Trends in Plant Science

CellPress
REVIEWS

Feature Review

CRISPR/Cas System: Recent Advances and Future Prospects for Genome Editing

Hakim Manghwar,[1,3] Keith Lindsey,[2] Xianlong Zhang,[1,3,*] and Shuangxia Jin[1,3,*]

Genome editing (GE) has revolutionized biological research through the new ability to precisely edit the genomes of living organisms. In recent years, various GE tools have been explored for editing simple and complex genomes. The clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 system has widely been used in GE due to its high efficiency, ease of use, and accuracy. It can be used to add desirable and remove undesirable alleles simultaneously in a single event. Here, we discuss various applications of CRISPR/Cas9 in a range of important crops, compare it with other GE tools, and review its mechanism, limitations, and future possibilities. Various newly emerging CRISPR/Cas systems, including base editing (BE), xCas9, and Cas12a (Cpf1), are also considered.

Highlights

Genome editing (GE) has modernized the biological world by providing a means to edit genomes of living organisms, including humans, plants, animals, and microbes.

CRISPR/Cas9 is an RNA guided endonuclease targeting the DNA.

CRISPR/Cas9 has high efficiency, accuracy, and ease of use for GE.

Until now, the CRISPR/Cas9 system has been the best choice for GE, but despite its extensive use and applications, there are still some limitations to its more widespread application.

Newly emerging CRISPR/Cas systems (i.e., spCas9-NG, base editing, xCas9, Cpf1, Cas13, Cas14) are now being used for GE.

Base editing (BE) introduces precise and reproducible nucleotide changes at genomic targets without requiring donor DNA templates, double-stranded breaks (DSBs), or dependency on homology-directed repair (HDR) and nonhomologous end-joining (NHEJ).

Genome Editing

Genome editing (GE) (see Glossary) is a technique which introduces DNA mutations in the form of insertions and/or deletions (**indels**) or base substitutions in target sequences. GE comprises various techniques, such as the use of zinc finger nucleases (ZFNs), transcriptional activator-like effector nucleases (TALENs), and the most recently developed **clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated nuclease 9 (Cas9)** system. ZFNs are targetable DNA cleavage proteins used to cut DNA sequences at any site. TALENs induce double-stranded breaks (DSBs) in target sequences, which trigger DNA damage response pathways, leading to genome modification [1]. Although ZFNs and TALENs have been widely used (since 2002 and 2011, respectively) for GE in human, animal, and plant cells, there are still some limitations which hinder their effective use. The specificity of ZFN is limited and it frequently introduces off-target mutations [2]. Vector construction for ZFNs and TALENs is time- and labor-consuming [3]. Therefore, since 2013, attention has been diverted towards the use of CRISPR/Cas9 and more recently towards several newly emerging CRISPR/Cas variants. CRISPR/Cas9 is an RNA-guided endonuclease that specifically targets DNA sequences via nucleotide base pairing (Box 1). Here, we review the applications of CRISPR/Cas9 in crop plants and its comparisons with other GE tools, such as ZFNs and TALENS. We focus on target efficiency and specificity, mechanism, and challenges and limitations, but also discuss prospects for the use of newly emerging GE tools, such as **base editing (BE)**, xCas9, Cas12a (Cpf1), and Cas 13 in plants.

Emerging CRISPR/Cas Systems for GE

To overcome the limitations of the CRISPR/Cas9 system (Box 2), a variety of CRISPR systems have been generated for efficient GE. The Cas9 variant CjCas9, derived from *Campylobacter jejuni*, is composed of 984 amino acid residues (2.95 kbp) and has been used for efficient GE *in vitro* and *in vivo*. CjCas9 is highly specific and cuts only a limited number of sites in the genomes of mouse or human. Delivered through adeno-associated virus (AAV), it has been shown to induce targeted mutations at high frequencies in retinal pigment epithelium (RPE) cells or mouse muscle cells. For example, it was used to target the *Vegfa* or *Hif1a* gene in RPE cells, which reduced the size of laser-induced choroidal neovascularization, so providing a new option for the treatment of age-related macular degeneration [4].

Cas13 is a recently identified CRISPR effector and CRISPR/Cas13 can target specific viral RNAs and endogenous RNAs in plants cells [5]. The Cas13 system has high RNA target specificity and efficiency [6]. Cas13 was used to direct ADAR2 deaminase for the modification of RNA (changing adenosine to inosine) in human cells for the recovery of functional proteins to halt disease progression [7]. Recently, CRISPR/Cas13a has been considered as an entirely new CRISPR type that belongs to class II type VI.

[1]National Key Laboratory of Crop Genetic Improvement, Huazhong Agricultural University, Wuhan, Hubei 430070, P.R. China

[2]Department of Biosciences, Durham University, Durham DH1 3LE, UK

[3]http://cotton.hzau.edu.cn/EN/

*Correspondence:
xlzhang@mail.hzau.edu.cn,
jsx@mail.hzau.edu.cn

Box 1. The Mechanism of the CRISPR/Cas9 System

CRISPR/Cas9 has become a vital tool in biological research for both understanding gene function and the improvement of crops, due to its simplicity, versatility, and specificity [32]. CRISPR/Cas systems are classified as types I to VI [97]. Type I systems are characterized based on the occurrence of signature protein Cas3, a protein which contains both DNase and helicase domains used to degrade the target. Type II CRISPR/Cas systems utilize Cas1, Cas2, Cas9, and a fourth protein (Csn2 or Cas4), whereas the type III CRISPR/Cas systems comprise the Cas10 with an indistinct role [98]. The type II CRISPR/Cas system originates from *S. pyogenes* and comprises three components: the CRISPR RNA (crRNA), **trans-activating crRNA (tracrRNA)**, and a Cas9 protein [99]. Cas9 has the DNA cleavage domains RuvC and His-Asn-His (HNH), which break the double-stranded DNA (dsDNA) site primarily located 3 bp upstream of protospacer adjacent motif (PAM) sequences (5′ NGG or 5′-NAG for *S. pyogenes* Cas9) in the target DNA [100]. The HNH domain cuts a complementary strand of crRNA, whereas the **RuvC-like domain** cuts an opposite strand of dsDNA [101]. As a result, DNA is repaired *in vivo* using error-prone nonhomologous end-joining (NHEJ) or homology-directed repair (HDR).

The NHEJ frequently leads to random DNA indels at the cleavage position, whereas HDR executes the precise sequence insertion or gene replacement by adding a donor DNA template with sequence homology at the predicted DSB site [100]. The Cas9 establishes a ribonucleoprotein (RNP) complex with crRNA and tracrRNA to cleave DNA efficiently. The crRNA plays an essential role in matching and recognizing the target DNA. It contains a sequence that guides the Cas9 RNP to a specific locus through base pairing with the target DNA, to form an R loop. The formation of the R-loop activates HNH and RuvC-like endonuclease domains for cleavage of the target and nontarget strands of the DNA, respectively; this results in a DSB [102]. The tracrRNA binds to the crRNA and Cas9 protein that is recruited to the complex [2]. A gRNA is formed as a chimeric molecule consisting of tracrRNA and crRNA anteceded by an 18–20-nt spacer sequence complementary to target DNA adjacent to the PAM. The PAM is a 3-nt (NGG) sequence located immediately downstream of the single-guide RNA (sgRNA) target site, which plays an essential role in binding and for Cas9-mediated DNA cleavage [103]. CRISPR/Cas9, therefore, works in three steps for gene editing: the first step is the expression of nuclear localized Cas9 protein, the second is the generation of gRNA containing 20 nt complementary to the target gene, and the third requires an NGG PAM site recognition located close to the 3′ end of the target site. Guided by the sgRNA, the sgRNA and Cas9 search for the target across the genome and create blunt-ended DSBs at about 3 bp upstream of the PAM site (Figure 1A) [76].

Due to the presence of higher eukaryotes and prokaryotes nucleotide-binding (HEPN) domains, it is associated with RNase activity [5]. The CRISPR/Cas9 and CRISPR/LshCas13a systems have each been used to create resistance against potyvirus (an RNA virus) in plants, which indicates that this system can be used in agricultural and biotechnological applications [8]. Recently, a study by Abudayyeh *et al.* [9] reported a C to U RNA editor referred to as RNA Editing for Specific C to U Exchange (RESCUE), by the directed evolution of ADAR2 into a cytidine deaminase. The RESCUE system has the ability to double the number of pathogenic mutations targetable by RNA editing and enables modification of phospho-signaling-relevant residues. In this study, RESCUE was applied to drive β-catenin activation and cellular growth. RESCUE retains A to I editing activity that enables multiplexed C to U and A to I editing through the use of tailored **guide RNAs (gRNAs)**.

Phage-assisted continuous evolution was used to develop an SpCas9 variant, xCas9(3.7), which recognizes a broader range of **protospacer adjacent motifs (PAMs)** [10]. xCas9 possesses a higher DNA specificity and editing efficiency, lower off-target activity, and broader PAM compatibility (including NG, GAA, and GAT) than does SpCas9, from which it is derived [11]. Using xCas9(3.7) to replace Cas9 in the BE3 vector led to the creation of a construct known as CBE, which can edit loci containing various PAMs such as NGN, GAT, and GAA [11]. The xCas9 variant mediates BE or DNA cleavage at various non-NGG PAMs. The editing efficiency of xCas9 is variable between different target sites. As with other evolved or engineered Cas9 variants, xCas9 requires high precision matching between gRNA and the target sequence, which must include a G at the 5′ end of gRNA and the corresponding first position of the protospacer [12].

Cas9-NG recognizes a minimal NG PAM and has been used for GE in human cells [13]. Recently, Ren and coworkers [14] evaluated the nuclease activity of Cas9-NG towards various NGN PAMs by

Glossary

Base editing (BE): a genome editing system that introduces precise and highly predictable nucleotide changes at genomic targets without requiring donor DNA templates or DSBs and are not dependent on HDR and NHEJ.
Clustered regularly interspaced short palindromic repeat (CRISPR): a specialized region of DNA with two distinct characteristics: the presence of nucleotide repeats and spacers. CRISPR/Cas9 is an RNA guided endonuclease that targets specific DNA sequence explicitly via nucleotide base pairing.
CRISPR-associated nuclease 9 (Cas9): the endonuclease derived from diverse bacterial species. Cas9 protein is used in genetic engineering to cut the DNA and ultimately alter the cell's genome.
CRISPR RNA (crRNA): The crRNA is transcribed from interval spacer sequences that correlate to the sequences on plasmid or phage (prospacer). The crRNA plays a vital role in matching and recognizing the target DNA.
Genome editing (GE): a technique that introduces mutations in the form of insertions and/or deletions (indels) or base substitutions in targeted sequences, so causing DNA modification.
Guide RNA (gRNA): a chimeric molecule that consists of tracrRNA and crRNA, anteceded by an 18–20-nt spacer sequence complementary to target DNA before PAM.
His-Asn-His (HNH) domain: one of the two endonuclease domains of Cas9 that functions to cleave the complementary strand of CRISPR RNA (crRNA).
Homology-directed repair (HDR): a repair pathway that executes the precise sequence or insertion, or gene replacement, by adding a donor DNA template with sequence homology at a predicted DSB site. In the presence of oligo template, HDR induces the specific replacement of genes or allows foreign DNA knock-ins.
Indels: a general term used for insertion or deletion mutations.
Nonhomologous end-joining (NHEJ): a pathway that repairs DSBs and creates indels or mismatches leading to gene

Box 2. The Beneficial Features and Limitations of the CRISPR/Cas9 System

Until recently, the CRISPR/Cas9 system has been considered the best choice for GE in plant species, but there are still some limitations that restrict its widespread application (Figure I). Recently, much research has been focused on the modification of this system to improve efficiency and reliability, with new emerging CRISPR/Cas variants (spCas9-NG, base editing, xCas9, etc.). Key problems with CRISPR/Cas9, and beneficial features of the variants, are summarized as follows:

i. The large size of the CRISPR/Cas9 system hinders its editing efficiency, and it is not suitable for packing into viral vectors for delivery to somatic tissues. A smaller-sized CRISPR/Cas is required for efficient GE of plants.

ii. SpCas9 requires a 5′-NGG-3′ PAM immediately adjacent to a 20-nt DNA target sequence where it only recognizes the NGG PAM site, and this can limit its effectiveness compared with new CRISPR/Cas variants. However, the xCas9 variant has more target efficiency, high DNA specificity, low off-target activity, and broad PAM compatibility (such as, with NG, GAT, and GAA).

iii. CRISPR/Cas9 can introduce multiple random off-target mutations in the genome [42]. However, new CRISPR/Cas variants have improved editing efficiency of target bases in the sequence of interest by recognizing different PAMs [16].

iv. CRISPR/Cas9 introduces mutations at nonspecific loci which are similar, but not identical, in homology to target sites.

v. CRISPR/Cas9 needs an *Agrobacterium*-mediated transformation system for creating a mutant plant, which is costlier and time and resource consuming. However, the use of tissue culture-free genome editing systems offers potential improvements to efficiency.

vi. Difficulties exist for the commercialization of transgenic crops expressing CRISPR/Cas9 in various countries, primarily because of the development costs and constraints imposed by regulatory systems for the field release of genetically modified organisms.

