit the targeted genes and there are many uncertain outcomes. Thus, since scientists approved the application of CRISPR/Cas genome editing tools in plants, the technology has attracted lots of attention from scientific communities and biotechnological industries. In the past eight years, there have been many reports of creating genome-edited plants with resistance to viral, bacterial, and fungal diseases in different plant species using CRISPR/Cas technology (Zhang et al., 2021). These include CRISPR/Cas9 knock-outs of the following genes: *mlo* for plant resistance to powdery mildew in wheat (Wang et al., 2014), tomato (Nekrasov et al., 2017; Martínez et al., 2020) and grapevine (Wan et al., 2020), *pmr4* for plant resistance to powdery mildew in tomato (Santillán Martínez et al., 2020), 14-3-3 gene for resistance to *Verticillium dahlia* in cotton (Zhang ZN et al., 2018), *crt1a* for resistance to *Verticillium longisporum* in both

Table 3 Establishment of CRISPR/Cas genome editing systems in agriculturally important crops*

Common name	Scientific name	Transformation	CRISPR/Cas	Targeted gene	Promotor for sgRNA	Knock-out efficiency	Off-target	Reference
Wheat	*Triticum aestivum*	Protoplast transformation, gene gun	CRISPR/Cas9	*TaMLO*	U6			Shan et al., 2013
Rice	*Oryza sativa*	Protoplast transformation, gene gun	CRISPR/Cas9	OsPDS, *OsBADH2*, *Os02g23823*, and *OsMPK2*	U3	Varied, >15%	Not found	Shan et al., 2013
Maize	*Zea mays*	Protoplast transformation, *Agrobacterium* transformation	CRISPR/Cas9	*ZmIPK*	U3	13.1%		Liang et al., 2014
Soybean	*Glycine max*	*Agrobacterium rhizogenes* transformation	CRISPR/Cas9	*GFP Glyma07g14530*, *DDM1*, and miRNA	U6	Varied, up to 95%	Low	Jacobs et al., 2015
Cotton	*Gossypium hirsutum*	*Agrobacterium* mediated	CRISPR/Cas9	*MYB*	U6		Not found	Li et al., 2017
Sorghum	*Sorghum bicolor*	*Agrobacterium*-mediated embryo transformation	CRISPR/Cas9	*DsRED2*	U6			Jiang et al., 2013
Sweet potato	*Ipomoea batatas*	*Agrobacterium* mediated	CRISPR/Cas9	*IbGBSSI* and *IbGBSSII*	U6	62%–92%		Wang HX et al., 2019
Potato	*Solanum tuberosum*	*Agrobacterium* mediated	CRISPR/Cas9	*StIAA2*	StU6			Wang et al., 2015
Tomato	*Solanum lycopersic*	*Agrobacterium*-mediated transformation	CRISPR/Cas9	*GFP* and *SlSHR*	U6			Ron et al., 2014
Yam	*Dioscorea alata*	*Agrobacterium* mediated	CRISPR/Cas9	*PDS*	DaU6	83.3%		Syombua et al., 2020
Cassava	*Manihot esculenta*	*Agrobacterium* mediated	CRISPR/Cas9	*MePDS*	AtU6	90%–100%		Odipio et al., 2017
Switchgrass	*Panicum virgatum*	*Agrobacterium* mediated	CRISPR/Cas9	*Pv4cl1*	U3			Park et al., 2017
Switchgrass	*Panicum virgatum*	*Agrobacterium* mediated	CRISPR/Cas9	*tb1a*, *tb1b*, and *pgm*	U6	Varied, up to 95.5%		Liu Y et al., 2018
Rapeseed	*Brassica napus*	*Agrobacterium* mediated	CRISPR/Cas9	*Alc*	U6	High	Not detected	Braatz et al., 2017
Jatropha	*Jatropha curcas*	*Agrobacterium* mediated	CRISPR/Cas9	*JcCYP735A*	U3			Cai et al., 2018
Apple	*Malus prunifolia*	*Agrobacterium* mediated	CRISPR/Cas9	*PDS*	AtU6	31.8%		Nishitani et al., 2016
Sweet orange	*Citrus sinensis*	*Xcc*-facilitated agroinfiltration	CRISPR/Cas9	*PDS*		3.2%–3.9%		Jia and Wang, 2014
Pear	*Pyrus communis*	*Agrobacterium* mediated	CRISPR/Cas9	*PDS* and *TFL1*	U3 and U6			Charrier et al., 2019
Poplar	*Populus*	*Agrobacterium* mediated	CRISPR/Cas9	*4CL*	U6	25%		Zhou et al., 2015
Sweet basil	*Ocimum basilicum*	*Agrobacterium* mediated	CRISPR/Cas9	*ObDMR1*		Varied		Navet and Tian, 2020

* There are many reports on different plant species for CRISPR/Cas-based genome editing. We attempted to list the first reports on each crop. If we missed some important studies, we apologize to the authors. CRISPR: clustered regularly interspaced short palindromic repeats; Cas: CRISPR-associated protein; sgRNA: single guide RNA; miRNA: microRNA; *Xcc*: *Xanthomonas citri* subsp. *citri*.

