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# Plant base editing and prime editing: The current status and future perspectives[FA]

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## ABSTRACT

Precise replacement of an allele with an elite allele controlling an important agronomic trait in a predefined manner by gene editing technologies is highly desirable in crop improvement. Base editing and prime editing are two newly developed precision gene editing systems which can introduce the substitution of a single base and install the desired short indels to the target loci in the absence of double-strand breaks and donor repair templates, respectively. Since their discoveries, various strategies have been attempted to optimize both base editor (BE) and prime editor (PE) in order to improve the precise editing efficacy, specificity, and expand the targeting scopes. Here, we summarize the latest development of various BEs and PEs, as well as their applications in plants. Based on these progresses, we recommend the appropriate BEs and PEs for both basic plant research and crop improvement. Moreover, we propose the perspectives for further optimization of these two editors. We envision that both BEs and PEs will become the routine and customized precise gene editing tools for both plant biological research and crop improvement in the near future.

Keywords: base editing, CRISPR/Cas, plants, precision genome editing, prime editing

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## INTRODUCTION

Favorable alleles derived from local cultivars or wild relatives or even orthologs from other plant species, which are conferred by differences of one or several single nucleotide polymorphisms (SNPs), or insertion and deletions (indels) in either promoter region or encoding region of a target gene, often account for major differences in agriculturally important traits (Jiao et al., 2010; Hu et al., 2015; Wang et al., 2018a, 2018b; Shang et al., 2022). Introducing these elite alleles into commercialized cultivars has been a major goal of crop breeding programs. However, it is very difficult, laborious, and time-consuming to introduce these favorable alleles into commercialized cultivars without any linkage drag from the parent lines. Overall, it usually takes more than decades for crop breeders to introduce a favorable allele into commercial cultivars through traditional breeding. Moreover, it is difficult or even impossible to eliminate the undesired genes/agronomic traits derived from the parent lines by crossing if they are closely linked to the target genes/alleles. Thus, if we could introduce these elite alleles into commercialized crop varieties without introducing deleterious genes/alleles through precision genome editing in a short period of time, it would greatly facilitate plant basic biological research and speed up the crop breeding process.

The Clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) system, as a a robust, versatile and simple system, has dominated the genome editing field and holds a great potential either for plant functional genomics or crop improvement over the past decade (Gasiunas et al., 2012; Jinek et al., 2012; Knott and Doudna, 2018; Mao et al., 2019; Wang et al., 2019;

Xu et al., 2019; Gao, 2021; Huang et al., 2021; Li et al., 2021c; Xia et al., 2021; Zhan et al., 2021; Puchta et al., 2022). To date, three major CRISPR/Cas mediated precision genome editing systems have been developed and successfully applied in plants such as homology-directed DNA repair (HDR)-mediated targeted gene replacement or gene targeting (Sun et al., 2016; Li et al., 2019; Li and Xia, 2020; Lu et al., 2020; Chen et al., 2022a; Puchta et al., 2022), base editing (Komor et al., 2016; Nishida et al., 2016; Gaudelli et al., 2017; Li et al., 2017; Lu and Zhu, 2017; Shimatani et al., 2017; Zong et al., 2017; Hua et al., 2018; Wei et al., 2021; Tian et al., 2022) (Figure 1), and prime editing (Anzalone et al., 2019; Butt et al., 2020; Jiang et al., 2020; Hua et al., 2020a; Li et al., 2020c; Lin et al., 2020; Tang et al., 2020; Xu et al., 2020a, 2020c; Lu et al., 2021; Wang et al., 2021b; Perroud et al., 2022) (Figure 2). Among these three precise editing technologies, HDR enables the installation or replacement of all kinds of mutations or various lengths of fragments in a predefined manner, representing the holy grail of genome editing. However, although various strategies have been attempted in the past decade (for review, please check Zhan et al., 2021; Puchta et al., 2022; Chen et al., 2022a), HDR remains challenging in plants due to the facts that once the double-strand breaks (DSBs) are generated by CRISPR/Cas nucleases the predominant repair mechanism in cells is nonhomologous end joining (NHEJ) which usually results in random indels, as well as the obstacles in delivery of sufficient donor repair template (DRT) into the vicinity of the DSB and competition with the original DNA strand/fragment to be replaced in plant cells (Li et al., 2019; Lu et al., 2020; for review, please check Li and Xia, 2020; Zhan et al., 2021; Chen et al., 2022a). In contrast, base editing and prime editing are two alternative promising strategies for precise genome editing without a DSB and a DRT. Whereas base editing has emerged as an alternative and effective tool to HDR-mediated gene replacement for precise single base substitution of an allele with a single SNP, facilitating precise gene editing by transition of one single base to another in a programmable manner (Komor et al., 2016; Nishida et al., 2016; Gaudelli et al., 2017) (Figure 1A–C), prime editing enables the installation of all 12 types of base substitutions and small indels, and substantially expands the scope and capabilities of precision genome editing (Anzalone et al., 2019) (Figure 2A).

Since the development of the first generation of base editor (BE) and prime editor (PE) for base editing and prime editing in mammalian cells (Komor et al., 2016; Nishida et al., 2016; Gaudelli et al., 2017; Anzalone et al., 2019), diverse strategies have been exploited to optimize these two editors in order to improve the precise editing efficiency and specificity, and to expand targeting scopes in plants (Li et al., 2020d; Molla et al., 2021; Hua et al., 2022). Here, we summarize the latest developments of various BEs and PEs, as well as their applications in plants. We also provide recommendations in selection of the proper BEs or PEs in practical applications in plants. Moreover, we propose the perspectives for further optimization of these two editors. We truly believe this review will provide a valuable clue to the readers on how to select the appropriate BEs and PEs, as well as future perspectives to streamline these two editors into the routine and customized platform for both fundamental biological studies and crop improvement.

## BASE EDITORS AND THEIR APPLICATIONS IN PLANTS

Base editing is a breakthrough technology that can precisely and efficiently achieve single base transition or transversion at target sites without inducing DSBs and the need for a DRT. Three BEs are currently in use: cytosine base editors (CBEs) for C:G to T:A transition (Figure 1A), adenine base editors (ABEs) for A:T to G:C transition (Figure 1B) and C-to-G base editors (CGBEs) for C:G to G:C transversion (Figure 1C). Precise base editing enables a single nucleotide substitution in a specific target gene to generate either loss-of-function or gain-of-function mutations, thus greatly accelerating functional annotation, crop improvement, de novo domestication or directed evolution of target genes in crop plants (Ren et al., 2018; Bharat et al., 2020; Kuang et al., 2020; Zeng et al., 2020; Xu et al., 2021a; Yan et al., 2021; Tan et al., 2022). Since the report of the first generation of CBE and ABE in 2016 (Komor et al., 2016) and in 2017 (Gaudelli et al., 2017) in mammalian cells, respectively, many efforts have been attempted in order to optimize and upgrade these two BEs in plants.

### Cytosine base editors

The first-generation CBE was engineered by fusing a rat cytidine deaminase rAPOBEC1 to the N-terminus of an impaired dead Cas9 (dCas9) (Cas9 with D10A and H840A mutations) to generate rAPOBEC1-dCas9 and designated as CBE1 (Komor et al., 2016) (Figure 1D). The substitution of C to T in DNA is created by deaminating the cytosine (C) into uracil (U) in the exposed non-target DNA strand, and the subsequent DNA repair and replication results in C to T base conversion (Figure 1A). The cellular base excision repair (BER) mechanism enables C:G to T:A transition in vivo, while recognizes any G:U base pair as a mismatch. The BER activity eliminates the uracil with the help of uracil *N*-glycosylase (UNG), resulting in a low efficiency of the CBE1 system (Komor et al., 2016). To improve base editing efficiency, the second-generation BE, CBE2 (rAPOBEC1-dCas9-UGI), was constructed by binding a uracil DNA glycosylase inhibitor (UGI) to the C-terminal of CBE1 to prevent the activity of UNG (Komor et al., 2016) (Figure 1E). CBE2 improves editing efficiency by three times and creates few unexpected indels (<0.1%). Subsequently, in order to further improve the editing efficiency, a third-generation CBE, CBE3, with a architecture of rAPOBEC1-nCas9(D10A)-UGI, was engineered by fusing the Cas9 nickase, nCas9(D10A), to rAPOBEC1 and UGI (Komor et al., 2016) (Figure 1F). CBE3 cannot cut dsDNA but can create a nick in the target strand to incite the cellular repair process. Furthermore, in order to improve the deamination activity, a fourth-generation CBE,