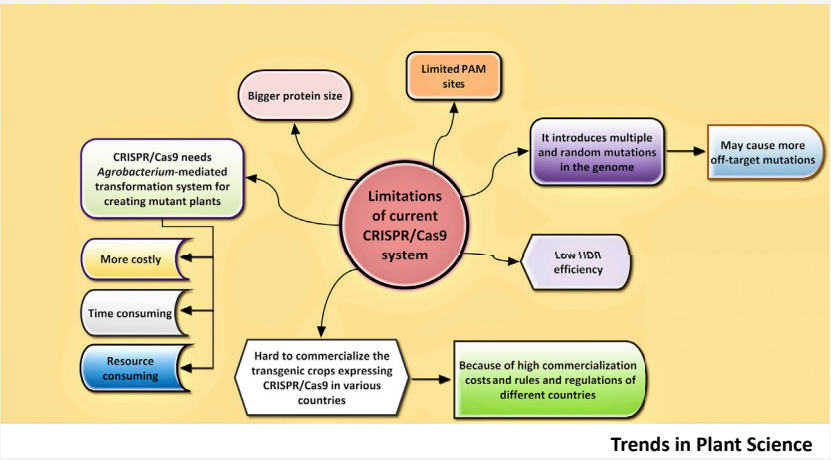

Figure I. Limitations of the Current CRISPR/Cas9 System.

Abbreviations: Cas9, CRISPR-associated nuclease 9; CRISPR, clustered regularly interspaced short palindromic repeat; HDR, homology-directed repair; PAM, protospacer adjacent motif.

knockout and loss-of-function mutants. NHEJ-mediated repair can be used to generate point mutations via gene replacement when the target sequences of CRISPR/Cas9 are located in introns.

Protospacer adjacent motif (PAM): a 3-nt sequence located immediately downstream of the single guide RNA (sgRNA) target site, which plays an essential role in binding and for Cas9-mediated DNA cleavage. The PAMs are the various extended conserved bases at the 5′ or 3′ end of the protospacer.

RuvC-like domain: one of the two endonuclease domains of Cas9 that functions to cleave the complementary strand of dsDNA.

Trans-activating crRNA (tracrRNA): a small trans-encoded RNA that stabilizes the structure and then activates the Cas9 for cleavage of the target DNA.

targeting endogenous genes in transgenic rice. Cas9-NG recognizes NAC, NTG, NTT, and NCG in addition to NG PAM. The Cas9-NG-engineered base editors have been successfully used to generate *OsBZR1* gain-of-function plants that cannot be created by other available Cas9-engineered base editors. Moreover, the Cas9-NG-based transcriptional activator was shown to efficiently upregulate the expression of endogenous target genes in rice. SpCas9-NG has been used for gene disruption and cytosine BE in rice [15].

In another study, Hua and coworkers [16] used stable transgenic lines for the evaluation of the efficacies of xCas9 and SpCas9-NG to perform gene editing and BE in rice. xCas9 was found to efficiently induce mutations at target sites in rice having NG and GAT PAM sequences. However, base editors using xCas9 failed to edit most of the tested target sites. Nevertheless, SpCas9-NG exhibited a robust editing activity at sites with a variety of NG PAMs without showing any preference for the third nucleotide after NG. xCas9 and SpCas9-NG were observed to have higher specificity than SpCas9 at the CGG PAM site. Different forms of adenine or cytosine base editors containing SpCas9-NG were shown to work efficiently in rice with broadened PAM compatibility.

CRISPR/Cas12a (Cpf1) is an RNA-guided system, classified as type V of class II CRISPR system. The *Francisella novicida U112* (FnCpf1) is analogous to CRISPR/Cas9 but exhibits some unique characteristics [17]. The CRISPR system from *Prevotella* and *Francisella 1* (Cpf1) relies on a T-rich sequence at the 5′-end of the protospacer sequence (5′-TTTN-3′ or 5′-TTTV-3′; V = A, C, or G, in some cases), as opposed to the G-rich, NGG sequence for Cas9. While Cas9 produces blunt-ended DNA breaks, Cpf1 generates DSBs with staggered ends at the distal position of a PAM, which may deliver further benefits, especially for knock-in strategies, and might improve the efficiency for **nonhomologous end-joining (NHEJ)**-based gene insertion [18]. These cohesive DNA ends may enhance the efficiency of DNA fragment insertion by using complementary DNA ends through **homology-directed repair (HDR)**. In addition, Cpf1 is considered as a more suitable GE tool than CRISPR/Cas9 because it only requires a 42-nt **CRISPR RNA (crRNA)**, whereas Cas9 utilizes ~100-nt gRNA. However, Cpf1-mediated GE is cheaper than SpCas9 because it requires only short synthetic single-guide RNA (sgRNA) sequences [19]. Cpf1 consists of a RuvC-like endonuclease domain along with a Nuc domain to cleave the DNA [20]. However, it lacks the **His-Asn-His (HNH) domain** and it may include a single active site in the RuvC domain [21]. Interestingly, Cpf1 proteins have RNase activity and have been used to process crRNA arrays for GE in plants [21]. These features increase the insertion efficiency at the Cpf1-cleaved site (Figure 1B) [22]. Recently, an enhanced *Acidaminococcus* sp. Cas12a variant (enAsCas12a) has been engineered with a substantially expanded targeting range that enables the targeting of various PAMs that were formerly inaccessible. On average, enAsCas12a exhibits a twofold higher GE activity at sites with canonical TTTV PAMs compared with wild type AsCas12a. The enAsCas12a has been found to improve the efficiency of multiplex gene editing, C to T BE, and endogenous gene activation. A high-fidelity version of enAsCas12a (enAsCas12a-HF1) has also been engineered to reduce off-target effects [23].

FnCpf1 is composed of approximately ~1300 amino acids, expressed specifically from the Cas9 locus. The Cpf1 array contains nine spacer sequences separated by repeating sequences where each sequence comprises 36 nt [24]. Although various Cas9 orthologs, such as SaCas9 [25] and CjCas9 [4], are relatively small, the Cpf1 is usually smaller than most of the Cas9 orthologs. Cpf1 targets sequences which possess a high frequency of PAM sequence and so may be particularly suitable for clinical purposes. The wide range of Cpf1 targetable genes has been extended by engineering Cpf1 variants [26].

CRISPR/Cas14a is a highly compact protein which can be utilized as a guided GE tool for the cleavage of single-stranded DNA (ssDNA) [27]. Recently isolated from nonculturable archaea, it has become an ideal tool for engineering resistance against economically important plant ssDNA viruses because of its sequence-independent and unrestricted cleavage [27]. Based on comparative sequence analysis, 24 different *Cas14* gene variants have been identified and these are clustered into three subgroups (*Cas14a*, *Cas14b*, and *Cas14c*) [28]. The size of CRISPR/Cas14a is almost half that of the previously characterized RNA-guided DNA-targeting Cas9 protein. Unlike other known class II systems, Cas14a does not require a flanking sequence (PAM) near the target site [28]. It has been shown by *in vitro* validation of the PAM requirement that Cas14a can cleave target sites irrespective of the different sequences adjacent to the targets of these different guides [27]. CRISPR/Cas14a is a potential system for engineering resistance against plant ssDNA viruses belonging to the *Geminiviridae* and *Nanoviridae* families [29].

BE is the most recent and a quite different GE system, which introduces precise and highly predictable nucleotide changes at genomic targets without any requirement for donor DNA templates,

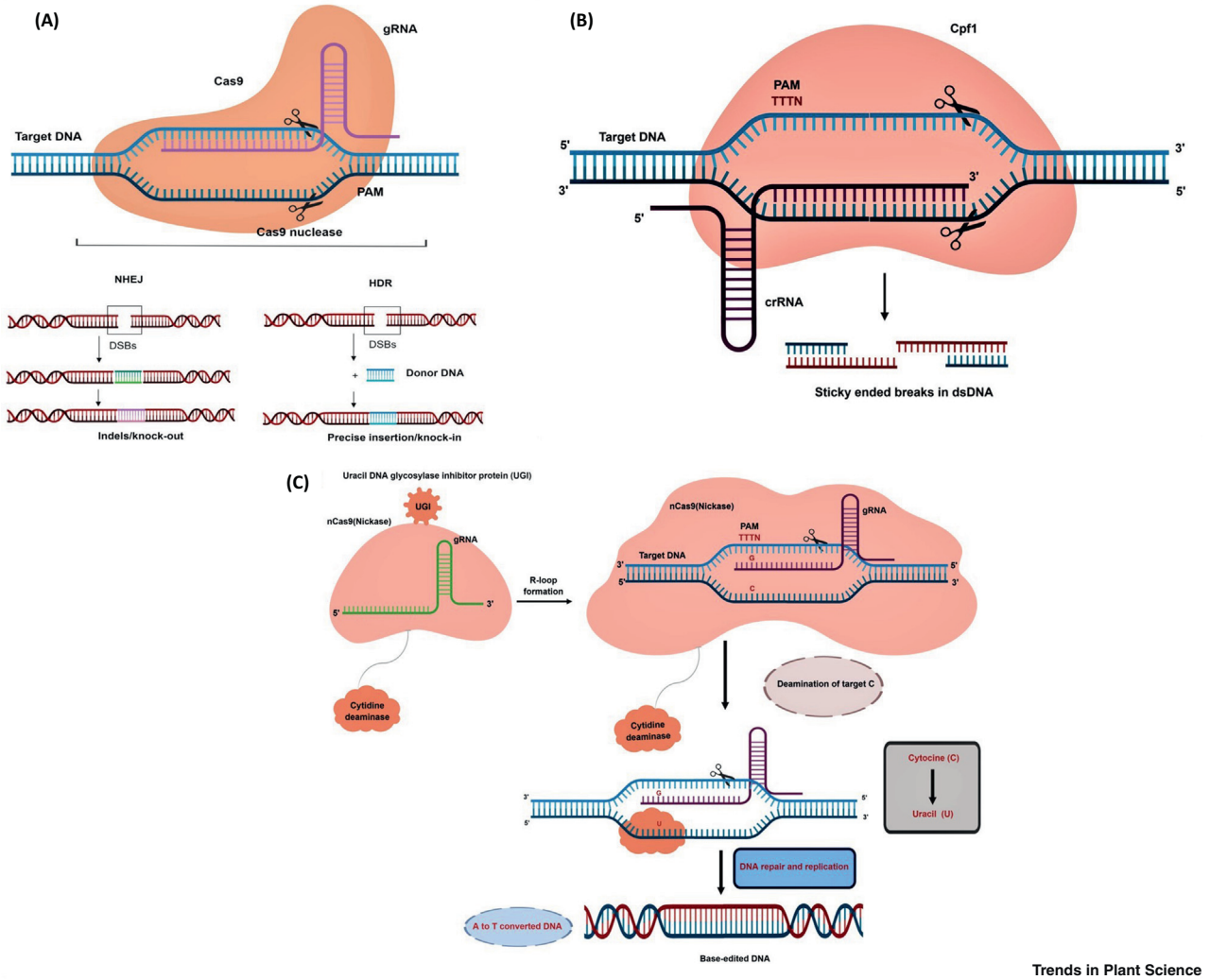

Figure 1. Comparison of the Mechanisms of Two Gene Editing Tools.