Arabidopsis thaliana and oilseed rape (Pröbsting et al., 2020), *OsERF922* for resistance to *Magnaporthe oryzae* in rice (Wang et al., 2016), *Clpsk1* for resistance to *Fusarium oxysporum* f. sp. *niveum* in watermelon (Zhang et al., 2020), *eif4e* for resistance to *Cucumber vein yellowing virus*, *Zucchini yellow mosaic virus*, and *Papaya ring spot mosaic virus-W* in cucumber (Chandrasekaran et al., 2016), *CsWRKY22* for resistance to *Xanthomonas citri* subsp. *citri* (*Xcc*) in orange (Wang LJ et al., 2019), *CsLOB1* for resistance to *Xcc* in citrus (Peng

et al., 2017), *SlJAZ2* for resistance to *Pseudomonas syringae* pv. *tomato* (*Pto*) DC3000 in tomato (Ortigosa et al., 2019), *Os8N3* for resistance to *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) in rice (Kim et al., 2019), and *LIPOXYGENASE 3* (*lox3*) for resistance to *Ustilago maydis* in maize (Pathi et al., 2020).

Compared with pathogen infection, fewer susceptibility genes have been identified in plants in response to abiotic stresses. Thus, progress has been much slower in creating CRISPR/Cas genome-edited lines for plant resistance to various abiotic stresses, such as drought, salinity, extreme temperature (cold and heat), and environmental pollution. However, as more negative genes have been identified, more progress has been made in creating genome-edited plants with high tolerance to abiotic stresses. During long-term studies, scientists found that many structural and regulatory genes are associated with a plant's response to different environmental abiotic stresses. Many genes controlling plant development, particularly root development, also contribute to the plant's response to abiotic stresses. For example, arginase and nitric oxide synthase (NOS) are two important enzymes regulating nitric oxide (NO) synthesis and root development, particularly lateral root development (Correa-Aragunde et al., 2004). These two enzymes compete for arginine which is important for NO biosynthesis. Reduced arginase activity increased NO accumulation in *Arabidopsis* arginase gene (*arg*) mutants and enhanced lateral and adventitious root development (Flores et al., 2008). In contrast, overexpression of the *arg* gene inhibited NO accumulation and further repressed lateral root development in transgenic cotton (Meng et al., 2015). Using CRISPR/Cas9 genome editing technology, Wang et al. (2017) successfully knocked out the *arg* gene in cotton. This significantly increased lateral root differentiation and development as shown by an increase in root number of more than 25%, and of root surface area by more than 50%, in both low- and high-nitrogen conditions (Wang et al., 2017). This suggests that CRISPR/Cas9-edited *arg* knock-out plants have better root development, enhancing plant growth and tolerance to different abiotic stresses, including nitrogen deficiency and drought stress (Zhang et al., 2021; Peng et al., 2021). Knock-out of the G protein genes, *gs3* and *dep1*, significantly improved rice tolerance to different abiotic stresses, including drought, chilling, and salinity stresses. Under salinity treatment, all tested genome-edited lines showed enhanced tolerance compared to the controls (Cui et al., 2020). Abiotic stresses induced aberrant expression of many transcription factors and non-coding RNAs that play important roles in the plant's response to these environmental stresses. There are many classes of transcription factors that regulate lots of downstream genes for controlling plant growth, development, and response to various environmental stresses. Knock-out or overexpression of these transcription factor genes alters the expression profiles of many other genes and then affects plant development and response to different stresses. Auxin response factor (ARF) is one family of functionally distinct DNA-binding transcription factors found widely in all plant species (Li SB et al., 2016). CRISPR/Cas9 knock-out of the *arf4* transcription factor gene improved plant tolerance to salinity and osmotic stresses in tomato (Bouzroud et al., 2020). Using the CRISPRa epigenome editor, Roca Paixão et al. (2019) successfully fused dCas9 protein with a histone acetyltransferase (AtHAT1) and used this fused CRISPR/dCas9 system to target the abscisic acid (ABA)-responsive element-binding protein 1 (AREB1)/ABRE-binding factor 2 (ABF2). Their results showed that the CRISPRa dCas9^{HAT} system activated the endogenous promoter of AREB1 and enhanced plant tolerance to drought stress in *Arabidopsis*. Under salinity stress, the overexpression technology combined with the CRISPR-Cas9 system demonstrated that the transcription factor gene *NAC06* caused proline and glycine accumulation to alleviate or avoid reactive oxygen species (ROS)-induced oxidative stress and maintained ionic homeostasis and Na^+/K^+ ratio in soybean hairy roots. Consequently, soybean plant tolerance to salinity stress was enhanced (Li et al., 2021).

