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

Chao LI1, Eleanor BRANT2, Hikmet BUDAK3**, Baohong ZHANG4**

1Oil Crops Research Institute, Chinese Academy of Agricultural Sciences, Key Laboratory for Biology and Genetic Improvement of Oil Crops, Ministry of Agriculture and Rural Affairs, Wuhan 430062, China
2Agromony Department, University of Florida, Gainesville, FL 32611, USA
3Montana BioAgriculture, Inc., Missoula, MT 59802, USA
4Department of Biology, East Carolina University, Greenville, NC 27858, USA

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 endonucleases, 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
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, 2015).

### 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 FokI DNA cleaving domain and a DNA-binding domain | No | Yes | Low | Very high |
| TALEN               | Protein–DNA | Long             | TALEN with a FokI 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.
Two years later similarly. (accidently cloned a sequence which contained cloning a gene in, whilst. In recent years, elucidated, scientists had been building protein to cleave the target site, and is introduced alongside a Cas RNA, which can be designed to target any tracrRNA complex was replaced with a single guide. When Jinek et al. 2012 (creating a DSB as a guide for the Cas endonuclease to initiate sequencing), Gasiunas et al. 2012, Nakata et al. 1991, and 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, II, 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/Cas9, 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, 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 StCas9 and SsCas9, 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'-NNNACAC-3' or 5'-NNNNRYAC-3' (R and Y represent purines (A or G) and pyrimidines (C or T), respectively) (Kim E et al., 2017), and ScCas9, derived from Streptococcus canis, prefers 5'-NGG-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'-NNGRRRT-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'-NNRRT-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 NRH, SpCas9 NRCH, and SpCas9 NRTH, which collectively show preference for the 5'-NNRH-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., Franciscella 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/B3-YE2/B3-YEE/B3-EE, and BE4 (Komor et al., 2016; Kim YB et al., 2017; Gehrk 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 enzyme     | Cas        |                   | DSB               | No                  | sgRNA | Gene knock-out, inducing silence mutations |
| Regular CRISPR/Cas enzyme 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 SKO3 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 promoter 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 realtime 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; Cher- tow, 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, breakdown, 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, hypoaactivity 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 *hegI* 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 *hegI* 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 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 immunotherapies, 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 haplinsufficient 7q tumor suppressor in acute myeloid leukemia (AML) in sh*NF1; 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, Bungsby 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) (Bungsby 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 m6A 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 ren7 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 malignancies. 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). Stadtmayer 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 (Stadtmayer 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 widely 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-genomes) 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; Martinez et al., 2020) and grapevine (Wan et al., 2020), pmr4 for plant resistance to powdery mildew in tomato (San-tilán Martinez 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 | Promoter for sgRNA | Knock-out efficiency | Off-target | Reference |
|-------------|-----------------|----------------|-------------|---------------|---------------------|----------------------|------------|-----------|
| Wheat       | Triticum aestivum | Protoplast transformation, CRISPR/ Cas9 | TaMLO | U6 | Shan et al., 2013 |
| Rice        | Oryza sativa     | Protoplast transformation, CRISPR/ OsPDS, OsBADH2, Os02g23823, and OsMPK2 | U3 | Varied, >15% | Not found Shan et al., 2013 |
| Maize       | Zea mays         | Protoplast transformation, CRISPR/ ZmIPK | U3 | 13.1% | Liang et al., 2014 |
| Soybean     | Glycine max      | Agrobacterium rhizogenes transformation | GFP/Gm40714530, DDMI, and miRNA | U6 | Varied, up to 95% | Low | Jacobs et al., 2015 |
| Cotton      | Gossypium hirsutum | Agrobacterium mediated | MYB | U6 | Not found Li et al., 2017 |
| Sorghum     | Sorghum bicolor  | Agrobacterium-mediated embryo transformation | DsRED2 | U6 | Jiang et al., 2013 |
| Sweet potato | Ipomoea batatas | Agrobacterium mediated | kgGSS3 and kGBSSII | U6 | Wang HX et al., 2019 |
| Potato      | Solanum tuberosum | Agrobacterium mediated | STIA2 | U6 | Wang et al., 2015 |
| Tomato      | Solanum lycopersicum | Agrobacterium-mediated transformation | GFP and SISH | U6 | Ron et al., 2014 |
| Yarn        | Dioscorea alata  | Agrobacterium mediated | PDS | DaU6 | Syombua et al., 2020 |
| Cassava     | Manihot esculenta | Agrobacterium mediated | MePDS | AtU6 | Oidjio et al., 2017 |
| Switchgrass | Panicum virgatum | Agrobacterium mediated | P44c1l | U3 | Park et al., 2017 |
| Switchgrass | Panicum virgatum | Agrobacterium mediated | th1a, th1b, and pgm | U6 | Liu Y et al., 2018 |
| Rapeseed    | Brassica napus   | Agrobacterium mediated | Alc | U6 | Braatz et al., 2017 |
| Jatropha    | Jatropha curcas  | Agrobacterium mediated | Jc-CYP735A | U3 | Cai et al., 2018 |
| Apple       | Malus prunifolia | Agrobacterium mediated | PDS | AtU6 | Nishitani et al., 2016 |
| Sweet orange | Citrus sinensis | Xcc-facilitated | PDS | 3.2%–3.9% | Jia and Wang, 2014 |
| Pear        | Pyrus communis   | Agrobacterium mediated | PDS and TFL1 | U3 and 4CL | Charrier et al., 2019 |
| Poplar      | Populus communis | Agrobacterium mediated | U6 | 25% | Zhou et al., 2015 |
| Sweet basil | Ocimum basilicum | Agrobacterium mediated | 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), Cipk1 for resistance to Fusarium oxysporum f. sp. niveum in watermelon (Zhang et al., 2020), elf4e 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 *depl*, 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)/AREB-binding factor 2 (ABF2). Their results showed that the CRISPRa dCas9 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/Cas9 to improve crop yield (Table 4). 

\[ \text{Gb1, dep1, and gs3 are three negative regulators associated with grain number per panicle, grain size, and seed size in rice (Ashikari et al., 2005). Gb1 is a gene for cytokinin oxidase/dehydrogenase (OsCKX2). Reduced expression of gb1 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 (gw6) 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/Cas9 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 amylase benefit human health by lowering the potential risk of certain serious diseases. Improving the content of resistant starch and amylase

\[ \text{\begin{tabular}{|l|l|l|l|}
| Crop species & Targeted gene & Improved trait & CRISPR/Cas editor & Reference \\
|-----------|-------------|-----------------|-----------------|-------|
| Rice      & gb1a, dep1, and gs3 & Grain number, dense erect panicles, and larger grain size & CRISPR/Cas9 & Li MR et al., 2016 \\
| Rice      & gw2, gw5, and tgw6 & Grain size and crop yield & CRISPR/Cas9 Xu et al., 2016 \\
| Rice      & OsPAO5     & Grain weight, grain numbers, and yield & CRISPR/Cas9 Lv et al., 2021 \\
| Switchgrass & tb1     & Tillers and fresh biomass & CRISPR/Cas9 Liu et al., 2020 \\
| Rapeseed  & BnaMAX1    & Plant architecture and yield & CRISPR/Cas9 Zheng et al., 2020 \\
| Soybean   & GmLHY      & Plant height and internode length & CRISPR/Cas9 Cheng et al., 2019 \\
| Soybean   & AP1        & Flowering time and plant height & CRISPR/Cas9 Chen et al., 2020 \\
| Soybean   & GmNMHC5    & Flowering and maturity & CRISPR/Cas9 Wang WT et al., 2020 \\
| Wheat     & TaGW2      & Grain weight and protein content & CRISPR/Cas9 Zhang Y et al., 2018 \\
| Rice      & OsGcs1     & High-quality sugar production & CRISPR/Cas9 Honma et al., 2020 \\
| Rice      & Ehd1       & Basic vegetative growth & CRISPR/Cas9 Wu et al., 2020 \\
\end{tabular}} \]

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 CRISPR/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 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-promoting 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 SGRI (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       | GBSSI         | Amylose content | CRISPR/Cas9 | Kusano et al., 2018 |
| 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 |
| Rapseed      | fad2 genes   | High oleic/low linoleic acid | CRISPR/Cas9 | Huang et al., 2020 |
| Rapseed      | 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 |
| Rapseed      | BnPAT and BnPAT | Oil and starch content | CRISPR/Cas9 | Zhang et al., 2019 |
| Rapseed      | BnTT8         | Oil and protein  | CRISPR/Cas9 | Zhai et al., 2020 |
| Rapseed      | BnITPK        | Phytic acid content | CRISPR/Cas9 | Sashidhar et al., 2020 |
| Rapseed      | BnSFAR4 and BnSFAR5 | Seed oil content | CRISPR/Cas9 | Karunaraithna et al., 2020 |
| Rapseed      | BnTT2         | Oil and ac 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 |
| Grape        | IdnDH         | Reduced tartaric acid content | CRISPR/Cas9 | Ren et al., 2016 |
| Potato       | St16DOX       | Reduced steriald glycoalkaloid content | CRISPR/Cas9 | Nakayasu et al., 2018 |
| Maize        | ZmIPK         | Reduced phytic acid content | CRISPR/Cas9 | Liang et al., 2014 |
| Functional metabolites |         |                |                   |           |
| Rice         | rc            | Roanthocyanin and anthocyanin content | CRISPR/Cas9 | Zhu et al., 2019 |
| Potato       | SPPPO2        | Enzymatic browning | CRISPR/Cas9 | González et al., 2020 |
| Tomato       | ANT1          | Anthocyanin content | CRISPR/Cas9 | Čermák et al., 2015 |
| Tomato       | SIGAD2 and GIGAD3 | 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
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 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 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.