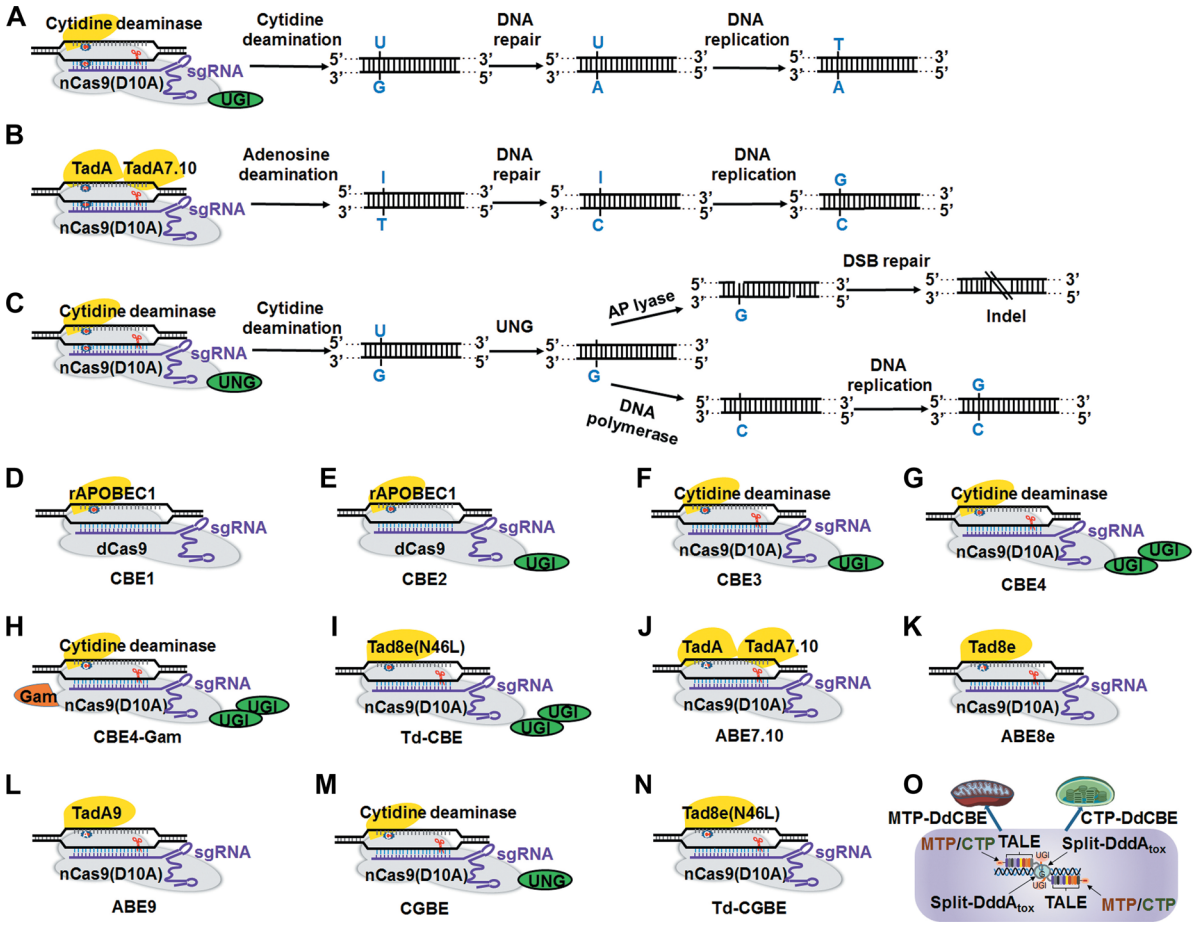


**Figure 1. Structural representations of clustered regularly interspaced short palindromic repeats (CRISPR)/nicking CRISPR-associated protein 9 (nCas9)-mediated base editing and the so far developed base editors**

(**A**) CRISPR/nCas9-mediated cytosine base editing. A cytosine base editor (CBE), which is composed of a catalytically impaired nCas9(D10A) and a cytidine deaminase, binds to the target sequence in the genomic DNA in a guide RNA (gRNA)-programmed manner. The cytidine deaminase catalyzes the deamination of cytosine (C) in a narrow window of the non-target and makes the base change from C to U (uracil) at a target site. U is recognized as thymine (T) during DNA replication, resulting in a C·G to T·A transition. (**B**) CRISPR/nCas9-mediated adenine base editing. An adenine base editor (ABE) is composed of an adenosine deaminase and nCas9(D10A) fusion binding to the target site in a gRNA-programmed manner. The adenosine deaminase catalyzes an A (adenine) to I (inosine) change at the target site. During replication, the original A is replaced with G (guanine). Finally, A·T to G·C conversion is achieved in the non-target DNA strand. (**C**) CRISPR/nCas9-mediated C-to-G base editing. The C-to-G base editor (CGBE) is composed of a cytidine deaminase, nCas9(D10A), and uracil *N*-glycosylase (UNG), and binds to the target site in a gRNA-programmed manner. The cytidine deaminase catalyzes the deamination of cytosine (C) and makes the base change from C to U (uracil). UNG can remove U from the DNA double strands and an error-prone DNA polymerase replaces G with C at the target site. The C·G to-G·C transversion occurs during DNA replication. As nCas9(D10A) nicks the target strand, a DSB is formed when the abasic site on the non-target strand is converted into a nick by an apurinic or apyrimidinic site lyase (AP lyase). The DSB results in indel formation at the target site. (**D**) The first-generation cytosine base editor, CBE1, was engineered by fusing cytidine deaminase, rAPOBEC1 to the N-terminus of a dead Cas9 (dCas9, a mutant of Cas9 containing both D10A and H840A mutations). (**E**) The second-generation base editor, CBE2, was engineered by fusing rAPOBEC1 to the N-terminus of dCas9 and fusing a uracil DNA glycosylase inhibitor (UGI) to the C-terminus of dCas9. (**F**) The third-generation cytosine base editor, CBE3, was engineered by fusing different deaminases to the N-terminus of nCas9(D10A), and fusing UGI to the C-terminus of nCas9 (D10A), respectively. The deaminases that have been successfully applied in plants include rAPOBEC1 (Li et al., 2017; Lu and Zhu, 2017; Zong et al., 2017), PmCDA1 (Shimatani et al., 2017; Zhong et al., 2019), hAID (Ren et al., 2018; Wang et al., 2020a), APOBEC3A (Zong et al., 2018), and evoFENRY (Zeng et al., 2020). (**G**) The fourth-generation cytosine base editor, CBE4, was developed by fusing two UGI molecules to the C-terminal of nCas9 on the basis of CBE3. (**H**) CBE4-Gam cytosine base editor was generated by adding bacteriophage Mu Gam protein to the N-terminus of nCas(D10A), the basis of CBE4. (**I**) The new cytosine base editor, Td-CBE or TadCBEs, was developed by fusing TadA8e (N46L) to the N-terminal of nCas9(D10A) and two UGI molecules to the C-terminal of nCas9(D10A). (**J**) The adenine base editor, ABE7.10, was engineered by fusing a dimer composed of a wild-type adenine deaminase TadA and a directed-evolved adenine deaminase TadA7.10 to the N-terminus of nCas9 (D10A). (**K**) The adenine base editor, ABE8e, was engineered by fusing a more efficient adenine deaminase variant, TadA8e to the N-terminus of nCas9(D10A). (**L**) The adenine base editor, ABE9, was engineered by fusing TadA9 to the N-terminus of nCas9(D10A). TadA9 was obtained by incorporating two mutations, V82S and Q154R, into TadA8e. (**M**) The C-to-G base editor, CGBE, consists of cytosine deaminase, nCas9(D10A) and UNG. (**N**) The C-to-G base editor, Td-CGBE, consists of TadA8e (N46L) and nCas9(D10A). (**O**) Base editors are assembled to edit organelle DNA. For base editing in organelles, DdCBE is split into two parts. Each part consists of a mitochondrion transit peptide (MTP) or a CTP, a transcriptional activator-like effector (TALE) array for binding to a specific organelle DNA target, an inactive Split-DddAtox that works on double-stranded DNA, and a UGI. After the MTP/CTP transports the two parts into the mitochondrial/chloroplast matrix, the bindings of two TALE arrays to the nearby target organelle DNAs, respectively, lead to the two inactive Split-DddAtox in proximity to work as an active form. Then, it performs deamination of C in the double-stranded DNA and finally induces a C·G to T·A transition.

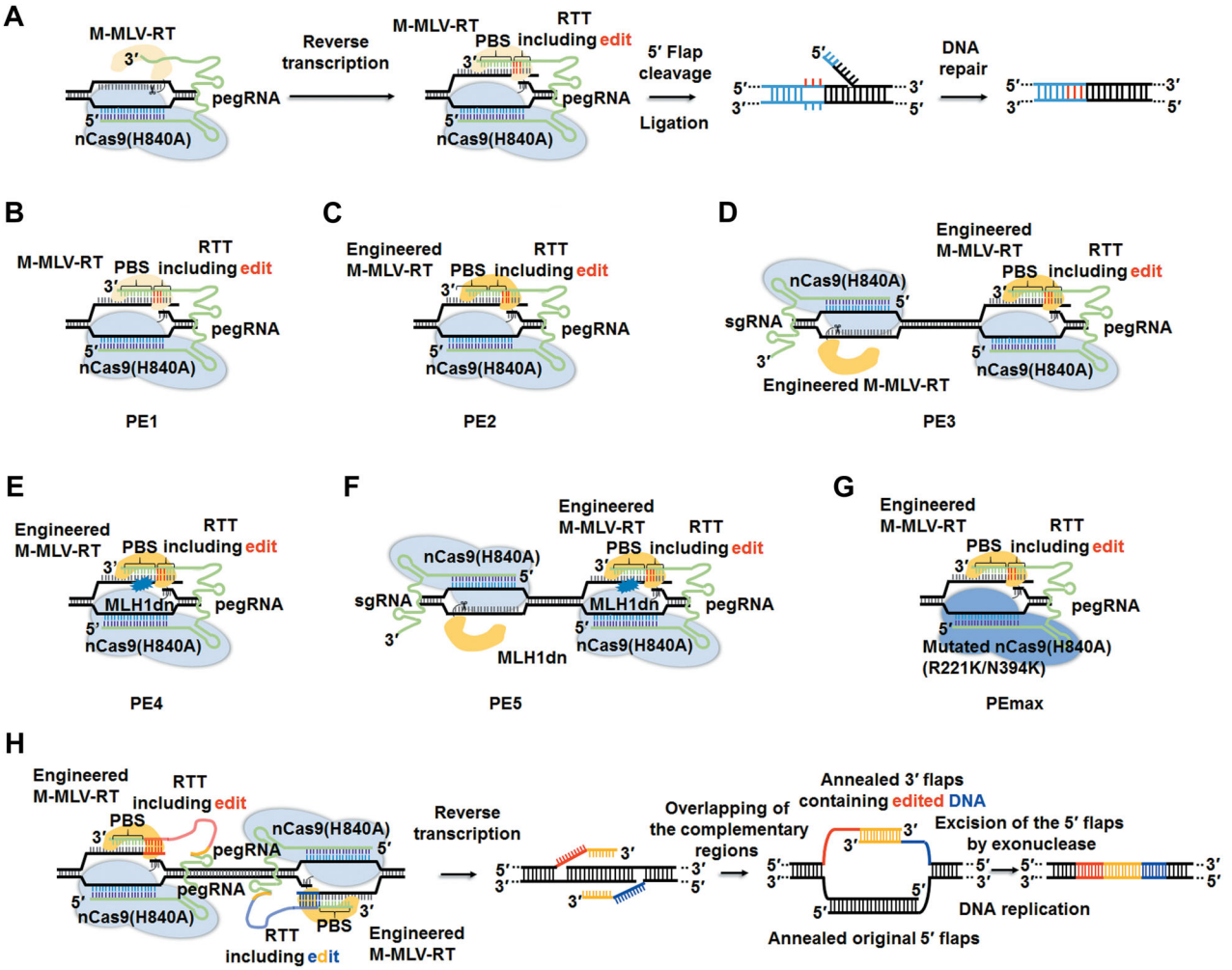


**Figure 2. The principle of clustered regularly interspaced short palindromic repeats (CRISPR)/nicking CRISPR-associated protein 9-mediated prime editing and schematic diagrams of the so far developed prime editors**