5.3 CRISPR/Cas systems have been widely used in improving crop yield and quality

Improving crop yield and quality is the ultimate goal for precision crop breeding and can be achieved directly or indirectly, including improving crop tolerance to various environmental stresses. Crop yield is generally controlled by multiple genes, and single genes that significantly control crop yield alone are hard to find. Although great progress has been achieved in obtaining transgenic plants, the main focus has been on insect-resistance and herbicide tolerance. These transgenic crops, particularly insect-resistant biotech crops (such as Bt cotton), have brought huge economic and social benefits, and in certain cases the crop yield has

also significantly increased because of reduced losses caused by different pests. However, as more modern techniques, such as high-throughput deep sequencing, have been used to investigate the molecular mechanisms controlling crop yield, more genes associated with crop yield have been identified. Among them, there is one class of genes, called negative regulators, that negatively affect crop yield. These negative regulators of crop yield, just like *S* genes in the plant's response to pathogen infection, provide a great potential target for CRISPR/Cas to improve crop yield (Table 4). *Gn1a*, *dep1*, and *gs3* are three negative regulators associated with grain number per panicle, grain size, and seed size in rice (Ashikari et al., 2005). *Gn1a* is a gene for cytokinin oxidase/dehydrogenase (OsCKX2). Reduced expression of *gn1a* increased the number of reproductive organs and enhanced grain yield (Ashikari et al., 2005). Li MR et al. (2016) used CRISPR/Cas9 technology to successfully knock out all three of these genes. Their CRISPR/Cas9 genome-edited rice showed enhanced grain number, dense erect panicles, and larger grain size. It is also well known that grain width 2 (*gw2*), *gw5*, and thousand-grain weight 6 (*tgw6*) negatively regulate grain weight (Zuo and Li, 2014; Xu et al., 2016). Knock-out of these three genes significantly increased grain weight in rice, and simultaneous knock-out of two or more genes resulted in a larger grain size and crop yield (Xu et al., 2016). Also, CRISPR knock-out of the *OsPAO5* gene enhanced the grain weight, grain number, and yield potential in rice (Lv et al., 2021). In the biofuel crop switchgrass, CRISPR/Cas9 knock-out of the teosinte branched 1 (*tb1*) gene increased the number of plant tillers and fresh biomass (Liu et al., 2020). In rapeseed, simultaneous knock-out of all four *BnaMAX1* alleles resulted in semi-dwarf and increased branching phenotypes with more siliques. These traits contributed to increased yield compared with their wild-type controls (Zheng et al., 2020). There are three *GW2* homeologs (TaGW2-A1, -B1, and -D1) in hexaploid wheat. CRISPR/Cas9 knock-out of an individual homeolog affected wheat grain width and length and thousand-grain weight and yield. Double CRISPR knock-out of *TaGW2* showed a stronger effect on these traits than a single mutation (Zhang Y et al., 2018). By targeted editing of the early heading date 1 (*Ehd1*) gene by CRISPR/Cas9, Wu et al. (2020) generated both frame-shift and in-frame deletion mutants in four rice cultivars. The mutants showed significantly longer basic vegetative growth periods and significantly improved yield potential compared with wild types when planted at low-latitude stations.

Sugar is an important component in our daily life and in biofuel production. However, sugarcane and sugar beet are the only two crops that produce significant amounts of sugar. Recently, Honma et al. (2020) knocked out the *OsGcs1* gene in rice by CRISPR/Cas and obtained sugary-rice grains containing a high percentage of high-quality sugar. The CRISPR/Cas-edited rice ovules contained 10%−20% sugar with an extremely high sucrose content (98%) (Honma et al., 2020). This may provide a new way to develop novel sugar-producing plants.