Acknowledgments

We greatly appreciate the scientific community for making such rapid progress in this field. We apologize to authors whose wonderful work was not cited in this paper. Baohong ZHANG is supported in part by Cotton Incorporated and the National Science Foundation (award 1658709). This work was supported by the National Natural Science Foundation of China (No. 31700316), the Fundamental Research Funds for the Central Nonprofit Scientific Institution (No. 160172018009), and the Natural Science Foundation of Hubei Province (No. 2018CFB543), China.

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.

This article does not contain any studies with human or animal subjects performed by any of the authors.

References

Abe K, Araki E, Suzuki Y, et al., 2018. Production of high oleic/low linoleic rice by genome editing. Plant Physiol
Addington AM, Gormick M, Duckworth J, et al., 2005. GADI (2q31.1), which encodes glutamic acid decarboxylase (GAD65), is associated with childhood-onset schizophrenia and cortical gray matter volume loss. *Mol Psychiatry*, 10(6): 581-588. https://doi.org/10.1038/sj.mp.4001599

Adli M, 2018. The CRISPR tool kit for genome editing and beyond. *Nat Commun*, 9:1911. https://doi.org/10.1038/s41467-018-04252-2

Ai JW, Zhou X, Xu T, et al., 2019. CRISPR-based rapid and ultra-sensitive diagnostic test for *Mycobacterium tuberculosis*. *Energ Microbes Infect*, 8(1):1361-1369. https://doi.org/10.1080/22221751.2019.1664939

al Amin N, Ahmad N, Wu N, et al., 2019. CRISPR-Cas9 mediated targeted disruption of *FAD2-2 microsomal omega-6 desaturase* in soybean (*Glycine max.* L.). *BMC Biotechnol*, 19:9. https://doi.org/10.1186/s12896-019-0501-2

Ali Z, Aman R, Mahas A, et al., 2020. iSCAN: an RT-LAMP-coupled CRISPR-Cas12 module for rapid, sensitive detection of SARS-CoV-2. *Virus Res*, 288:198129. https://doi.org/10.1016/j.viruses.2020.198129

Altenbach SB, Chang HC, Yu XB, et al., 2019. Elimination of omega-1,2 gliadins from bread wheat (*Triticum aestivum*) flour: effects on immunogenic potential and end-use quality. *Front Plant Sci*, 10:580. https://doi.org/10.3389/fpls.2019.00580

Andersson M, Turesson H, Olsson N, et al., 2018. Genome editing in potato via CRISPR-Cas9 ribonuclease protein delivery. *Physiol Plant*, 164(4):378-384. https://doi.org/10.1111/ppl.12731

Anzalone AV, Randolph PB, Davis JR, et al., 2019. Search-and-replace genome editing without double-strand breaks or donor DNA. *Nature*, 576(7785):149-157. https://doi.org/10.1038/s41586-019-1711-4

Aravalli RN, Steer CJ, 2016. Gene editing technology as an approach to the treatment of liver diseases. *Exp Opin Biol Ther*, 16(5):595-608. https://doi.org/10.1517/14712598.2016.1158808

Ashikari M, Sakakibara H, Lin SY, et al., 2005. Cytokinin oxidase regulates rice grain production. *Science*, 309(5735): 741-745. https://doi.org/10.1126/science.1113373

Aznar-Moreno JA, Durrett TP, 2017. Simultaneous targeting of multiple gene homeologs to alter seed oil production in *Camelina sativa*. *Plant Cell Physiol*, 58(7):1260-1267. https://doi.org/10.1093/pcp/pcx058

Baker M, 2012. Gene-editing nucleases. *Nat Methods*, 9(1): 23-26. https://doi.org/10.1038/nmeth.1807

Bañuls L, Pellicer D, Castillo S, et al., 2020. Gene therapy in rare respiratory diseases: what have we learned so far? *J Clin Med*, 9(8):2577. https://doi.org/10.3390/jcm9082577

Barro F, Iehisa JCM, Giménez MJ, et al., 2016. Targeting of prolamins by RNAi in bread wheat: effectiveness of seven silencing-fragment combinations for obtaining lines devoid of coeliac disease epitopes from highly immunogenic gliadins. *Plant Biotechnol J*, 14(3):986-996. https://doi.org/10.1111/pbi.12455

Becker D, Wieser H, Koehler P, et al., 2012. Protein composition and techno-functional properties of transgenic wheat with reduced α-gliadin content obtained by RNA interference. *J Appl Bot Food Qual*, 85(1):23-33.

Bogdanović O, Lister R, 2017. DNA methylation and the preservation of cell identity. *Curr Opin Genet Dev*, 46:9-14. https://doi.org/10.1016/j.gde.2017.06.007

Bouzrourd S, Gasparini K, Hu GJ, et al., 2020. Down regulation and loss of Auxin Response Factor 4 function using CRISPR/Cas9 alters plant growth, stomatal function and improves tomato tolerance to salinity and osmotic stress. *Genes*, 11(3):272. https://doi.org/10.3390/genes111030272

Braatz J, Harloff HJ, Mascher M, et al., 2017. CRISPR-Cas9 targeted mutagenesis leads to simultaneous modification of different homoeologous gene copies in polyploid oil-seed rape (*Brassica napus*). *Plant Physiol*, 174(2):935-942. https://doi.org/10.1104/pp.17.00426

Brandsma E, Verhagen HM, van de Laar TJW, et al., 2021. Rapid, sensitive, and specific severe acute respiratory syndrome coronavirus 2 detection: a multicenter comparison between standard quantitative reverse-transcriptase polymerase chain reaction and CRISPR-based DETECTR. *J Infect Dis*, 223(2):206-213. https://doi.org/10.1093/infdis/jiaa641

Broughton JP, Deng XD, Yu GX, et al., 2020. CRISPR-Cas12-based detection of SARS-CoV-2. *Nat Biotechnol*, 38(7):870-874. https://doi.org/10.1038/s41587-020-0513-4

Bungsy M, Palmer MCL, Jeuesset LM, et al., 2021. Reduced RBXI expression induces chromosome instability and promotes cellular transformation in high-grade serous ovarian cancer precursor cells. *Cancer Lett*, 500:194-207. https://doi.org/10.1016/j.canlet.2020.11.051

Burstein D, Harrington LB, Strutt SC, et al., 2017. New CRISPR-Cas systems from uncultivated microbes. *Nature*, 542(7640):237-241. https://doi.org/10.1038/nature21059

Büschges R, Hollricher K, Panstruga R, et al., 1997. The barley mlo gene: a novel control element of plant pathogen resistance. *Cell*, 88(5):695-705. https://doi.org/10.1016/s0092-8674(00)81912-1

Cai L, Zhang L, Fu QT, et al., 2018. Identification and expression analysis of cytokinin metabolic genes *IPTh*, *CYP735A* and *CKX*s in the biofuel plant *Jatropha curcas*. *PeerJ*, 6:e4812. https://doi.org/10.7717/peerj.4812

Cao XS, Kouyama-Suzuki E, Pang B, et al., 2020. Inhibition of DNA ligase IV enhances the CRISPR/Cas9-mediated knock-in efficiency in mouse brain neurons. *Biochem Biophys Res Commun*, 553(3):449-457. https://doi.org/10.1016/j.bbrc.2020.09.053

Čermák T, Baltes NJ, Cegan R, et al., 2015. High-frequency, precise modification of the tomato genome. *Genome Biol*, 16:232. https://doi.org/10.1186/s13059-015-0796-9

Chandrasekar J, Brumin M, Wolf D, et al., 2016. Development of broad virus resistance in non-transgenic
cucumber using CRISPR/Cas9 technology. *Mol Plant Pathol*, 17(7):1140-1153.
https://doi.org/10.1111/mpp.12375

Charrrier A, Vergne E, Dousset N, et al., 2019. Efficient targeted mutagenesis in apple and first time edition of pear using the CRISPR-Cas9 system. *Front Plant Sci*, 10:40.
https://doi.org/10.3389/fpls.2019.00040

Chatterjee P, Jakimo N, Jacobson JM, 2018. Minimal PAM specificity of a highly similar SpCas9 ortholog. *Sci Adv*, 4(10):eaau0766.
https://doi.org/10.1126/sciadv.aau0766

Chavez A, Scheiman J, Vora S, et al., 2015. Highly efficient Cas9-mediated transcriptional programming. *Nat Methods*, 12(4):326-328.
https://doi.org/10.1038/nmeth.3312

Chen C, Liu Y, Rappaport AR, et al., 2014. MLL3 is a haploinsufficient 7q tumor suppressor in acute myeloid leukemia. *Cancer Cell*, 25(5):652-665.
https://doi.org/10.1016/j.cccr.2014.03.016

Chen HR, Gao SS, Liu WX, et al., 2021. RNA N6-methyladenosine methyltransferase METTL3 facilitates colorectal cancer by activating the m6A-GLUT1-mTORC1 axis and is a therapeutic target. *Gastroenterology*, 160(4):1284-1300.e16.
https://doi.org/10.1053.j.gastro.2020.11.013

Chen JS, Ma EB, Harrington LB, et al., 2018. CRISPR-Cas12a target binding unleashes indiscriminate single-stranded DNAse activity. *Science*, 360(6387):436-439.
https://doi.org/10.1126/science.aar6245

Chen KL, Wang YP, Zhang R, et al., 2019. CRISPR/Cas genome editing and precision plant breeding in agriculture. *Annu Rev Plant Biol*, 70:667-697.
https://doi.org/10.1146/annurev-arplant-050718-100049

Chen LY, Nan HY, Kong LP, et al., 2020. Soybean *AP1* homologs control flowering time and plant height. *J Integr Plant Biol*, 62(12):1868-1879.
https://doi.org/10.1111/jipb.12988