(**A**) The CRISPR/nCas9-mediated prime editing system. A prime editor mainly consists of a catalytically impaired nCas9(H840A), a M-MLV-RT (Moloney murine leukemia virus reverse transcriptase), and a prime editing guide RNA (pegRNA). pegRNA is composed of three components, including a single-guide RNA (sgRNA) targeting the specific site, a reverse transcription template (RTT) encoding the desired edit, and a primer binding site (PBS) initiating RT. The nCas9(H840A)-M-MLV-RT and pegRNA complex bind to the target sequence in the genomic DNA in a sequence-specific manner. The M-MLV-RT helps the 3' DNA end from the PBS to prime the reverse transcription of an edit-encoding extension from pegRNA directly into the target site. (**B**) The first-generation prime editor, PE1, was engineered by fusing a wild M-MLV-RT to the N-terminus of nCas9(H840A). (**C**) The second-generation prime editor, PE2, was engineered by fusing an engineered M-MLV-RT with six amino acid mutations to the N-terminus of nCas9(H840A). (**D**) The third-generation prime editor, PE3, was engineered by using an additional sgRNA on the non-targeting strand. (**E**) The fourth-generation prime editor, PE4, was developed with co-expression of a dominant negative mismatch repair (MMR) protein (MLH1dn) on the basis of PE2. (**F**) The fifth-generation prime editor, PE5, was developed with transient co-expression of a dominant negative MMR protein (MLH1dn) on the basis of PE3. (**G**) PEmax was engineered by replacing nCas9(H840A) with a mutated version which harbors R221K and N394K mutations. (**H**) Overview of the design of twinPE or GRAND editor and the sequence replacement process. The single-strand DNAs (red and blue lines) produced by the paired pegRNAs containing RTTs highlighted in light red and light blue, respectively, bind to each other through their complementary ends highlighted in orange. The original 5' flaps were replaced by the aannealed 3' flaps containing the edited DNA following DNA replication and repair.

CBE4, was developed by fusing two UGI molecules to the C-terminal of Cas9 nickase on the basis of CBE3 to enhance the inhibition of UNG (Komor et al., 2017) (Figure 1G). Compared with CBE3, CBE4 not only improves the base editing efficiency but also reduces the frequency of C to A or G transversions by 2.3 times. In addition, bacteriophage Mu Gam protein was added on the basis of CBE4 to construct a BE CBE4-Gam, in order to further improve the product purity and reduce the occurrence of indels (Komor et al., 2017) (Figure 1H).

CBEs, especially CBE3 and CBE4, have been widely used in plants. Initially, a base editing system was developed using a rat APOBEC1 in rice. To validate and test the feasibility of CBEs in plants, by fusing a rat APOBEC1 to the N-terminus of nCas9 (D10A) to form a structure of rAPOBEC1-nCas9 (D10A), two agriculturally important genes of rice, *OsNRT1.1B* and *OsSLR1*, were edited at editing efficiencies of 2.7% and 13.3%, respectively (Lu and Zhu, 2017) (Table 1). Simultaneously, three targets in rice, one target (P2)

**Table 1. Base editing in plants**

| Base editors | Structures of various base editors | Species | Target gene | Transformation | Selection | Editing efficiency (%) | Editing window | Trait improvement | References |
|---|---|---|---|---|---|---|---|---|---|
| APOBEC1-CBE2/CBE3 | APOBEC1-XTEN-nCas9 | Rice | *OsNRT1.1B, OsSLR1* | *Agrobacterium* | Hygromycin | 2.70–13.30 | C4–C8 | High nitrogen use efficiency; Dwarf | Lu and Zhu (2017) |
| | APOBEC1-XTEN-nCas9-UGI | Rice | *OsSBEIIb, OsPDS* | Bombardment | Hygromycin | 20.00 | C4–C8 | High amylose | Li et al. (2017) |
| | APOBEC1-XTEN-nCas9/dCas9-UGI | Rice, Wheat Maize | *OsCDC48, OsNRT1.1B, OsSPL14, TaLOX2, ZmCENH3* | *Agrobacterium* /Bombardment | Herbicide | 0–43.48 | C3–C9 | High nitrogen use efficiency; High yield | Zong et al. (2017) |
| | APOBEC1-XTEN-nCas9-UGI | Rice | *OsCERK1, OsSERK1, OsSERK2, ipa1, Pi-ta* | *Agrobacterium* | Hygromycin | 10.50–38.90 | C4–C8 | High yield; Blast resistance | Ren et al. (2017) |
| | APOBEC1-XTEN-nCas9-UGI | Wheat | *TaALS-P174* | Bombardment | Herbicide | 33.00–75.00 | C4–C8 | Herbicide resistance | Zhang et al. (2019) |
| | APOBEC1-XTEN-nCas9-UGI | Cotton | *GhCLA, GhPEBP* | *Agrobacterium* | Hygromycin | 0–57.78 | C4–C8 | – | Qin et al. (2020) |
| hAID-CBE3 | hAID-XTEN-nCas9 | Rice | *OsFLS2, OsAOS1, OsJAR1, OsJAR2, OsCOI2, OsPi-D2* | *Agrobacterium* | Hygromycin | 8.30–73.30 | C3–C8 | Blast resistance | Ren et al. (2018) |
| | hAID-nSpCas9-NG/nCas9-UGI | Rice | *OsBZR1, OsSERK2* | *Agrobacterium* | Hygromycin | 4.44–27.08 | C3–C8 | Enhance fruit quality | Ren et al. (2019) |
| | hAID-nScCas9-UGI<br>hAID-nSpCas9-UGI | Rice | *OsMPK9, OsMPK17, OsCPK5, OsMPK15, OsMPK16, OsCPK6, OsCPK7, OsCPK8* | *Agrobacterium* | Hygromycin | 2.56–97.92<br>0–95.83 | C3–C8 | – | Wang et al. (2020a) |
| | hAID-XTEN-nSpRY-UGI | Rice | *OsCOI2, OsBSR, OsMPK13, OsGS1, OsGSK4* | Agrobacterium | Hygromycin | 26.00–34.15 | C3–C8 | – | Xu et al. (2021c) |
| APOBEC3A-CBE3/CBE4 | A3A-XTEN-nCas9-UGI; Gam-XTEN-A3A-nCas9-UGI-UGI | Rice , Wheat Potato | *OsAAT, OsCDC48, OsDEP1, OsNRT1, OsOD, TaALS, TaMTL, TaLOX2, StGBSS-T6* | *Agrobacterium* /Bombardment | Herbicide/Hygromycin | 0–82.90<br>1.20–20.00 | C1–C17 | Herbicide resistance | Zong et al. (2018) |
| PmCDA1-CBE2/CBE3/CBE4 | nCas9/dCas9-PmCDA1 | Rice , Tomato | *ALS, DELLA, ETR1.* | *Agrobacterium* | Herbicide/Hygromycin | 26.20–53.80 | C2–C5 | Herbicide resistance | Shimatani et al. (2017) |
| | PmCDA1-xCas9-UGI<br>PmCDA1-nSpCas9-NG-UGI | Rice | *OsDEP1, OsCDC48, OsGS3, OsPDS* | *Agrobacterium* | Hygromycin | 0–21.10<br>3.50–56.30 | C2–C5 | – | Zhong et al. (2019) |
| | nCas9-NG-PmCDA1-UGI | Tomato potato | *SlALS* | *Agrobacterium* | Hygromycin | 32.00 | C2–C5 | Herbicide resistance | Veillet et al. (2020) |
| | PmCDA1-nScCas9$^{+}$$^{+}$-UGI-UGI | Rice | *OsWaxy, OsEUI1* | *Agrobacterium* | Hygromycin | 8.3–86.1 | C1–C17 | Reduced amylose content | Liu et al. (2021b) |
| CBE4 | FENRY-nCas9-NG-UGI-UGI | Rice | *OsCKX2, OsWaxy, OsEUI1, OsSPL4, OsSPL7, OsSPL14,* | *Agrobacterium* | Hygromycin | 0–86.30<br>0–59.40 | C4–C12<br>C2–C5 | – | Zeng et al. (2020) |

