

## Review

# The Continuous Improvement of the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)–CRISPR-Associated Protein System Has Led to Its Highly Efficient Application in Plants

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**Abstract:** The creation of the CRISPR–Cas system has provided unprecedented opportunities in plant genome research and crop genetic improvement. In recent years, this system has been continuously improved to meet human needs through the expansion and modification of Cas proteins, the diversification of targeting locations, and the optimization of CRISPR vectors. In this review, we systematically describe the Class II Cas proteins that have been used in plants, deactivated Cas9 (dCas9) and its role in transcriptional regulation, precision editing systems, Cas9 protein variants, as well as methods and examples of CRISPR–Cas systems targeting various regions with different breadths. In addition, we outline the optimization plans based on CRISPR constructs that can overcome the pleiotropy of genes or accelerate the generation of transgene-free plants and the applications of CRISPR systems in plant breeding practices. Finally, we discuss the theory and development of “CRISPR plus”, and the integrated application of existing systems in more species.

**Keywords:** CRISPR–Cas; precise editing; CRISPR screen; transgene-free; crop improvement



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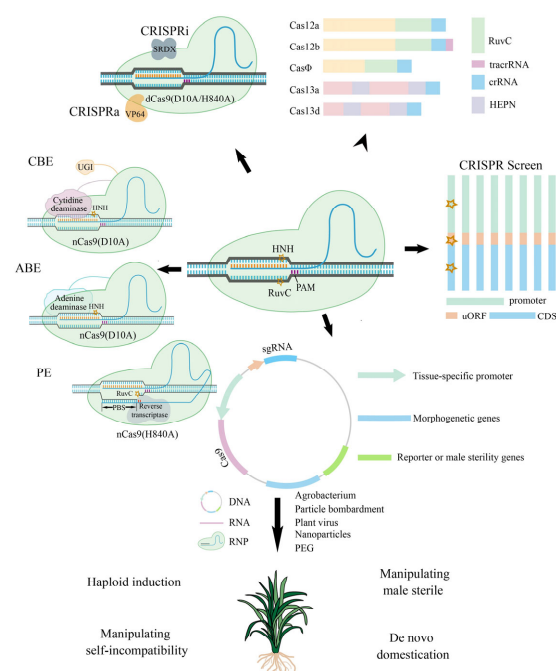
## 1. Introduction

The increasing climate alterations and the diversity of human needs require an efficient system to achieve rapid plant improvement [1]. Since 2013, the clustered regularly interspaced short palindromic repeats (CRISPR)–CRISPR-associated protein (Cas) system, which is based on an adaptive phage immune system in prokaryotes, was first applied in plants [2,3]. It has been favored by researchers for its advantages of easy operation, high efficiency, stability, and its application has achieved genetic improvement in many species [4,5].

To promote the application of the CRISPR–Cas system, constant improvements and evolutions of the system have been developed through the efforts of many research teams [4]. It involved the expansion and modification of Cas proteins, the diversity of editing regions, and the addition of cofactors, among others. Different Cas proteins can target different templates and provide a smaller volume [6,7]. Based on the modifications of the Cas9 protein, the generated base editors, including cytosine base editors (CBEs) and adenine base editors (ABEs), can achieve accurate base editing. And the generation of prime editors (PEs) achieves accurate editing in a wider range [8,9]. Specific variants of *Streptococcus pyogenes* Cas9 (SpCas9), such as SpCas9-NG and SpRY, enable gene editing without protospacer adjacent motif (PAM) restrictions, which could contribute to the design of more efficient single guide RNAs (sgRNAs) [10–13]. Deactivated Cas9 (dCas9) retains its

ability to target specific regions, enabling the transcriptional regulation of genes [14–17]. In addition, the characteristics of the CRISPR–Cas system allow for the expression of multiple sgRNAs simultaneously, enabling targeted editing of multiple genes, which is convenient for the study of functional redundancy between genes. Moreover, the editing region is not limited to the coding sequences (CDSs) of genes; cis-regulatory elements (CREs) in the promoter and upstream open reading frames (uORFs) can also be targeted [18,19]. To understand the gene functions better and faster, CRISPR screening has also been performed in multiple species, resulting in the generation of many mutants with different phenotypes [20,21]. In CRISPR–Cas constructs, the use of tissue-specific promoters aids in the improvement of editing efficiency and the study of pleiotropic genes [22,23]. Moreover, different applications can be achieved through the addition of cofactors, including conventional reporter genes to identify transgenic plants, morphogenetic genes to promote regeneration, and male sterility (MS) or embryonic lethal genes to reduce or directly block the generation of transgenic plants in offspring [24–27]. These strategies, which are constantly undergoing improvement, provide guidance for the efficient use of the CRISPR–Cas system in plants. After the construction of a suitable CRISPR–Cas system, how to obtain regenerated plants is also the focus of researchers, and the delivery methods of editing reagents and regeneration strategies are also being innovated [28,29]. These findings indicate that CRISPR–Cas systems show great potential, especially in species for which functional studies are just beginning, and a detailed application guide is needed.

This review focuses on the integrated applications of the CRISPR–Cas system, and that are relatively easy to implement in most laboratories. The expansion and modification of Cas proteins, the various target regions with different breadths, the optimization of the CRISPR toolset, the upgrade of delivery methods and the application of the CRISPR–Cas system in plant breeding practices are systematically summarized in this review (Figure 1), to provide readers with a navigation map of a comprehensive CRISPR–Cas system. This review also discusses new opportunities for the application of CRISPR systems in the future, with less focus on the specific functions of genes. Our goal is to provide a reference for researchers focusing on gene function or crop genetic improvement and to promote better application of the CRISPR system in more plant species.



**Figure 1.** Overview of CRISPR system extensions, modifications, upgrades, and optimizations, and its delivery methods and applications in breeding practice.

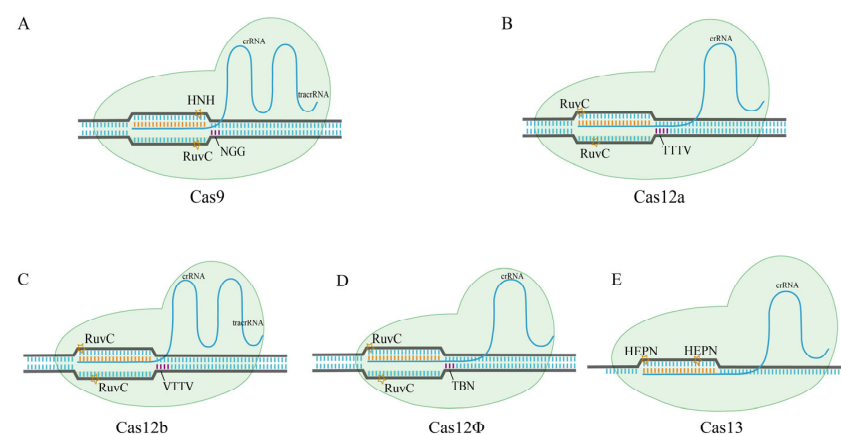
## 2. Diversity and Modification of Cas Proteins

Archaea and bacteria achieve adaptive immunity through a series of CRISPR–Cas systems, each consisting of the combination of Cas proteins and CRISPR RNA (crRNA) [30]. The different CRISPR–Cas systems are divided into two categories: Class I (types I, III, and IV) systems use multiple Cas proteins and crRNA to form complexes, whereas Class II (types II, V, and VI) systems combine large single-component Cas proteins with crRNA to mediate targeted cleavage, such as the well-characterized Cas9 [7,31].

Since the dCas9 protein still retains its targeting ability, transcriptional regulation can be achieved by binding dCas9 to transcriptional activators or suppressors [32,33]. Most of the changes caused by double-strand breaks (DSBs) are knockout mutations, and the resulting edits are often random, which makes it difficult to meet the demands of breeding improvement. To solve this problem, base editors that combine Cas9 nickase (Cas9n) with cytidine or adenosine deaminase to produce targeted base editing have been proposed [34,35], and the creation of PEs addresses the limited number of base transitions and enables precise insertions and deletions [8]. The SpCas9 is the most commonly used Cas protein, but due to its characteristic of recognizing NGG PAMs (where N is any nucleotide), suitable or efficient sgRNAs for the required region cannot be designed for numerous genes. Hence, several Cas9 variants targeting a wider range of PAMs have been developed [12].

### 2.1. Class II Cas Proteins

Cas9 belongs to Class II type II, type V, and type VI Cas proteins, and has also been utilized in plants, providing new opportunities for CRISPR–Cas systems because of the differences in structures, sizes, targeting abilities, and specificities (Figure 2). Cas9 contains an HNH nuclease domain inserted into the RuvC domain, which together cleaves double-stranded DNA (dsDNA) [6]. Cas12 proteins, which belong to type V, contain a single RuvC nuclease domain that targets and collaterally cleaves single-stranded RNA (ssRNA) and ssDNA, and can also perform dsDNA nicking and cleavage [7]. The type VI protein Cas13 targets ssRNAs, which provides unique advantages in the regulation of RNA levels and virus defense [36].