Chen YZ, Fu MC, Li H, et al., 2021. High-oleic acid content, nontransgenic allotropelid oilseed (*Gossypium hirsutum* L.) generated by knockout of GhFAD2 genes with CRISPR/Cas9 system. *Plant Biotechnol J*, 19(3):424-426.
https://doi.org/10.1111/pbi.13507

Chen AW, Wang HY, Yang H, et al., 2013. Multiplexed activation of endogenous genes by CRISPR-on, an RNA-guided transcriptional activator system. *Cell Res*, 23(10):1163-1171.
https://doi.org/10.1038/cr.2013.122

Cheng Q, Dong LD, Su T, et al., 2019. CRISPR/Cas9-mediated targeted mutagenesis of *GmlLHY* genes alters plant height and internode length in soybean. *BMC Plant Biol*, 19:562.
https://doi.org/10.1186/s12870-019-2145-8

Chertow DS, 2018. Next-generation diagnostics with CRISPR. *Science*, 360(6387):381-382.
https://doi.org/10.1126/science.aat4982

Choi BD, Yu XL, Castano AP, et al., 2019. CRISPR-Cas9 disruption of PD-1 enhances activity of universal EGRFrIII CAR T cells in a preclinical model of human glioblastoma. *J Immunother Cancer*, 7(1):304.
https://doi.org/10.1186/s40425-019-0806-7

Correa-Aragunde N, Graziano M, Lamattina L, 2004. Nitric oxide plays a central role in determining lateral root development in tomato. *Planta*, 218(6):900-905.
https://doi.org/10.1007/s00425-003-1172-7

Cui Y, Jiang N, Xu ZJ, et al., 2020. Heterotrimmeric G protein are involved in the regulation of multiple agronomic traits and stress tolerance in rice. *BMC Plant Biol*, 20:90.
https://doi.org/10.1186/s12870-020-2289-6

Dar AA, Choudhury AR, Kanchcharla PK, et al., 2017. The FAD2 gene in plants: occurrence, regulation, and role. *Front Plant Sci*, 8:1789.
https://doi.org/10.3389/fpls.2017.01789

Deng H, Tan SW, Gao QX, et al., 2020. Cas9 knocking out mediated by CRISPR-Cas9 genome editing for PD-L1 attenuation and enhanced antitumor immunity. *Acta Pharm Sin B*, 10(2):358-373.
https://doi.org/10.1016/j.apsb.2019.07.004

Dickinson DJ, Ward JD, Reiner DJ, et al., 2013. Engineering the *Caenorhabditis elegans* genome using Cas9-triggered homologous recombination. *Nat Methods*, 10(10):1028-1034.
https://doi.org/10.1038/nmeth.2641

Ding X, Yin K, Li ZY, et al., 2020. Ultrasensitive and visual detection of SARS-CoV-2 using all-in-one dual CRISPR-Cas12a assay. *Nat Commun*, 11:4711.
https://doi.org/10.1038/s41467-020-18575-6

Doman JL, Raguram A, Newby GA, et al., 2020. Evaluation and minimization of Cas9-independent off-target DNA editing by cytosine base editors. *Nat Biotechnol*, 38(5):620-628.
https://doi.org/10.1038/s41587-020-0414-6

Doudna JA, 2020. The promise and challenge of therapeutic genome editing. *Nature*, 578(7794):229-236.
https://doi.org/10.1038/s41586-020-1978-5

Du J, Duan S, Wang H, et al., 2008. Comprehensive analysis of polymorphisms throughout *GAD1* gene: a family-based association study in schizophrenia. *J Neural Transm (Vienna)*, 115(3):513-519.
https://doi.org/10.1007/s00702-007-0844-z

Ellis BL, Hirsch ML, Porter SN, et al., 2013. Zinc-finger nucleic-acid-mediated gene correction using single AAV vector transduction and enhancement by food and drug administration-approved drugs. *Gene Ther*, 20(1):35-42.
https://doi.org/10.1038/gt.2011.211

Endo M, Mikami M, Endo A, et al., 2019. Genome editing in plants by engineered CRISPR-Cas9 recognizing NG PAM. *Nat Plants*, 5(1):14-17.
https://doi.org/10.1038/s41477-018-0321-8

Esvelt KM, Mali P, Braff JL, et al., 2013. Orthogonal Cas9 proteins for RNA-guided gene regulation and editing. *Nature Methods*, 10(11):1116-1121.
https://doi.org/10.1038/Nmeth.2681

Feng YL, Liu SC, Chen RD, et al., 2021. Target binding and residence: a new determinant of DNA double-strand break repair pathway choice in CRISPR/Cas9 genome editing. *J Zhejiang Univ-Sci B (Biomed & Biotechnol)*, 22(1):73-86.
https://doi.org/10.1631/jzus.B2000282

Ferrari G, Thrasher AJ, Aiuti A, 2021. Gene therapy using haematopoietic stem and progenitor cells. *Nat Rev Genet*, 22:216-234.
https://doi.org/10.1038/s41576-020-00298-5
Filiz E, Vatansever R, 2018. Genome-wide identification of mildew resistance locus O (MLO) genes in tree model poplar (Populus trichocarpa): powdery mildew management in woody plants. *Eur J Plant Pathol*, 152(1):95-109. https://doi.org/10.1007/s10658-018-1454-3

Flores T, Todd CD, Tovar-Mendez A, et al., 2008. Arginase-negative mutants of Arabidopsis exhibit increased nitric oxide signaling in root development. *Plant Physiol*, 147(4):1936-1946. https://doi.org/10.1104/pp.108.121459

Fu Y, Rocha PP, Luo VM, et al., 2016. CRISPR-dCas9 and sgRNA scaffolds enable dual-colour live imaging of satellite sequences and repeat-enriched individual loci. *Nat Commun*, 7:11707. https://doi.org/10.1038/ncomms11707

Fujihara K, Yamada K, Ichitani Y, et al., 2020. CRISPR/Cas9-engineered Gad1 elimination in rats leads to complex behavioral changes: implications for schizophrenia. *Transl Psychiatry*, 10:426. https://doi.org/10.1038/s41398-020-01108-6

Furukawa T, Maekawa M, Oki T, et al., 2007. The Rc and Rd genes are involved in proanthocyanidin synthesis in rice pericarp. *Plant J*, 49(1):91-102. https://doi.org/10.1111/j.1365-313X.2006.02958.x

Gao LY, Cox DBT, Yan WX, et al., 2017. Engineered Cpf1 variants with altered PAM specificities. *Nat Biotechnol*, 35(8):789-792. https://doi.org/10.1038/nbt.3900

Gao SP, Kilili AJ, Zhang K, et al., 2021. AKT1 E17K inhibits cancer cell migration by abrogating β-catenin signaling. *Mol Cancer Res*, online. https://doi.org/10.1158/1541-7786.Mcr-20-0623

Garneau JE, Dupuis ME, Villion M, et al., 2010. The CRISPR/Cas bacterial immune system cleaves bacteriophage and plasmid DNA. *Nature*, 468(7320):67-71. https://doi.org/10.1038/nature09523

Gasinuaus G, Barrangou R, Horvath P, et al., 2012. Cas9-crRNA ribonucleoprotein complex mediates specific DNA cleavage for adaptive immunity in bacteria. *Proc Natl Acad Sci USA*, 109(39):E2795-E2856.

Gaudelli NM, Komor AC, Rees HA, et al., 2017. Programmable base editing of A·T to G·C in genomic DNA without DNA cleavage. *Nature*, 551(7681):464-471. https://doi.org/10.1038/nature24644

Gaudelli NM, Lam DK, Rees HA, et al., 2020. Directed evolution of adenase base editors with increased activity and therapeutic application. *Nat Biotechnol*, 38(7):892-900. https://doi.org/10.1038/s41587-020-0491-6

Ge ZX, Zheng LQ, Zhao YL, et al., 2019. Engineered xCas9 and SpCas9-NG variants broaden PAM recognition sites to generate mutations in Arabidopsis plants. *Plant Biotechnol J*, 17(10):1865-1867. https://doi.org/10.1111/pbi.13148

Geber LFR, MacRae IJ, 2019. Regulation of microRNA function in animals. *Nat Rev Mol Cell Biol*, 20(1):21-37. https://doi.org/10.1038/s41580-018-0045-7

Gehrke JM, Cervantes O, Clement MK, et al., 2018. An APOBEC3A-Cas9 base editor with minimized bystander and off-target activities. *Nat Biotechnol*, 36(10):977-982. https://doi.org/10.1038/nbt.4199

Gilbert LA, Larson MH, Morsut L, et al., 2013. CRISPR-mediated modular RNA-guided regulation of transcription in eukaryotes. *Cell*, 154(2):442-451. https://doi.org/10.1016/j.cell.2013.06.044

Gilbert LA, Horlbeck MA, Adamson B, et al., 2014. Genome-scale CRISPR-mediated control of gene repression and activation. *Cell*, 159(3):647-661. https://doi.org/10.1016/j.cell.2014.09.029

Gilm-Humanes J, Pistón F, Hernando A, et al., 2008. Silencing of γ-gliadins by RNA interference (RNAi) in bread wheat. *J Cereal Sci*, 48(3):565-568. https://doi.org/10.1016/j.jcs.2008.03.005

Gilm-Humanes J, Pistón F, Tollefsen S, et al., 2010. Effective shutdown in the expression of celiac disease-related wheat gliadin T-cell epitopes by RNA interference. *Proc Natl Acad Sci USA*, 107(39):17023-17028. https://doi.org/10.1073/pnas.1007773107

Gleditsch S, Pausch P, Müller-Esparza H, et al., 2019. PAM identification by CRISPR-Cas effector complexes: diversified mechanisms and structures. *RNA Biol*, 16(4):504-517. https://doi.org/10.1080/15476268.2018.1504546