Continued

**Table 1. Continued**

| Base editors | Structures of various base editors | Species | Target gene | Transformation | Selection | Editing efficiency (%) | Editing window | Trait improvement | References |
|---|---|---|---|---|---|---|---|---|---|
| | PmCDA1-nCas9-NG-UGI-UGI APOBEC1-nCas9-NG-UGI-UGI | | *LF1, OsIAA13, OsMADS57, OsGBSSI* | | | 0–66.70 | C4–C8 | | |
| ABE7.10 | TadA-TadA7.10-nCas9(D10A) | Rice , Wheat | *OsALS, OsCDC48, OsAAT, OsDEP1, OsACC, OsNRT1.1B, OsEV, OsOD, TaDEP1, TaEPSPS, TaGW2* | *Agrobacterium* /Bombardment | Herbicide/ Hygromycin | 3.20–59.10 | A4–A8 | Herbicide resistance | Li et al. (2018) |
| | TadA-TadA7.10-nCas9 | Rice | *OsSERK2, OsMPK6, OsWRKY45, OsMPK13* | *Agrobacterium* | Hygromycin | 6.45–62.26 | A4–A8 | – | Yan et al. (2018) |
| | TadA-TadA7.10-nSpCas9-NG TadA-TadA7.10-nSaCas9 | Rice | *OsSPL7, OsSPL14, OsSLR1, OsSPL4, OsSPL16, OsSPL17, OsSPL18* | *Agrobacterium* | Hygromycin | 0–26.00 4.80–61.30 | A4–A8 | High yield | Hua et al. (2018) |
| | TadA-TadA7.10-nScCas9 | Rice | *OsMPK14, OsCPK9, OsMPK15, OsCPK10* | *Agrobacterium* | Hygromycin | 50.00–94.12 | A4–A8 | – | Wang et al. (2020a) |
| ABE-P1S | TadA7.10-nSpCas9 TadA7.10-nSaCas9 | Rice | *OsSERK2, OsSPL14, SLR1, Tms9-1, OsNRT1.1B, OsACC1, OsDEP1* | *Agrobacterium* | Hygromycin | 4.50–96.30 0–61.10 | A1–A12 | High yield; Herbicide resistance | Hua et al. (2020b) |
| ABE8e | TadA8e(V106W)-nCas9 TadA8e(V106W)-nCas9-NG | Rice | *OsEPSPS, OsALS, OsWaxy* | *Agrobacterium* | Herbicide/ Hygromycin | 4.00–100.00 0–100.00 | A4–A8 | Herbicide resistance | Wei et al. (2021) |
| | TadA8e-XTEN-nSpRY | Rice | *OsCOI2, OsBSR, OsMPK13, OsGS1, OsGSK4* | *Agrobacterium* | Hygromycin | 27.79–93.75 | A3–A10 | Herbicide resistance | Xu et al. (2021c) |
| | TadA8e-DBD-nCas9-NG TadA8e-DBD-nSpG TadA8e-DBD-nSpRY | Rice | *OsSPL14, OsIAA13, OsSPL7, OsLF1, OsGBSSI, OsCKS2, OsEUI1, OsTS* | *Agrobacterium* | Hygromycin | 0–90.50 0–92.50 0–100.00 | A1–A14 A1–A14 A1–A14 | – | Tan et al. (2022) |
| ABE9 | TadA9-XTEN-nSpCas9 TadA9-XTEN-nSpCas9-NG TadA9-XTEN-nSpRY TadA9-XTEN-nScCas9 | Rice | *OsMPK6, OsMPK13, OsSERK2, OsWRKY45, OsDEP2, OsETR2, OsGSK4, OsJAR1, OsGS1, OsALS1* | *Agrobacterium* | Herbicide/ Hygromycin | 0–97.92 0–100.00 0–37.50 0–68.75 | A1–A12 A4–A10 A3–A10 A4–A12 | – | Yan et al. (2021) |
| pDuBE1 | TadA8e-nCas9-CDA1-UGI | Rice | *OsALS, OsBADH2, OsLAZY1, OsPDS* | *Agrobacterium* | Herbicide/ Hygromycin | 0.40–87.60 | C2–C5 A4–A8 | – | Xu et al. (2021a) |

**Table 1. Continued**

| Base editors | Structures of various base editors | Species | Target gene | Transformation | Selection | Editing efficiency (%) | Editing window | Trait improvement | References |
|---|---|---|---|---|---|---|---|---|---|
| CGBE | Anc689-nCas9-OsUNG<br>Anc689(R33A)-nCas9-OsUNG | Rice | *OsIPA1*, *OsBZIP5*, *OsSLR1*, *OsALS1*, *OsNRT1.1B* | *Agrobacterium* | Herbicide/ Hygromycin | 0–40.60<br>9.70–52.50 | C4–C9 | High nitrogen use efficiency; Herbicide resistance | Tian et al. (2022) |
| DdCBE | cTP-TALE-L-nDdA-UGI-cTP-TALE-R-cDdA-UGI | Rice | *OspsaA* | *Agrobacterium* | Hygromycin | 97.50 | C3–C13 | High photosynthetic efficiency, High yield | Li et al. (2021b) |
| | PTP-TALE-L-nDdA-UGI-PTP-TALE-R-cDdA-UGI | Arabidopsis | 16s *rRNA*, *rpoC1*, *psbA* | *Agrobacterium* | Hygromycin | 0–86.40 | C3–C13 | – | Nakazato et al. (2021) |

in *OsPDS*, which encodes a phytoene desaturase, and two targets (S3 and S5) in *OsSBEIIb*, which encodes a starch branching enzyme IIb, were successfully edited in rice by using CBE3 with the efficiencies of 19.2%, 10.5%, and 1.0% at the S5, S3, and P2 targets, respectively (Li et al., 2017) (Table 1). Meanwhile, targeted C·G to T·A transitions in *OsCDC48*, *OsSPL14*, *OsNRT1.1B*, *TaLOX2*, *ZmCENH3* genes were achieved at frequencies of up to 43.48% from position 3 to 9 within the protospacer in the genomes of rice, wheat and maize by using a nCas9-cytidine deaminase fusion (Zong et al., 2017) (Table 1). Although the editing efficiencies of CBEs has been improved to a certain extent, its application is limited by the narrow editing window and some gene loci cannot be effectively edited.

To further improve editing efficiency and expand the editing window, many deaminases from different species or the evolved deaminases have been investigated in different crop species. For example, the human AID (hAID) mutant version (hAID*Δ), another type of cytosine deaminase with a deamination priority on GC and AC, was applied in CBE optimization. The *OsFLS2* gene was successfully edited at 57.0% editing efficiency in rice and novel rice blast resistance germplasm was obtained by precise editing of the *pi-d2* gene at 30.8% editing efficiency (Ren et al., 2018) (Table 1). Another research reported that the introduction of multiple herbicide resistance point mutations in acetolactate synthase (ALS) was induced in rice and tomato by employing the BE comprising of either dCas9 or nCas9(D10A) fused to *Petromyzon marinus* cytidine deaminase (PmCDA1) with editing frequencies from 26.2% to 53.8% (Shimatani et al., 2017) (Table 1). A BEACON base editing system by fusing dCas12a with a human deaminase APOBEC3A (hA3A) was developed to achieve enhanced deamination efficiency and editing specificity (Wang et al., 2020b). Consequently, A3A-PBE, composed of a nCas9(D10A) and hA3A, was reported to improve the efficiency of base editing in wheat, rice, and potato with a 17-nucleotide editing window at all examined sites, independent of sequence context (Zong et al., 2018). Later on, a BE, PhieCBE, consisting of a codon-optimized evolved cytidine deaminase, evoFERNY, which is derived from a wild-type deaminase Anc656, exhibited the highest editing efficiency (86.3%) at the center region (C3-C10) in editing windows of the respective the target genes (Zeng et al., 2020) (Table 1). Collectively, the PhieCBE has a superior editing efficiency compared with the other reported plant CBEs.

Recently, a novel CBE, designated as Td-CBEs or TadCBEs, was developed by either re-engineering or by phage-assisted continuous evolution of the adenine deaminase TadA-8e for efficient and specific CRISPR-based cytosine base editing, respectively (Neugebauer et al., 2022; Chen et al., 2022c). Introduction of a N46L mutation in TadA-8e eliminated its adenine deaminase activity. By fusion of a series of TadA-8e mutants with UGIs, several Td-CBEs were developed either with a high activity similar to that of CBE4max or with higher accuracy compared to previously reported CBEs for C:G to T:A base editing (Figure 1I) (Chen et al., 2022c). At the same time, an evolved TadA8e which

enables cytidine deamination was obtained by phage-assisted continuous evolution (Neugebauer et al., 2022). This evolved TadA cytidine deaminases containing mutations at DNA-binding residues that alter enzyme selectivity to strongly favor deoxycytidine over deoxyadenosine deamination. Compared to commonly used CBEs, TadA-derived cytosine BEs (TadCBEs) (Figure 1I) offer similar or higher on-target activity, smaller size and substantially reduced Cas-independent DNA and RNA off-target activity (Neugebauer et al., 2022).