**Figure 2.** Different Cas systems of class II applied in plants. **(A)** The Cas9 system contains an HNH nuclease domain inserted into the RuvC domain, and requires the combination of crRNA and transactivating crRNA (tracrRNA), ultimately producing blunt ends proximally upstream of the NGG PAMs. **(B)** The Cas12a system only requires crRNA, and produces staggered ends and large deletions located distally downstream of the TTTV PAM (V = A, C, or G). **(C)** The Cas12b system requires both crRNA and tracrRNA, and produces staggered ends and large deletions located distally downstream of the VTTV PAM. **(D)** The Cas $\Phi$  is approximately half the size of Cas9, and recognizes a TBN PAM (B = G, T or C). **(E)** The Cas13 system contains two structurally distinct higher eukaryote and prokaryote nucleotide-binding (HEPN) domains that provide RNase activity, recognizes and cleaves ssRNA but cannot cleave dsDNA.

### 2.1.1. Cas12a

CRISPR–Cas12a (Cpf1) is another well-studied system in addition to Cas9 [7]. This system utilizes a T-rich PAM (TTTV) to identify target DNA, which facilitates the targeting of gene promoters and other AT-rich sites [37]. Unlike Cas9, which requires the combination of crRNA and tracrRNA into a sgRNA, Cas12a only requires crRNA and has RNase activity to perform its own crRNA processing. Thus, the CRISPR–Cas12a system is a good multimodal editing platform [38–41]. The Cas12a cleavage site is located distally downstream of the PAM sequence rather than proximally upstream like the Cas9 cleavage site; Cas12a produces staggered ends and large deletions, whereas Cas9 produces blunt-end DSBs, resulting in small insertions and deletions [42]. The DNA interrogation, cleavage, and product release activities of Cas12a have been further characterized by single-molecule fluorescence analysis and biochemical assays [43]. The analysis of editing activity at different temperatures indicates that the Cas12a system is temperature sensitive, with high temperatures improving its editing efficiency [44]. Among some improvements, the editing efficiency of Cas12a has also been improved. A modified tRNA–crRNA array enabled the editing of multiple genes and the successful editing of target sites that were missed by crRNA arrays [45]. A total of 17 novel Cas12a orthologs were investigated, and Ev1Cas12a and Hs1Cas12a were proved to have high editing efficiency, with Hs1Cas12a showing lower temperature sensitivity [46]. Moreover, a more efficient variant of LbCas12a was obtained through saturation mutagenesis, and LbCas12a-RRV combined with RV and D156R presented greater editing efficiency in rice and poplar [47].

### 2.1.2. Cas12b

CRISPR–Cas12b (C2c1) contains RuvC and Nuc domains. This system also tends to target T-rich PAMs and produces staggered ends. But like Cas9, Cas12b requires both crRNA and tracrRNA [48–50]. Moreover, Cas12b is considered an attractive option because of its small size and high specificity, but the optimal temperature for DNA cleavage by Cas12b is generally greater than 40 °C, complicating its use in plant cells [51]. The editing efficiency of AaCas12b under different temperature conditions and durations was evaluated in cotton (*Gossypium hirsutum*) because of its resistance to high temperatures, and the results revealed that the editing efficiency was greatest at a temperature of 45 °C and a duration of 4 days. AaCas12b induced 1–16 base pair (bp) deletions, with most deletions having a length of 9–14 bp; these deletions are larger than those induced by Cas9 (1–5 bp) [52]. Interestingly, it was found that the editing efficiency of this system was affected by the chromatin structure [53]. In rice, the editing efficiencies of four Cas12b proteins were compared; among them, AaCas12b, which preferentially recognized ATTV and GTTG PAMs, was the most efficient [54]. A comparison of five guide RNA scaffolds revealed that Aac and Aa1.2 significantly increased editing efficiency with no off-target activity, and the target mutations were largely germline transmitted [55]. In *Arabidopsis*, Cas12b most commonly induced 5–13 bp deletions, which is consistent with the results in cotton and rice. Moreover, Cas12b can successfully induce multiple genome editing, resulting in a deletion of approximately 1 kb between the two target sites [56]. The Cas12i3-based Multiplex direct repeats (DR)-spacer Array Genome Editing (iMAGE) system, which utilizes another Cas12 protein, also produces a greater frequency of chromosomal structural variation compared to Cas9 [57].

### 2.1.3. CasΦ

CasΦ (Cas12j) is a hypercompact Cas protein discovered in huge phages [58]. CasΦ is approximately half the size of SpCas9 and Cas12a (~70 kDa), which means that it can be packaged in virus-based vectors [7]. The CasΦ protein recognizes a TBN PAM, especially

NTTV; employs a single RuvC domain for DNA cleavage and can also be used for crRNA processing. Cas $\Phi$  was delivered as ribonucleoproteins (RNPs) for targeting the *PDS3* gene in *Arabidopsis* protoplasts, and deletions of 8–10 bp were induced at the target site [58]. In rice and tomato cells, a 7–13 bp deletion frequently occurs at the target site distal to the PAM, and protospacers of 18 bp or longer are more efficient. Moreover, protoplast regeneration may be more effective for Cas $\Phi$ -mediated editing in plants [59]. Cas $\Phi$  is not sensitive to temperature but is sensitive to the chromatin environment, and editing efficiency was increased when the target site was not methylated. Compared with wild-type (WT) Cas $\Phi$ , the vCas $\Phi$  and nCas $\Phi$  variants both exhibit greater efficiency and specificity [60].

#### 2.1.4. Cas13

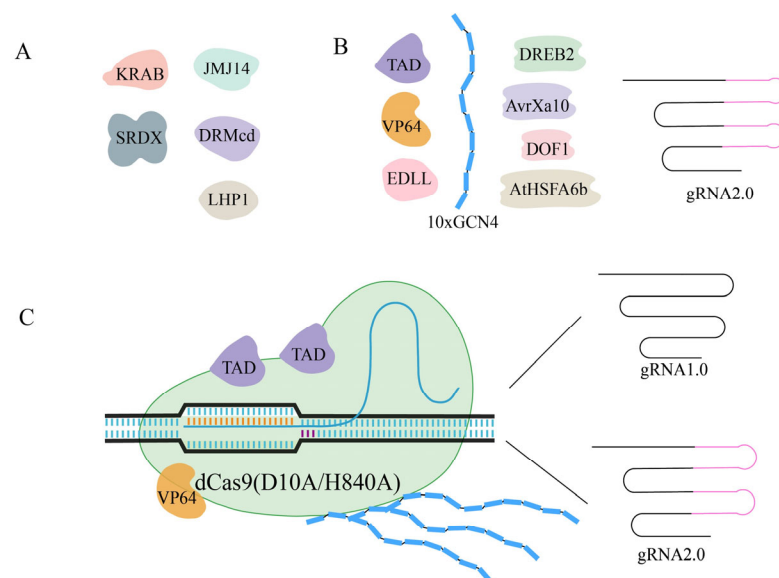
In the type VI CRISPR–Cas system, the Cas13 protein, which contains two structurally distinct higher eukaryote and prokaryote nucleotide-binding (HEPN) domains that provide RNase activity, recognizes and cleaves ssRNA but cannot cleave dsDNA; Cas13 can also target pre-crRNA and process it to create functional crRNA [61,62]. This system is divided into six subtypes: VI-A (Cas13a, C2c2), VI-B (Cas13b, C2c4), VI-C (Cas13c, C2c7), VI-D (Cas13d), VI-X (Cas13X), and VI-Y (Cas13Y) [36]. Cas13X and Cas13Y (775 to 803 amino acids [aa]) are two compact families derived from metagenomic datasets [63]. Abudayyeh et al. [64] used LwaCas13a to target *EPSPS*, *HCT*, and *PDS* in rice protoplasts, and most of the guides achieved over 50% knockdown. And the application of this system in crops holds particular promise for plant virus interference [65]. CRISPR–Cas13a was used to interfere with Turnip Mosaic Virus (TuMV) in *Nicotiana benthamiana* and *Arabidopsis*, and the reduction levels were influenced by the RNA targets [66,67]. In addition, CRISPR–Cas13 efficiently interfered with Potato Virus Y (PVY), Southern Rice Black-Streaked Dwarf Virus (SRBSDV), Rice Stripe Mosaic Virus (RSMV), and Sweet Potato Chlorotic Stunt Virus (SPCSV)-RNase3 [68–70]. The CasRX variant (Cas13d from *Ruminococcus flavefaciens*) is the smallest of the Cas13a–d variants but has shown the greatest interference activity in *N. benthamiana* [71,72]. Simultaneous targeting of multiple potato RNA viruses was achieved with an endogenous tRNA-processing system (polycistronic tRNA-gRNA [PTG]) [73]. Cas13d has also been used to target a variety of RNA targets, including microRNAs (miRNAs), long noncoding RNAs (lncRNAs), and circular RNAs (circRNAs) [74]. Moreover, surprisingly, crRNA designed to guide Cas13 could cause a significant reduction in RNA levels without the Cas13 protein. Cas13-independent guided induced gene silencing (GIGS) has been observed in tobacco, tomato, and *Arabidopsis*, and can be extended to crRNAs designed by Cas9 with more than 28 bp [75]. In addition, CRISPR–Cas13 has been used to detect and quantify genes [76].