(A) Diagrammatic representation of the clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated nuclease 9 (Cas9) system. CRISPR/Cas induces double-stranded breaks (DSBs) in DNA strands. The CRISPR RNA (crRNA) guides the Cas9 protein. The trans-activating CRISPR RNA (tracrRNA) is used to stabilize the structure and then activates Cas9 for cleavage of the target DNA. The single-guide RNA [sgRNA (pink)] identifies the target gene and then the Cas9 protein (orange) cleaves both strands of the target DNA through its RuvC and His-Asn-His (HNH) domains. The protospacer adjacent motif (PAM) sequence is primarily required for the Cas9 to cleave DNA. A stretch of 20 bases of sgRNA defines the binding specificity. Two methods repair the DSBs in DNA: homology-directed repair (HDR), which is activated in the presence of a template and results in knock-in or gene replacement; and nonhomologous end-joining (NHEJ), which is not precise and permanently results in a gene knockout. (B) The Cpf1 system. Cpf1 is a CRISPR-associated two-component RNA programmable DNA nuclease. The PAM for Cpf1 is TTTN (i.e., a T-rich region). Cpf1 cleaves the target DNA and introduces DSBs, a 5-nt potential staggered cut distal to a 5′ T-rich PAM. (C) CRISPR/Cas9 nickase introduces breaks only in strands complementary to the sgRNA. Paired nickase with two sgRNAs introduces staggered DSBs in DNA and then the HDR repairs the DSBs in the DNA. The uracil DNA glycosylase inhibitor (UGI) protein blocks removal of uracil in DNA and the subsequent repair pathway and helps to improve mutation frequency. This figure was created using BioRender (https://biorender.com/).

DSBs, or dependency on HDR and NHEJ [30]. BE technology has been widely used in various organisms and cell lines [30,31]. It is considered more effective than HDR-mediated base pair substitution because it induces fewer unwanted mutations at the target locus [31]. There are several BE systems that have been used for editing living organisms, including BE3 [32], BE4 [33], Targeted-AID [22], and dCpf1-BE [34]. These systems utilize Cas9 or Cpf1 variants for the recruitment of cytidine deaminases,

which generate specific C to T alterations by using DNA mismatch repair pathways. Moreover, the adenine base editors, developed by fusion of an evolved tRNA adenosine deaminase with SpCas9 nickase (D10A), generates the conversion of A•T to G•C when directed by sgRNAs to genomic targets in human cells [35]. A cytidine deaminase fusion of Cas9 nickase (Cas-D10A) converts base pairs from C to T or G to A, with a uracil glycosylase inhibitor (UGI) for blocking base excision repair of base changes, so converting CRISPR to a single-base editor [36]. The BE3 system is a base editor consisting of rat cytidine deaminase APOBEC1 linked to a Cas9 nickase [nCas9 (D10A)] and the UGI (Figure 1C). BE3 plays a critical role in the conversion of targeted cytidine to thymidine in DNA [37], and both the efficiency and specificity of its PAM have been increased [38]. The BE systems can use predicted gRNAs for converting codons CAG, TGG, CAA, and CAG into stop codons via C to T conversions [39].

A study by Kocak and coworkers [40] showed that the engineering of a hairpin secondary structure onto the spacer region of sgRNAs (hp-sgRNAs) can increase the specificity by several orders of magnitude when combined with numerous CRISPR effectors. The hp-sgRNAs can fine-tune the activity of a trans-activator based on Cas9 from *Streptococcus pyogenes* (SpCas9). Furthermore, the hp-sgRNAs enhance the specificity of gene editing when using five different Cas9 or Cas12a variants. The secondary structure of the RNA is a fundamental parameter for tuning the activity of various CRISPR systems, and gRNA structures can affect the cleavage of on- and off-target sites [41]. The CRISPR/Cas variants and their potential roles in GE are presented in Table 1.

Methodology for the Screening of CRISPR/Cas9 and Other CRISPR/Cas System-Induced Mutants

The first 20 nt of chimeric sgRNA and the PAM determine the target specificity of the CRISPR/Cas9 system [42]. Efficient screening methods are crucial for the identification of induced mutations to analyze various genome-edited regenerated plants. The path used from selecting the target gene to genetic transformation by CRISPR/Cas9 system is illustrated in Figure 2.

qPCR

Mutated DNA sequences may be easily determined by amplifying the locus and sequencing the PCR products. qPCR can be used to distinguish homozygous and heterozygous mutations, and this approach has been validated in several plant species, including *Arabidopsis* (*Arabidopsis thaliana*), maize (*Zea mays*), sorghum (*Sorghum bicolor*), and rice (*Oryza sativa*). It is an efficient, simple, and rapid method to detect induced mutations [43].

Surveyor Nuclease and T7 Endonuclease I (T7EI) Assays

Surveyor™ nuclease (Transgenomic Inc., Omaha, NE, USA) belongs to the CEL family of mismatch-specific nucleases obtained from celery (*Apium graveolens*). It identifies and cleaves mismatches because of the occurrence of small indels or SNPs and cleaves both DNA strands downstream of the mismatch and detects indels of up to 12 nt [44]. The Surveyor nuclease and T7EI assays are extensively used and considered appropriate for any target sequence. They recognize and digest mismatched heteroduplex DNA. However, the detection sensitivity of these methods is much lower than PCR/RE (restriction enzyme) assays and are more time and labor consuming [45]. T7E1 can, however, recognize and cleave various dsDNA molecules if their structure is curved and able to bend further [46].

High-Resolution Melting Analysis (HRMA)-Based Assay

The HRMA assay involves DNA sequence amplification by qPCR covering about 90–200 bp of the genomic target, incorporating fluorescent dye followed by amplicon melt curve analysis [47]. HRMA is considered the most sensitive and simple method and compatible with a high-throughput screening format (96-well microliter plates). The whole procedure for genomic DNA preparation and mutation detection takes less than 2 hours, because of the nondestructive nature of the method. Further sequencing and gel electrophoresis might be used to analyze amplicons [48]. However, HRMA has some limitations because it is unable to detect larger indels and the costs are also very

Table 1. Different CRISPR/Cas Systems and Their Potential Functions in Various Hosts

Class name	Size (bp)	PAM	Host	sgRNA size (bp)	Cut site	Target	Function	Refs
SpCas9	1368	5′-NGG-3′	*Streptococcus pyogenes*	20	5′ of PAM	Target dsDNA	In SpCas9 system, the PAM corresponds to an NGG consensus sequence which contains two G:C base pairs	[104]
SpCas9-NG	–	5′-NAC, NTG, NTT, and NCG apart from NG-3′ PAM	*S. pyogenes*	–	5′ of PAM	Target DNA	Cas9-NG can greatly expand the targeting scope of genome-editing tools, showing great potential for targeted genome editing, base editing, and genome regulation in plants	[14]
FnCas9	1629	5′-NGG-3′	*Francisella novicida*	20	5′ of PAM	Target DNA	The FnCas9 identifies 5′-NGG-3′ PAM and uses structural information for creating a variant which can identify more relaxed 5′-YG-3′ PAM	[105]
SaCas9	1053	5′-NNGRRT-3′	*Staphylococcus aureus*	21	5′ of PAM	Target DNA	SaCas9 facilitates efficient *in vivo* genome editing which identifies a 5′-NNGRRT-3′ PAM (where R represents a purine such as A or G)	[106]
Nme Cas9	1082	5′-NNNNGATT-3′	*Neisseria meningitidis*	24 and 20	5′ of PAM	Target DNA	*Nme*Cas9 system requires a longer PAM for site-specific cleavage. NmCas9 can use an sgRNA to direct its activity	[107]
St1Cas9	1121	NNAGAAW	*Streptococcus thermophilus*	20	5′ of PAM	Target DNA	St1Cas9 and SaCas9 play a critical role in bacterial positive selection system. St1Cas9 functions as a nuclease in human cells	[108]
St3Cas9	1409	5′-NGGNG-3′	*S. thermophilus*	20	5′ of PAM	Target DNA	St3Cas9 is a large-sized protein with multiple domains. The Cas9–crRNA complex of St3Cas9 system introduces *in vitro* dsDNA breaks at a specific site containing a sequence complementary to crRNA	[45]
CjCas9	984	NNNNACAC and NNNRYAC	*Campylobacter jejuni*	22	5′ of PAM	Target DNA	CjCas9 cleaves only a limited number of sites in mouse or human genome and	[4]

(Continued on next page)

Table 1. *Continued*

Class name	Size (bp)	PAM	Host	sgRNA size (bp)	Cut site	Target	Function	Refs
							generates mutations at target site at high frequencies	
Cpf1	–	TTTV	*Prevotella* and *Francisella* 1	20	5′ of PAM	Target DNA	Cpf1 relies on a T-rich PAM sequence at the 5′-end of the protospacer sequence	[18]
Cpf1 (AsCpf1)	1307 bp	5′-TTTN-3′	Acidaminococcus sp.	24	3′ of PAM	Target DNA	AsCpf1 identifies crRNA scaffold and the 5′-TTTN-3′ PAM in structure- and sequence-dependent manners. AsCpf1 contains two domains located at the positions appropriate to generate staggered DSBs in DNA	[20]
Cas12a	–	Thymine-rich PAM sequences	Acidaminococcus sp.	–	5′ of PAM	Target DNA	Cas12a (also known as Cpf1) is a type V-A CRISPR effector RNA-guided DNA endonuclease which generates double-stranded cuts with staggered ends in the region downstream and distal to T-rich PAM sequences	[21]
Cas14	400–700	–	Uncultivated archaea	–	–	Target ssDNA	Cas14 proteins are approximately 400–700 amino acids and are able to cut ssDNA without the requirements of restrictive sequence	[28]
Cas13	1440	Non-G nucleotide at the 3′ protospacer flanking site (PFS)	Multiple orthologs; *Leptotrichia shaii*	28	–	Target ssRNA	Cas13 (also called as C2c2) is effector protein of type VI system, which is used as RNA-guided ribonuclease, the nonspecific, trans-acting RNase activity of which is activated by base pairing of the crRNA guide to an ssRNA target.	[109]
xCas9	–	NG, GAA, and GAT	–	–	–	–	The xCas9 is a Cas9 variant which identifies various types of PAMs, including NG, GAA, and GAT	[110]
AacC2c1	1277	T-rich PAM	*Alicyclobacillus acidoterrestris*	20	5′ of PAM	Target DNA	AacC2c1 is a C2c1-a type V-B CRISPR/Cas endonuclease with a bi-lobed architecture that consists of an REC and NUC lobe	[111]