Gonzalez MN, Massa GA, Andersson M, et al., 2020. Reduced enzymatic browning in potato tubers by specific editing of a polyphenol oxidase gene via ribonucleoprotein complexes delivery of the CRISPR/Cas9 system. *Front Plant Sci*, 10:1649. https://doi.org/10.3389/fpls.2019.01649

Gootenberg JS, Abudayyeh OO, Lee JW, et al., 2017. Nucleic acid detection with CRISPR-Cas13a/C2c2. *Science*, 356(6336):438-442. https://doi.org/10.1126/science.aam9321

Gootenberg JS, Abudayyeh OO, Kellner MJ, et al., 2018. Multiplexed and portable nucleic acid detection platform with Cas13, Cas12a, and Csm6. *Science*, 360(6387):439-444. https://doi.org/10.1126/science.aqz0179

Haas KT, Wightman R, Meyerowitz EM, et al., 2020. Pectin homogalacturonan nanofilament expansion drives morphogenesis in plant epidermal cells. *Science*, 367(6481):1003-1007. https://doi.org/10.1126/science.aaz5103

Han LQ, Zhang ML, Xing ZY, et al., 2020. Knockout of butyrophilin subfamily 1 member A1 (BTNL1) alters lipid droplet formation and phospholipid composition in bovine mammary epithelial cells. *J Anim Sci Biotechnol*, 11:72. https://doi.org/10.1186/s40104-020-00479-6

Hanzawa N, Hashimoto K, Yuan XM, et al., 2020. Targeted DNA demethylation of the Fgt21 promoter by CRISPR/dCas9-mediated epigenome editing. *Sci Rep*, 10:5181. https://doi.org/10.1038/s41598-020-62035-6

Hayward D, Cole PA, 2016. LSD1 histone demethylase assays and inhibition. *Methods Enzymol*, 573:261-278. https://doi.org/10.1016/bs.mie.2016.01.020

He SH, 2020. The first human trial of CRISPR-based cell therapy clears safety concerns as new treatment for late-stage lung cancer. *Signal Transduct Target Ther*, 5:168. https://doi.org/10.1038/s41392-020-00283-8

Hermans PW, van Soolingen D, Bik EM, et al., 1991. Insertion element IS987 from *Mycobacterium bovis* BCG is located in a hot-spot integration region for insertion elements in *Mycobacterium tuberculosis* complex strains. *Infect Immun*, 59(8):2695-2705.
Heyer WD, Ehmse KT, Liu J, 2010. Regulation of homologous recombination in eukaryotes. Annu Rev Genet, 44: 113-139.
https://doi.org/10.1146/annurev-genet-051710-150955

Hirano S, Nishimatsu H, Ishitani R, et al., 2016. Structural basis for the altered PAM specificities of engineered CRISPR-Cas9. Mol Cell, 61(6): 886-894.
https://doi.org/10.1016/j.molcel.2016.02.018

Honma Y, Adhikari PB, Kuwata K, et al., 2020. High-quality sugar production by osgscl1 rice. Commun Biol, 3: 617.
https://doi.org/10.1038/s42003-020-01329-x

Horii T, Morita S, Hino S, et al., 2020. Successful generation of epigenetic disease model mice by targeted demethylation of the epigenome. Genome Biol, 21: 77.
https://doi.org/10.1186/s13059-020-01991-8

Hou ZG, Zhang Y, Propson NE, et al., 2013. Efficient genome engineering in human pluripotent stem cells using Cas9 from Neisseria meningitidis. Proc Natl Acad Sci USA, 110(39): 15644-15649.
https://doi.org/10.1073/pnas.1313587110

Hu JH, Miller SM, Geurts MH, et al., 2018. Evolved Cas9 variants with broad PAM compatibility and high DNA specificity. Nature, 556(7699): 57-63.
https://doi.org/10.1038/nature26155

Hu XX, Wang C, Fu YP, et al., 2016. Expanding the range of CRISPR-Cas9 genome editing in rice. Mol Plant, 9(6): 943-945.
https://doi.org/10.1093/mp/olw086

Hu XX, Meng XB, Liu Q, et al., 2018. Increasing the efficiency of CRISPR-Cas9-VQR precise genome editing in rice. Plant Biotechnol J, 16(1): 292-297.
https://doi.org/10.1111/pbi.12771

Hua K, Tao XP, Liang WY, et al., 2020. Simplified adenine base editors improve adenine base editing efficiency in rice. Plant Biotechnol J, 18(3): 770-778.
https://doi.org/10.1111/pbi.13244

Huang H, Liu RE, Niu QF, et al., 2019. Global increase in DNA methylation during orange fruit development and ripening. Proc Natl Acad Sci USA, 116(4): 1430-1436.
https://doi.org/10.1073/pnas.1803824

Huang HB, Cui TT, Zhang LL, et al., 2020. Modifications of fatty acid profile through targeted mutation at BnaFAD2 gene with CRISPR/Cas9-mediated gene editing in Brassica napus. Theor Appl Genet, 133(8): 2401-2411.
https://doi.org/10.1007/s00122-020-03607-y

Hunziker J, Nishida K, Kondo A, et al., 2020. Multiple gene substitution by target-AID base-editing technology in tomato. Sci Rep, 10: 20471.
https://doi.org/10.1038/s41598-020-77379-2

Huo WY, Zhao GN, Yin JG, et al., 2017. Lentiviral CRISPR/Cas9 vector mediated miR-21 gene editing inhibits the epithelial to mesenchymal transition in ovarian cancer cells. J Cancer, 8(1): 57-64.
https://doi.org/10.7150/jca.16723

Ishino Y, Shinagawa H, Makino K, et al., 1987. Nucleotide sequence of the iap gene, responsible for alkaline phosphatase isozyme conversion in Escherichia coli, and identification of the gene product. J Bacteriol, 169(12): 5429-5433.
https://doi.org/10.1128/jb.169.12.5429-5433.1987

Islam KU, Iqbal J, 2020. An update on molecular diagnostics for COVID-19. Front Cell Infect Microbiol, 10: 560616.
https://doi.org/10.3389/fcimb.2020.560616

Ito Y, Nishizawa-Yokoi A, Endo M, et al., 2015. CRISPR/Cas9-mediated mutagenesis of the RIN locus that regulates tomato fruit ripening. Biochem Biophys Res Commun, 467(1): 76-82.
https://doi.org/10.1016/j.bbrc.2015.09.117

Jacobs TB, Lafayette PR, Schmitz RJ, et al., 2015. Targeted genome modifications in soybean with CRISPR/Cas9. BMC Biotechnol, 15: 16.
https://doi.org/10.1186/s12896-015-0131-2

Jansen R, van Embden JDA, Gaaster W, et al., 2002. Identification of genes that are associated with DNA repeats in prokaryotes. Mol Microbiol, 43(6): 1565-1575.
https://doi.org/10.1046/j.1365-2958.2002.02839.x

Javalkote VS, Kancharla N, Bhadra B, et al., 2020. CRISPR-based assays for rapid detection of SARS-CoV-2. Methods, in press.

Jia HG, Wang N, 2014. Targeted genome editing of sweet orange using Cas9/sgRNA. PLoS ONE, 9(4): e93806.
https://doi.org/10.1371/journal.pone.0093806

Jiang WN, Liang YX, Wei W, et al., 2020. Functional classification of prostate cancer-associated miRNAs through CRISPR/Cas9-mediated gene knockout. Mol Med Rep, 22(5): 3777-3784.
https://doi.org/10.3892/mmr.2020.11491

Jiang WZ, Zhou HB, Bi HH, et al., 2013. Demonstration of CRISPR/Cas9/sgRNA-mediated targeted gene modification in Arabidopsis, tobacco, sorghum and rice. Nucleic Acids Res, 41(20): e188.
https://doi.org/10.1093/nar/gkt780

Jiang WZ, Henry IM, Lynagh PG, et al., 2017. Significant enhancement of fatty acid composition in seeds of the allohexaploid, Camellina sativa, using CRISPR/Cas9 gene editing. Plant Biotechnol J, 15(5): 648-657.
https://doi.org/10.1111/pbi.12663

Jin Y, Datsomor AK, Olsen RE, et al., 2020. Targeted mutagenesis of Δ5 and Δ6 fatty acyl desaturases induce dysregulation of lipid metabolism in Atlantic salmon (Salmo salar). BMC Genomics, 21: 805.
https://doi.org/10.1186/s12864-020-07218-1

Jinek M, Chylinski K, Fonfara I, et al., 2012. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. Science, 337(6096): 816-821.
https://doi.org/10.1126/science.1225829

Jouanin A, Schaart JG, Boyd LA, et al., 2019. Outlook for coeliac disease patients: towards bread wheat with hypoimmunogenic gluten by gene editing of α- and γ-gliadin gene families. BMC Plant Biol, 19: 333.
https://doi.org/10.1186/s12870-019-1889-5

Kojoung, N, Engreitz JM, Konermann S, et al., 2017. Genomescalar activation screen identifies a lncRNA locus regulating a gene neighbourhood. Nature, 548(7667): 343-346.
https://doi.org/10.1038/nature23451

Juhász A, Colgrave ML, Howitt CA, 2020. Developing gluten-free cereals and the role of proteomics in product...
safety. J Cereal Sci, 93:102932.  
https://doi.org/10.1016/j.ces.2020.102932

Jung YJ, Lee GJ, Bae S, et al., 2018. Reduced ethylene production in tomato fruits upon CRISPR/Cas9-mediated leMADS-RIN mutagenesis. Hortic Sci Technol, 36(3):396-405.  
https://doi.org/10.12927/kjbst.20180039