#### 2.2. dCas9 and Transcriptional Regulation

Overexpression is the most commonly used method for exploring gene function. However, owing to the large cloning workload, limited vector capacity, and need for multiple promoters, achieving multigene overexpression via traditional methods is challenging and inefficient [77]. The RuvC D10A and HNH H841A targeted mutations generate the dCas9 protein. When combined with guide RNAs and transcriptional effectors, the system can achieve gene activation or inhibition (Figure 3). The combination of dCas9 and transcriptional activators, such as the EDLL activation domain (dCas9-EDLL), TAL activation domain (dCas9-TAD), and VP64, a fusion of four tandem repeats of VP16 (dCas9-VP64), constitutes the CRISPR activation (CRISPRa) system [32,33]. However, the activation of the target gene by these systems was less effective, so the second generation of CRISPRa systems was generated. Among them, dCas9-TV relies on six copies of the transcription activator-like effector (TALE) TAD motif coupled with VP128 [77], dCas9-SunTag combines



a tandem array of GCN4 peptides that can recruit VP64 transcriptional activators [78], and CRISPR-Act2.0 is based on the recruitment of VP64 and the modification of guide RNA scaffold [79]. Differences in the ability of these CRISPRa systems to activate target genes led to the creation of CRISPR-Act3.0, which combines dCas9-VP64, gRNA2.0, 10xGCN4 SunTag, and 2xTAD activator [16]. And the derived CRISPR-Combo platform, using gRNA1.0 and gRNA2.0 scaffolds with protospacers of different lengths, achieved simultaneous editing and activation [15,80]. Recent studies have focused on the application of plant transcriptional activation domains (ADs). Zinselmeier et al. [14] noted that the system in which VP64 is replaced with plant-derived ADs, such as programmable transcriptional activators (PTAs), has considerable room for improvement. Using *Arabidopsis* and *Seteria* protoplasts as dicotyledon and monocotyledon models, respectively, multiple ADs were tested, and it was found that *DREB2*, *AvrXa10*, *DOF1*, *AtHSA6b*, and *DREB1* had activation efficiencies equal to or greater than those of the commonly used VP64. However, further validation is needed. Moreover, plant transcriptional ADs have been identified at the genome scale, and combining these ADs with continuously improving CRISPR–Cas systems could drive further developments in transcriptional regulation [81].



**Figure 3.** The combination of the dCas9 protein with transcriptional effectors and epigenetic modifications enables transcriptional regulation of target genes. (A) These transcriptional effectors and epigenetic modifications fused with dCas9 can achieve transcription inhibition. (B) Transcriptional effectors, plant-derived ADs, and some strategies that can enhance the activation of target genes. (C) A CRISPR–Combo platform with gRNA1.0 and gRNA2.0 scaffolds can achieve editing and activation simultaneously.

Currently, the CRISPRa system has been practiced in many plant species. One potential application of CRISPRa is customizing the plant metabolome by activating selected enzymes in specific metabolic pathways, for example, selective enrichment of naringenin, eriodictiol, kaempferol, and quercetin was achieved in *N. benthamiana* leaves via dCas9-EV2.1 [82]. In grape cells, both the dCas9-VP64 and dCas9-TV systems effectively activated the UDP-glucose flavonoid glycosyltransferase (*UGT*) gene, and the expression level of *CBF4* was increased 3.7- to 42.3-fold via the dCas9-TV system [83]. When CRISPR-Act3.0 was used to target the promoters of seven genes in pears, four of the seven genes were activated at least ten times [84]. The CRISPRa system has also been applied in *Populus*, and the expression of target genes increased 1.2- to 7.0-fold [85]. Recently, parthenogenesis and epigenetic factory-mediated transcriptional activation have also been achieved

in maize and tomato via the CRISPRa tool through specific experimental designs [86,87]. Three new cotton germplasms were also produced with the dCas9-TV system [88]. Moreover, a chemically inducible CRISPRa tool called ER Tag was developed by combining  $\beta$ -estradiol-inducible XVE (LexA-VP16-ER) with dCas9-SunTag, and the system is capable of temporarily controlling gene expression [89].

When dCas9 is coexpressed with guide RNA, a DNA recognition complex that can specifically interfere with transcription elongation, RNA polymerase binding, or transcription factor (TF) binding is produced; this repurposed CRISPR–Cas system was designated as CRISPR interference (CRISPRi). CRISPRi can avoid genome-wide uncertainty of RNA interference (RNAi) and inhibit multiple genes simultaneously [90]. When dCas9 was fused with the Krüppel associated box (KRAB) domain of Kox1, the GFP signal decreased five-fold [91]. Combining dCas9 with the SRDX inhibitory domain of ERF TFs (dCas9:SRDX or dCas9-3X) achieved transcription inhibition [32,33], and deactivated Cas12a fused with SRDX also exhibited powerful transcription inhibition effects in plants [42]. On the other hand, the epigenetic strategy is a common implementation of CRISPRi; this strategy uses the SunTag system with the *N. tabacum* DRM methyltransferase catalytic domain (DRMcd) to effectively target DNA methylation to the *FWA* and *SUPERMAN* promoters and achieve gene silencing [78]. Different factors, including SUVH2, JM14, LHP1, HD2C, ELF7, and CPL2, which represent different epigenetic pathways, were added to the SunTag system. And target gene silencing was observed in the application of JM14 and LHP1, while the remaining factors resulted in weak silencing [17]. However, compared with CRISPRa, the application of CRISPRi in plants is relatively limited, and the inhibitory efficiency varies greatly. Therefore, taking CRISPRa as a reference, more effective strategies, such as screening for highly effective transcriptional suppressors and modifying sgRNAs, are needed to increase the inhibition efficiency of CRISPRi [19].

### 2.3. Precise Editing

The classical CRISPR–Cas system uses nucleases such as Cas9, in which the HNH domain cleaves the complementary strand (target strand) and the RuvC-like domain cleaves the noncomplementary one (nontarget strand) [92], resulting in DSBs that serve as substrates of cellular DNA repair mechanisms, including homologous directed repair (HDR) and nonhomologous end joining (NHEJ) [93]. NHEJ often results in imprecise mutations, whereas HDR can produce precise modifications but is limited by the inherent inefficiency in higher organisms [19,34,94]. In addition, editing through the creation of DSBs usually result in cell damage [95]. Efficient genome modification tools require more precise editing and need to be controllable. An alternative strategy is the use of base editors that utilize a Cas9ncontaining an Asp10Ala (D10A) point mutation, which inactivates the RuvC nuclease domain of SpCas9, and generates a single-strand break (“nick”) in the target strand, [9]. nCas9 (D10A) binds to different deaminases to achieve accurate base conversion. The PE system, which represents a landmark advance in precise editing, utilizes a clever combination of nCas9 (H840A) with other tools, that inactivate the HNH domain [8].

#### 2.3.1. Cytosine Base Editing

The commonly used CBE system BE3 consists of nCas9 (D10A), cytidine deaminase, and uracil DNA glycosylase (UDG) inhibitors (UGIs) [34,94]. The cytidine deaminase deaminates cytidine to uridine on the nontarget strand, and the UGI prevents UDG from deaminating cytidine to the apyrimidinic (AP) site. When nCas9 (D10A) induces a nick in the target strand, the DNA repair pathway is activated, preferentially replacing the U:G mismatch with the desired U:A, resulting in a C:G-to-T:A base transition after DNA replication [1].

In plants, the original CBE system, which was based on the rat cytidine deaminase APOBEC1, generated targeted mutations at positions three to nine in the protospacer and had a preference for TC motifs [96]. Different cytidine deaminases were used subsequently, and the use of *Petromyzon marinus* cytidine deaminase 1 (PmCDA1) improved editing efficiency [97]. Moreover, the base editing window was extended to 17 bp by using human APOBEC3A (hAPOBEC3A), and targeted C-to-T mutations were generated very efficiently in wheat, rice, and potato [98]. Two CBE variants based on A3Bctd deaminase (a truncated human APOBEC3B cytidine deaminase) eliminated sgRNA-independent off-target editing in rice and showed improved specificity [99]. Moreover, Ren et al. [100] compared 21 different CBE systems and found that PmCDA1-CBE\_V04 and A3A/Y130F-CBE\_V04 had high editing efficiency. Several tools have also been used to optimize CBE systems. AlphaFold2 is a structure-based protein clustering method [101]; since a protein's structure determines its function, AlphaFold2 provides a simple way to discover and engineer novel deaminases, which expands the usefulness of CBE systems to more species [102].