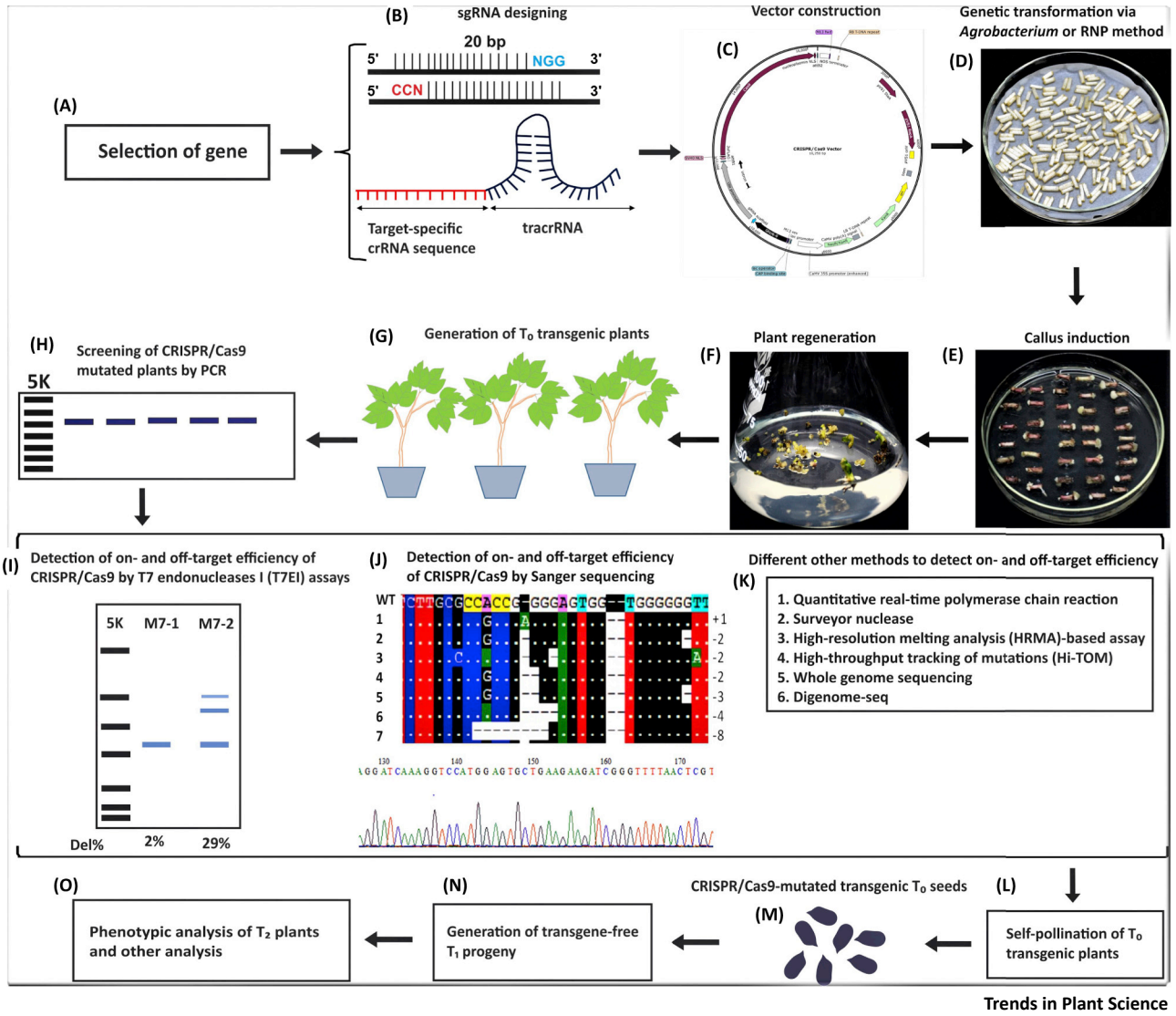

Figure 2. Pipeline of CRISPR/Cas9 Genetic Transformation of Genes from Gene Selection to Plant Analysis.
(A) Selection of the target gene. (B) Designing the single-guide RNA (sgRNA) for the target gene. (C) Vector construction. (D) Genetic transformation via *Agrobacterium*/ribonucleoprotein (RNP) for the delivery of CRISPR/Cas9. (E) Tissue culture (callus induction). (F) Plant regeneration from CRISPR/Cas9-mutated tissues. (G) Generation of T_0 CRISPR/Cas9-mutated transgenic plants. (H) Screening of transgenic plants by PCR. (I) Detection of on- and off-target efficiency of CRISPR/Cas9-mutated plants by T7E1. (J) Detection of on- and off-target efficiency by Sanger sequencing. (K) Different methods to detect on- and off-target efficiency. (L) Self-pollination of T_0 transgenic plants for generation of homozygous T_1 plants. (M) CRISPR/Cas9-mutated T_0 seeds. (N) Generation of transgene-free T_1 progeny. (O) Phenotypic analysis of T_1 plants and other analysis. Abbreviations: Cas9, CRISPR-associated nuclease 9; CRISPR, clustered regularly interspaced short palindromic repeat; crRNA, CRISPR RNA; tracrRNA, trans-activating CRISPR RNA.

high. This cost can be reduced by pairing the qPCR machine with online HRMA software (e.g., https://dna.utah.edu/uv/uanalyze.html) [49].

High-Throughput Tracking of Mutations (Hi-TOM)

Hi-TOM is an online tool (http://www.hi-tom.net/hi-tom/) that is used for the precise and quantitative detection of mutations caused by the CRISPR system. Hi-TOM does not require any additional data analysis or complex parameter configuration. It is easy to use and requires no specialist expertise in

bioinformatics or next-generation sequencing (NGS). It has been found to be a more reliable and sensitive tool through analysis of human cells and rice tissues. Because of its convenience and simplicity, this tool has become the most suitable high-throughput detection methodology for mutations induced by CRISPR/Cas systems [50].

Whole-Genome Sequencing (WGS) to Detect On- and Off-Targets

It is crucial to understand the scope of on- and off-target mutations in edited crops by any GE technology. WGS is a most effective technique for the identification of various kinds of mutations, such as small indels, SNPs, and structural variations, including major deletions, inversions, duplications, and rearrangements [51], and has already been exploited for detecting off-target mutations caused by Cas9 in various crops. WGS has a high cost due to the requirement for relatively high sequencing depth (>50) [48]. Moreover, the detection of low-frequency mutations when sequencing depth is low remains challenging [52].

Applications of CRISPR/Cas9 and Other CRISPR/Cas Systems in Diverse Plant Species

CRISPR/Cas9 technology has been used to modify a wide range of plant species, including *Arabidopsis* [53], rice [54], wheat (*Triticum aestivum*) [55], maize [56], soybean (*Glycine max*) [57], sorghum [58], cotton (*Gossypium hirsutum* L.) [52,59], rapeseed (*Brassica napus* L.) [60], barley (*Hordeum vulgare* L.) [61], *Nicotiana benthamiana* [62], tomato (*Solanum lycopersicum* L.) [63], potato (*Solanum tuberosum*) [64], sweet orange (*Citrus sinensis* L.) [65], cucumber (*Cucumis sativus* L.) [66], wild cabbage (*Brassica oleracea* L.) [61], wild legume (*Lotus japonicus* L.) [67], lettuce (*Lactuca sativa* L.) [68], *Medicago truncatula* [69], *Marchantia polymorpha* [70], tobacco (*Nicotiana tabacum* L.) [71], *Nicotiana attenuata* [68], *Petunia hybrida* [72], grape (*Vitis vinifera* L.) [73], apple (*Malus pumila*) [74], tropical staple cassava (*Manihot esculenta*) [75], watermelon (*Citrullus lanatus*) [76], and others (Table 2). There have been multiple examples of the application of CRISPR/Cas9 editing, as follows.

Targeted Mutagenesis

As described above, the CRISPR/Cas system can induce sequence-specific mutagenesis to interrupt genes to evaluate their functions and be used for trait improvement in crops [77]. By mutation of its nuclease domains, Cas9 can be transformed into a DNA-binding protein. The consequence is that its DNA binding activity remains intact, whereas the DNA cleavage activity is deactivated. Direct or indirect fusion of this 'dead' Cas9 (dCas9) nuclease to an effector domain can be utilized to guide fusion proteins to specific sites in the genome [78]. This allows the exploitation of CRISPR/Cas for various site-specific modifications, including epigenetic changes [79], regulation of gene expression [3], and BE without induction of DSB, such as facilitated by fusion with deaminases in rice, wheat, and maize [36] or imaging of genomic loci in live leaf cells of *N. benthamiana* [80].

Multiplex GE

CRISPR has the potential to create mutations simultaneously at more than one genomic site by using multiple sgRNAs, in any organism. CRISPR/Cas9 has also been used for multiplex GE, which enables the rapid stacking of multiple traits in an elite variety background [81]. Multiplex gene editing also provides a powerful tool for targeting multiple members of multigene families. It can be achieved in two ways, by either constructing multiple gRNA expression cassettes in separate vectors or assembling various sgRNAs in a single vector [82].

Recently, a study by Wang *et al.* [82] reported the strategy of enabling the clonal reproduction of F_1 rice hybrids through seeds. Heterozygosity of F_1 hybrid rice was fixed by multiplex CRISPR/Cas9 GE of three meiotic genes (*REC8*, *PAIR1*, and *OSD1*) for the production of clonal diploid gametes and tetraploid seeds. Furthermore, the editing of the *MATRILINEAL (MTL)* gene, which is involved in fertilization, could induce the formation of haploid seeds in hybrid rice. Combining fixation of heterozygosity and haploid induction by simultaneous editing of these genes (*REC8*, *PAIR1*, *OSD1*, and *MTL*) in hybrid rice resulted in plants that could propagate clonally via seeds. Another report by Khanday

Table 2. The Applications of the CRISPR/Cas9 System in Major Crops

Crop/fruit/ vegetable	Gene	Technology	Mutation type	Editing efficiency (%)	Reason for transformation	Method of Cas9 system delivery	Phenotype	Gene function	Refs
Rice (*Oryza sativa* L.)	*TMS5*	CRISPR/Cas9 system	Single-nucleotide insertions, deletion, and substitutions	From 46.2 to 88.2 and 69.2 to 94.1	To develop commercial 'transgene clean' TGMS rice lines	*Agrobacterium*-mediated delivery method	The offspring were observed to be stronger and provided a greater yield compared with control variety	*TMS5* is thermo-sensitive genic male sterility (TGMS) gene in China which encodes the endonuclease RNase Z^{S1} in AnS-1	[112]
Rice (*O. sativa* L.)	*ALS*	CRISPR/Cas9 system	Point mutations	–	To introduce various distinct point mutations in the rice *ALS* gene. To increase resistance against herbicide	*Agrobacterium*-mediated transformation method	Knock-in and resistant against sulfonylurea herbicides	Encodes acetolactate synthase, which is involved in the biosynthesis of the branched amino acid	[113]
Wheat (*Triticum aestivum* L.)	*TaMLO* homologs	TALENs and CRISPR/Cas9 systems	Insertion and deletion (indels) mutations	23–38	To increase resistance against powdery mildew in wheat	Particle bombardment method	Knockout and resistance to powdery mildew	Involved to inhibit resistance pathway to powdery mildew	[114]
Wheat (*T. aestivum* L.)	*TaGW2*	CRISPR/Cas9 RNPs	Insertion and deletion (indels) mutations	41.2	For efficient and specific genome editing	Cas9-RNP-mediated GE method	–	*TaGW2* gene plays a vital role in grain weight control	[115]
Upland cotton (*Gossypium hirsutum* L.)	*GhCLA1* (*Chloroplasts alterados 1*)	CRISPR/Cas9 system	Nucleotide insertion and substitution	47.6–81.8	For targeted mutagenesis of cotton genome	*Agrobacterium*-mediated transformation method	Mutations were detected in cotton protoplast	–	[116]
Upland cotton (*G. hirsutum* L.)	*GhVP* (*vacuolar* H^+ *-pyrophosphatase*)	CRISPR/Cas9 system	Nucleotide deletion and substitution	47.6–81.8	For targeted mutagenesis of cotton genome	*Agrobacterium*-mediated transformation method	Mutations were detected in cotton protoplast	–	[116]

(Continued on next page)

Table 2. *Continued*

Crop/fruit/ vegetable	Gene	Technology	Mutation type	Editing efficiency (%)	Reason for transformation	Method of Cas9 system delivery	Phenotype	Gene function	Refs
Allotetraploid cotton (*G. hirsutum* L.)	An endogenous gene *GhCLA1* and *DsRed2* (*Discosoma red fluorescent protein2*)	CRISPR/Cas9 system	Nucleotide insertions and deletions (indels)	66.7–100	For targeted mutagenesis of cotton genome	*Agrobacterium tumefaciens*-mediated transformation method	Disappeared red fluorescence and showed albino phenotype	*AtCLA1* is involved in the development of chloroplast. *DsRed2* protein is utilized as a reporter due to its different benefits over other report proteins	[117]
Cotton (*G. hirsutum* L.)	*GhMYB 25-like*	CRISPR/Cas9 system	Nucleotide insertion and deletion mutations (indels)	100 and 98.8	For efficient and specific genome editing	*Agrobacterium*-mediated transformation and somatic embryogenesis method	–	*GhMYB25*-like is involved in the development of cotton fiber	[118]
Maize (*Zea mays* L.)	*ZmAgo18a* and *ZmAgo18b* (*Argonaute* 18) and *Dihydroflavonol* 4-reductase or *anthocyaninless* (a1 and a4)	CRISPR/Cas9 system	Showed mono- or diallelic mutations of one locus and various allelic variations of two loci	70	For mutagenesis frequency and heritability	*Agrobacterium*-mediated transformation method	–	Involved in the biosynthesis of 24-nt phasiRNA in anthers	[100]
Soybean (*Glycine max* L. Merr.)	*GmPPD1* and *GmPPD2*	CRISPR/Cas9 system	Heterozygous and chimeric mutations	68 in *GmPPD1* and 88 in *GmPPD2*	Inheritable site-directed mutagenesis	*Agrobacterium*-mediated transformation method	Rippled trifoliate leaves were observed thicker with deeper green color, longer petioles, and bigger pods	*PPD* protein is involved in the transcriptional regulation of cell division in *Arabidopsis*	[119]
Soybean (*G. max* L. Merr.)	*GmFT2a*	CRISPR/Cas9 system	Site-directed and insertion and deletion (indels) mutations	48, 53 and 37	To induce targeted mutagenesis of *GmFT2a*	*Agrobacterium tumefaciens*-mediated transformation method	Showed late flowering under both short-day and long-day conditions	*GmFT2a* is an integrator in the photoperiod flowering pathway	[120]