It is worth noting that the strict requirement of protospacer adjacent motif (PAM) of canonical NGG for Cas9 (refers to SpCas9 from *Streptococcus pyogene* hereafter unless specified) impedes the wide application of the base editing system due to the limitation of targetable sites in a specific gene in plants. To expand the range of PAM sites and achieve efficient and highly specific genome editing, the efficacy of ScCas9, xCas9, and SpCas9-NG was evaluated in performing base editing in different plants (Hua et al., 2019; Ren et al., 2019; Zhong et al., 2019; Wang et al., 2020a; Liu et al., 2021b). An orthologous Cas9 protein from *Streptococcus canis* (ScCas9), which broadens the targeting range of BEs, enabled highly efficient target gene mutagenesis at NAG sites compared to NGG, NTG, and NCG sites (Wang et al., 2020a). Furthermore, codon-optimized ScCas9$^{++}$ fused PmCDA1 achieved stable and efficient multi-site base editing at NNG-PAM sites with a wider editing window (C1-C17) and without target sequence context preference (Liu et al., 2021b) (Table 1). In addition, xCas9 can efficiently induce mutations at target sites with NG and GAT PAM sequences with higher target specificity than the nSpCas9 (Hua et al., 2019; Zhong et al., 2019). Furthermore, nSpCas9-NG showed a powerful editing activity at sites with a variety of NG PAMs, and had much higher editing efficiency than xCas9 at NG-PAM sites (Hua et al., 2019; Zhong et al., 2019) (Table 1). The broad PAM compatibility of SpCas9-NG enables its related genome editing tools to be more efficient (Ren et al., 2019; Zhong et al., 2019). Therefore, SpCas9-NG is a preferred variant for targeting relaxed PAMs in plant genome editing. Shortly afterward, two structurally engineered SpCas9 variants, SpG and SpRY (Walton et al., 2020), greatly further expand the scope of base editing with highly flexible PAM recognition but reduced editing efficiency to a certain extent in rice (Li et al., 2021a; Ren et al., 2021; Xu et al., 2021c; Zhang et al., 2021) (Table 1). Collectively, the CBE tools composed of Cas9 variants fused with different cytidine deaminases will certainly expand the targeting scope of base editing in rice and other crop plants.

## Adenine base editors

Similar to CBE in both structure and base editing mechanisms, ABE is composed of nCas9 (D10A) fused with an artificially evolved adenosine deaminase, which helps to convert adenine (A) to inosine (I), and then DNA repair and replication to create A:T to G:C base substitution (Gaudelli et al., 2017) (Figure 1B). First, ABE7.10 was engineered by fusing nCas9 (D10A) with a dimer composed of wild-type adenine deaminase TadA and an evolved adenine deaminase TadA7.10, with the editing window at positions of 4–8 nt in the protospacer region (counting the PAM as positions 21–23 nt) (Figure 1J). Subsequently, the editing efficiency of the ABE7.10 was increased by codon optimization and adding an additional nuclear localization sequence (NLS) in mammalian cells (Koblan et al., 2018). Furthermore, ABEmax was developed by adding an additional NLS at both ends of ABE7.10, with the editing efficiency of less than 50% at most target sites (Hua et al., 2018; Li et al., 2018; Yan et al., 2018). ABEmax introduced an A:T to G:C conversion in *OsACC* (Li et al., 2018), *OsMPK6*, *OsSERK2*, and *OsWRKY45* at frequencies of 17.6%, 32.1%, and 62.3%, in rice plants, respectively (Yan et al., 2018) (Table 1). Moreover, a simplified BE ABE-P1S containing TadA7.10-nCas9 (D10A) showed much higher editing efficiency in rice than the widely used TadA-TadA7.10-nCas9 (D10A) fusion (Hua et al., 2020b). Recently, ABE8e was further developed by using a more efficient adenine deaminase variant, TadA8e, which has been artificially evolved from TadA7.10 (Gaudelli et al., 2020; Richter et al., 2020) (Figure 1K). ABE8e deaminates the target base over a thousand times faster than the previous ABE7.10, and significantly improves the efficiency of A-to-G conversion (Richter et al., 2020). Further, the mutation of V106W was also introduced in TadA8e to reduce the off-target effects (Richter et al., 2020). Later on, a high-efficiency ABE, rABE8e (rice ABE8e), was developed in rice by combining the codon-optimized monomeric TadA8e and bis-bpNLS (NLS at both the N and C termini) (Wei et al., 2021). The rABE8e substantially increased editing efficiencies on NG-PAM and NGG-PAM target sequences compared with ABEmax in rice. For most targets, rABE8e exhibited nearly 100% editing efficiency and higher homozygous ratio substitution were achieved within the editing window, especially at positions A5 and A6 (Wei et al., 2021) (Table 1). Recently, a more efficient ABE toolbox (PhieABE) was developed based on hyTadA8e by fusing TadA8e and a single-stranded DNA-binding domain (DBD). The PhieABE has significantly higher base editing activity and broader editing windows compared with the general ABE8e systems (Tan et al., 2022). At last, a more efficient adenosine deaminase, TadA9, was obtained in rice by incorporating two mutations, V82S and Q154R, into TadA8e (Yan et al., 2021). TadA9 is compatible with nSpCas9, nSpCas9-NG, and nScCas9, as well as near-PAM-less SpRY (Table 1). Importantly, in comparison with TadA8e, TadA9 expands the editing window, especially for previously difficult-to-edit endogenous target sites, showing strong editing capabilities in commercial rice cultivars (Yan et al., 2021) (Figure 1L).

Further, a CRISPR-based system named simultaneous and wide editing induced by a single system (SWISS) was also developed, which could induce multiplexed and simultaneous base editing in rice (Li et al., 2020b). The SWISS works in the principle that an RNA aptamer added at the end of a single-guide RNA (sgRNA) scaffold could recruit its binding proteins fused with cytidine deaminase or adenine deaminase to the nCas9 (D10A) to function at target sites. This facilitates the generation of both C:G to T:A and A:T to G:C transitions within the editing window at a specific target of a gene. However, the efficiency of SWISS remains to be improved in plants.

Selecting the base-edited cells from massive transformed calli for regeneration is time-consuming and labor-intensive during plant tissue culture, especially for the low-efficient editing events. To improve the screening efficiency of base-edited callus, a Discriminated sgRNAs-based SurroGate system (DisSUGs) was established (Xu et al., 2020b). This system enables the enrichment of base-edited events at the target site on the hygromycin selection medium when a mutated hygromycin resistance gene in the transforming vector is corrected to be the functional wild-type by CBE or ABE.

## The C-to-G base editor

Although CBEs and ABEs have been broadly applied in various organisms including plants, CBEs and ABEs only induce base transition rather than base transversion. An uracil DNA glycosylase, UNG, widely exists in animal and plant cells, as well as in bacteria, and can remove U from the DNA double strands and initiate BER (Figure 1C). CGBE, a C-to-G or C to A BE, consisting of a rAPOBEC1 cytidine deaminase variant (R33A), nCas9 (D10A), and an UNG, has recently been reported that enables efficient C-to-G editing in bacteria and mammalian cells (Kurt et al., 2021; Zhao et al., 2021) (Figure 1M). By optimizing the codon of UNG, the OsCGBE03 BE was generated, which could achieve C-G editing in rice. The efficiency of OsCGBE03 was tested on five endogenous genes (*OsIPA1*, *OsbZIP5*, *OsSLR1*, *OsALS1*, and *OsNRT1.1B*), and C-to-G base transversion was achieved at an average frequency of 21.3% in rice plants (Tian et al., 2022) (Table 1). Interestingly, introduction of a N46L mutation in TadA-8e eliminated its adenine deaminase activity and resulted in a TadA-8e-derived C-to-G BE (Td-CGBE) capable of highly efficient and precise C·G to G·C editing (Figure 1N) (Chen et al., 2022c). However, the feasibility of Td-CGBE in plants remains to be investigated. Together, CGBE expands the base editing tools, providing a powerful tool for generating more base substitution types in precise crop breeding as well as creating new germplasm resources.

## Applications of BEs in plants

Since the first report of CBEs and ABEs, base editing has been successfully applied in various crop plants, including rice (Li et al., 2017; Ren et al., 2017; Shimatani et al., 2017; Zong et al., 2017; Ren et al., 2018; Ren et al., 2019; Liu et al., 2021b), wheat (Zong et al., 2017; Zong et al., 2018), maize (Zong et al., 2017), cotton (Qin et al., 2020), potato (Zong et al., 2018; Veillet et al., 2020), tomato (Veillet et al., 2020) and other plant species (Nakazato et al., 2021).

CBE systems with APOBEC1 were first successfully applied in improvement of important crop traits simultaneously by some laboratories. In rice, two targets in *OsSBEIIb* were successfully edited, and disruption of an intron-exon boundary in *OsSBEIIb* gene resulted in high-amylose rice (Li et al., 2017) (Table 1). Simultaneously, successfully edited *OsNRT1.1B* improved nitrogen use efficiency, and edited *OsSLR1* led to obviously dwarfed rice plants (Lu and Zhu, 2017; Zong et al., 2017) (Table 1). Meanwhile, targeted C·G to T·A transition in *OsSPL14* increased the grain yield of rice (Zong et al., 2017) (Table 1). It was reported that the *ALS* gene has also been successfully edited in rice, wheat, tomato and potato plants using CBEs with APOBEC1, APOBEC3A and PmCDA1, making these crops resistant to herbicides (Shimatani et al., 2017; Zong et al., 2018; Zhang et al., 2019; Veillet et al., 2020) (Table 1). The *pi-d2* gene was successfully edited by CBE3 with hAID and novel rice blast resistance germplasm was obtained in rice (Ren et al., 2018) (Table 1). CBE3 with engineered nCas9-NG enabled C·G to T·A conversion in *OsBZR1* and *OsSERK2* genes and grain quality of rice was enhanced (Ren et al., 2019) (Table 1). In allotetraploid cotton, a CBE3 system has relatively high specificity and accuracy for the generation of targeted point mutations in *GhCLA* and *GhPEBP* genes (Qin et al., 2020) (Table 1). Additionally, editing *Oswaxy* gene by PmCDA1 fused with nScCas9$^{++}$ generated rice lines with increased amylopectin content (Liu et al., 2021b) (Table 1). Recently, it was shown that CGBE could work in crop plants (Tian et al., 2022). Targeted C·G to G·C transversion in *OsALS1* and *OsNRT1.1B* endowed rice plants with high nitrogen use efficiency and herbicide resistance (Table 1). Therefore, the CBE or CGBE tools composed of nickases of different Cas9 variants fused with different cytidine deaminases will certainly expand the target scope in precise crop breeding and creating new germplasm resources for crop improvement.