In CBEs, UDG activity is inhibited by the UGI, whereas UDG overexpression triggers base excision repair (BER); UDG recognizes the U: G mismatch, excises the uracil, and creates a basic site that is nicked by AP lyase. This nick, combined with the one formed by Cas9 near the DSB, should produce a precise deletion [1,103]. On the basis of this rationale, APOBEC–Cas9 fusion-induced deletion systems (AFIDs), including cytidine deaminase, Cas9, UDG, and AP lyase, have been developed to generate precise deletions [103]. In addition to deletion, ssDNA extensions produced by AFIDs may also be used to create precise insertions or replacements [9].

### 2.3.2. Adenine Base Editing

The combination of adenosine deaminase with nCas9 (D10A) generates ABEs. Adenosine deaminase deaminates adenosine to inosine, which is recognized by DNA polymerase as guanosine during DNA repair and replication, realizing the conversion of A:T to G:C. Natural adenine deaminase is rare, and highly active ABEs with broad sequence compatibility (ABE6s and ABE7s) were obtained after several rounds of directed evolution [34]. Subsequently, the ABEs were rapidly applied to rice, wheat, and rapeseed [104,105]. Compared with CBEs, ABEs showed greater targeting specificity [106,107]. The addition of three SV40 nuclear localization sequences to the C-terminus of nCas9, enhanced sgRNA by modifying the sgRNA scaffold, and the *RPS5A* gene promoter was used to improve editing efficiency [104,105]. Compared with the widely used ABE-P1, ABE-P1S (Plant version 1 Simplified) is more effective in rice, possibly because of its greater protein expression [108]. Through phage-assisted non-continuous and continuous evolution (PANCE and PACE) of deaminase, ABE8e containing eight additional mutations was obtained. Compared with ABE7.10, the activity ( $k_{app}$ ) of ABE8e was increased 590-fold, and the ability to catalyze DNA deamination was increased up to 1100-fold [109,110]. Rice ABE8e (rABE8e), which combines monomeric TadA8e, bis-bpNLS, and codon optimization, exhibited extremely high editing efficiency in the specific editing window [111]. Moreover, TadA9 was established by incorporating V82S/Q154R mutations into TadA8e, and it achieved comparable or improved editing compared with TadA8e [112].

### 2.3.3. Dual-Base Editing

CBEs and ABEs can induce only one type of modification [113]; therefore, dual-base editors were created. The saturated targeted endogenous mutagenesis editor (STEME) combines nCas9 (D10A) with cytidine deaminase, adenosine deaminase, and UGI. In rice protoplasts, STEME-1 achieved simultaneous C-to-T and A-to-G substitutions with efficiency up to 15.10% [114]. Simultaneous wide-editing induced by a single system



(SWISS), multiplexed orthogonal base editor (MoBE), and plant high-efficiency dual base editors (PhieDBEs) system are also powerful tools for generating multiplexed base editing [35,115,116]. However, these systems rely on the simultaneous application of both cytidine and adenosine deaminases, thus enlarging the size of the vector. TadDE, a dual base editor derived from TadA-8e, can generate dual base editing with a more compact structure [117].

#### 2.3.4. Transversion Editing

CBEs and ABEs have been extensively developed in plants, but the types of base conversion are limited. However, in ABE and CBE applications, researchers have observed C-to-G transversions [118,119]. To increase the efficiency of this transversion, a C-to-G base editor (CGBE), composed of nCas9, the cytidine deaminase rAPOBEC1 or its R33A variant, and *Escherichia coli*-derived uracil DNA N-glycosylase (eUNG) or rXRCC1 sourced from rats, was created. eUNG converts uracil to an AP site and initiates BER [119,120]. CGBEs achieved C-to-G editing with different editing efficiencies in rice, tomato, and poplar. C-to-T edits and insertions and deletions (indels) were the major byproducts [121,122].

To achieve efficient adenine transversion, ABE8e was combined with mouse alkyladenine DNA glycosylase (mAAG) variants, which enabled efficient A-to-C and A-to-T base transversions [123]. In rice, the fusion of the hypoxanthine excision protein N-methylpurine DNA glycosylase (MPGv3, also named AAG) with rBE49b generated a novel system called pAKBE (K = G or T). Compared with mammalian cells, pAKBE exhibited frequent A-to-G transitions and poor A-to-T transversion efficiency in rice [124,125].

#### 2.3.5. Prime Editing

A revolutionary technology was developed in 2019 and its emergence solved the problem of the limited number of base transitions. This system can produce all kinds of base substitutions and allow targeted indels of DNA fragments without the need for DSBs or donor DNA templates [1,8]. The PE system combines nCas9 (H840A), reverse transcriptase (RT), and primer editing guide RNA (pegRNA), which consists of an RT template containing the genetic information for the desired mutation and a primer binding site (PBS). The PBS pairs with the ssDNA nicks created by nCas9 (H840A), triggering reverse transcription and integrating genetic information into the genome [1]. Different strategies have been adopted to improve PE1; PE2 uses engineered RT, and PE3 adds an additional gRNA to create another nick in the target strand, which stimulates DNA repair [8].

Since its creation, the PE system has been rapidly implemented in plants such as rice, wheat, maize, and tomato. Through the optimization of codon, promoter, and editing conditions, selection-assisted enrichment of base-edited cells, the design of enhanced sgRNA (esgRNA) and T<sub>m</sub>-directed PBS length, fusion gene transformation with P2A, and the use of dual-pegRNA, the efficiency of PE in plants has improved [126–131]. Moreover, the assessment of off-target activity showed the high specificity of this system [132]. ePPEplus achieved greater improvements in efficiency through the introduction of V223A substitution into the RT of the engineered plant prime editor (ePPE), varying nuclear localization signals (NLSs) and increasing Cas9 activity [133,134]. The more compact and efficient PE6 has been generated through phage-assisted evolution and protein engineering [135]. Moreover, the practice of multiplex prime editing in wheat and rice improved the flexibility and applicability of PEs [136]. Compared with sgRNAs used with Cas9 or base editors, pegRNAs are more difficult to design and play an important role in determining editing efficiency. Supporting design and analysis tools have also been developed, such as PlantPegDesigner (<http://www.plantgenomeediting.net/>) accessed on 24 December 2024 [131], PE-Designer

(<http://www.rgenome.net/pe-designer/>) accessed on 24 December 2024, and PE-Analyzer (<http://www.rgenome.net/pe-analyzer/>) accessed on 24 December 2024 [137].

Efficient editing is required for accurate genome manipulation, and the DNA mismatch repair (MMR) system has been shown to promote unwanted indel byproducts. When a dominant-negative MMR protein (MLH1dn) was coexpressed with the PE2/PE3 system, the average editing efficiency of PE4/PE5 increased from 2.8- to 7.7-fold [138]. However, the effect in plants was poor [139], while direct knockdown of *OsMLH1* in the ePE5c system increased the efficiency from 1.30- to 2.11-fold. At the same time, a conditional excision system was introduced to overcome the partial sterility caused by *OsMLH1* knockdown [140].

Prime editing also provides guidance for precise large fragment deletions; an editing method based on a pair of pegRNAs, PRIME-Del, can effectively induce deletions of up to 10 kb and simultaneously insert short fragments into the genome [141]. Another PE-Cas9-based deletion and repair (PEDAR) system was generated by fusing Cas9 nuclease with RT and combining this complex with two pegRNAs, enabling the efficient creation of large precise genomic alterations [142]. On the other hand, PrimeRoot (prime editing-mediated recombination of opportune targets) can also produce large DNA insertions [143].

#### 2.4. Cas9 Protein Variants

In the practical applications of CRISPR-Cas technology, the requirement of PAMs such as NGG or TTN for target recognition limits the genomic sites that can be targeted [144]. As a result, several variants of the Cas9 protein that target a wider range of sites have been developed, enabling nearly PAM-free targeted editing. Two SpCas9 variants, xCas9 and Cas9-NG, recognize more permissive NG PAMs. xCas9 exhibits nearly the same editing efficiency as Cas9 at most typical NGG PAMs but shows limited activity at atypical NGH PAMs (H = A, C, or T). In the case of sgRNA mismatch, xCas9 has better targeting specificity than Cas9 [10,11,145]. Two Cas9-NG variants, Cas9-NGv1 and Cas9-NG, were designed to target NGN PAMs [146], with crystal structures indicating that the loss of the third nucleobase base-specific interaction is compensated with newly introduced non-base-specific interactions, enabling NG PAM recognition [144]. The editing efficiency of Cas9-NG is much greater than that of Cas9-NGv1 at NG PAM sites, but the editing efficiency at NGG PAMs is relatively poor. Cas9-NG also has higher specificity than Cas9 at NGG PAMs [10,11].