(Continued on next page)

Table 2. *Continued*

Crop/fruit/vegetable	Gene	Technology	Mutation type	Editing efficiency (%)	Reason for transformation	Method of Cas9 system delivery	Phenotype	Gene function	Refs
Sorghum (*Sorghum bicolor* L. Moench)	Whole *k1C* gene family	CRISPR/Cas9 system	Insertion and deletion (indels) mutations	92.4	To create kafirin variants for the improvement of protein digestibility and quality	*A. tumefaciens*-mediated transformation method	Vitreousness and α-kafirin levels were reduced, whereas an increase in the grain protein digestibility and lysine content was observed	Kafirins are proteins that are used as storage in *Sorghum* grains and form protein bodies with poor digestibility	[121]
Barley (*Hordeum vulgare* L.)	*HvPM19*	CRISPR/Cas9 system	Insertion and deletion (indels) mutations	23 and 10	To induce targeted mutagenesis of barley genes	*Agrobacterium*-mediated transformation method	Dwarf phenotype	*HvPM19* encodes an ABA-inducible plasma membrane protein that is involved in the positive regulation of grain dormancy in wheat	[61]
Brassica oleracea	*BolC.GA4.a*	CRISPR/Cas9 system	Insertion and deletion (indels) mutations	10	To induce targeted mutagenesis of *B. oleracea* genes	*Agrobacterium*-mediated transformation method	Dwarf phenotype	*GA4* is involved in the gibberellin biosynthesis pathway	[61]
Rapeseed (*Brassica napus* L.)	*RGAs*, *FULs*, *DAs*, and *A2.DA2*	CRISPR/Cas9 system	Homozygotes, bialleles, and heterozygotes	65.3	To induce targeted genome modifications at multiple loci	*Agrobacterium*-mediated transformation method	Dwarf phenotype	*RGAs* act as a master repressor in gibberellic signaling. The *BnaFULs* are involved in the regulation of silique dehiscence during flower development. The *da2* and *da1* are serving as negative regulators of organ size	[122]

(Continued on next page)

Table 2. *Continued*

Crop/fruit/ vegetable	Gene	Technology	Mutation type	Editing efficiency (%)	Reason for transformation	Method of Cas9 system delivery	Phenotype	Gene function	Refs
Oilseed Rape (*B. napus* L.)	Rapeseed *SPL3* homologous gene copies	CRISPR/Cas9 system	Insertion and deletion (indels) mutations	96.8–100	To rapidly generate and identify simultaneously mutagenesis of multiple gene homologs	*Agrobacterium*-mediated transformation method	Showed developmental delay phenotype	*SPL3* is key floral activator which acts upstream of *AP1* in *Arabidopsis*	[123]
Arabidopsis thaliana	*ADH1* and *TT4*	CRISPR/Cas9 system	DSB induction	2.5 up to 70	Induction of DSB plants to promote NHEJ and homologous recombination	*Agrobacterium*-mediated transformation method	–	–	[124]
Tropical staple cassava (*Manihot esculenta*)	*MePDS* (phytoene desaturase) gene	CRISPR/Cas9 system	Insertions, deletions, and substitutions	100	To induce targeted mutagenesis in cassava	*Agrobacterium*-mediated delivery method	–	*PDS* is involved in the carotenoid biosynthesis pathway	[75]
Watermelon	*ClPDS* (phytoene desaturase)	CRISPR/Cas9 system	Insertions or deletions	100	To effectively create knockout mutations in watermelon	*Agrobacterium*-mediated transformation method	Clear or mosaic albino phenotype	*ClPDS* introduces obvious albino phenotype	[76]
A traditional Chinese medicinal herb (*Salvia miltiorrhiza*)	Diterpene synthase gene (*SmCPS1*)	CRISPR/Cas9 system	DNA insertions, deletions, and point mutations	11.5 and 30.8 for homozygous and chimeric mutants, respectively	To precisely knockout the *SmCPS1* in *S. miltiorrhiza*	*Agrobacterium rhizogenes*-mediated transformation method	The roots of homozygous mutants were white in appearance whereas wild type plants had red colored roots	Tanshinone biosynthesis	[125]

(Continued on next page)

Table 2. *Continued*

Crop/fruit/ vegetable	Gene	Technology	Mutation type	Editing efficiency (%)	Reason for transformation	Method of Cas9 system delivery	Phenotype	Gene function	Refs
Lotus japonicus	*SYMRK* (symbiosis receptor-like kinase), *LjLb1*, *LjLb2*, and *LjLb3*	CRISPR/Cas9 system	Diverse insertion, deletion, and substitution mutations, biallelic homozygous mutations	35	To efficiently and effectively target single and multiple SNF genes	*Agrobacterium*-mediated a stable or hairy root transformation method	Shoot and root mass of transgenic plant reduced compared with wild type. The triple mutant produced white nodules, whereas control plants formed pink nodules. The symbiosomes of the plant seemed smaller, showed the abnormal shape, and degraded compared with rod-shaped bacteroids, radial-distributed, and the regularized symbiosomes in control plants	SYMRK is involved in symbioses of legumes. The *LjLbs* genes establish high energy status (ATP/ ADP) and low free-oxygen concentration within nodules for active SNF	[67]
Grape cultivar (*Vitis vinifera* L.)	*MLO-7* (*Mildew Locus O*)	CRISPR/Cas9 ribonucleoproteins (RNPs) system	Insertions and deletions (indels) mutations	0.1	To increase tolerance against powdery mildew	Protoplast transformation, direct delivery of CRISPR RNPs to the protoplast of grapevine	–	MLO genes are conserved and their loss of function results in resistance against powdery mildew. MLOs are membrane proteins involved in various physiological processes in different tissues	[74]

(Continued on next page)

Table 2. *Continued*

Crop/fruit/ vegetable	Gene	Technology	Mutation type	Editing efficiency (%)	Reason for transformation	Method of Cas9 system delivery	Phenotype	Gene function	Refs
Apple (*Malus pumila*)	*DIPM-1*, *DIPM-2*, and *DIPM-4*	CRISPR/Cas9 RNPs system	Insertions and deletions (indels) mutations	0.5–6.9	To increase resistance to fire blight disease	Protoplast transformation, direct delivery of CRISPR RNPs to apple protoplast	–	*DIPM*s exhibit direct physical interaction with the disease-specific gene of *Erwinia amylovora*, which may act as a susceptible factor	[126]
Wanjincheng orange (*Citrus sinensis* Osbeck)	Gene *CsLOB1* (*LATERAL ORGAN BOUNDARIES1*) promoter	CRISPR/Cas9 system	Insertions, deletions, and substitutions	11.5–64.7	Exhibited enhanced tolerance against citrus canker as compared with wild type	*Agrobacterium*-mediated epicotyl transformation method	Mutant plants showed tolerance against citrus canker	*CsLOB1* promotes the growth of pathogen and formation of an erumpent pustule	[65]
Duncan grapefruit (*Citrus paradise* Macf.)	*CsLOB1*(*C. sinensis Lateral Organ Boundaries*) gene	CRISPR/Cas9 system	Insertion and short deletions	15.63, 14.29, 54.54, and 81.25	For generating canker-resistant grapefruit	*Agrobacterium*-mediated epicotyl transformation method	Exhibited canker symptoms same as wild type	*CsLOB1* is the disease susceptible gene for bacterial canker disease of citrus	[127]
Banana (*Musa acuminata*)	*PDS* (phytoene desaturase)	CRISPR/Cas9 system	Insertions and deletions (indels) mutations	59	Basic methodology	*Agrobacterium*-mediated transformation method	Slower germination growth on the selection medium. Leaves showed either spots or small streaks in early stages and later chimeric phenotype with green and albino phenotype	*PDS* is used as a marker to demonstrate genome editing in several plant species	[128]

(Continued on next page)

Table 2. *Continued*

Crop/fruit/ vegetable	Gene	Technology	Mutation type	Editing efficiency (%)	Reason for transformation	Method of Cas9 system delivery	Phenotype	Gene function	Refs
Tobacco (*Nicotiana tabacum* L.)	*NtPDS* and *NtPDR6*	CRISPR/Cas9 system	Insertion and deletion (indels) mutations, a large deletion and inversion of genomic structure	81.8 for *NtPDS* gRNA4 and 87.5 for *NtPDR6* gRNA2	For targeted mutagenesis and examination of transient genome editing activity	*Agrobacterium*-mediated transformation method	The *psd* mutant showed etiolated leaves and *pdr6* mutant displayed more branches	*PDR6* is an ABC transporter which plays a role in strigolactone transport	[129]
Tobacco (*N. tabacum* L.)	*XylT* (β(1,2)-*xylosyltransferase*) and *FucT* (a(1,3)-*fucosyltransferase*)	CRISPR/Cas9 system	Small indels and deletions between the target sites	–	To remove plant type glycans by inactivation of those two enzymes	*A. tumefaciens* LBA4404virG method	Devoid and strong reduction of α(1,3)-fucose and β(1,2)-xylose	These two enzymes have the function in the addition of plant-specific glycans	[130]
Tobacco (*N. benthamiana* L.) and *Arabidopsis*	*EDS1* and *PAD4*	CRISPR/Cas9 system	Small deletions	–	For induction of inheritable chromosomal deletions	*Agrobacterium*-mediated transformation-compatible method	–	*EDS1*-family genes are essential regulators of plant innate immunity	[131]
Petunia (*Petunia hybrida*)	*PDS* (phytoene desaturase)	CRISPR/Cas9 system	Insertions, deletions, and replacement	55.6–87.5	For targeted genome mutagenesis	*Agrobacterium*-mediated transformation method	Albino phenotype	*PDS* is involved in the biosynthesis of carotenoid and chlorophyll	[132]
Petunia (*P. hybrida*)	*NR* (nitrate reductase)	CRISPR/Cas9, RNPs system	Site-specific mutations, insertions, and deletions	21 by T7E1 and 17.8 by deep DNA sequencing	For site-directed mutagenesis	Direct delivery of purified Cas9 protein and sgRNA into protoplasts cells	–	NR genes are involved in facilitating nitrogen uptake and nitrate metabolism	[72]

(Continued on next page)

Table 2. *Continued*

Crop/fruit/ vegetable	Gene	Technology	Mutation type	Editing efficiency (%)	Reason for transformation	Method of Cas9 system delivery	Phenotype	Gene function	Refs
Tomato (*Solanum lycopersicum* L.)	*SlPDS* (phytoene desaturase) and *SlPIF4* (phytochrome interacting factor)	CRISPR/Cas9 system	Insertions and deletions (indels)	83.56	For targeted mutagenesis in tomato plants	*Agrobacterium tumefaciens*-mediated transformation method	The mutants of *SlPDS* showed an albino phenotype, the mutants of *SlPIF4* did not show any obvious abnormal phenotype	*SlPDS* are involved in carotenoid biosynthesis. The *SlPIF4* is a homologous gene of *Arabidopsis* PIF4, which belongs to the basic helix-loop-helix multigene family	[133]
Tomato (*S. lycopersicum* L.)	*SlIAA9* (auxin-induced 9)	CRISPR/Cas9 system	Insertions and deletions (indels)	100	To generate parthenocarpic tomato plants	*Agrobacterium*-mediated leaf disk method	Morphological changes in seedless fruit and leaf shape	*SlIAA9* is a key gene controlling parthenocarpy	[134]
Potato (*Solanum tuberosum*)	*GBSS* (granule-bound starch synthase)	CRISPR/Cas9 system	Mutation is alleles, small insertions, and/or deletions (indels) mutations	Up to 67	In order to alter the starch quality	Transient transfection and regeneration from isolated protoplasts	Reduction of amylose content in starch	*GBSS* is responsible for the synthesis of amylose	[135]
Cucumber (*Cucumis sativus* L.)	*eIF4E* (eukaryotic translation initiation factor 4E) gene	CRISPR/Cas9 system	Small deletions and single nucleotide polymorphisms (SNPs)	–	In order to enhance tolerance against the virus in cucumber	*A. tumefaciens*-mediated transformation method	Homozygous *if4e* mutants showed enhanced tolerance against the infection of *Papaya ring spot mosaic virus-W* (PRSV-W)	*eIF4E* is a plant cellular translation factor which plays the crucial role in the *Potyviridae* life cycle	[66]
Lettuce (*Lactuca sativa*)	*LsBIN2* (*A. thaliana BRASSINOSTEROID INSENSITIVE 2*)	CRISPR/Cas9 system	Germline-transmissible small insertions or deletions	46	For DNA-free genome editing	Polyethylene glycol (PEG)-mediated protoplast transfection method	Calli contained monoallelic and biallelic mutations	*BIN2* gene encodes a negative regulator in the signaling pathway of brassinosteroid (BR)	[68]

et al. [83] showed that expression of *BABY BOOM1* (*BBM1*) in sperm cells of rice and its ectopic expression in the egg cell is adequate for parthenogenesis. The zygotic expression of *BBM1* is normally limited to the paternal allele, but biparentally expressed later. Triple knockout of the *BBM1*, *BBM2*, and *BBM3* genes causes embryo abortion and arrest, whereas male-transmitted *BBM1* fully rescues embryo development.