Individual components, such as amino acids or carbohydrates, significantly affect crop quality. Thus, targeting the specific biosynthetic pathway of an individual component is a promising strategy to improve crop quality. Resistant starch and amylose benefit human health by lowering the potential risk of certain serious diseases. Improving the content of resistant starch and amylose

Table 4 Improvement of crop yield and associated traits by using CRISPR/Cas

Crop species	Targeted gene	Improved trait	CRISPR/Cas editor	Reference
Rice	*gn1a*, *dep1*, and *gs3*	Grain number, dense erect panicles, and larger grain size	CRISPR/Cas9	Li MR et al., 2016
Rice	*gw2*, *gw5*, and *tgw6*	Grain size and crop yield	CRISPR/Cas9	Xu et al., 2016
Rice	*OsPAO5*	Grain weight, grain numbers, and yield	CRISPR/Cas9	Lv et al., 2021
Switchgrass	*tb1*	Tillers and fresh biomass	CRISPR/Cas9	Liu et al., 2020
Rapeseed	*BnaMAX1*	Plant architecture and yield	CRISPR/Cas9	Zheng et al., 2020
Soybean	*GmLHY*	Plant height and internode length	CRISPR/Cas9	Cheng et al., 2019
Soybean	*AP1*	Flowering time and plant height	CRISPR/Cas9	Chen et al., 2020
Soybean	*GmNMHC5*	Flowering and maturity	CRISPR/Cas9	Wang WT et al., 2020
Wheat	*TaGW2*	Grain weight and protein content	CRISPR/Cas9	Zhang Y et al., 2018
Rice	*OsGcs1*	High-quality sugar production	CRISPR/Cas9	Honma et al., 2020
Rice	*Ehd1*	Basic vegetative growth	CRISPR/Cas9	Wu et al., 2020

CRISPR: clustered regularly interspaced short palindromic repeats; Cas: CRISPR-associated protein.

is a long-standing goal for breeders and scientists. By using CRISPR/Cas9 genome editing technology, Li JY et al. (2020) knocked out the *TaSBEIIa* gene in both winter and spring wheat cultivars. The genome-edited wheat contained a significantly higher amount of amylose, resistant starch, protein, and soluble pentosan than the wild type (Li JY et al., 2020). By knocking out the starch branching enzyme genes, *SBEI* and *SBEIIb*, Sun et al. (2017) obtained transgene-free rice with an amylose content as high as 25.0% and a resistant starch content of 9.8%. By using CRISPR/Cas9 genome editing, Zhang JS et al. (2018) knocked out the *Waxy* gene in two widely cultivated elite japonica rice cultivars. Their results showed that *Waxy* genome-edited rice contained lower amylose and converted the rice into glutinous rice without affecting other desirable agronomic traits.

Gluten is a family of storage proteins, which plays an important role in cooking, particularly for making bread and noodles, the most commonly consumed foods. Gluten is widely found in certain cereal grains, including wheat, barley, and rye. However, many people are sensitive or even allergic to gluten, and show celiac disease. Thus, breeding gluten-free crops is necessary and important. In breeding programs, scientists and breeders have used traditional breeding technology and mutation breeding (e.g., chemically or physically induced amastigogenesis) to obtain low-gluten-content cultivars (van den Broeck et al., 2009; Juhász et al., 2020). Although some progress has been made, the traditional methods are time-, lab-, and cost-intensive and usually require the generation of a huge breeding population and from which to select plants with the desired traits. As scientists understand more about gluten biosynthesis and the development of RNA interference (RNAi) technology, by targeting a specific gene during gluten biosynthesis, several different laboratories have knocked down an individual gene and obtained RNAi plants with a reduced gluten content (Gil-Humanes et al., 2008, 2010; Becker et al., 2012; Barro et al., 2016; Altenbach et al., 2019). However, RNAi technology cannot completely eliminate gluten biosynthesis in plants, and in certain cases this technology is hard to handle due to its instability. The rapid development of CRISPR/Cas systems provides a perfect technology for controlling gluten biosynthesis, and opens a new era for breeding gluten-free cultivars. By targeting the α-gliadin genes, Sánchez-León et al. (2018) successfully obtained low-gluten transgene-free wheat using CRISPR/Cas9 genome editing technology. All 21 CRISPR mutant lines showed a significant reduction (up to 85%) in gluten content. Using the same technology, another research group targeted both α- and γ-gliadins and observed clear changes in the gluten profiles of CRISPR/Cas9 genome-edited bread wheat lines (Jouanin et al., 2019).