To enable broader PAM targeting, a variant called SpG capable of targeting an expanded set of NGN PAMs was developed; further optimizing developed a nearly PAM-free variant called SpRY (NRN and NYN PAMs; R = A or G and Y = C or T) [12]. In plants, SpG supports NGD PAMs (D = A, G, or T); although it is less efficient than SpCas9-NG, SpRY achieves efficient editing at a wide range of genomic loci, indicating a preference for NGD and NAN PAMs [13,147,148]. Several other SpCas9 variants, such as SpCas9-NRRH, SpCas9-NRCH, and SpCas9-NRTH, recognize NRNH PAMs, thus further broadening the editing range of the SpCas9 system in plants [149]. Happily, at the same time that different Cas9 variants were developed, corresponding transcriptional regulatory systems and base editors were also developed, and their effectiveness was demonstrated in different plants [16,149].

### 3. CRISPR-Cas Systems Targeting Different Locations with Different Breadths

#### 3.1. Targeting the CDS

Editing the CDS of genes is the most common use of CRISPR. Since the functions of different members of the same gene family are often redundant, it is necessary to produce

mutants that contain multiple genes mutated simultaneously [150]. CRISPR–Cas systems provide many effective ways to express Cas9 with multiple gRNAs. Based on Golden Gate ligation or Gibson Assembly, tandem sgRNA expression cassettes have been produced, each containing a polymerase (Pol) III promoter and terminator. Through this approach, up to eight sgRNA expression cassettes were inserted into a single binary CRISPR–Cas9 vector, and seven of the eight target genes were mutated in three T0 plants [151]. The endogenous tRNA-processing system performs multiplex editing through the endogenous tRNA-processing RNA enzymes (RNase P and RNase Z) that recognize tRNA components and excise them from the PTG into individual gRNAs [152]. CRISPR system *yersinia* (Csy)-type ribonuclease 4 (Csy4) was also used to simultaneously express multiple gRNAs, and the combination of Csy4 and tRNA expression systems was nearly twice as effective as the single Pol III promoter for gRNA expression. And the mutagenesis capacity was improved by the addition of the Trex2 exonuclease [153]. Through crRNA processing activity of Cas12a, intronic crRNAs can also be cleaved for editing multiple genes [154]. Moreover, sgRNAs driven by different Pol III promoters were assembled by multiple restriction enzymes [155], and isocaudomers are widely used, such as Spe I/Nhe I, BamH I/Bgl II, Xba I/Nhe I, and Xho I/Sal I. The intermediate vectors were digested and finally introduced into the editing vector [156]. In theory, this system could be used to edit an unlimited number of genes simultaneously [156]. By reducing the length of the sgRNA promoter, 12 sgRNA expression cassettes were assembled [157]. Moreover, structure-guided site mutagenesis and random screening were conducted; more than 35 sgRNA scaffold variants were selected, and numerous functional variants were identified. Using these variants and different tRNAs, spacer-scaffold-tRNA-spacer units containing up to 9 targets can be synthesized via fusion PCR; in conjunction with Golden Gate ligation, up to 24 target sites can be efficiently cloned in one step [158].

The selection of sgRNAs is an important factor in determining the efficiency and specificity of CRISPR–Cas systems. To date, many plant genome sgRNA design websites have been established (Table 1), including CRISPR-P 2.0 (<http://cbi.hzau.edu.cn/CRISPR2/>) accessed on 24 December 2024 [159], the local sgRNA design tool CRISPR-Local (<http://crispr.hzau.edu.cn/CRISPR-Local/>) accessed on 24 December 2024 [160], and the integrated toolkit CRISPR-GE (<http://skl.scau.edu.cn/>) accessed on 24 December 2024 [161]. Other comprehensive tools, including Cas designer (<http://rgenome.net/cas-designer/>) accessed on 24 December 2024 and CRISPOR (<http://crispor.org>) accessed on 24 December 2024, also cover a substantial amount of plant genome information, and comprehensive consideration of the results from multiple websites would aid in the selection of better sgRNAs [162–164].

**Table 1.** Tool sets of sgRNA design and editing result analysis.

| Tools        | Websites                                                                                                                     | References |
|--------------|------------------------------------------------------------------------------------------------------------------------------|------------|
| CRISPR-P 2.0 | <a href="http://cbi.hzau.edu.cn/CRISPR2/">http://cbi.hzau.edu.cn/CRISPR2/</a> , accessed on 24 December 2024/                | [159]      |
| CRISPR-Local | <a href="http://crispr.hzau.edu.cn/CRISPR-Local/">http://crispr.hzau.edu.cn/CRISPR-Local/</a> , accessed on 24 December 2024 | [160]      |
| CRISPR-GE    | <a href="http://skl.scau.edu.cn/">http://skl.scau.edu.cn/</a> , accessed on 24 December 2024                                 | [161]      |
| Cas designer | <a href="http://rgenome.net/cas-designer/">http://rgenome.net/cas-designer/</a> , accessed on 24 December 2024               | [162]      |
| CRISPOR      | <a href="http://crispor.org">http://crispor.org</a> , accessed on 24 December 2024                                           | [163]      |
| TIDE         | <a href="http://tide.nki.nl">http://tide.nki.nl</a> , accessed on 24 December 2024                                           | [4]        |
| ICE          | <a href="https://ice.synthego.com">https://ice.synthego.com</a> , accessed on 24 December 2024                               | [4]        |

Table 1. Cont.

| Tools        | Websites                                                                                                                                 | References |
|--------------|------------------------------------------------------------------------------------------------------------------------------------------|------------|
| Hi-TOM2      | <a href="http://www.hi-tom.net/hitom2/">http://www.hi-tom.net/hitom2/</a> , accessed on 24 December 2024                                 | [165]      |
| CRISPResso2  | <a href="https://github.com/pinellolab/CRISPResso2">https://github.com/pinellolab/CRISPResso2</a> , accessed on 24 December 2024         | [166]      |
| CRISPR-GRANT | <a href="https://github.com/fuhuancheng/CRISPR-GRANT">https://github.com/fuhuancheng/CRISPR-GRANT</a> , accessed on 24 December 2024     | [167]      |
| CrisprStitch | <a href="https://zhangtaolab.org/software/crisprstitch">https://zhangtaolab.org/software/crisprstitch</a> , accessed on 24 December 2024 | [168]      |

### 3.2. CRISPR Screen

The CRISPR–Cas9 system, with its specificity only at the 20 nucleotide (nt) target sites, enables high-throughput editing, which contributes to functional genomic research and can provide valuable genetic resources for crop improvement [169]. High quality, high coverage, and uniform distribution are guarantee of the efficient application of CRISPR screens [21]. Two popular CRISPR screening methods have been developed, arrayed screens and pooled screens; owing to the application characteristics of array screens, pooled sgRNAs are more widely used in plants [170]. To date, CRISPR screens have been performed in many plants (Table 2). The practice of large CRISPR screening was initially carried out in rice, that contains 88,541 sgRNAs to target 34,234 genes, and obtained 91,004 transgenic plants with an editing efficiency of about 83.9%. Phenotypic changes in fertility, tiller angle, and leaf color were observed in these transgenic lines [171]. In the same year, a library containing 25,604 sgRNAs targeting 12,802 genes highly expressed in rice stem tissue was also reported, generating 14,000 T0 plants [172]. In tomato, 4379 sgRNAs were designed for 990 TFs, and 487 positive plants were obtained, only 92 of which contained a single sgRNA, with an editing efficiency of 23%. The proportion of plants containing a single sgRNA was increased to 42% by the transformation of a small library targeting 30 TFs [173]. In rapeseed, a CRISPR library targeting 10,480 genes through 18,414 sgRNAs was also constructed, and 1 104 T0 plants were obtained with an editing efficiency of 55.80% [174].

In fact, current genome-wide CRISPR library construction is still focused mainly on rice. In other species, CRISPR libraries with small scopes or specific functions have been constructed, and mutant libraries of appropriate size exhibit advantages in the study of special functions. Initially, in tomato, 15 mutant lines were obtained by targeting 54 immune-associated leucine-rich repeat subfamily XII genes with 165 sgRNAs; phenotypic analysis revealed that *Fls2.1* plays a major role in the leaf response to flg22 [175]. Using seventy CRISPR–Cas9 vectors to target candidate genes with potential functions in nodulation or seeds in soybeans, a total of 407 T0 lines containing all the sgRNAs were obtained, with an average mutation frequency of 59.2%, and of which 35.6% lines carried multiple mutations [176]. In maize, 1368 sgRNAs targeting 1244 genes identified from genetic mapping and comparative genomic analysis were pooled and produced 4356 glyphosate-resistant T0 plants containing 743 target genes, and many unexpected phenotypic changes were observed [177]. In rice, CRISPR-specific libraries related to MS, receptor-like kinase (RLK), and seed-preferring genes have also been established [178–180]. Interestingly, CRISPR libraries based on different traits have also been constructed in a woody plant (cotton), including genes that are differentially expressed during plant development, endogenous insect resistance-related genes, and the calcium-dependent protein kinase (CDPK) gene family [181–183]. The finding of multitarget insertion in these studies also indicated the importance of building specific CRISPR libraries, which is advantageous when analyzing the same trait.