Gene Regulation: CRISPR Interference and Activation

The CRISPR interfering (CRISPRi) system is used as an orthogonal system in a variety of living organisms; the requirements are only a coexpression of a catalytically inactive Cas9 protein and a modified sgRNA, designed with a complementary region to any gene of interest. The CRISPRi system is derived from the *S. pyogenes* CRISPR pathway. The complex comprising Cas9 and sgRNA binds to DNA elements complementary to the sgRNA and causes a steric block that stops transcript elongation by RNA polymerase, so repressing the target gene. Therefore, CRISPRi has been considered as an effective and precise genome-targeting platform for transcription control without changing the target DNA sequence [84]. dCas9 is a useful and robust tool for the regulation of transcription levels of any target gene. The gRNA directs the binding of dCas9 to any genomic locus that can efficiently stop the progress of RNA polymerase to the downstream gene. In bacterial cells, CRISPRi is a more useful genetic engineering tool for gene knockdown compared with RNAi, because it avoids cell death through disruption of the genome [85].

dCas9 is fused to a transcriptional activator domain VP-16 and uses an sgRNA sequence to direct it to the target site. VP-16 interacts with TF-IIH, which is one of the main constituents of the basal transcriptional apparatus and enhances gene expression [86]. In various plant species, an efficient multiplex transcriptional activation has been successfully developed using the CRISPRAct2.0 and mTALE-Act systems. These tools can activate more than four genes at the same time and can be used to evaluate positive feedback transcriptional loops and the control of tissue-specific gene activation [87]; however, it does introduce more off-target effects [88]. To solve this problem, a potent transcriptional activation tool termed dCas9-TV has been developed using VP128 (which possesses an additional VP64 moiety, which is an activation domain) that was joined to six copies each of plant-specific activation domains (ethylene response factor 2m and EDLL) and guided by a single sgRNA. This assembly promoted up to 55-fold activation of the target gene compared with the conventional dCas9-VP64 system [89].

Epigenetic Modifications

Epigenetic and post-translational protein modifications, for example, DNA and histone acetylation/methylation, ubiquitination, SUMOylation, and phosphorylation, can alter chromatin structure and regulate gene expression patterns [90]. The dCas9 fusion proteins can be used as sequence-specific synthetic epigenome converters, which alter local epigenetic status and the expression of related genes. dCas9 fused to epigenetic regulatory factors involved in histone acetylation, or methylation of DNA, can be used to modulate chromatin activity and gene expression patterns involved in plant development and environmental adaptation [86]. Recently, targeted DNA methylation or demethylation has been achieved in *Arabidopsis* [91]. The histone demethylase Lys-specific histone demethylase 1 (LSD1) fused to *Neisseria meningitidis* dCas9 has been used for experimentally controlling gene repression [92].

Gene Replacement and Gene Knock-in

DSBs at targeted genome sites are repaired either by HDR (also known as targeted integration [93]) or NHEJ, which can allow gene replacement or gene knockout, respectively [81]. CRISPR/Cas has successfully been used for gene replacement in plants [94]. One example is the replacement of the endogenous 5-enolpyruvylshikimate-3-phosphate synthase (*OsEPSPS*) in rice with a gene encoding a form of the protein tolerant to the herbicide glyphosate. Gene replacement frequency was about 2.0% [95]. HDR-mediated gene replacement has also been achieved in *N. benthamiana* protoplasts [96].

Key Figure

Future Recommendations/Perspectives for the CRISPR/Cas System

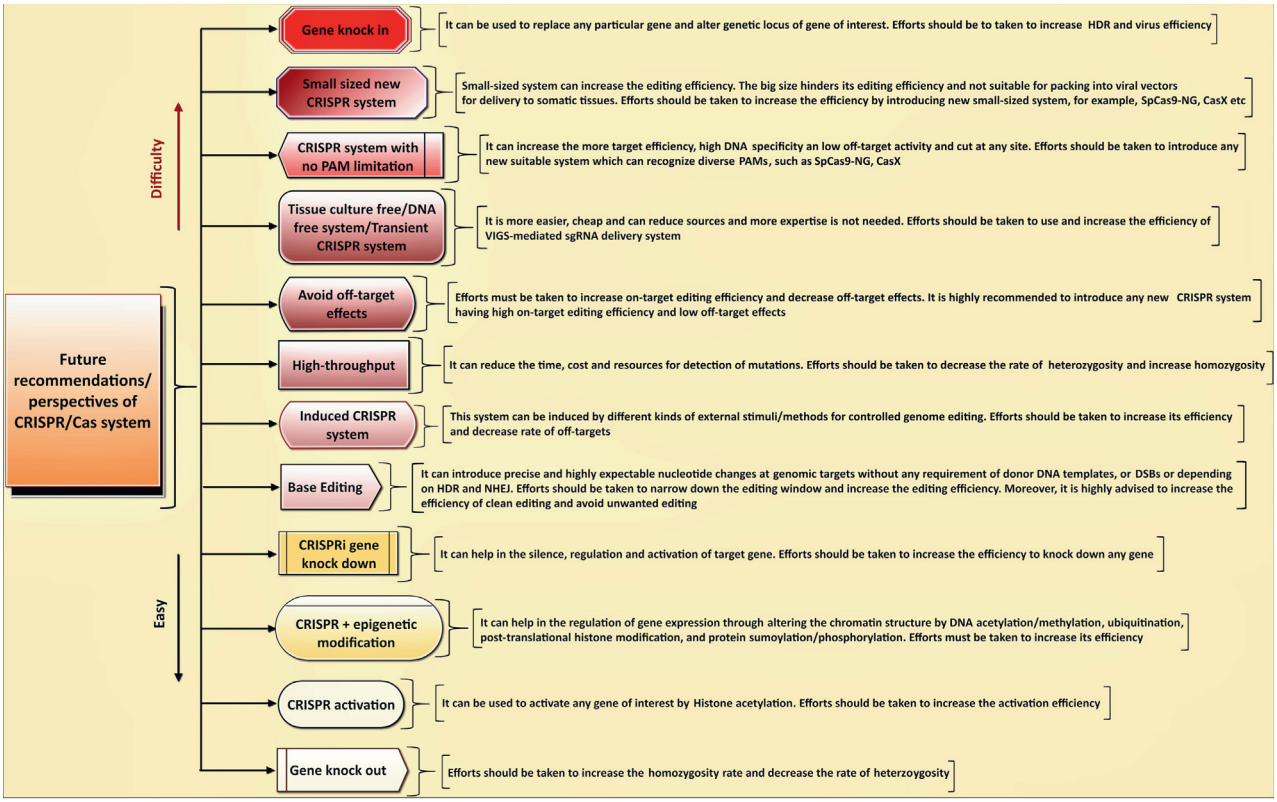

Figure 3. The black and red arrows indicate 'easy' and 'difficult', respectively. The off-white to red colors indicate the easiest to more difficult experiments. Abbreviations: Cas, CRISPR-associated nuclease; CRISPR, clustered regularly interspaced short palindromic repeat; CRISPRi, CRISPR interference; DSB, double-stranded break; HDR, homology-directed repair; NHEJ, nonhomologous end-joining; PAM, protospacer adjacent motif; sgRNA, single guide RNA; VIGS, virus-induced gene silencing.

The CRISPR/Cas9 and other CRISPR/Cas systems represent major technological developments for GE, but efforts must be made to improve their efficiency for a range of organisms, including major crops (Figure 3, Key Figure).

Concluding Remarks and Future Perspectives

CRISPR/Cas9 has been considered one of the most powerful tools for GE of various important crops, because of its high efficiency, relatively low cost, and ease of use compared with other GE techniques, such as ZFNs and TALENs. CRISPR/Cas9 has begun to revolutionize biological research, as the method of choice for targeting specific genome sequences in simple or complex organisms. Although significant progress has been made to increase its efficiency and target specificity, more work remains to be done to improve it (see Outstanding Questions). Although some limitations of the CRISPR/Cas9 system limit its widespread use, different strategies are being developed to improve its effectiveness for editing human, animal, and plant cells.

i. The size of the CRISPR/Cas9 system is relatively large, and so it is not suitable for packing into viral vectors. A smaller-sized CRISPR system is required for efficient GE in crop species.

ii. The requirement of a PAM site at the target sequence limits the application of Cas9 because canonical spCas9 only recognizes the NGG PAM site once every 8–16 bp. A multiple PAM site selection system is needed to increase target scope.

iii. Plant GE technology based on type II CRISPR/Cas system depends upon *Agrobacterium tumefaciens* or direct gene transfer, using cultured plant tissues. However, in many crops or other plant species, genetic transformation and/or regeneration from tissue culture is not efficient; it takes a long time to select and characterize mutants and can generate somaclonal variation, which creates additional mutations. Therefore, to overcome this problem, the use of tissue culture-free GE systems, such as ribonucleoproteins (RNPs), viral delivery, and nanoparticle systems provide alternatives that can accelerate the GE process. Tissue culture-free GE systems are likely to be easier, cheaper, and less expertise-intensive. At the same time, it can increase the efficiency of CRISPR/Cas and reduce the time required to generate edited plants.

iv. There is a need to increase the efficiency of knock-in homologous recombination. This requires research effort to be directed to improving HDR and viral vector efficiencies. New strategies or systems should be considered that involve inhibiting endogenous NHEJ activity or the application of CRISPR-associated transposases.

v. It is necessary to avoid off-target effects by CRISPR/Cas9, and new and improved strategies are required, not only to avoid, but also to detect more easily, off-target effects.

Outstanding Questions

How can the efficiency of the CRISPR/Cas9 system to edit from simple to complex genomes of important plant species be increased?

How can the off-target effects caused by the CRISPR/Cas9 system be decreased?

How can the requirement of PAM sites at target sequences be decreased?

How can knock-in homologous recombination efficiency be increased?

How can the cost and time for the screening of CRISPR/Cas9-mutant plants be decreased?

How can a small-sized CRISPR/Cas system for efficient genome editing of important plant species be introduced?

What are the effective methods to accelerate the tissue culture-free genome editing of plants?

Acknowledgments

This research was supported by grants from National R&D Project of Transgenic Crops of Ministry of Science and Technology of China (2016ZX08010001-006), Consulting Project of China Academy of Engineering (No. 2018-XY-63), and Fundamental Research Funds for the Central Universities (2013PY064, 2662015PY028, 2662015PY091, and 0900206328) to Dr Shuangxia Jin.