There are many different types of plant oils, and different oils have different quality. Generally speaking, oleic oil has better quality because of its high content of healthier fats (monounsaturated and polyunsaturated) (Zhou et al., 2020). Thus, scientists have been attempting to modify oil components to improve oil quality by using advanced CRISPR/Cas genome editing. The fatty acid desaturase 2 (FAD2) enzyme is the enzyme that controls the biological switch between oleic acid and linoleic acid (Dar et al., 2017). Knock-out of *fad2* genes significantly increases oleic acid content and improves oil quality. Thus, *fad2* genes have been become a target gene for using CRISPR/Cas to modify oil quality in different plant species. By targeting the *fad2* gene using CRISPR/Cas9, Jiang et al. (2017) obtained CRISPR/Cas genome-edited *Camelina sativa* plants with oleic acid content increased from 16% to >50% of the fatty acid composition (Jiang et al., 2017). They also observed that CRISPR/Cas genome-edited *fad2* plant seeds had a significantly reduced content of the less desirable polyunsaturated fatty acids, linoleic acid (a decrease from about 16% to <4%) and linolenic acid (a decrease from about 35% to <10%). *fad2* gene was also knocked out by CRISPR/Cas in other plant species, including rice (Abe et al., 2018), rapeseed (Huang et al., 2020), tobacco (Tian et al., 2020), cotton (Chen YZ et al., 2021), soybean (al Amin et al., 2019), and peanut (Yuan et al., 2019), to obtain high-quality oil with high oleic/low linoleic acid content. In tobacco, CRISPR/Cas9 knock-out of the *fad2* gene dramatically increased the oleic acid content from 11% to >79%, whereas linoleic acid was reduced from 72% to 7% (Tian et al., 2020).

Many plant oils contain significant amounts of long-chain fatty acids that are undesirable for many different purposes. Using CRISPR/Cas9, Ozseyhan et al. (2018) successfully knocked out the fatty acid elongase 1 (*FAE1*) gene in *C. sativa*. Their results showed that C20−C24 very long-chain fatty acids (VLCFAs) were reduced to less than 2% of the total fatty acids from over 22% in the wild type (Ozseyhan et al., 2018).

CRISPR/Cas genome editing technology has also been used to modify lipid content quality. For example, Lin and Ng (2020) used CIRSPR/Cas9 to knock out the *fad3* gene and obtained a 46% higher accumulation of lipids in *Chlorella vulgaris* FSP-E. In bovine mammary epithelial cells, CRISPR/Cas knock-out of the butyrophilin subfamily 1 member A1 (*BTN1A1*) gene changed the lipid droplet formation and phospholipid composition. The percentage of phosphatidylethanolamine (PE) increased, while the percentage of phosphatidylcholine (PC) decreased, which resulted in a lower PC/PE ratio (Han et al., 2020). CRISPR/Cas9 knock-out of the *fad2* gene in Atlantic salmon also altered their lipid metabolism (Jin et al., 2020).

During long evolutionary history, plants have evolved certain pathways to synthesize specific components, some of which are good for human health, while others are not. For example, red rice contains high levels of proanthocyanidins and anthocyanins that are health-promoting nutrients. The red pericarp found widely in cultivated rice ancestors and wild rice species is controlled by two complementary genes, *Rc* and *Rd* (Furukawa et al., 2007). However, during rice evolution and domestication, a 14-bp frame-shift deletion in the *Rc* gene enabled the selection of white rice (Sweeney et al., 2007). In a recent study, Zhu et al. (2019) used CRISPR/Cas9 genome editing technology to successfully restore the function of the *Rc* gene by reverting the 14-bp frame-shift deletion to in-frame mutations (Zhu et al., 2019). Using this strategy, they successfully converted three elite white pericarp rice cultivars into red pericarp types, all of which can accumulate high levels of proanthocyanidins and anthocyanins in their red grains without affecting other important agronomic traits (Zhu et al., 2019). GABA is a non-proteinogenic amino acid, which has beneficial human health effects. Increasing the GABA content of fruit enhances its health benefit by lowering blood pressure. GABA biosynthesis is controlled mainly by GAD. By targeting *SlGAD2* and *GlGAD3*, CRISPR/Cas9-edited tomato plants accumulated 7- to 15-fold more GABA in their fruits (Nonaka et al., 2017). By targeting genes associated with GABA metabolism, including *GABA-TP1*, *GABA-TP2*, *GABA-TP3*, *CAT9*, and *SSADH*, Li et al. (2018b) obtained CRISPR genome-edited tomatoes with GABA content up to 19-fold higher than that of the wild type. Hunziker et al. (2020) used the CRISPR/Cas9 system fused with target activation-induced cytidine deaminase (Target-AID) base-editing technology to knock out the *SlDDB1*, *SlDET1*, and *SlCYC-B* genes to alter carotenoid accumulation. Lycopene is a plant nutrient with antioxidant properties which have been linked to beneficial effects on several diseases, including heart and cardiovascular diseases and certain cancers. Increasing lycopene content in fruits has significant benefits. By targeting genes associated with lycopene biosynthesis, such as *SGR1* (GenBank accession No. DQ100158), lycopene ε-cyclase (*LCY-E*; GenBank accession No. EU533951), β-lycopene cyclase (*Blc*; GenBank accession No. XM_010313794), lycopene β-cyclase 1 (*LCY-B1*; GenBank accession No. EF650013), and *LCY-B2* (GenBank accession No. AF254793), Li XD et al. (2018) obtained CRISPR-edited tomatoes with up to a 5.1-fold increase in lycopene content using a bidirectional strategy: promoting the biosynthesis of lycopene, while inhibiting its conversion to β- and α-carotene. Using similar technology, Kaur et al. (2020) obtained β-carotene-enriched Cavendish bananas with β-carotene content increased by up to 6-fold (about 24 μg/g) compared with unedited plants.