**Table 2.** A brief summary of CRISPR screen practice in plants.

| Species  | Description                                            | Number |        |        | Mutation Efficiency | Refs  |
|----------|--------------------------------------------------------|--------|--------|--------|---------------------|-------|
|          |                                                        | Gene   | sgRNA  | Plant  |                     |       |
| Rice     | genome-scale                                           | 34,234 | 88,541 | 91,004 | 83.90%              | [171] |
|          | highly expressed genes in shoot base tissue            | 12,802 | 25,604 | 14,000 | 65.30%              | [172] |
|          | anther-specific genes                                  | 73     | 73     | 333    | 82.30%              | [178] |
|          | receptor-like kinase (RLK) family                      | 1072   | 1166   | 5039   | 92.10%              | [179] |
|          | seed-preferred genes                                   | 310    | 375    | 2688   | 84.06%              | [180] |
| Tomato   | LRR-XII gene family                                    | 54     | 165    | 31     | 62.50%              | [175] |
|          | TFs                                                    | 990    | 4379   | 487    | 23%                 | [173] |
| Soybean  | genes with potential functions in nodulation and seeds | 102    | 70     | 407    | 59.20%              | [176] |
| Maize    | genes related to agronomy and nutrition traits         | 1244   | 1368   | 4356   | 81%                 | [177] |
| Cotton   | plant development-related genes                        | 112    | 116    | 718    | 75%                 | [181] |
|          | endogenous insect-resistant genes                      | 502    | 969    | 2000   | 97.29%              | [182] |
|          | CDPK gene family                                       | 82     | 246    | 518    | 89.49%              | [183] |
| Rapeseed | genes related to the reproductive organs               | 10,480 | 18,414 | 1104   | 55.80%              | [174] |

The identification of the sgRNAs of T0 plants is mostly based on targeted amplification sequencing. Chen et al. [179] developed fragment-length markers for distinguishing gRNAs (FLASH) pipeline; an aligned CRISPR library based on the FLASH pipeline was used to target 1072 members of the RLK family in rice, and rapid identification of gRNAs was achieved without sequencing. Recently, many new systems have been proposed to provide guidance for better use of CRISPR libraries. To overcome functional redundancy, a toolbox named multi-knock was developed. A total of 59,129 optimal sgRNAs each targeting 2 to 10 genes within a family, for a total of 16,152 genes, were designed and divided into 10 functional sublibraries to allow flexible and targeted genetic screens; by transforming one of these sublibraries, novel TRPs with hidden functions due to genetic redundancy were discovered [150]. By combining breeding with gene editing in maize, a BREEDIT system based on multiplex CRISPR-mediated genome editing and hybridization schemes was proposed to establish the link between traditional breeding and genetic engineering [184]. All of these systems can be extended to other species.

CRISPR screens have also been used for the CDS of single genes by designing a wide range of sgRNAs, which were shown to enable saturation mutagenesis and promote protein evolution. Butt et al. [185] designed 119 sgRNAs based on sites adjacent to the PAM in the CDS of SF3B1 and produced mutants with varying degrees of resistance to splicing inhibitors. Owing to the randomness of mutations introduced by CRISPR–Cas9, precise editing systems were subsequently applied. In conjunction with gRNA libraries, the directed evolution of herbicide resistance genes, including acetyl-CoA carboxylase (ACC) [186], acetolactate synthase 1 (ALS1) [187], and 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) [100] has been generated. Dual-base editors and the prime editing library-mediated saturation mutagenesis (PLSM) system were also used to generate the direct evolution of OsACC [114,188]. More recently, Wang et al. [189] established an efficient germline-specific system based on an improved base editor and the ability of *Arabidopsis* to produce many seeds; through this system, herbicide-resistant EPSPS, ALS, and HPPD variants were produced.

Sanger sequencing is a common method for detecting mutations, and bioinformatics tools such as Tracking of Indels by Decomposition (TIDE; <http://tide.nki.nl>) accessed on 24 December 2024 and Inference of CRISPR Edits (ICE; <https://ice.synthego.com>) accessed on 24 December 2024 can be used to decode Sanger sequencing data containing different types of mutations [4]. However, the sensitivity of Sanger sequencing is only



approximately 15%; therefore, edits with a low frequency are likely to be ignored. And it is cumbersome and expensive when applying pooled strategies, whereas barcode-based high-throughput next-generation sequencing (NGS) has shown unique advantages in CRISPR screens. Analytical tools such as Hi-TOM2 [165], CRISPResso2 [166], CRISPR-GRANT [167], and CrisprStitch [168] have also been developed (Table 1). These tools enable convenient analysis of the large datasets generated by NGS.

### 3.3. Targeting CRE in the Promoter and uORFs

CRISPR–Cas systems are able to generate many kinds of genetic variants. For pleiotropic genes that are active in multiple organs or involved in multiple regulatory pathways, CRE editing can achieve significantly better effects than CDS editing. In combination with CRISPR screening, a variety of cis-regulatory alleles with decoupled regulatory traits can be produced, which can contribute to crop domestication and quantitative trait improvement [18].

Many CRE editing practices have been carried out in plants. By targeting the effector binding element (EBEP<sub>thA4</sub>) of *CsLOB1*, increased resistance to citrus canker can be achieved [190]. *CLAVATA 3* (*CLV3*) and *WUSCHEL* (*WUS*) maintains a highly conserved negative feedback loop to regulate cell proliferation. In tomato, CRISPR–Cas9-mediated CRE mutation was used to characterize the relationships between *CLV3* and quantitative traits, and elucidate the additive and synergistic relationships between conserved sequences [191,192]. Moreover, Hendelman et al. [193] engineered many *WUSCHEL* *HOMEODOMAIN* (*WOX9*) promoter alleles in tomato, revealing the hidden pleiotropic role of *WOX9*, and extended it to other species. The *CLAVATA3/EMBRYO SURROUNDING REGION-RELATED* (*CLE*) peptide signal in the *CLV3*–*WUS* feedback pathway also regulates meristem size. The use of CRISPR–Cas9 to generate weak *CLE* alleles improved yield-related traits [194]. In addition, fine-tuned amylose levels and heading dates were realized through targeted editing of the promoter of *Wx* and three major heading date-related genes [195,196].

In rice, *Ideal Plant Architecture 1* (*IPA1*) mutation increased the number of grains per panicle but reduced the number of tillers. By transforming 39 vectors containing 2–4 sgRNAs in the promoter, untranslated regions (UTRs) and downstream regions, tiling deletion was performed. A 54 bp deletion in the promoter simultaneously increased the number and size of panicles [197]. This method represents a new type of CRISPR screen compared with the standard one, involving two sgRNAs simultaneously targeting a sequence that result in a large deletion. The phenotypic regulation achieved by targeted editing at specific sites also provides guidance for identifying TF binding sites [197]. Meanwhile, a deep learning model, BPNet, which uses DNA sequences to predict the base-resolution chromatin immunoprecipitation (ChIP)-nexus binding profile of pluripotent TFs, was validated by CRISPR-mediated point mutation [198]. These results suggest that promoter-based editing plays an important role in uncoupling gene pleiotropy and characterizing the quantitative relationships between CRE and quantitative traits. However, some properties of promoters, including AT base enrichment, low sequence complexity, and strong element repeatability, impose certain requirements on the CRISPR–Cas system [199]. The Cas12a protein and precision editing tools are highly effective alternative tools [18,200]. In rice, a CRISPR–Cas12a promoter editing (CAPE) system introduced quantitative trait variation (QTV) continuums for multiple traits through the editing of gene promoters [201]. Thus, editing systems that target promoter regions have great potential.

Gene functions are rich and diverse, and in order to create desired traits, in addition to knocking out, promoting gene expression also plays an important role. As effective CREs in mRNAs, uORFs play important roles in controlling the translation initiation of

downstream primary ORFs (pORFs) [202], and they are emerging as a general mechanism that inhibits protein levels [203,204]. Editing uORF sequences has been shown to achieve overexpression [19]. In lettuce, editing the uORF of *LsGGP2*, which is involved in vitamin C biosynthesis, improved the antioxidant ability and ascorbate content [203]. By editing the highly conserved uORF of *FvebZIPs1.1*, the sugar content was increased in strawberries [205]. In addition, when the uORF of *SlGGP1* was edited, ascorbate enrichment was observed, which was linked to MS in tomatoes [206]. Multiple mutagenesis of the uORF of the *PSBS1* gene in rice revealed an overexpression phenotype and a two- to three-fold increase in OsPsbS1 protein abundance [207]. These results suggest that editing uORFs provides a universal and effective way to manipulate mRNA translation, that can be applied to dissect the mechanisms of biological processes or improve the quality and yield of crops.