ABEs have also been widely applied in breeding in recent years due to their improved efficiency in plants. Targeted A·T to G·C conversion in *OsSPL14* increased the yield of rice grain (Hua et al., 2018) (Table 1). Meanwhile, an OsACC with C2186R substitution endowed rice plants with herbicide tolerance and was generated using the ABE7.10 system (Li et al., 2018) (Table 1). Moreover, a simplified BE ABE-P1S successfully edited *OsSPL14* and *OsALS*, leading to high yield and herbicide tolerance, respectively (Hua et al., 2020b) (Table 1). Later on, *OsWaxy* and *OsALS* were edited more efficiently at frequencies up to 100% in rice by using ABE8e (Wei et al., 2021) (Table 1). Successful applications of ABEs in plants will certainly benefit biological research and crop improvement.

Plant organelles, including chloroplasts and mitochondria, contain DNA independent of the nuclear genome, encoding many genes necessary for photosynthesis and respiration, respectively. Base editors have been widely used in nuclei, but there are few BEs used in organelles. In a previous study, it was found that a kind of modified genomic RNA (gRNA) with a 20-bp stem-loop element of nuclear ribonuclease P added in the 5′ terminal of the sgRNA could guide the mitochondrial localization signal peptide labeled Cas9 to mediate sequence-specific DNA cleavage in mitochondria (Hussain et al., 2021) (Figure 1O). Further, the CRISPR-independent mitochondrial genome editing technology had been developed based on the TALEN technology (Kazama et al., 2019). For example, the BE for editing the human mitochondrial genome was developed by fusing a split half of the deaminase domain of the bacterial toxin DddA (DddAtox), transcriptional activator-like effectors (TALEs) and UGI (Mok et al., 2020). This DddAtox-derived CBEs (DdCBE) can catalyze the deamination of cytosines of

dsDNA, resulting in efficient C:G to T:A conversion in the human mitochondrial genome with high target specificity (Mok et al., 2020). Similar research was performed in lettuce (*Lactuca sativa*) and rapeseed (*Brassica napus*) protoplasts with up to 23% efficiencies (Kang et al., 2021). Recently, using the DdCBE linked to a plastid-targeting signal peptide (PTP) of AtRecA1 protein at its N-terminus, three target genes (16s rRNA, rpoC1, psbA) located in the plastid genome were successfully edited without leaving any foreign genes in either the plastid or nuclear genomes in *Arabidopsis* (Nakazato et al., 2021) (Figure 1O; Table 1). Furthermore, an efficient DdCBE system was constructed by fusing a chloroplast transition peptide (CTP) to its N-terminus. This CTP-DdCBE achieved a conserved chloroplast gene chlorophyll A of photosystem I (*psaA*), for C to T transitions in rice chloroplasts (Li et al., 2021b) (Figure 1O; Table 1). The edited *psaA* could potentially improve photosynthetic efficiency and grain yield of crops. The successful implementation of DdCBEs (CRISPR-independent organelle BEs) in plant organelle cells increases the possibility of precise manipulation of organelle genomes for crop improvement (Kang et al., 2021).

Single nucleotide variation is the genetic basis for the improvement of important crop traits. Random mutagenesis by physical or chemical methods has long been applied to improve traits in plants, but it is labor-intensive and time-consuming. The base editing system can enable the artificial evolution of agriculturally important genes in current crop varieties to develop novel gene resources and germplasm. A base editing (CBE and/or ABE) mediated gene evolution (BEMGE) strategy was developed to obtain novel allelic variants in *OsALS* and *OsACC* in rice (Kuang et al., 2020; Liu et al., 2020; Wang et al., 2022b) (Table 1). At the same time, dual-base editor named saturated targeted endogenous mutagenesis editor (STEME) is capable of simultaneously performing C to T and A to G transitions by a single sgRNA in rice (Li et al., 2020a), albeit at a relatively low efficiency. Later on, another efficient plant dual-base editor, DuBE (pDuBE1), was engineered by fusing eCDAL with TadA8e (Xu et al., 2021a). The editing efficiency of pDuBE1 was up to 87.6%, and the frequency of simultaneous A to G and C to T conversions were as high as 49.7% in stably transformed rice plants (Xu et al., 2021a) (Table 1). This dual-BE enables robust dual editing in plant genomes, providing a powerful tool for direct evolution in crops.

### Off-targeting of BEs

Certain genomic sites, homology to the target sequence of sgRNA, can also be bound and edited during the base editing, resulting in unpredictable off-target effects. Currently, genome-wide and unbiased analyses of the off-target effects of BEs in vivo have been conducted via whole-genome sequencing of rice plants generated by CBEs and ABEs. The whole-genome sequencing analysis revealed that CBEs, but not ABEs, induce substantial genome-wide off-target mutations, mainly C to T single nucleotide variants, and overexpression of cytosine deaminase, and UGI increases global C to T conversion (Jin et al., 2019). Further, 27 predicted potential off-target sites were analyzed by targeted deep sequencing, and it was found that some C > T substitutions were detected in the editing windows of these sites in allotetraploid cotton (Qin et al., 2020). To avoid off-target effects, some web tools can be employed to predict them. For example, CRISPR-GE (http://skl.scau.edu.cn/) provides a set of tools for the design of target-sgRNAs (targetDesign), prediction of off-target sites (offTarget) (Xie et al., 2017). Moreover, deep sequencing of edited plants can facilitate the identification of off-target mutants. However, it is worth noting that in crop breeding practice, the poor agronomic traits caused by off-target mutations and spontaneous mutations will be eliminated following by phenotype selection during the breeding process. Therefore, in contrast to base editing in mammalian cells for gene correction and gene therapy, from a technical point of view, off-target effects of CBEs and ABEs in plants do not actually pose any threat in crop improvement (Tang et al., 2019; Manghwar et al., 2020).

## PRIME EDITORS AND THEIR APPLICATIONS IN PLANTS

### The current developed major PEs

A search-and-replace genome editing method, also known as prime editing, has been developed to install precise small indels, all kinds of single or multiple base(s) substitutions (transitions and transversions) and their combinations at a target site in mammalian cells without requiring DSBs and DRT (Anzalone et al., 2019). Prime editor is composed of a catalytically impaired nCas9(H840A) fused with a reverse transcriptase, a M-MLV-RT (Moloney murine leukemia virus reverse transcriptase), at the C-terminus (Figure 2A). A prime editing guide RNA (pegRNA) is constituted of three components, including a sgRNA targeting the specific site, a reverse transcript encoding the desired edit as template (RTT), and a primer binding site (PBS) initiating reverse transcription (Figure 2A). In prime editing, the protein complex binds the target DNA and induces a nick at the non-target strand, from which the resulting 3′ DNA terminal hybridizes to the PBS and then starts reverse transcription, and to eventually copy the desired mutation into the genomic DNA following DNA replication and repair (Figure 2A). Protein engineering and elaborated guide RNA designs contributed to the advent of several generation of PEs, from PE1 to PE2 and then to PE3 and PE3b as well as the following generations, with a gradual improvement in editing efficiency and/or product purity (Figure 2). For example, PE1 was first generated by fusing a wild-type M-MLV-RT to the C-terminus of nCas9(H840A) (Figure 2B). To enhance the prime editing efficacy, PE2 was generated by replacing the original M-MLV-RT with an engineered M-MLV-RT with six mutations H9Y+D200N+T306K+W313F+T330P+L603W (Figure 2C). Then PE3 was further developed by using another nicking sgRNA at various distances from the nicks induced by pegRNA to direct a second cut on the non-edited strand to further increase the editing efficiency (Figure 2D). However, as two single-strand breaks

were induced nearby on opposite DNA strands, the PE3 system may induce NHEJ and thus exhibited higher indel frequencies. To suppress unwanted indels, PE3b uses a specific sgRNA complementary to the edited DNA strand, which induces the second nick after the edited flap is incorporated into the genomic DNA. Furthermore, manipulation of DNA repair pathway is an alternative method to obtain efficient prime editing. It is reported that inhibiting the key factors involved in DNA mismatch repair (MMR) pathway, such as MSH2, MSH6, MLH1 and PMS2, can effectively enhance the prime editing efficiency (Chen et al., 2021). Based on this fact, PE4 and PE5 were developed with fusing a dominant negative MMR protein (MLH1dn, an endonuclease-impaired MLH1 protein) at the C-terminus of PE2 or PE3 to evade DNA mismatch repair, and thus improve the capacity of prime editing (Figure 2E, F). Compared to PE2 and PE3 systems, PE4 and PE5 enhance the efficiency by an average of 7.7-fold and 2.0-fold in mammalian cells, respectively (Chen et al., 2021). Moreover, nicking activity in the non-target strand is critical for successful prime editing. SpCas9max, which contains R221K and N394K mutations in SpCas9, had been shown to improve Cas9 nuclease activity (Spencer and Zhang, 2017). Replacing the original nCas9 (H840A) with nCas9(R221K/N394K/H840A) in PE2 generated PEmax (Figure 2G) significantly improves the prime editing efficiency (Chen et al., 2021; Jiang et al., 2022b; Li et al., 2022b). At last, in order to enable the replacement of large indels or knock-in of gene of interest, twinPE (Anzalone et al., 2022) and GRAND editor (genome editing by RTTs partially aligned to each other but nonhomologous to target sequences within dual pegRNAs) (Wang et al., 2022c) (Figure 2H) were developed by using a pair of specially designed pegRNAs, in which the two RTTs were nonhomologous to the target sites but partially complementary to each other (Anzalone et al., 2022; Wang et al., 2022c). For example, twinPE can achieve precise deletions of up to 780 bp with up to 28% efficiency in mammalian cells (Anzalone et al., 2022). GRAND editor can precisely insert large fragments of DNA at target sites, ranging from 20 bp to ~1 kb. It exhibited an efficiency of up to 63.00% for a 150-bp insertion with minor by-products and 28.40% for a 250-bp insertion in mammalian cells, respectively (Wang et al., 2022c) (Figure 2H). Overall, prime editing offers several advantages in terms of improving precise genome editing efficiency over conventional HDR strategies and overcoming the shortage of BEs by enabling all kinds of base substitutions and replacement of short indels in a specific target gene of interest, respectively (Anzalone et al., 2019; Bharat et al., 2020; Jiang et al., 2020; Li et al., 2020d; Anzalone et al., 2022; Hua et al., 2022; Wang et al., 2022c).