CRISPR/Cas genome editing is also being used to improve crop storage and post-harvest quality (Table 5). The storage and transportation of certain fruits, such as tomato and peach, are long-standing problems. When fruits are fully mature with great taste, they become soft and difficult to store long-term and transport. Yu et al. (2017) used CRISPR/Cas genome editing technology to obtain both *ALC* gene mutagenesis and replacement in tomato. As in other plant species, CRISPR HDR-mediated gene replacement is much more difficult than CRISPR/Cas knock-out mutagenesis (Yu et al., 2017). The CRISPR-edited tomato demonstrated improved storage performance and long-shelf life without affecting other agronomic traits, such as plant size and fruit firmness (Yu et al., 2017). Using a CRISPR/Cas9 knock-out of a tomato ripening-related lncRNA, *lnRNA1459*, Li R et al. (2018a) obtained tomato plants with altered fruit ripening in tomato.

6 Current challenges of CRISPR/Cas and its future directions

In the past decade, significant progress has been made in discovering, modifying, and adopting CRISPR/Cas systems in gene function studies, clinical research,

Table 5 Improvement of crop quality and associated traits using CRISPR/Cas genome editing technology

Crop species	Targeted gene	Improved trait	CRISPR/Cas editor	Reference
Carbohydrate				
Wheat	*TaSBEIIa*	Amylose and resistant starch content	CRISPR/Cas9	Li JY et al., 2020
Rice	*SBEI* and *SBEIIb*	Amylose and resistant starch content	CRISPR/Cas9	Sun et al., 2017
Rice	*Waxy*	Amylose content	CRISPR/Cas9	Zhang JS et al., 2018
Potato	*GBSS*	Amylose content	CRISPR/Cas9	Andersson et al., 2018
Potato	*GBSS1*	Amylose content	CRISPR/Cas9	Kusano et al., 2018
Protein				
Wheat	*α-gliadin*	Low-gluten	CRISPR/Cas9	Sánchez-León et al., 2018
Wheat	*α-gliadin* and *γ-gliadin*	Low-gluten	CRISPR/Cas9	Jouanin et al., 2019
Oil content and quality				
Camelina sativa	*fad2* genes	Oil quality	CRISPR/Cas9	Jiang et al., 2017
Rice	*fad2* genes	High oleic/low linoleic acid	CRISPR/Cas9	Abe et al., 2018
Peanut	*fad2* genes	High oleic/low linoleic acid	CRISPR/Cas9	Yuan et al., 2019
Rapeseed	*fad2* genes	High oleic/low linoleic acid	CRISPR/Cas9	Huang et al., 2020
Rapeseed	*fad2* genes	High oleic/low linoleic acid	CRISPR/Cas9	Okuzaki et al., 2018
Tobacco	*fad2* genes	High oleic/low linoleic acid	CRISPR/Cas9	Tian et al., 2020
Cotton	*fad2* genes	High oleic/low linoleic acid	CRISPR/Cas9	Chen YZ et al., 2021
Soybean	*fad2* genes	High oleic/low linoleic acid	CRISPR/Cas9	al Amin et al., 2019
Camelina sativa	*fae1* genes	Oil quality	CRISPR/Cas9	Ozseyhan et al., 2018
Camelina sativa	*CsDGAT1* and *CsPDAT1*	Seed oil production and fatty acid composition	CRISPR/Cas9	Aznar-Moreno and Durrett, 2017
Camelina sativa	*CsCRUC*	Seed protein and oil profile	CRISPR/Cas9	Lyzenga et al., 2019
Rapeseed	*BnPAT* and *BnLPAT*	Oil and starch content	CRISPR/Cas9	Zhang et al., 2019
Rapeseed	*BnTT8*	Oil and protein	CRISPR/Cas9	Zhai et al., 2020
Rapeseed	*BnITPK*	Phytic acid content	CRISPR/Cas9	Sashidhar et al., 2020
Rapeseed	*BnSFAR4* and *BnSFAR5*	Seed oil content	CRISPR/Cas9	Karunarathna et al., 2020
Rapeseed	*BnTT2*	Oil and atty acid composition with higher linoleic acid (C18:2) and linolenic acid (C18:3)	CRISPR/Cas9	Xie T et al., 2020
Wheat	*TaGW2*	Grain weight and protein content	CRISPR/Cas9	Zhang Y et al., 2018
Anti-nutrient				
Grape	*IdnDH*	Reduced tartaric acid content	CRISPR/Cas9	Ren et al., 2016
Potato	*St16DOX*	Reduced steroidal glycoalkaloid content	CRISPR/Cas9	Nakayasu et al., 2018
Maize	*ZmIPK*	Reduced phytic acid content	CRISPR/Cas9	Liang et al., 2014
Functional metabolites				
Rice	*rc*	Roanthocyanidin and anthocyanin content	CRISPR/Cas9	Zhu et al., 2019
Potato	*StPPO2*	Enzymatic browning	CRISPR/Cas9	González et al., 2020
Tomato	*ANT1*	Anthocyanin content	CRISPR/Cas9	Čermák et al., 2015
Tomato	*SlGAD2* and *GlGAD3*	γ-Aminobutyric acid (GABA) content	CRISPR/Cas9	Nonaka et al., 2017
Tomato	*GABA-TP1*, *GABA-TP2*, *GABA-TP3*, *CAT9*, and *SSADH*	GABA content	CRISPR/Cas9	Li R et al., 2018b