Kang BC, Yun JY, Kim ST, et al., 2018. Precision genome engineering through adenine base editing in plants. Nat Plants, 4:427-431.  
https://doi.org/10.1038/s41477-018-0178-x

Kanitchnida S, Srisala I, Suebsaeng R, et al., 2020. CRISPR-Cas fluorescent cleavage assay coupled with recombinase polymerase amplification for sensitive and specific detection of Enteroctocysto-zoon hepatoenzepaei. Biotechnol Rep (Amst), 27:e00485.  
https://doi.org/10.1016/j.btre.2020.e00485

Karunaratna NL, Wang HY, Harloff HJ, et al., 2020. Elevating seed oil content in a polyploid crop by induced mutations in SEED FATTY ACID REDUCER genes. Plant Biotechnol J, 18(11):2251-2266.  
https://doi.org/10.1111/pbi.13381

Kaur N, Alok A, Shivani, et al., 2020. CRISPR/Cas9 directed editing of lycopene epsilon-cyclase modulates metabolic flux for β-carotene biosynthesis in banana fruit. Metab Eng, 59:76-86.  
https://doi.org/10.1016/j.meben.2020.01.008

Kaya H, Mikami M, Endo A, et al., 2016. Highly specific targeted mutagenesis in plants using Staphylococcus aureus Cas9. Sci Rep, 6:26871.  
https://doi.org/10.1038/srep26871

Kearns NA, Pham H, Tabak B, et al., 2015. Functional annotation of native enhancers with a Cas9-histone demethylase fusion. Nat Methods, 12(5):401-403.  
https://doi.org/10.1038/nmeth.3325

Kellner MJ, Koob JG, Gootenberg JS, et al., 2019. SHERLOCK: nucleic acid detection with CRISPR nucleases. Nat Protoc, 14(10):2986-3012.  
https://doi.org/10.1038/s41596-019-0210-2

Khosravi S, Ishii T, Dreissig S, et al., 2020. Application and prospects of CRISPR/Cas9-based methods to trace defined genomic sequences in living and fixed plant cells. Chromosome Res, 28(1):1-17.  
https://doi.org/10.1007/s10577-019-09622-0

Kim E, Koo T, Park SW, et al., 2017. In vivo genome editing with a small Cas9 orthologue derived from Campylobacter jejuni. Nat Commun, 8:14500.  
https://doi.org/10.1038/s41467-019-14500

Kim H, Kim ST, Ryu J, et al., 2017. CRISPR/Cpf1-mediated DNA-free plant genome editing. Nat Commun, 8:14406.  
https://doi.org/10.1038/s41467-019-14406

Kim YA, Moon H, Park CJ, 2019. CRISPR/Cas9-targeted mutagenesis of OsxN3 in rice to confer resistance to Xanthomonas oryzae pv. oryzae. Rice, 12:67.  
https://doi.org/10.1186/s12284-019-0325-7

Kim YB, Komor AC, Levy JM, et al., 2017. Increasing the genome-targeting scope and precision of base editing with engineered Cas9-ctidine deaminase fusions. Nat Biotechnol, 35(4):371-376.  
https://doi.org/10.1038/nbt.3803

Kitamoto K, Taketani Y, Fujii W, et al., 2020. Generation of mouse model of TGFBI-R124C corneal dystrophy using CRISPR/Cas9-mediated homology-directed repair. Sci Rep, 10:2000.  
https://doi.org/10.1038/s41598-020-58876-w

Kleinstiver BP, Prew MS, Tsai SQ, et al., 2015a. Broadening the targeting range of Staphylococcus aureus CRISPR-Cas9 by modifying PAM recognition. Nat Biotechnol, 33(12): 1293-1298.  
https://doi.org/10.1038/nbt.3404

Kleinstiver BP, Prew MS, Tsai SQ, et al., 2015b. Engineered CRISPR-Cas9 nucleases with altered PAM specificities. Nature, 523(7561):481-485.  
https://doi.org/10.1038/nature14592

Komor AC, Kim YB, Packer MS, et al., 2016. Programmable editing of a target base in genomic DNA without double-stranded DNA cleavage. Nature, 533(7603):420-424.  
https://doi.org/10.1038/nature17946

Komor AC, Zhao KT, Packer MS, et al., 2017. Improved base excision repair inhibition and bacteriophage Mu Gam protein yields C: G-to-T: A base editors with higher efficiency and product purity. Sci Adv, 3(8):eaa4774.  
https://doi.org/10.1126/sciadv.aao4774

Konermann S, Brigham MD, Trevino AE, et al., 2013. Optical control of mammalian endogenous transcription and epigenetic states. Nature, 500(7463):472-476.  
https://doi.org/10.1038/nature12466

Konermann S, Brigham MD, Trevino AE, et al., 2015. Genome-scale transcriptional activation by an engineered CRISPR-Cas9 complex. Nature, 517(7536):583-588.  
https://doi.org/10.1038/nature14136

Kumar P, Malik YS, Ganesh B, et al., 2020. CRISPR-Cas system: an approach with potentials for COVID-19 diagnosis and therapeutics. Front Cell Infect Microbiol, 10:576875.  
https://doi.org/10.3389/fcimb.2020.576875

Kurilova T, Koyama-Suzuki E, Satoga M, et al., 2020. DNA repair protein RAD51 enhances the CRISPR/Cas9-mediated knock-in efficiency in brain neurons. Biochem Biophys Res Commun, 524(3):621-628.  
https://doi.org/10.1016/j.bbrc.2020.01.132

Kurt IC, Zhou RH, Iyer S, et al., 2020. CRISPR C-to-G base editors for inducing targeted DNA transversions in human cells. Nat Biotechnol, 39(1):41-46.  
https://doi.org/10.1038/s41587-020-0609-x

Kusano H, Ohnuma M, Matsuuro-Aoki H, et al., 2018. Establishment of a modified CRISPR/Cas9 system with increased mutation frequency using the translational enhancer dMac3 and multiple guide RNAs in potato. Sci Rep, 8:13753.  
https://doi.org/10.1038/s41598-018-32049-2

Lau CH, Suh Y, 2018. In vivo epigenome editing and transcriptional modulation using CRISPR technology. Transgenic Res, 27(6):489-509.  
https://doi.org/10.1007/s11248-018-0096-8

Lawhorn IEB, Ferreira JP, Wang CL, 2014. Evaluation of sgRNA target sites for CRISPR-mediated repression of TP53. PLoS ONE, 9(11):e113232.  
https://doi.org/10.1371/journal.pone.0113232

Li C, Unver T, Zhang BH, 2017. A high-efficiency CRISPR/Cas9 system for targeted mutagenesis in cotton (Gossypium hirsutum L.). Sci Rep, 7:43902.  
https://doi.org/10.1038/srep43902
Li C, Zhang R, Meng XB, et al., 2020. Targeted, random mutagenesis of plant genes with dual cytosine and adenine base editors. Nat Biotechnol, 38(7):875-882. https://doi.org/10.1038/s41587-019-0393-7

Li HY, Li JY, Chen JL, et al., 2020. Precise modifications of both exogenous and endogenous genes in rice by prime editing. Mol Plant, 13(5):671-674. https://doi.org/10.1016/j.molp.2020.03.011

Li JF, Norville JE, Aach J, et al., 2013. Multiplex and homologous recombination-mediated genome editing in Arabidopsis and Nicotiana benthamiana using guide RNA and Cas9. Nat Biotechnol, 31(8):688-691. https://doi.org/10.1038/nbt.2654

Li JY, Jiao GA, Jiang QY, et al., 2013. Multiplex and targeted mutagenesis of lncRNA and GnDEP. J Genet Genomics, 41(2):63-68. https://doi.org/10.1016/j.jgg.2013.12.001

Liao TT, Lin CC, Jiang JK, et al., 2020. Harnessing stemness and PD-L1 expression by AT-rich interaction domain-containing protein 3B in colorectal cancer. Theranostics, 10(14):6095-6112. https://doi.org/10.7150/thno.44147

Lin QP, Zong Y, Xue CX, et al., 2020. Prime genome editing in rice and wheat. Nat Biotechnol, 38(5):582-585. https://doi.org/10.1038/s41587-020-0455-x

Lin S, Staahl BT, Alla RK, et al., 2014. Enhanced homology-directed human genome engineering by controlled timing of CRISPR/Cas9 delivery. elife, 3:e04766. https://doi.org/10.7554/eLife.04766

Lin WR, Ng IS, 2020. Development of CRISPR/Cas9 system in Chlorella vulgaris FSP-E to enhance lipid accumulation. Enzyme Microb Technol, 133:109458. https://doi.org/10.1016/j.enzmictect.2019.109458

Liu HW, Zhang BH, 2020. Virus-based CRISPR/Cas9 genome editing in plants. Trends Genet, 36(11):810-813. https://doi.org/10.1016/j.tig.2020.08.002

Liu JW, Sun MY, Cho KB, et al., 2021. A CRISPR-Cas9 repressor for epigenetic silencing of KRAS. Pharmacol Res, 164:105304. https://doi.org/10.1016/j.phrs.2020.105304

Liu XS, Wu H, Ji X, et al., 2016. Editing DNA methylation in the mammalian genome. Cell, 167(1):233-247.e17. https://doi.org/10.1016/j.cell.2016.08.056

Liu Y, Merrick P, Zhang ZZ, et al., 2018. Targeted mutagenesis in tetraploid switchgrass (Panicum virgatum L.) using CRISPR/Cas9. Plant Biotechnol J, 16(2):381-393. https://doi.org/10.1111/pbi.12778

Liu Y, Wang WL, Yang B, et al., 2020. Functional analysis of the teosinte branched 1 gene in the tetraploid switchgrass (Panicum virgatum L.) by CRISPR/Cas9-directed mutagenesis. Front Plant Sci, 11:572193. https://doi.org/10.3389/fpls.2020.572193