## Optimization of pegRNA to improve prime editing efficiency

The pegRNA is a key determinant of PE efficiency, and therefore optimization of the canonical pegRNA (Figure 3A) is very important for improving the performance of PE. To achieve efficient prime editing, multiple parameters should be considered, such as the length of the PBS, the sequence of the RTT, the location of the desired mutations on the RTT, as well as the stability of pegRNA.

One of the key points in designing a canonical pegRNA for prime editing is the optimal length of the PBS and RTT. Priming regions with lower G/C content generally require longer PBS sequences, consistent with the energetic requirements of hybridization of the nicked DNA strand to the PBS from pegRNA (Anzalone et al., 2019). A recent study in rice suggests that the editing frequency of prime editing systems is highest when the melting temperature (Tm) of PBS is 30 °C (Lin et al., 2021). Perhaps, the PBS with this Tm ensures efficient annealing with the nicked non-target strand while maximally avoiding self-misfolding of pegRNA in rice. As for the RTT, the first thing to notice is that the "C" should be avoided at the end of the RTT, because this cytidine can pair with G81 in the sgRNA scaffold, which affects the non-canonical base pair with A68 of sgRNA or the interaction between Cas9 and sgRNA. These intermolecular interaction defects may abolish the editing activity of prime editing (Anzalone et al., 2019). Moreover, introducing multiple synonymous mutations at proper positions in the RTT of a pegRNA could improve prime editing efficiency up to 29.22-fold (Chen et al., 2021; Li et al., 2022c; Xu et al., 2022) (Table 2). Although it was recommended a 13-nt PBS and a 12-nt RTT for initial testing, it is worth noting that the optimal combination of the PBS and RTT lengths is dependent on the target sequences and needs to be further tested case-by-case (Anzalone et al., 2019; Kim et al., 2021).

Altering the secondary structure of pegRNA can improve the efficiency of prime editing (Li et al., 2022c; Liu et al., 2021c). So far, two strategies have been developed by altering the pegRNA secondary structure to stabilize the pegRNA and increase prime editing efficiency (Li et al., 2022c; Liu et al., 2021c) (Figure 3B, C). A pegRNA with C/G pair at the bottom of the small hairpin, designated as apegRNA (Figure 3B), in coordination with PE3 or PE5, resulted in significantly improved prime editing efficiency (Li et al., 2022c). Furthermore, mutating the fourth uracil of consecutive uracils in the scaffold of pegRNA into cytosine can eliminate a putative transcription termination signal, thus increasing the pegRNA expression and its capability of prime editing (Liu et al., 2021c) (Figure 3C).

Different from the canonical sgRNAs protected by an associated Cas9 protein, the 3′ terminal extensions of pegRNAs are more susceptible to exonucleolytic degradation in cells (Nelson et al., 2022). It is noted that the PBS from the 3′ end of pegRNA, which plays a major role in initiating reverse transcription, is complementary to part of the spacer at the 5′ end of pegRNA, and their annealing is likely to cause the circularization of pegRNA, which can potentially impede prime editing (Nelson et al., 2022). To address this issue, a 20-nt Csy4 recognition site was fused to the 3′ end of canonical pegRNA to prevent its circularization and thus remarkably boost the prime editing efficiency in mammalian cells (Liu et al., 2021c) (Figure 3C). Similar to the above strategy, the structured RNA motif, such as evopreQ$_1$ or mpknot, was incorporated into the 3′ terminus of pegRNAs, enhancing their stability and preventing

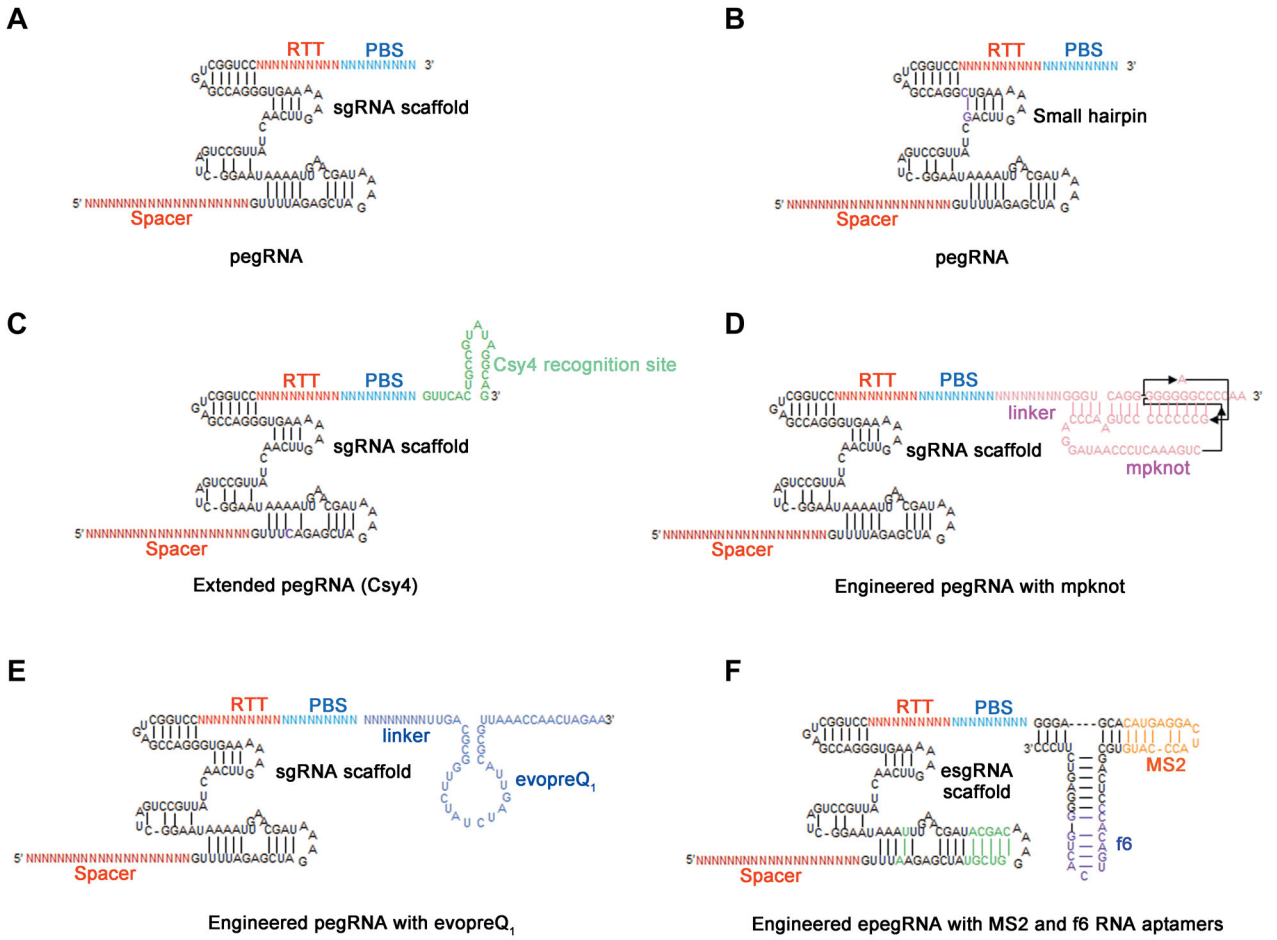


**Figure 3. Optimizations of different prime editing guide RNAs (pegRNAs)**

(**A**) The schematic diagram of a canonical pegRNA. A pegRNA is composed of three components, including a single-guide RNA (sgRNA) targeting the specific site, a reverse transcriptase (RT) template (RTT) encoding the desired edit, and a primer binding site (PBS) initiating RT. The RTT sequence is highlighted in red, the PBS sequence is highlighted in blue, and the spacer sequence is highlighted in dark red. (**B**) The schematic diagram of apegRNA, which has a C/G pair at the bottom of the small hairpin. The C/G base pair is highlighted in purple. (**C**) The schematic diagram of a Csy4-processed pegRNA, which protects the 3′ extension from degradation by exonucleases. Csy4 is a specialized ribonuclease that selects clustered regularly interspaced short palindromic repeats (CRISPR) transcripts from the cellular milieu for binding and cleavage. With Csy4 processing, the hairpin Csy4 recognition site remains at the 3′ end of the pegRNA as an extension. At the same time, mutation of the fourth one of the consecutive uracils (highlighted in purple) was introduced to the scaffold of pegRNA. The Csy4 recognition site sequence is highlighted in green. (**D**) An engineered pegRNA with a structured RNA pseudoknot (mpknot), protects its 3′ extension from degradation by exonucleases. The mpknot is a frameshifting pseudoknot from Moloney murine leukemia virus (M-MLV), and it is an endogenous template for the M-MLV-RT from which the RT in canonical prime editors was engineered, raising the possibility that mpknot might help recruit the RT. The mpknot sequence is highlighted in pink. (**E**) An engineered pegRNA with a structured RNA pseudoknot evopreQ$_1$, which protects the 3′ extension from degradation by exonucleases. evopreQ$_1$, as a modified prequeosine1-1 riboswitch aptamer composed of 42 nucleotides (nt) in length, is one of the smallest naturally derived RNA structural motifs with a defined tertiary structure. The evopreQ$_1$ sequence is highlighted in dark slate. (**F**) A representative engineered epegRNA with MS2 and f6 RNA aptamers. esgRNA, enhanced sgRNA with modifications highlighted in green. RNA aptamers can recruit their respective effector proteins for efficient gene editing. The MS2 sequence is highlighted in orange. The f6 sequence is highlighted in purple.

degradation of the 3′ extension, and eventually improving the efficiency of prime editing by 3- to 4-fold in human cells without increasing off-target editing activity (Nelson et al., 2022) (Figure 3D, E). Extension of this strategy in plants significantly enhanced prime editing efficiency (Jiang et al., 2022b; Li et al., 2022b; Zou et al., 2022) (Table 2). In addition, MS2-based PE (MS2-PE) has also been developed to improve the prime editing efficiency by using RNA aptamers (MS2 and f6) in pegRNA and fusion of their binding protein MCP with the PE2 system (Figure 3F), and achieved up to 10.1-fold increase in editing efficiency at five of six targets in transgenic rice lines (Chai et al., 2021).