To be continued

Table 5

Crop species	Targeted gene	Improved trait	CRISPR/Cas editor	Reference
Tomato	*SlDDB1*, *SlDET1*, and *SlCYC-B*	Carotenoid accumulation	CRISPR/Cas9-AID base editor	Hunziker et al., 2020
Tomato	*SGR1*, *LCY-E*, *Blc*, *LCY-B2*, and *LCY-B1*	Lycopene content	CRISPR/Cas9	Li XD et al., 2018
Tomato	*CycB*, *FW2.2*, etc.	Lycopene content	CRISPR/Cas9	Zsögön et al., 2018
Tomato	*GGP1*, *GLV3*, etc.	Vitamin C content	CRISPR/Cas9	Li TD et al., 2018
Banana	*LCYε*	β-Carotene	CRISPR/Cas9	Kaur et al., 2020
Chinese kale	*BoaCRTISO*	Color-related chlorophyll and carotenoid content	CRISPR/Cas9	Sun et al., 2020
Rice	*OsCYP97A4*, *OsDSM2*, *OsCCD4a*, *OsCCD4b*, and *OsCCD7*	Carotenoid accumulation	CRISPR/Cas9	Yang et al., 2017
Other traits				
Tomato	*ALC*	Improved storage performance and long-shelf life	CRISPR/Cas9 (NHEJ and HDR)	Yu et al., 2017
Tomato	*LncRNA1459*	Fruit ripening	CRISPR/Cas9	Li R et al., 2018a
Tomato	*RIN*	Fruit ripening	CRISPR/Cas9	Ito et al., 2015
Tomato	*LeMADS-RIN*	Fruit ripening	CRISPR/Cas9	Jung et al., 2018

CRISPR: clustered regularly interspaced short palindromic repeats; Cas: CRISPR-associated protein; AID: activation-induced cytidine deaminase; NHEJ: non-homologous end joining; HDR: homology-directed repair.

and crop improvement. However, there are still several major issues associated with this advanced technology that need to be resolved before it will be widely used in biomedicine and precision breeding.

6.1 CRISPR/Cas delivery is still one bottleneck hindering its wider usage

Whether treating human genetic diseases or carrying out precision crop breeding, the first important step is to deliver CRISPR/Cas reagents into the target cells. In plants, CRISPR/Cas-based genome editing is highly dependent on plant tissue culture-based gene transformation. Currently, the most efficient way to obtain genome editing events is by using *Agrobacterium*-mediated gene transformation, which is limited to a small number of plant species. Although plants of some species, such as agriculturally important crops, can be regenerated, this is limited to only a few genotypes or cultivars. Thus, developing a highly efficient genotype-independent plant tissue culture and plant regeneration system, or developing a new transformation method avoiding plant regeneration, is urgently needed. It is possible to improve plant regeneration capacity through testing different explants, plant growth hormones/regulators, different active chemicals, and combinations of these effectors. At first, it was extremely difficult to obtain regenerated plants from rice and maize tissue culture. However, after many years of hard work, this problem was solved in rice and maize, mainly by selecting immature embryos as explants (Peng et al., 2021). Now, rice has been become a model plant species for both genetic and applied research. One of the reasons is that there is now a highly efficient tissue culture and transformation system for rice. Cotton is also an important crop, and is thought to be one of the most difficult plant species for obtaining somatic embryogenesis and plant regeneration. However, modifying the plant growth medium and culture strategies, including starvation and drought treatment, significantly improved the capacity of cotton tissue culture and plant regeneration, and regenerated plants have now been obtained from almost all tested cotton genotypes (Zhang et al., 2009).