Liu YZ, Qi XW, Zeng ZZ, et al., 2017. CRISPR/Cas9-mediated p53 and Pten dual mutation accelerates hepatocarcinogenesis in adult hepatitis B virus transgenic mice. Sci Rep, 7:2796. https://doi.org/10.1038/s41598-017-03070-8

Liu QZ, Chen M, Chen SY, et al., 2018. Highly efficient RNA-guided base editing in rabbit. Nat Commun, 9:2717. https://doi.org/10.1038/s41467-018-05232-2

Lottfi M, Rezaei N, 2020. CRISPR/Cas13: a potential therapeutic option of COVID-19. Biomed Pharmacother, 131:110738. https://doi.org/10.1016/j.biopharma.2020.110738

Lu SX, Hu MY, Wang ZH, et al., 2020. Generation and application of the zebrafish heg1 mutant as a cardiovascular disease model. Biomolecules, 10(11):1542.
https://doi.org/10.3390/biom10111542
Lu Y, Xue JX, Deng T, et al., 2020. Safety and feasibility of CRISPR-edited T cells in patients with refractory non-small-cell lung cancer. *Nat Med.*, 26(5):732-740.
https://doi.org/10.1038/s41591-020-0840-5
Lv YS, Shao GN, Jiao GJ, et al., 2021. Targeted mutagenesis of *POLYAMINE OXIDASE 5* that negatively regulates mesocotyl elongation enables the generation of direct-seeding rice with improved grain yield. *Mol Plant*, 14(2):344-351.
https://doi.org/10.1016/j.molp.2020.10.007
Lyzenga WJ, Harrington M, Bekkaoui D, et al., 2019. CRISPR/Cas9 editing of three CRUCIFERIN C homoeologues alters the seed protein profile in *Camelina sativa*. *BMC Plant Biol.*, 19:292.
https://doi.org/10.1186/s12870-019-1873-0
Ma HH, Naseri A, Reyes-Gutierrez P, et al., 2015. Multicolor CRISPR labeling of chromosomal loci in human cells. *Proc Natl Acad Sci USA*, 112(10):3002-3007.
https://doi.org/10.1073/pnas.1420024112
Ma XN, Zhang XY, et al., 2015. Multicolor DNA-free plant genome editing using virally delivered CRISPR-Cas9. *Nat Plants*, 6(7):773-779.
https://doi.org/10.1038/s41477-020-0704-5
Maddalò D, Manchado E, Concepcion CP, et al., 2014. In vivo engineering of oncogenic chromosomal rearrangements with the CRISPR/Cas9 system. *Nature*, 516(7531):423-427.
https://doi.org/10.1038/nature13902
Maeder ML, Linder SI, CASCIO VM, et al., 2013. CRISPR RNA-guided activation of endogenous human genes. *Nat Methods*, 10(10):977-979.
https://doi.org/10.1038/nmeth.2598
Magadán AH, Dupuis ME, Villion M, et al., 2012. Cleavage of phage DNA by the *Streptococcus thermophilus* CRISPR/Cas9 system. *PLoS ONE*, 7(7):e40913.
https://doi.org/10.1371/journal.pone.0040913
Mahoney KM, Freeman GJ, McDermott DF, 2015. The next immune-checkpoint inhibitors: PD-1/PD-L1 blockade in melanoma. *Clin Ther*, 37(4):764-782.
https://doi.org/10.1016/j.clinthera.2015.02.018
Makarova KS, Wolf YI, franzo J, et al., 2020. Evolutionary classification of CRISPR-Cas systems: a burst of class 2 and derived variants. *Nat Rev Microbiol*, 18(2):67-83.
https://doi.org/10.1038/s41579-019-0299-x
Malina A, Mills JR, Cencic R, et al., 2013. Repurposing CRISPR/Cas9 for in situ functional assays. *Genes Dev*, 27(23):2602-2614.
https://doi.org/10.1101/gad.227132.113
Martínez MS, Bracuto V, Koseoglu E, et al., 2020. CRISPR/Cas9-targeted mutagenesis of the tomato susceptibility gene *PM4* for resistance against powdery mildew. *BMC Plant Biol.*, 20:284.
https://doi.org/10.1186/s12870-020-02497-y
Meng ZG, Meng ZH, Zhang R, et al., 2015. Expression of the rice arginine gene *OsARG* in cotton influences the morphology and nitrogen transition of seedlings. *PLoS ONE*, 10(11):e0141530.
https://doi.org/10.1371/journal.pone.0141530
Miller SM, Wang TN, Randolph PB, et al., 2020. Continuous evolution of SpCas9 variants compatible with non-G PAMs. *Nat Biotechnol.*, 38(4):471-481.
https://doi.org/10.1038/s41587-020-0412-8
Mojica FJM, Montoliu L, 2016. On the origin of CRISPR-Cas technology: from prokaryotes to mammals. *Trends Microbiol.*, 24(10):811-820.
https://doi.org/10.1016/j.tim.2016.06.005
Mojica FJM, Juez G, Rodriguez-Valera F, 1993. Transcription at different salinities of *Halofexus mediterranei* sequences adjacent to partially modified PstI sites. *Mol Microbiol.*, 9(3): 613-621.
https://doi.org/10.1111/j.1365-2958.1993.tb01721.x
Mojica FJM, Ferrer C, Juez G, et al., 1995. Long stretches of short tandem repeats are present in the largest replicons of the archaea *Halofexus mediterranei* and *Halofexus volcanii* and could be involved in replicon partitioning. *Mol Microbiol.*, 17:85-93.
https://doi.org/10.1111/j.1365-2958.1995.mmi_17010085.x
Mojica FJM, Diez-Villaseñor C, García-Martínez J, et al., 2005. Intervening sequences of regularly spaced prokaryotic repeats derive from foreign genetic elements. *J Mol Evol.*, 60(2):174-182.
https://doi.org/10.1007/s00239-004-0046-3
Morita S, Noguchi H, Horii T, et al., 2016. Targeted DNA demethylation in vivo using dCas9-peptide repeat and scFv-TET1 catalytic domain fusions. *Nat Biotechnol.*, 34(10):1060-1065.
https://doi.org/10.1038/nbt.3658
Müller M, Lee CM, Gasunas G, et al., 2016. *Streptococcus thermophilus* CRISPR-Cas9 systems enable specific editing of the human genome. *Mol Ther*, 24(3):636-644.
https://doi.org/10.1038/m.2015.218
Mustafa MI, Makhawi AM, 2021. SHERLOCK and DETECTR: CRISPR-Cas systems as potential rapid diagnostic tools for emerging infectious diseases. *J Clin Microbiol.*, 59(3): e00745-20.
https://doi.org/10.1128/jcm.00745-20
Myhrvold C, Freije CA, Gootenberg JS, et al., 2018. Field-deployable viral diagnostics using CRISPR-Cas13. *Science*, 360(6387):444-448.
https://doi.org/10.1126/science.aas8836
Nakada A, Amenura M, Makino K, 1989. Unusual nucleotide arrangement with repeated sequences in the *Escherichia coli* K-12 chromosome. *J Bacteriol.*, 171(6):3553-3556.
https://doi.org/10.1128/jb.171.6.3553-3556.1989
Nakayasu M, Akiyama R, Lee HJ, et al., 2018. Generation of α-solanine-free hairy roots of potato by CRISPR/Cas9 mediated genome editing of the *St16DOX* gene. *Plant Physiol Biochem.*, 131:70-77.
https://doi.org/10.1016/j.plaphy.2018.04.026
Nature Methods, 2012. Method of the year 2011. *Nat Methods*, 9:1.
https://doi.org/10.1038/nmeth.1852
Navet N, Tian MY, 2020. Efficient targeted mutagenesis in allopolyploid sweet basil by CRISPR/Cas9. *Plant Direct*, 4(6):e00233.
https://doi.org/10.1002/pld3.233
Nekrasov V, Wang CM, Win J, et al., 2017. Rapid generation of a transgene-free powdery mildew resistant tomato by genome deletion. *Sci Rep*, 7:482.
https://doi.org/10.1038/s41598-017-00578-x
Nishioka K, Arazoe T, Yachie N, et al., 2016. Targeted nucleotide editing using hybrid protokaryotic and vertebrate adaptive immune systems. *Science*, 353(6305):aaf8729. https://doi.org/10.1126/science.aaf8729

Nishimatsu H, Cong L, Yan WX, et al., 2015. Crystal structure of *Staphylococcus aureus* Cas9. *Cell*, 162(5):1113-1126. https://doi.org/10.1016/j.cell.2015.08.007

Nishimatsu H, Shi X, Ishiguro S, et al., 2018. Engineered CRISPR-Cas9 nuclease with expanded targeting space. *Science*, 361(6408):1259-1262. https://doi.org/10.1126/science.aas9129

Nishitani C, Hirai N, Komori S, et al., 2016. Efficient genome editing in apple using a CRISPR/Cas9 system. *Sci Rep*, 6:31481. https://doi.org/10.1038/srep31481

Nonaka S, Arai C, Takayama M, et al., 2017. Efficient increase of γ-aminobutyric acid (GABA) content in tomato fruits by targeted mutagenesis. *Sci Rep*, 7:7057. https://doi.org/10.1038/s41598-017-06400-y

Odipo J, Alici T, Engelbrecht I, et al., 2017. Efficient CRISPR/Cas9 genome editing of *Phytoene desaturase* in cassava. *Front Plant Sci*, 8:1780. https://doi.org/10.3389/fpls.2017.01780

Okuzaki A, Ogawa T, Koizuka C, et al., 2018. CRISPR/Cas9-mediated genome editing of the fatty acid desaturase 2 gene in *Brassica napus*. *Plant Physiol Biochem*, 131:63-69. https://doi.org/10.1016/j.plaphy.2018.04.025