## 4. Optimization of CRISPR–Cas Vectors

### 4.1. Application of Tissue-Specific Promoters

*Cauliflower mosaic virus* (CaMV) 35S and ubiquitin promoters are often used to drive the expression of Cas9, but the application of this system in *Arabidopsis* is relatively inefficient compared with rice, and many mutations are not inherited to the next generation [151]. To achieve efficient delivery of targeted mutations to progeny, the Cas9 protein was driven by tissue-specific promoters, including the *INCURVATA2* and *YAO* promoters, both are preferentially expressed in actively dividing cells [208,209]. The *SPOROCTELESS* (*SPL*), *DD45*, and tomato *LAT52* genes are expressed in sporogenous cells and microsporocytes, egg cells, and pollen cells, respectively. The promoters of these genes were used to drive Cas9 expression in germline cells, resulting in the increasing rate of heritable gene mutations [210]. Miki et al. [211] reported a sequential transformation method with which they compared four germline-specific promoters (*DD45*, *Lat52*, *YAO*, and *CDC45*), it was found that the *DD45* promoter improved the frequency of sequence replacements. The egg cell promoter (*EC1*) has also been used for efficient gene targeting (GT) [212]. Homozygous *try cpc* (*TRIPTYCHON* and *CAPRICE*) double mutants were observed in the T1 generation via *pWUS* and showed improved editing efficiency compared with the *EC1.2* promoter [213].

Owing to the pleiotropy of genes, traditional editing systems cannot effectively analyze the function of genes in specific tissues. Therefore, Decaestecker et al. [214] proposed a CRISPR-based tissue-specific knockout (CRISPR-TSKO) system, which can generate somatic mutations in specific cells, tissues, and organs. In tomatoes, genome editing for fruit-specific gene using the *phosphoenolpyruvate carboxylase 2* (*PPC2*) promoter was shown to be an effective tool to help bypass the pleiotropic effects of genes [215]. In addition, cotton pollen was transformed with Cas9 expression vectors driven by the *GhPLIM2b* and *GhMYB24* promoters via *Agrobacterium* vacuum infiltration, and targeted gene editing in cotton pollen was successfully achieved [216]. On the other hand, plants are sessile and rely on a myriad of signals and defense mechanisms to respond to the external environment. Many genes are involved in these mechanisms, and most of them have important biological functions in plant growth and development [22]. The knock-out of these genes is likely to generate major effects on morphology and yield, while tissue-specific knockout would be an excellent way to elucidate the underlying mechanisms of defense responses [22,23]. Moreover, for many reported negative regulatory genes, tissue-specific genome editing (TSGE) could be used to optimize their application. The application of CRISPR-TSGE requires promoters that function well in various tissues, and many tissue-specific promoters and several TSGE systems that provide guidance for these applications have been described [23]. Another major application of TSGE is the improvement of pathogen resistance. Viruses systematically infect and replicate in specific tissues of plants, using virus-inducible or tissue-specific promoters where viruses replicate to express Cas9 for locally knocked

down plant interaction partners can limit the systematic transmission and replication of viruses [22]. In combination with BEs, PEs, and other tools, TSGE can be better utilized.

#### 4.2. Acceleration of the Generation of Transgene-Free Plants

The use of the CRISPR–Cas9 system has led to revolutionary improvements in crops [1]. However, owing to the existence of transgenes, the continuous editing activity of the Cas9 protein makes it difficult to distinguish between mutations that are inherited or newly generated and increases off-target effects. This limitation poses a challenge to its further application, and assessing the heritability and phenotypic stability of CRISPR-edited plants requires the elimination of constructs [217]. In addition, removing these constructs may be a prerequisite for edited crops for commercial applications. PCR amplification is a common way to isolate transgene-free plants from progeny, but this process is time-consuming and laborious. Commonly used reporter genes such as mCherry, GFP, and its derivative RFP can be added to Cas9 constructs [218,219], making it easy to distinguish plants that contain transgenes from those that do not, but these fluorescent markers require additional tools. The  $\beta$ -glucuronidase (GUS) reporter gene is also widely used in plants, but expensive substrates are required [220].

The emergence of RUBY, which converts tyrosine into the vivid red betalain, makes the presence of transgenes clearly visible to the naked eye [221,222]. In maize, a ViMe-Box system that enhances the expression of DsRED2 was developed, and through tissue-specific promoters, makes Cas9-containing seeds visible to the naked eye under natural light [223]. Similarly, the seed fluorescence reporter (SFR) system, which expresses DsRED in endosperm, embryo, or both tissues, was established in maize [224]. Moreover, one co-expression system, Cas9-PF, expressed *PRODUCTION OF ANTHOCYANIN PIGMENTS 1 (PAP1)* and *Flowering Locus T (FT)* through the promoters of *Cestrum yellow leaf curling virus (CmYLCV)* and *Arabidopsis ubiquitin 10 (AtUbi10)*, respectively. This system can accelerate the generation of targeted mutants and transgene-free plants [225]. Other morphogenetic genes, such as *GLABRA1 (GL1)* and *GL2*, play roles in the formation of trichomes, and mutations in these genes result in hairless leaves and stems; a system that relies on *GL2* mutation-based visible selection (GBVS) has been established [226]. These marker genes can be driven by a single promoter or linked in tandem with the Cas9 protein via the P2A peptide to indicate the expression level of the Cas9 protein [219].

The addition of reporter genes provides a convenient way to distinguish plants with and without transgenes, and the direct elimination of transgenic offspring through suicide genes or MS genes would have a significant impact on the use of CRISPR technology for crop improvement. By introducing REG2-BARNASE and 35S-CMS2 expression cassettes into the conventional CRISPR–Cas9 vector, He et al. [217] constructed a Transgene Killer CRISPR (TKC) system; when a plant reached the reproductive stage, toxic proteins would kill male gametophytes and embryos containing the constructs. However, specific applications of genome editing, such as haploid induction-coupled editing, require the retention of transgenes [227,228]. In maize, a pollen self-elimination CRISPR–Cas (PSEC) system was proposed, and a pollen-specific promoter (*polygalacturonase 47, ZmPG47*) was used to drive the  $\alpha$ -amylase gene *AA1* to prevent transgene transmission [26]. The fluorescence marker and pollen killer-assisted CRISPR–Cas9 system (FMPKC) used DsRed2 to distinguish transgenic offspring and pollen killers (*RAmy1A* or *orfH79*) to reduce the proportion of transgenic seeds [27], establishing a balance between the separation of transgene-free seeds and the acquisition of transgenic seeds.

#### 4.3. Application of Morphogenetic Genes

For most plants, tissue culture mediated by *Agrobacterium* is still the main method of regeneration, and high regeneration efficiency is an important guarantee for achieving targeted editing. Activation of the AP2/ERF TF *WOUND INDUCED DEDIFFERENTIATION 1* (*WIND1*) strongly promoted bud regeneration in *Brassica napus* and *Arabidopsis*, but inhibited leaf growth [229]. *Baby boom* (*BBM*) and *WUS* have been shown to increase regeneration efficiency in many species, including *Zea mays*, *Sorghum bicolor*, *Saccharum officinarum*, *Oryza sativa*, *Malus domestica*, and so on [230–232], but these genes may also cause some abnormal phenotypes, such as dysplasia, distortion, and decreased fertility. *GROWTH-REGULATING FACTOR* (*GRF*) genes belong to a small family of plant-specific TFs that form a transcription complex with *GRF INTERACTING FACTOR* (*GIF*), which plays a role in regulating plant growth and development [233,234]. The overexpression of *GRF5* improved the transformation efficiency of dicot and monocot species, such as canola, soybean, sunflower, and maize [235]. The overexpression of wheat *GRF4-GIF1* fusion protein significantly improved regeneration efficiency and speed in wheat, triticale, rice, and even dicotyledon citrus [236]. The transgenic plants were fertile without obvious developmental defects, and efficient regeneration could be induced without exogenous cytokinins [236]. Comparing several developmental regulators (DRs) in tomato, including *PLETHORA 5* (*PLT5*), *WIND1*, *ENHANCED SHOOT REGENERATION 1* (*ESR1*), *WUS* and a fusion of *WUS*, and *BBM* (*WUS-P2A-BBM*), revealed that plants overexpressing *PLT5* had the highest regeneration efficiency. *PLT5* also promoted regeneration efficiency in *B. rapa* and *Capsicum annum*, but some morphological changes were also observed [237]. Recently, some genes that can improve regeneration efficiency, including *WOX5*, *DOF5.6*, *DOF3.4*, and *LAX1*, were identified in wheat, some of which are also effective in maize, soybean and barley [25,238,239]. Several genes, including *LAX2*, *LAX1*, and *LOX3*, have also been identified in cotton via single-cell sequencing technology combined with genetic analysis [240].