Although we can obtain genome-edited plants through plant tissue culture-based methods, many issues, such as induced mutations during plant tissue culture and the lengthy process, have limited the application of CRISPR/Cas technology. Thus, developing a tissue culture-independent delivery method will be the best choice for CRISPR/Cas genome editing. Recently, Ma et al. (2020) used *Sonchus yellow net rhabdovirus* (SYNV) to successfully deliver both Cas9 protein and sgRNAs (transfer RNA (tRNA)−guide RNA (gRNA)−tRNA fusion) into plant cells, and obtained CRISPR/

Cas genome editing events. Further modifications of this virus-based CRISPR/Cas reagent delivery system will provide a tissue culture-independent transgenic and genome editing system that can be used for any plant species without the need for a complicated laboratory process (Liu and Zhang, 2020).

6.2 Off-target effects are still a big challenge for clinical treatments

Off-target impacts are a big issue for CRISPR/Cas-based genome editing, particularly for clinical treatment that does not allow any errors. Although it is not so critical for genome editing in plants, it may affect precision breeding by affecting other agriculturally important traits. Off-targets occur in CRISPR/Cas-based genome editing, for all types of genome editors, particularly in animals. Although scientists have been attempting to limit off-target impacts, it seems that it is hard to eliminate them completely. Currently, there are several efficient strategies used for reducing CRISPR/Cas-based genome editing off-targets. (1) Selecting an appropriate CRISPR/Cas reagent delivery system. Different delivery systems may have significantly different effects, not only for genome editing efficiency and outcomes, but also in relation to federal regulations. No matter what delivery strategy, the Cas DNA/RNA/protein and sgRNAs need to be delivered into plant/animal cells. If Cas/sgRNA genes are inserted into the genome, this will cause those genes to be permanently expressed in the cells and cause potential off-target effects. However, if the Cas/sgRNA genes are not inserted into the plant/animal genome, and exist in the target cells for only a short period, the rate of off-target effects may be minimized. Many studies show that delivery of Cas-sgRNA ribonucleoprotein (RNP) complex into cells reduces off-target impacts mainly because the CRISPR/Cas9 system is not inserted into the host genome and the RNA/protein has a short life time (Doudna, 2020). (2) Designing high-fidelity sgRNA and selecting the right Cas enzymes and genome editing tools can also reduce the rate of off-target effects. If we can fuse a proofreading enzyme, like DNA polymerase, with the Cas enzyme, when an off-target event occurs, the proofreading enzyme will correct any errors (Zhang and Zhang, 2020b). This will eliminate the off-target impact of CRISPR/Cas genome editing. Another potential way to reduce off-target impact is to deliver the CRISPR/Cas/gRNA reagents only to the targeted cells.

6.3 CRISPR/Cas multiplex genome editing: good or bad?

In recent years, multiplex CRISPR/Cas genome editing has attracted attention from many research laboratories, and has been used to edit genes in certain plant and animal genomes. Although it can be used to edit multiple genes simultaneously, it also causes lots of potential problems, including more off-target impacts and removal of long DNA fragments. Thus, multiplex CRISPR/Cas genome editing is not a robust and high-tech technology, and adds only one or more gRNAs into the constructs. Higher off-target impacts and removal of long DNA fragments will cause more serious outcomes. Therefore, multiplex CRISPR/Cas genome editing does not yet have general practical application in clinical treatment or precision breeding. Even for gene function studies, it may take a lot more time to select and identify CRISPR/Cas mutations after genome editing. To reduce potential side effects, when using multiplex CRISPR/Cas genome editing technology the targeted sites should not be located on the same chromosome. This will help reduce the frequency of long DNA fragment deletions.

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Author contributions

All authors were actively involved in summarizing the literature and writing the manuscript. All authors have read and approved the final manuscript.

Compliance with ethics guidelines

Chao LI, Eleanor BRANT, Hikmet BUDAK, and Baohong ZHANG declare that they have no conflict of interest.

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