Ortigosa A, Gimenez-Ibáñez S, Leonhardt N, et al., 2019. Design of a bacterial speck resistant tomato by CRISPR/Cas9-mediated editing of SLAZ2. *Plant Biotechnol J*, 17(3):665-673. https://doi.org/10.1111/pbi.13006

Ozseyhan ME, Kang JL, Mu XP, et al., 2018. Mutagenesis of the *FAE1* genes significantly changes fatty acid composition in seeds of *Camelina sativa*. *Plant Physiol Biochem*, 123:1-7. https://doi.org/10.1016/j.plaphy.2017.11.021

Park JI, Yoo CG, Flanagan A, et al., 2017. Defined tetra-allelic gene disruption of the 4-coumarate: coenzyme A ligase 1 (*Pv4CL1*) gene by CRISPR/Cas9 in switchgrass results in lignin reduction and improved sugar release. *Biotechnol Biofuels*, 10:284. https://doi.org/10.1186/s13068-017-0972-0

Pathi KM, Rink P, Budhagatapalli N, et al., 2020. Engineering smut resistance in maize by site-directed mutagenesis of *LIPOXYGENASE 3*. *Front Plant Sci*, 11:54385. https://doi.org/10.3389/fpls.2020.54385

Pausch P, Al-Shayeb B, Bisson-Rapp E, et al., 2020. CRISPR-Cas9 from huge phages is a hypercompact genome editor. *Science*, 369(6501):333-337. https://doi.org/10.1126/science.abb1400

Peng AH, Chen SC, Lei TG, et al., 2017. Engineering canker-resistant plants through CRISPR/Cas9-targeted editing of the susceptibility gene *CsLOB1* promoter in citrus. *Plant Biotechnol J*, 15(12):1509-1519. https://doi.org/10.1111/pbi.12733

Peng RH, Jones DC, Liu F, et al., 2021. From sequencing to genome editing for cotton improvement. *Trends Biotechnol*, 39(3):221-224. https://doi.org/10.1016/j.tibtech.2020.09.001

Pröbsting M, Schenke D, Hossain R, et al., 2020. Loss of function of CRT1A (calreticulin) reduces plant susceptibility to *Verticillium longisporum* in both *Arabidopsis thaliana* and oilseed rape (*Brassica napus*). *Plant Biotechnol J*, 18(11):2328-2344. https://doi.org/10.1111/pbi.13394

Qi LS, Larson MH, Gilbert LA, et al., 2013. Repurposing CRISPR as an RNA-guided platform for sequence-specific control of gene expression. *Cell*, 152(5):1173-1183. https://doi.org/10.1016/j.cell.2013.02.022

Quan J, Langelier C, Kuchta A, et al., 2019. FLASH: a next-generation CRISPR diagnostic for multiplexed detection of antimicrobial resistance sequences. *Nucleic Acids Res*, 47(14):e83. https://doi.org/10.1093/nar/gkz418

Ran FA, Cong L, Yan WX, et al., 2015. *In vivo* genome editing using *Staphylococcus aureus* Cas9. *Nature*, 520(7546):186-191. https://doi.org/10.1038/nature14299

Ren B, Liu L, Li SF, et al., 2019. Cas9-NG greatly expands the targeting scope of the genome-editing toolkit by recognizing NG and other atypical PAMs in rice. *Mol Plant*, 12(7):1015-1026. https://doi.org/10.1016/j.molp.2019.03.010

Ren C, Liu XJ, Zhang Z, et al., 2016. CRISPR/Cas9-mediated efficient targeted mutagenesis in chardonnay (*Vitis vinifera L*.). *Sci Rep*, 6:32289. https://doi.org/10.1038/srep32289

Richter MF, Zhao KT, Eton E, et al., 2020. Phage-assisted evolution of an adenine base editor with improved Cas domain compatibility and activity. *Nat Biotechnol*, 38:883-891. https://doi.org/10.1038/s41587-020-0453-z

Roca Paixão JF, Gillet FX, Ribeiro TP, et al., 2019. Improved drought stress tolerance in *Arabidopsis* by CRISPR/dCas9 fusion with a histone acetyltransferase. *Sci Rep*, 9:8080. https://doi.org/10.1038/s41598-019-44571-y

Ron M, Kajala K, Pauluzzi G, et al., 2014. Hairy root transformation using *Agrobacterium rhizogenes* as a tool for exploring cell type-specific gene expression and function using tomato as a model. *Plant Physiol*, 166(2):455-469. https://doi.org/10.1104/pp.114.239392

Sánchez-León S, Gil-Humanes J, Ozuna CV, et al., 2018. Low-gluten, nontransgenic wheat engineered with CRISPR/Cas9. *Plant Biotechnol J*, 16(4):902-910. https://doi.org/10.1111/pbi.12837

Santillán Martínez MI, Bracuto V, Koseoglou A, et al., 2020. CRISPR-Cas9-targeted mutagenesis of the tomato susceptibility gene *PMR4* for resistance against powdery mildew. *BMC Plant Biol*, 20:284. https://doi.org/10.1186/s12870-020-02497-y

Sashidhar N, Harloff HJ, Potgieter L, et al., 2020. Gene editing of three *BnITPK* genes in tetraploid oilseed rape leads to significant reduction of phytic acid in seeds. *Plant Biotechnol J*, 18(11):2241-2250. https://doi.org/10.1111/pbi.13380

Sauer NJ, Narváez-Vásquez J, Mozornuk J, et al., 2016. Oligonucleotide-mediated genome editing provides precision and function to engineered nucleases and antibiotics in plants. *Plant Physiol*, 170(4):1917-1928.
https://doi.org/10.1104/pp.15.01696
Shan QW, Wang YP, Li J, et al., 2013. Targeted genome modification of crop plants using a CRISPR-Cas system. *Nat Biotechnol*, 31(8):686-688. https://doi.org/10.1038/nbt.2650
Shi Y, Wang G, Cai XP, et al., 2020. An overview of COVID-19. *J Zhejiang Univ Sci B (Biomed & Biotechnol)*, 21(5): 343-360. https://doi.org/10.1631/jzus.B2000803
Shin JJ, Schröder MS, Caiado F, et al., 2020. Controlled cycling and quiescence enables efficient HDR in engraftment-enriched adult hematopoietic stem and progenitor cells. *Cell Rep*, 32(9):108093. https://doi.org/10.1016/j.celrep.2020.108093
Silva G, Poirot L, Galetto R, et al., 2011. Meganuclease and other tools for targeted genome engineering: perspectives and challenges for gene therapy. *Curr Gene Ther*, 11(1):11-27. https://doi.org/10.2174/156652311794520111
Soares F, Chen B, Lee JB, et al., 2020. CRISPR screen identifies genes that sensitize AML cells to double negative T cell therapy. *Blood*, online. https://doi.org/10.1182/blood.2019004108
Stadtmueller EA, Fraietta JA, Davis MM, et al., 2020. CRISPR-engineered T cells in patients with refractory cancer. *Science*, 367(6481):eaab7365. https://doi.org/10.1126/science.aba7365
Steinert J, Schiml S, Fauser F, et al., 2015. Highly efficient heritable plant genome engineering using Cas9 orthologues from *Streptococcus thermophilus* and *Staphylococcus aureus*. *Plant J*, 84(6):1295-1305. https://doi.org/10.1111/tpj.13078
Strahl BD, Allis CD, 2000. The language of covalent histone modifications. *Nature*, 403(6765):41-45. https://doi.org/10.1038/347412
Su S, Zou ZY, Chen FJ, et al., 2017. CRISPR-Cas9-mediated disruption of PD-1 on human T cells for adoptive cellular therapies of EBV positive gastric cancer. *Oncoimmunology*, 6(1):e1249558. https://doi.org/10.1080/2162402x.2016.1249558
Sun B, Jiang M, Zheng H, et al., 2020. Color-related chlorophyll and carotenoid concentrations of Chinese kale can be altered through CRISPR/Cas9 targeted editing of the carotenoid isomerase gene *BouCRTISO*. *Hortic Res*, 7:161. https://doi.org/10.1038/s41438-020-00379-w
Sun N, Petiwala S, Wang R, et al., 2019. Development of drug-inducible CRISPR-Cas9 systems for large-scale functional screening. *BMC Genomics*, 20:225. https://doi.org/10.1186/s12864-019-5601-9
Sun YW, Jiao GA, Liu ZP, et al., 2017. Generation of high-amylose rice through CRISPR/Cas9-mediated targeted mutagenesis of starch branching enzymes. *Front Plant Sci*, 8:298. https://doi.org/10.3389/fpls.2017.00298
Sweeney MT, Thomson MJ, Cho YG, et al., 2007. Global dissemination of a single mutation conferring white pericarp in rice. *PLoS Genet*, 3(8):e133. https://doi.org/10.1371/journal.pgen.0030133
Syombua ED, Zhang ZZ, Tripathi JN, et al., 2020. A CRISPR/Cas9-based gene-editing system for yam (*Dioscorea* spp.). *Plant Biotechnol J*, online. https://doi.org/10.1111/pbi.13515
Takahashi N, Cho P, Selfors LM, et al., 2020. 3D culture models with CRISPR screens reveal hyperactive NRF2 as a prerequisite for spheroid formation via regulation of proliferation and ferroptosis. *Mol Cell*, 80(5):828-844. e6. https://doi.org/10.1016/j.molcel.2020.10.010
Tan JJ, Zhang F, Karcher D, et al., 2020. Expanding the genome-targeting scope and the site selectivity of high-precision base editors. *Nat Commun*, 11:629. https://doi.org/10.1038/s41467-020-14465-z
Tan YY, Du H, Wu X, et al., 2020. Gene editing: an instrument for practical application of gene biology to plant breeding. *J Zhejiang Univ Sci B (Biomed & Biotechnol)*, 21(6):460-473. https://doi.org/10.1631/jzus.B1900633
Tannenbaum ME, Gilbert LA, Qi LS, et al., 2014. A protein-tagging system for signal amplification in gene expression and fluorescence imaging. *Cell*, 159(3):635-646. https://doi.org/10.1016/j.cell.2014.09.039
Tang X, Lowder LG, Zhang T, et al., 2017. A CRISPR-Cpf1 system for efficient genome editing and transcriptional repression in plants. *Nat Plants*, 3(3):17018. https://doi.org/10.1038/nplants.2017.18
Tang X, Sretenovic S, Ren QR, et al., 2020. Plant prime editors enable precise gene editing in rice cells. *Mol Plant*, 13(5):667-670. https://doi.org/10.1016/j.molp.2020.03.010
Tian XL, Gu TX, Patel S, et al., 2019. CRISPR/Cas9—an evolving biological tool kit for cancer biology and oncology. *NPJ Precis Oncol*, 3:8. https://doi.org/10.1038/s41469-019-0080-7
Tian YS, Chen K, Li X, et al., 2020. Design of high-oleic tobacco (*Nicotiana tabacum L.*) seed oil by CRISPR-Cas9-mediated knockout of *NtFAD2-2*. *BMC Plant Biol*, 20:233. https://doi.org/10.1186/s12870-020-02441-0
Travis J, 2015. Makes the cut: CRISPR genome-editing technology shows its power. *Science*, 350(6267):1456-1457. https://doi.org/10.1126/science.350.6267.1456
van den Broeck HC, van Herpen TWJM, Schuit C, et al., 2009. Reversing celiac disease-related gluten proteins from bread wheat while retaining technological properties: a study with Chinese spring deletion lines. *BMC Plant Biol*, 9:41. https://doi.org/10.1186/1471-2229-9-41
Vojta A, Dobrinič P, Tadić V, et al., 2016. Repurposing the CRISPR-Cas9 system for targeted DNA methylation. *Nucleic Acids Res*, 44(12):5615-5628. https://doi.org/10.1093/nar/gkw159
Walton RT, Christie KA, Whittaker MN, et al., 2020. Unconstrained genome targeting with near-PAMless engineered CRISPR-Cas9 variants. *Science*, 368(6488):290-296. https://doi.org/10.1126/science.aba8853
Wan DY, Guo Y, Cheng Y, et al., 2020. CRISPR/Cas9-mediated mutagenesis of *VvMLO3* results in enhanced resistance to powdery mildew in grapevine (*Vitis vinifera*). *Hortic Res*, 7:116. https://doi.org/10.1038/s41438-020-0339-8
Wang FJ, Wang CL, Liu PQ, et al., 2016. Enhanced rice blast resistance by CRISPR/Cas9-targeted mutagenesis of the ERF transcription factor gene OsERF922. PLoS ONE, 11(4): e0154027. https://doi.org/10.1371/journal.pone.0154027