The discovery of these morphogenetic genes provides guidelines for high-efficiency plant regeneration. For genes that may produce adverse growth, weak promoters or tandem reporter genes can be used to reduce negative phenotypes. The conditioned Cre/loxP SSR system can also be used with specific treatments to achieve the removal of unfavorable factors after bud formation [241]. In addition to the usual plant hormones, adenosine monophosphate (AMP) also has the ability to increase regeneration efficiency [242]. On the other hand, multiple research teams have applied a ternary vector system in which morphogenetic genes are added to further improve the regeneration efficiency of sorghum and maize [24,241,243].

## 5. CRISPR–Cas System and Plant Breeding Practices

### 5.1. Bypassing Tissue Culture or Transgene-Free

Although some DRs have been found to promote plant regeneration, the tissue culture process is still complex and genotype-dependent to a certain degree. Therefore, several new transformation strategies have been proposed. *Agrobacterium* carrying DRs and sgRNA cassettes was injected into Cas9-overexpressing plants from which the meristem was removed, and gene-edited plants were obtained directly from the generated buds in *N. benthamiana*, tomato, potato, and grape [244]. Since plant viruses have the ability to move between cells [29], some positive-strand RNA viruses, such as the Tobacco Rattle Virus (TRV) and Barley Stripe Mosaic Virus (BSMV), have been used to deliver sgRNA into Cas9-overexpressing plant cells [245–249]. Alternatively, Cas9 and sgRNA cassettes can be simultaneously inserted into negative-strand RNA viruses, including the Barley Yellow Striate Mosaic Virus (BYSMV) or Sonchus Yellow Net Rhabdovirus (SYNV) [250,251], all

of which successfully achieve targeted editing. On the other hand, RNA sequences such as tRNA and FT have the ability to travel long distances within the plant [248]; based on this characteristic, CRISPR reagents can be transferred from the rootstock to the scion by grafting. The researchers produced a rootstock containing Cas9-tRNA-like sequences (TLSs) and gRNA-TLS fusions, and targeted deletions in offspring were observed in the scions that were grafted onto the rootstock [252]. However, the manufacturing of stock is a speed-limiting step, and implementing this approach in monocotyledonous plants is challenging. In addition, nanoparticles are also considered as novel delivery vectors for genome editing but are limited by the size of nucleases [28].

There are three types of CRISPR–Cas9 delivery reagents: expression plasmids (DNA), *in vitro* transcripts (IVTs), and preassembled RNP complexes, which include Cas9 proteins and guide RNA [19]. Among them, RNPs degrade rapidly once they enter the cell, enabling the instantaneous expression of Cas nucleases and reducing off-target effects [253]. Polyethylene glycol (PEG)-mediated RNP transformation has been used in many important plant species, including rice, wheat, maize, grape, and apple [254–256]. Transfected protoplasts were used to produce regenerated plants with different editing efficiencies in different species [257]. Given the potential toxicity of PEG, several other transformation methods, including lipofection, particle bombardment, and electroporation, have also been used [258–260].

### 5.2. Haploid Induction

Haploid breeding can stabilize the genetic background of hybrids in two generations, while the traditional method requires six to eight generations, and the key factor for its successful implementation is efficient haploid induction (HI) [9]. Frameshift mutations in *MATRILINEAL* [*MTL*, also known as *NOT LIKE DAD* (*NLD*) or *PHOSPHOLIPASE A* (*PLA1*)], encoding pollen-specific phospholipase, can trigger the elimination of paternal chromosomes in zygotes [261–263], which leads to the formation of haploid maize embryos and further spread to rice and wheat [264,265]. Moreover, *DOMAIN OF UNKNOWN FUNCTION 679 MEMBRANE PROTEIN* (*DMP*), *PHOSPHOLIPASE D3* (*PLD3*), and *POD65* were also shown to trigger maternal HI [266–269]. In *Arabidopsis*, engineering centromere-specific histone 3 (*CENH3*) variants can trigger efficient paternal HI [270], that has also been applied in wheat [271]. New systems that combine HI techniques with CRISPR–Cas9, such as HI-Edit, Haploid-Inducer Mediated Genome Editing (IMGE), and gene editing followed by doubled haploid production (GEDH) [227,228,269], can overcome the barriers with genotype dependency and achieve rapid homozygosity.

### 5.3. MS and Self-Incompatibility Manipulation

Owing to hybrid vigor, hybridization is an important way to improve yield and quality in crop breeding. To produce commercial hybrid seeds, it is necessary to avoid self-pollination, and the establishment of MS lines is a practical and effective method. Although many MS lines have been recorded, the transfer of MS traits to elite varieties via traditional methods is time-consuming and laborious [1]. The CRISPR–Cas system offers a quick method for establishing MS. Editing *Male Sterility 1* (*MS1*, encoding a glycosylphosphatidylinositol-anchored lipid transfer protein) and *MS45* (encoding a strigosidine synthase-like enzyme) in wheat, *SISTR1* in tomato and other genes associated with MS [272–274] can rapidly produce MS germplasms; and these strategies can be extended to other species [275]. In addition, by targeting the thermosensitive genic male sterility (TGMS) gene *TMS5* or the photoperiod-sensitive genic male sterility (PGMS) gene *CARBON-STARVED ANTHELS* (*CSA*) in rice, more flexible MS lines have been established [275].



Self-incompatibility is also widespread in flowering plants, including diploid potato, rapeseed, and cabbage. This characteristic promotes cross-pollination to maintain a high level of heterozygosity within the population and reduces the presence of harmful homozygous recessive genes [9]. However, stable homozygous inbred lines are difficult to obtain in these species, hindering basic research and crop breeding. *S-RNase* controls self-incompatibility in Solanaceae, and a self-compatible potato was generated by editing *S-RNase* [276]. While the farnesyl pyrophosphate synthase (*FPS2*) gene functions in *S-RNase*-independent unilateral incompatibility, editing *FPS2* could restore self-incompatibility [277]. In rapeseed and cabbage, self-incompatibility is inhibited by editing the M-locus protein kinase (*MLPK*) and S-receptor kinase (*SRK*) genes, respectively [278,279]. Moreover, elucidation of the mechanism of plant self-incompatibility is expected to overcome the barrier of interspecific reproduction [1].

#### 5.4. De Novo Domestication

Selective breeding for high yield and high quality has resulted in the loss of genetic diversity in many domesticated crops, leaving them vulnerable to biological and abiotic stresses, whereas wild species retain a diverse gene pool and may have innate resistance to adverse conditions [280]. With adequate genome analysis, rapid domestication can be achieved. De novo domestication based on genome editing has achieved a good balance between productivity and environmental adaptation. Orphan crops, which are grown and consumed in limited regions and have undesirable characteristics similar to their wild relatives, are also important materials for de novo domestication [281–284]. This strategy was used to achieve rapid improvement of multiple traits in ground cherry (*Physalis pruinosa*), wild tomato and allopolyploid rice through simultaneous editing of multiple genes [285–288]. Wild species or orphan crops of other plants, such as quinoa (*Chenopodium quinoa*), wild potato (*Solanum* spp.) and alfalfa (*Medicago sativa*), also have prominent strengths and weaknesses, and targeting genes that regulate undesirable traits can promote the improvement of plant species [1]. The transformation systems for wild species and accurate identification of regulatory genes for target traits were the basis for the efficient use of de novo domestication.

## 6. Conclusions and Future Prospects

The continuously recognized Cas proteins, increased targeting efficiency, expanded targeting width and breadth, and optimized auxiliary measures, it can be seen that the continuous improvement of the CRISPR–Cas system provides strong support for its efficient application in plants, and the combination of different classifications in this review could further promote its application. In the future, the development of CRISPR systems can be divided into two aspects: further improvement and the integration of existing systems in more species.

To realize the efficient application of the CRISPR system in plants, in addition to comprehensive knowledge of the CRISPR system, clever design and modification according to experimental purposes are also essential. The use of CRISPR plus regulatory elements, multi-omics, or protein analysis tools could lead to new applications. And, a growing number of tools are available to identify and redesign the components of CRISPR systems. On the other hand, although CRISPR systems stand out in many transgenic technologies, and many excellent lines have been obtained, substantial research is still needed before mass production of these excellent lines. Relevant supervisory policies and public recognition are important factors for these entering the public eye. The full identification of the stability of editing lines and the establishment of CRISPR systems with more accurate and smaller off-target effects are goals that researchers need to make continuous efforts.

Large-scale CRISPR practice remains an important approach for quickly understanding gene function, especially for species with lagging basic research. However, there is no platform where all or most Cas proteins or variants can be obtained. Therefore, for those who want to explore this area for the first time, it is relatively difficult to obtain the appropriate reagents. In the future, a collection platform for published information may be needed, which would promote the efficient application of the CRISPR–Cas system in plants. On the other hand, it is expected that the combination of CRISPR and CRISPRa screening could improve the understanding of gene function. CRISPR screens that target specific gene families or specific traits have unique advantages. Owing to the significance of genome-wide CRISPR screening to a plant species, it may require the concerted efforts of many laboratories to obtain as many regenerated plants as possible and present them to the public in the form of a database. In the future, this database would be ready to serve as the basis for a new study.

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