References

1. Gaj, T. *et al.* (2013) ZFN, TALEN, and CRISPR/Cas-based methods for genome engineering. *Trends Biotech.* 31, 397–405
2. Puchta, H. (2017) Applying CRISPR/Cas for genome engineering in plants: the best is yet to come. *Curr. Opin. Plant Biol.* 36, 1–8
3. Tang, X. *et al.* (2017) A CRISPR–Cpf1 system for efficient genome editing and transcriptional repression in plants. *Nat. Plants* 3, 17018
4. Kim, E. *et al.* (2017) *In vivo* genome editing with a small Cas9 orthologue derived from. *Campylobacter jejuni*. *Nat. Commun.* 8, 14500
5. Wolter, F. and Puchta, H. (2018) The CRISPR/Cas revolution reaches the RNA world: Cas13, a new Swiss Army knife for plant biologists. *Plant J.* 94, 767–775
6. Abudayyeh, O.O. *et al.* (2017) RNA targeting with CRISPR–Cas13. *Nature* 550, 280–284
7. Gootenberg, J.S. *et al.* (2017) Nucleic acid detection with CRISPR-Cas13a/C2c2. *Science* 356, 438–442
8. Aman, R. *et al.* (2018) RNA virus interference via CRISPR/Cas13a system in plants. *Genome Biol.* 19, 1
9. Abudayyeh, O.O. *et al.* (2019) A cytosine deaminase for programmable single-base RNA editing. *Science* 365, 382–386
10. Rees, H.A. and Liu, D.R. (2018) Base editing: precision chemistry on the genome and transcriptome of living cells. *Nat. Rev. Genet.* 19, 770–788
11. Hu, J.H. *et al.* (2018) Evolved Cas9 variants with broad PAM compatibility and high DNA specificity. *Nature* 556, 57–63
12. Gehrke, J.M. *et al.* (2018) An APOBEC3A-Cas9 base editor with minimized bystander and off-target activities. *Nat. Biotechnol.* 36, 977–982
13. Nishimasu, H. *et al.* (2018) Engineered CRISPR-Cas9 nuclease with expanded targeting space. *Science* 361, 1259–1262
14. Ren, B. *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, 1015–1026
15. Endo, M. *et al.* (2019) Genome editing in plants by engineered CRISPR–Cas9 recognizing NG PAM. *Nat. Plants* 5, 14–17
16. Hua, K. *et al.* (2019) Genome engineering in rice using Cas9 variants that recognize NG PAM sequences. *Mol. Plant* 12, 1003–1014
17. Zetsche, B. *et al.* (2015) Cpf1 is a single RNA-guided endonuclease of a class 2 CRISPR-Cas system. *Cell* 163, 759–771
18. Moon, S.B. *et al.* (2018) Highly efficient genome editing by CRISPR-Cpf1 using CRISPR RNA with a uridinylate-rich 3′-overhang. *Nat. Commun.* 9, 3651
19. Zaidi, S.S. *et al.* (2017) CRISPR-Cpf1: a new tool for plant genome editing. *Trends Plant Sci.* 22, 550–553
20. Yamano, T. *et al.* (2016) Crystal structure of Cpf1 in complex with guide RNA and target DNA. *Cell* 165, 949–962

21. Jeon, Y. *et al.* (2018) Direct observation of DNA target searching and cleavage by CRISPR-Cas12a. *Nat. Commun.* 9, 2777
22. Safari, F. *et al.* (2019) CRISPR Cpf1 proteins: structure, function and implications for genome editing. *Cell Biosci* 9, 36
23. Kleinstiver, B.P. *et al.* (2019) Engineered CRISPR–Cas12a variants with increased activities and improved targeting ranges for gene, epigenetic and base editing. *Nat. Biotechnol.* 37, 276–282
24. Bayat, H. *et al.* (2018) The conspicuity of CRISPR-Cpf1 system as a significant breakthrough in genome editing. *Curr. Microbiol.* 75, 107–115
25. Ran, F.A. *et al.* (2015) *In vivo* genome editing using *Staphylococcus aureus* Cas9. *Nat. Rev. Genet.* 520, 186–191
26. Gao, L. *et al.* (2017) Engineered Cpf1 variants with altered PAM specificities. *Nat. Biotechnol.* 35, 789–792
27. Khan, M.Z. *et al.* (2019) Targeting plant ssDNA viruses with engineered miniature CRISPR-Cas14a. *Trends Biotech.* 37, 800–804
28. Harrington, L.B. *et al.* (2018) Programmed DNA destruction by miniature CRISPR-Cas14 enzymes. *Science* 362, 839–842
29. Ali, Z. *et al.* (2016) CRISPR/Cas9-mediated immunity to geminiviruses: differential interference and evasion. *Sci. Rep.* 6, 26912
30. Hess, G.T. *et al.* (2017) Methods and applications of CRISPR-mediated base editing in eukaryotic genomes. *Mol. Cell* 68, 26–43
31. Yang, B. *et al.* (2017) APOBEC: from mutator to editor. *J. Genet. Genomics* 44, 423–437
32. Doudna, J.A. and Charpentier, E. (2014) The new frontier of genome engineering with CRISPR-Cas9. *Science* 346, 1258096
33. Komor, A.C. *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, eaao4774
34. Li, X. *et al.* (2018) Base editing with a Cpf1-cytidine deaminase fusion. *Nat. Biotechnol.* 36, 324–327
35. Gaudelli, N.M. *et al.* (2017) Programmable base editing of A• T to G• C in genomic DNA without DNA cleavage. *Nature* 551, 464
36. Zong, Y. *et al.* (2017) Precise base editing in rice, wheat and maize with a Cas9-cytidine deaminase fusion. *Nat. Biotechnol.* 35, 438–440
37. Zong, Y. *et al.* (2018) Efficient C-to-T base editing in plants using a fusion of nCas9 and human APOBEC3A. *Nat. Biotechnol.* 36, 950–953
38. Kim, Y.B. *et al.* (2017) Increasing the genome-targeting scope and precision of base editing with engineered Cas9-cytidine deaminase fusions. *Nat. Biotechnol.* 35, 371–376
39. Billon, P. *et al.* (2017) CRISPR-mediated base editing enables efficient disruption of eukaryotic genes through induction of STOP codons. *Mol. Cell* 67, 1068–1079
40. Kocak, D.D. *et al.* (2019) Increasing the specificity of CRISPR systems with engineered RNA secondary structures. *Nat. Biotechnol.* 37, 657–666
41. Hsu, P.D. *et al.* (2013) DNA targeting specificity of RNA-guided Cas9 nucleases. *Nat. Biotechnol.* 31, 827–832
42. Zhang, X.H. *et al.* (2015) Off-target effects in CRISPR/Cas9-mediated genome engineering. *Mol. Ther. Nucleic Acids* 4, e264
43. Peng, C. *et al.* (2018) High-throughput detection and screening of plants modified by gene editing using quantitative real-time PCR. *Plant J.* 95, 557–567
44. Qiu, P. *et al.* (2004) Mutation detection using Surveyor™ nuclease. *Biotechniques* 36, 702–707
45. Cong, L. *et al.* (2013) Multiplex genome engineering using CRISPR/Cas systems. *Science* 339, 819–823
46. Déclais, A.C. *et al.* (2006) Structural recognition between a four-way DNA junction and a resolving enzyme. *J. Mol. Biol.* 359, 1261–1276
47. Wang, K. *et al.* (2015) Research of methods to detect genomic mutations induced by CRISPR/Cas systems. *J. Biotechnol.* 214, 128–132
48. Zischewski, J. *et al.* (2017) Detection of on-target and off-target mutations generated by CRISPR/Cas9 and other sequence-specific nucleases. *Biotechnol. Adv.* 35, 95–104
49. Talbot, J.C. and Amacher, S.L. (2014) A streamlined CRISPR pipeline to reliably generate zebrafish frameshifting alleles. *Zebrafish* 11, 583–585
50. Liu, Q. *et al.* (2018) Hi-TOM: a platform for high-throughput tracking of mutations induced by CRISPR/Cas systems. *Sci. China Life Sci.* 62, 1–7
51. Veres, A. *et al.* (2014) Low incidence of off-target mutations in individual CRISPR-Cas9 and TALEN targeted human stem cell clones detected by whole-genome sequencing. *Cell Stem Cell* 15, 27–30
52. Li, J. *et al.* (2018) Whole genome sequencing reveals rare off-target mutations and considerable inherent genetic or/and somaclonal variations in CRISPR/Cas9-edited cotton plants. *Plant Biotechnol. J.* 17, 858–868
53. Li, C. *et al.* (2018) Verification of DNA motifs in *Arabidopsis* using CRISPR/Cas9-mediated mutagenesis. *Plant Biotechnol. J.* 16, 1446–1451
54. Macovei, A. *et al.* (2018) Novel alleles of rice eIF4G generated by CRISPR/Cas9-targeted mutagenesis confer resistance to rice tungro spherical virus. *Plant Biotechnol. J.* 16, 1918–1927
55. Okada, A. *et al.* (2019) CRISPR/Cas9-mediated knockout of Ms1 enables the rapid generation of male-sterile hexaploid wheat lines for use in hybrid seed production. *Plant Biotechnol. J.* 17, 1905–1913
56. Lee, K. *et al.* (2018) Activities and specificities of CRISPR/Cas9 and Cas12a nucleases for targeted mutagenesis in maize. *Plant Biotechnol. J.* 17, 362–372
57. Li, Z. *et al.* (2015) Cas9-guide RNA directed genome editing in soybean. *Plant Physiol.* 169, 960–970
58. Che, P. *et al.* (2018) Developing a flexible, high-efficiency *Agrobacterium*-mediated sorghum transformation system with broad application. *Plant Biotechnol. J.* 16, 1388–1395
59. Qin, L. *et al.* (2019) High efficient and precise base editing of C• G to T• A in the allotetraploid cotton (*Gossypium hirsutum*) genome using a modified CRISPR/Cas9 system. *Plant Biotechnol. J.* Published online May 22, 2019. https://doi.org/10.1111/pbi.13168
60. Braatz, J. *et al.* (2017) CRISPR-Cas9 targeted mutagenesis leads to simultaneous modification of different homoeologous gene copies in polyploid oilseed rape (*Brassica napus*). *Plant Physiol.* 174, 935–942
61. Lawrenson, T. *et al.* (2015) Induction of targeted, heritable mutations in barley and *Brassica oleracea* using RNA-guided Cas9 nuclease. *Genome Biol.* 16, 258
62. Jansing, J. *et al.* (2018) CRISPR/Cas9-mediated knockout of six glycosyltransferase genes in *Nicotiana benthamiana* for the production of recombinant proteins lacking β-1, 2-xylose and core α-1, 3-fucose. *Plant Biotechnol. J.* 17, 350–361
63. Ortigosa, A. *et al.* (2019) Design of a bacterial speck resistant tomato by CRISPR/Cas9-mediated editing of SlJAZ2. *Plant Biotechnol. J.* 17, 665–673
64. Enciso-Rodriguez, F. *et al.* (2019) Overcoming self-incompatibility in diploid potato using CRISPR-Cas9. *Front. Plant Sci.* 10, 376