Wang HX, Wu YL, Zhang YD, et al., 2019. CRISPR/Cas9-based mutagenesis of starch biosynthetic genes in sweet potato (Ipomoea batatas) for the improvement of starch quality. Int J Mol Sci, 20(19):4702.

Wang JJ, Meng XB, Hu XX, et al., 2019. xCas9 expands the scope of genome editing with reduced efficiency in rice. Plant Biotechnol J, 17(4):709-711. https://doi.org/10.1111/pbi.13053

Wang LJ, Chen SC, Peng AH, et al., 2019. CRISPR/Cas9-mediated editing of CaWRKY22 reduces susceptibility to Xanthomonas citri subsp. citri in Wanjincheng orange (Citrus sinensis (L.) Osbeck). Plant Biotechnol Rep, 13(5):501-510. https://doi.org/10.1007/s11816-019-00556-x

Wang M, Zhang R, Li JM, 2020. CRISPR/Cas systems redefine nucleic acid detection: principles and methods. Biosens Bioelectron, 165:112430. https://doi.org/10.1016/j.bios.2020.112430

Wang SH, Zhang SB, Wang WX, et al., 2015. Efficient targeted mutagenesis in potato by the CRISPR/Cas9 system. Plant Cell Rep, 34(9):1473-1476. https://doi.org/10.1007/s00299-015-1816-7

Wang WT, Wang ZL, Hou WS, et al., 2020. GmM/HCC5, a neoteric positive transcription factor of flowering and maturity in soybean. Plants (Basel), 9(6):792. https://doi.org/10.3390/plants9060792

Wang XJ, Shang XY, Huang XX, 2020a. Next-generation pathogen diagnosis with CRISPR/Cas-based detection methods. Emerg Microbes Infect, 9(1):1682-1691. https://doi.org/10.1080/22221751.2020.1793689

Wang XJ, Zhong MT, Liu Y, et al., 2020b. Rapid and sensitive detection of COVID-19 using CRISPR/Cas12a-based detection with naked eye readout, CRISPR/Cas12a-NER. Sci Bull (Beijing), 65(17):1436-1439. https://doi.org/10.1007/s10428-020-4041-8

Wang YQ, Cheng X, Shan QW, et al., 2014. Simultaneous editing of three homeoalleles in hexaploid bread wheat confers heritable resistance to powdery mildew. Nat Biotechnol, 32(9):947-951. https://doi.org/10.1038/nbt.2969

Wu MJ, Liu HQ, Lin Y, et al., 2020. In-frame and frame-shift editing of the Ehd1 gene to develop Japonica rice with prolonged basic vegetative growth periods. Front Plant Sci, 11:307. https://doi.org/10.3389/fpls.2020.00307

Wu XJ, Mao SQ, Ying YC, et al., 2019. Progress and challenges for live-cell imaging of genomic loci using CRISPR-based platforms. Genom Proteom Bioinf, 17(2):119-128. https://doi.org/10.1016/j.gpb.2018.10.001

Wu Y, Xu W, Wang FP, et al., 2019. Increasing cytosine base editing scope and efficiency with engineered Cas9-pmCDAl fusions and the modified sgRNA in rice. Front Genet, 10:379. https://doi.org/10.3389/fgene.2019.00379

Wang HX, Ge XL, Yang FY, et al., 2020. High-fidelity SaCas9 identified by directional screening in human cells. PLoS Biol, 18(7):e3000747. https://doi.org/10.1371/journal.pbio.3000747

Xie T, Chen X, Guo T, et al., 2020. Targeted knockout of BrTT2 homologues for yellow-seeded Brassica napus with reduced flavonoids and improved fatty acid composition. J Agric Food Chem, 68(20):5676-5690. https://doi.org/10.1021/acs.jafc.0c01126

Xu JY, Lee YK, Wang Y, et al., 2014. Therapeutic application of endothelial progenitor cells for treatment of cardiovascular diseases. Curr Stem Cell Res Ther, 9(5):401-414. https://doi.org/10.2174/1574888x09666140619121318

Xu L, Wang J, Liu YL, et al., 2019. CRISPR-edited stem cells in a patient with HIV and acute lymphocytic leukemia. N Engl J Med, 381(13):1240-1247. https://doi.org/10.1056/NEJMoai1817426

Yang XY, He JX, Knopping out of...
Highly efficient engineered CRISPR/Cas9-mediated mutagenesis of Cpsk1 in watermelon to confer resistance to Fusarium oxysporum f. sp. niveum. *Plant Cell Rep.*, 39(5):589-595. https://doi.org/10.1007/s00299-020-02516-0

Zhang, Y., Li, D., Zhang, D.B., et al., 2018. Analysis of the functions of TuGW2 homologs in wheat grain weight and protein content traits. *Plant J.*, 94(5):857-866. https://doi.org/10.1111/tpj.13903

Zhang, Z.N., Ge, X.Y., Luo, X.Y., et al., 2018. Simultaneous editing of two copies of Gh14:3-3d confers enhanced transgene-clean plant defense against Verticillium dahliae in allotetraploid upland cotton. *Front Plant Sci.*, 9:842. https://doi.org/10.3389/fpls.2018.00842

Zhao, D.D., Li, J., Li, S.W., et al., 2020. Glycosylase base editors enable C-to-A and C-to-G base changes. *Nat Biotechnol.*, 39(1):35-40. https://doi.org/10.1038/s41598-020-0592-2

Zheng, M., Zhang, L., Tang, M., et al., 2020. Knockout of two BnaMAX1 homologs by CRISPR/Cas9-targeted mutagenesis improves plant architecture and increases yield in rapeseed (*Brassica napus L.*). *Plant Biotechnol J.*, 18(3):644-654. https://doi.org/10.1111/pbi.13228

Zhong, Z.H., Zhuo, W.W., Lai, Y., et al., 2020. Edible plant oil: global status, health issues, and perspectives. *Front Plant Sci.*, 11:1315. https://doi.org/10.3389/fpls.2020.03135

Zhu, Y.W., Lin, Y.R., Chen, S.B., et al., 2019. CRISPR/Cas9-mediated functional recovery of the recessive n allele to develop red rice. *Plant Biotechnol J.*, 17(11):2096-2105. https://doi.org/10.1111/pbj.13125

Zong, Y., Song, Q.N., Li, C., et al., 2018. Efficient C-to-T base editing in plants using a fusion of nCas9 and human APOBEC3A. *Nat Biotechnol.*, 36(10):950-953. https://doi.org/10.1038/nbt.4261

Zhao, C.Y., Li, J.Y., 2014. Molecular genetic dissection of quantitative trait loci regulating rice grain size. *Annu Rev Genet.*, 48:99-118. https://doi.org/10.1146/annurev-genet-120213-092138