65. Peng, A. *et al.* (2017) Engineering canker-resistant plants through CRISPR/Cas9-targeted editing of the susceptibility gene *CsLOB1* promoter in citrus. *Plant Biotechnol. J.* 15, 1509–1519
66. Chandrasekaran, J. *et al.* (2016) Development of broad virus resistance in non-transgenic cucumber using CRISPR/Cas9 technology. *Mol. Plant Pathol.* 17, 1140–1153
67. Wang, L. *et al.* (2016) Efficient inactivation of symbiotic nitrogen fixation related genes in *Lotus japonicus* using CRISPR-Cas9. *Front. Plant Sci.* 7, 1333
68. Woo, J.W. *et al.* (2015) DNA-free genome editing in plants with preassembled CRISPR-Cas9 ribonucleoproteins. *Nat. Biotechnol.* 33, 1162–1164
69. Michno, J.M. *et al.* (2015) CRISPR/Cas mutagenesis of soybean and *Medicago truncatula* using a new web-tool and a modified Cas9 enzyme. *GM Crops Food* 6, 243–252
70. Sugano, S.S. *et al.* (2014) CRISPR/Cas9-mediated targeted mutagenesis in the liverwort *Marchantia polymorpha* L. *Plant Cell Physiol.* 55, 475–481
71. Baltes, N.J. *et al.* (2014) DNA replicons for plant genome engineering. *Plant Cell* 26, 151–163
72. Subburaj, S. *et al.* (2016) Site-directed mutagenesis in *Petunia*× *hybrida* protoplast system using direct delivery of purified recombinant Cas9 ribonucleoproteins. *Plant Cell Rep.* 35, 1535–1544
73. Wang, X. *et al.* (2018) CRISPR/Cas9-mediated efficient targeted mutagenesis in grape in the first generation. *Plant Biotechnol. J.* 16, 844–855
74. Malnoy, M. *et al.* (2016) DNA-free genetically edited grapevine and apple protoplast using CRISPR/Cas9 ribonucleoproteins. *Front. Plant Sci.* 7, 1904
75. Odipio, J. *et al.* (2017) Efficient CRISPR/Cas9 genome editing of phytoene desaturase in cassava. *Front. Plant Sci.* 8, 1780
76. Tian, S. *et al.* (2017) Efficient CRISPR/Cas9-based gene knockout in watermelon. *Plant Cell Rep.* 36, 399–406
77. Scheben, A. *et al.* (2017) Towards CRISPR/Cas crops–bringing together genomics and genome editing. *New Phytol.* 216, 682–698
78. Konermann, S. *et al.* (2015) Genome-scale transcriptional activation by an engineered CRISPR-Cas9 complex. *Nature* 517, 583–588
79. Hilton, I.B. *et al.* (2015) Epigenome editing by a CRISPR-Cas9-based acetyltransferase activates genes from promoters and enhancers. *Nat. Biotechnol.* 33, 510–517
80. Dreissig, S. *et al.* (2017) Live-cell CRISPR imaging in plants reveals dynamic telomere movements. *Plant J.* 91, 565–573
81. Yin, K. *et al.* (2017) Progress and prospects in plant genome editing. *Nat. Plants* 3, 17107
82. Wang, C. *et al.* (2019) Clonal seeds from hybrid rice by simultaneous genome engineering of meiosis and fertilization genes. *Nat Biotechnol.* 37, 283–286
83. Khanday, I. *et al.* (2019) A male-expressed rice embryogenic trigger redirected for asexual propagation through seeds. *Nature* 565, 91–95
84. Larson, M.H. *et al.* (2013) CRISPR interference (CRISPRi) for sequence-specific control of gene expression. *Nat. Protoc.* 8, 2180–2196
85. Cho, S. *et al.* (2018) Applications of CRISPR/Cas system to bacterial metabolic engineering. *Int. J. Mol. Sci.* 19, 1089
86. Shrestha, A. *et al.* (2018) Cis-trans engineering advances and perspectives on customized transcriptional regulation in plants. *Mol. Plant* 11, 886–898
87. Lowder, L.G. *et al.* (2018) Robust transcriptional activation in plants using multiplexed CRISPR-Act2. 0 and mTALE-act systems. *Mol. Plant* 11, 245–256
88. Braun, C.J. *et al.* (2016) Versatile *in vivo* regulation of tumor phenotypes by dCas9-mediated transcriptional perturbation. *Proc. Natl. Acad. Sci. U. S. A.* 113, E3892–E3900
89. Li, Z. *et al.* (2017) A potent Cas9-derived gene activator for plant and mammalian cells. *Nat. Plants* 3, 930–936
90. Yamamuro, C. *et al.* (2016) Epigenetic modifications and plant hormone action. *Mol. Plant* 9, 57–70
91. Gallego-Bartolomé, J. *et al.* (2018) Targeted DNA demethylation of the *Arabidopsis* genome using the human TET1 catalytic domain. *Proc. Natl. Acad. Sci. U. S. A.* 115, E2125–E2134
92. Dominguez, A.A. *et al.* (2016) Beyond editing: repurposing CRISPR–Cas9 for precision genome regulation and interrogation. *Nat. Rev. Mol. Cell Biol.* 17, 5–15
93. Wilson, L.O. *et al.* (2018) The current state and future of CRISPR-Cas9 gRNA design tools. *Front. Pharmacol.* 9, 749
94. Schaeffer, S.M. and Nakata, P.A. (2015) CRISPR/Cas9-mediated genome editing and gene replacement in plants: transitioning from lab to field. *Plant Sci.* 240, 130–142
95. Li, J. *et al.* (2016) Gene replacements and insertions in rice by intron targeting using CRISPR–Cas9. *Nat. Plants* 2, 16139
96. Li, J.F. *et al.* (2013) Multiplex and homologous recombination–mediated genome editing in *Arabidopsis* and *Nicotiana benthamiana* using guide RNA and Cas9. *Nat. Biotechnol.* 31, 688–691
97. Makarova, K.S. *et al.* (2015) An updated evolutionary classification of CRISPR–Cas systems. *Nat. Rev. Microbiol.* 13, 722–736
98. Rath, D. *et al.* (2015) The CRISPR-Cas immune system: biology, mechanisms and applications. *Biochimie* 117, 119–128
99. Jinek, M. *et al.* (2014) Structures of Cas9 endonucleases reveal RNA-mediated conformational activation. *Science* 343, 1247997
100. Char, S.N. *et al.* (2017) An *Agrobacterium*-delivered CRISPR/Cas9 system for high-frequency targeted mutagenesis in maize. *Plant Biotechnol. J.* 15, 257–268
101. Liu, X. *et al.* (2017) Application of CRISPR/Cas9 in plant biology. *Acta Pharm. Sin. B* 7, 292–302
102. Mir, A. *et al.* (2018) Heavily and fully modified RNAs guide efficient SpyCas9-mediated genome editing. *Nat. Commun.* 9, 2641
103. Mao, Y. *et al.* (2017) Heritability of targeted gene modifications induced by plant-optimized CRISPR systems. *Cell. Mol. Life Sci.* 74, 1075–1093
104. Jinek, M. *et al.* (2012) A programmable dual-RNA–guided DNA endonuclease in adaptive bacterial immunity. *Science* 337, 816–821
105. Hirano, H. *et al.* (2016) Structure and engineering of *Francisella novicida* Cas9. *Cell* 164, 950–961
106. Nishimasu, H. *et al.* (2015) Crystal structure of *Staphylococcus aureus* Cas9. *Cell* 162, 1113–1126
107. Lee, C.M. *et al.* (2016) The *Neisseria meningitidis* CRISPR-Cas9 system enables specific genome editing in mammalian cells. *Mol. Ther.* 24, 645–654
108. Kleinstiver, B.P. *et al.* (2015) Engineered CRISPR-Cas9 nucleases with altered PAM specificities. *Nature* 523, 481–485
109. Garcia-Doval, C. and Jinek, M. (2017) Molecular architectures and mechanisms of Class 2 CRISPR-associated nucleases. *Curr. Opin. Struc. Biol.* 47, 157–166
110. Wang, J. *et al.* (2018) xCas9 expands the scope of genome editing with reduced efficiency in rice. *Plant Biotechnol. J.* 17, 709–711

111. Liu, L. *et al.* (2017) C2c1-sgRNA complex structure reveals RNA-guided DNA cleavage mechanism. *Mol. Cell* 65, 310–322
112. Zhou, H. *et al.* (2016) Development of commercial thermo-sensitive genic male sterile rice accelerates hybrid rice breeding using the CRISPR/Cas9-mediated TMS5 editing system. *Sci. Rep.* 6, 37395
113. Sun, Y. *et al.* (2016) Engineering herbicide-resistant rice plants through CRISPR/Cas9-mediated homologous recombination of acetolactate synthase. *Mol. Plant* 9, 628–631
114. Wang, Y. *et al.* (2014) Simultaneous editing of three homoeoalleles in hexaploid bread wheat confers heritable resistance to powdery mildew. *Nat. Biotechnol.* 32, 947–951
115. Liang, Z. *et al.* (2017) Efficient DNA-free genome editing of bread wheat using CRISPR/Cas9 ribonucleoprotein complexes. *Nat. Commun.* 8, 14261
116. Chen, X. *et al.* (2017) Targeted mutagenesis in cotton (*Gossypium hirsutum* L.) using the CRISPR/Cas9 system. *Sci. Rep.* 7, 44304
117. Wang, P. *et al.* (2018) High efficient multisites genome editing in allotetraploid cotton (*Gossypium hirsutum*) using CRISPR/Cas9 system. *Plant Biotechnol J.* 16, 137–150
118. Li, C. *et al.* (2017) A high-efficiency CRISPR/Cas9 system for targeted mutagenesis in cotton (*Gossypium hirsutum* L.). *Sci. Rep.* 7, 43902
119. Kanazashi, Y. *et al.* (2018) Simultaneous site-directed mutagenesis of duplicated loci in soybean using a single guide RNA. *Plant Cell Rep.* 37, 553–563
120. Cai, Y. *et al.* (2018) CRISPR/Cas9-mediated targeted mutagenesis of GmFT2a delays flowering time in soya bean. *Plant Biotechnol. J.* 16, 176–185
121. Li, A. *et al.* (2018) Editing of an alpha-kafirin gene family increases, digestibility and protein quality in Sorghum. *Plant Physiol.* 177, 1425–1438
122. Yang, H. *et al.* (2017) CRISPR/Cas9-mediated genome editing efficiently creates specific mutations at multiple loci using one sgRNA in *Brassica napus*. *Sci. Rep.* 7, 7489
123. Li, C. *et al.* (2018) An efficient CRISPR/Cas9 platform for rapidly generating simultaneous mutagenesis of multiple gene homoeologs in allotetraploid oilseed rape. *Front. Plant Sci.* 9, 442
124. Schiml, S. *et al.* (2016) Repair of adjacent single-strand breaks is often accompanied by the formation of tandem sequence duplications in plant genomes. *Proc. Natl. Acad. Sci. U. S. A.* 113, 7266–7271
125. Li, B. *et al.* (2017) Targeted mutagenesis in the medicinal plant. *Salvia miltiorrhiza*. *Sci. Rep.* 7, 43320
126. Pessina, S. *et al.* (2016) Knockdown of MLO genes reduces susceptibility to powdery mildew in grapevine. *Hort. Res.* 3, 16016
127. Jia, H. *et al.* (2016) Modification of the PthA4 effector binding elements in type I CsLOB1 promoter using Cas9/sg RNA to produce transgenic Duncan grapefruit alleviating XccΔpthA4:dCsLOB1.3 infection. *Plant Biotechnol. J.* 14, 1291–1301
128. Kaur, N. *et al.* (2018) CRISPR/Cas9-mediated efficient editing in phytoene desaturase (PDS) demonstrates precise manipulation in banana cv. Rasthali genome. *Funct. Integr. Genom* 18, 89–99
129. Gao, Y. *et al.* (2015) Auxin binding protein 1 (ABP1) is not required for either auxin signaling or *Arabidopsis* development. *Proc. Natl. Acad. Sci. U. S. A.* 112, 2275–2280
130. Mercx, S. *et al.* (2017) Inactivation of the β (1, 2)-xylosyltransferase and the α (1, 3)-fucosyltransferase genes in *Nicotiana tabacum* BY-2 cells by a multiplex CRISPR/Cas9 strategy results in glycoproteins without plant-specific glycans. *Front. Plant Sci.* 8, 403
131. Ordon, J. *et al.* (2017) Generation of chromosomal deletions in dicotyledonous plants employing a user-friendly genome editing toolkit. *Plant J.* 89, 155–168
132. Zhang, B. *et al.* (2016) Exploiting the CRISPR/Cas9 system for targeted genome mutagenesis in petunia. *Front. Plant Sci.* 6, 20315
133. Pan, C. *et al.* (2016) CRISPR/Cas9-mediated efficient and heritable targeted mutagenesis in tomato plants in the first and later generations. *Sci. Rep.* 6, 24765
134. Ueta, R. *et al.* (2017) Rapid breeding of parthenocarpic tomato plants using CRISPR/Cas9. *Sci. Rep.* 7, 507
135. Andersson, M. *et al.* (2017) Efficient targeted multiallelic mutagenesis in tetraploid potato (*Solanum tuberosum*) by transient CRISPR-Cas9 expression in protoplasts. *Plant Cell Rep.* 36, 117–128