While canonical PEs mainly enable base conversions and installation of small indels (Anzalone et al., 2019), development of PE capable of knock-in or replacement of large DNA fragments is highly desirable either for gene therapy or crop improvement. Recently, several powerful strategies have been developed to precisely replace, insert, and delete large DNA fragments in human cells, including twinPE (Anzalone et al., 2022) (Figure 2H), GRAND editing (Wang et al., 2022c) (Figure 2H), PRIME-Del (a prime editing-based method, which induces a deletion using a pair of pegRNAs that target opposite DNA strands) (Choi et al., 2022), and PEDAR (PE-Cas9-based

**Table 2. Clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associate protein 9 (Cas9) mediated prime editing in plants**

| PEs | PE features (PBS length (nt) and RT template length (nt)) | Target gene | Mutation type | Editing effiency | | Transformation | Selection | Trait improvement | References |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Desired (%) | Undesired (%) | | | | |
| PE2 | | | | | | | | | |
| PE2 | 13, 15-20 | *OsALS, OsIPA1, OsTB1* | 2–4 bp Subs, 2 bp Ins | 0.00–2.04 | NR | *Agrobacterium* | Hygromycin, Bispyribac sodium | Herbicide resistance, High yield | Butt et al. (2020) |
| Sp-PE2 | 13, 13 | *GFP* | 2 bp Subs | 15.60 | NR | *Agrobacterium* | Hygromycin | - | Hua et al. (2020a) |
| pPE2 | 10–13, 10–34 | *HPTII, OsPDS, OsACC, OsWx* | 1–3 bp Ins, 1 bp Subs | 0.00–59.90 | NR | *Agrobacterium* | Hygromycin | - | Xu et al. (2020a) |
| PE2 | 13,16; 13, 14–23 | *OsALS, OsACC* | 1–3 bp Subs | 1.00–7.60 | 0.00 | *Agrobacterium* | Hygromycin | Herbicide resistance | Jiang et al. (2022b) |
| pPE2 | 10–13, 11–18 | *OsPDS, OsACC, OsALS, OsCDC48* | 1 bp Ins, 1–2 bp Subs | 0.00–29.17 | NR | *Agrobacterium* | Hygromycin | Herbicide resistance | Li et al. (2022b) |
| PPE | 8, 17 | *OsALS* | 2 bp Subs | 2.10 | NR | Agrobacterium | Hygromycin | Herbicide resistance | Zong et al. (2022) |
| pPE2 (an engineered pegRNA with mpknot) | 10–13, 13–18 | *OsPDS, OsALS, OsCDC48* | 1 bp Ins, 1–2 bp Subs | 10.42–25.00 | NR | *Agrobacterium* | Hygromycin | Herbicide resistance | Li et al. (2022b) |
| pPE2 (an engineered pegRNA with evopre$Q_1$) | 10–13, 11–18 | *OsPDS, OsACC, OsALS, OsCDC48* | 1 bp Ins, 1–2 bp Subs | 2.08–50.00 | NR | *Agrobacterium* | Hygromycin | Herbicide resistance | Li et al. (2022b) |
| ePPE(replac-ing M-MLV-RT with M-MLV-RT-ΔRNaseH) | 8, 17 | *OsALS* | 2 bp Subs | 11.30 | NR | Agrobacterium | Hygromycin | Herbicide resistance | Zong et al. (2022) |
| pZ1WS (drived by the CaMV35S-CmYLCV-U6 composite promoter) | 13, 16 | *ZmALS1, ZmALS2* | 2–3 bp Subs | 4.80–53.20 | NR | *Agrobacterium* | Glufosinate ammonium | Herbicide resistance | Jiang et al. (2020) |
| PE3 | | | | | | | | | |
| Sp-PE3 | 13, 13–16 | *APO1, GFP, OsALS* | 1–2 bp Subs | 0.00–17.10 | NR | *Agrobacterium* | Hygromycin, Bispyribac sodium | Herbicide resistance | Hua et al. (2020a) |
| PE3 | 13, 28–59 | *HPTII, OsEPSPS* | 3–7 bp Subs | 2.22–9.38 | NR | Bombardment | Hygromycin | Herbicide resistance | Li et al. (2020c) |

Continued

**Table 2. Continued**

| PEs | PE features (PBS length (nt) and RT template length (nt)) | Target gene | Mutation type | Editing effiency | | Transformation | Selection | Trait improvement | References |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Desired (%) | Undesired (%) | | | | |
| PPE3 | 10–12, 9–17 | *OsCDC48, OsALS* | 1–3 bp Subs, 6 bp Del | 2.60–21.80 | NR | Bombardment | Hygromycin | Herbicide resistance | Lin et al. (2020) |
| pPE3 | 13, 10 | *OsWx, OsACC* | 1 bp Subs | 0.00–16.70 | NR | *Agrobacterium* | Hygromycin | Herbicide resistance | Xu et al. (2020a) |
| PE-P1 | 11–14, 14–23 | *OsDEP1, OsALS, OsACC* | 1–4 bp Subs | 0.00–1.40 | 0.00 | *Agrobacterium* | Hygromycin | Herbicide resistance | Xu et al. (2020c) |
| PE3 | 9–13, 9–28 | *OsSPL14, OsDHDPS, OsNR2* | 2–3 bp Subs | 0.00–1.00 | NR | Bombardment | Hygromycin | - | Li et al. (2022a) |
| PE3 | 13,16; 13, 14–23 | *OsALS, OsACC, OsEPSPS* | 1–3 bp Subs | 1.30–70.30 | 9.00–37.90 | *Agrobacterium* | Hygromycin | Herbicide resistance | Jiang et al. (2022b) |
| PPE3-unmodified | 11–14, 11–18 | *OsROC, OsALS, OsCDC48, OsDEP1* | 1–3 bp Subs, 3 bp Ins | 0.00–2.90 (Normal) 0.00–15.20 (HT) | NR | *Agrobacterium* | Hygromycin | Herbicide resistance | Zou et al. (2022) |
| pPE3b | 13, 10 | *OsACC* | 1 bp Subs | 6.25 | NR | *Agrobacterium* | Hygromycin | Herbicide resistance | Xu et al. (2020a) |
| PE-P2(nCas9 (H840A)-M-MLV-T2A-hpt) | 11–14, 14–23 | *OsDEP1, OsALS, OsACC* | 1–4 bp Subs | 1.70–26.00 | 0.00–8.00 | *Agrobacterium* | Hygromycin | Herbicide resistance | Xu et al. (2020c) |
| pCXPE03 (drived by the RPS5A promoter) | 14, 17–18 | *SlGAI, SlALS, SlPDS* | 2 bp Subs, 2 bp Ins | 0.00–6.70 | NR | *Agrobacterium* | Hygromycin | Herbicide resistance | Lu et al. (2021) |
| PPE3-evopreQ$_1$ | 11–14, 11–18 | *OsROC, OsALS, OsCDC48, OsDEP1* | 1–3 bp Subs, 3 bp Ins | 2.60–47.50 (Normal) 5.00–60.50 (HT) | NR | *Agrobacterium* | Hygromycin | Herbicide resistance | Zou et al. (2022) |
| PPE3-mpknot | 11–14, 11–18 | *OsROC, OsALS, OsCDC48, OsDEP1* | 1–3 bp Subs, 3 bp Ins | 0.00–4.20 (Normal) 0.00–6.30 (HT) | NR | *Agrobacterium* | Hygromycin | Herbicide resistance | Zou et al. (2022) |
| PE-P2-RT-S (N-terminal M-MLV + a single desired mutation in RTT) | 8–14, 13–23 | *OsGS3, OsALS, OsACC, OsChalk5, OsDEP1, OsWaxy, OsGRF4, OsSD1, OsEPSPS, OsCold1, OsPSR1* | 1 bp Subs | 0.00–61.40 | 0.00–15.00 | *Agrobacterium* | Hygromycin | - | Xu et al. (2022) |
| PE-P3-RT-M (C-terminal M-MLV + multiple synonymous base mutations in RTT) | 8–14, 13–23 | *OsGS3, OsALS, OsACC, OsChalk5, OsDEP1, OsWaxy, OsGRF4, OsSD1, OsEPSPS, OsCold1, OsPSR1* | 3–4 bp Subs | 0.00–82.60 | 0.00–18.00 | *Agrobacterium* | Hygromycin | - | Xu et al. (2022) |

**Table 2. Continued**

| PEs | PE features (PBS length (nt) and RT template length (nt)) | Target gene | Mutation type | Editing effiency | | Transformation | Selection | Trait improvement | References |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Desired (%) | Undesired (%) | | | | |
| PE-P3-RT-S (C-terminal M-MLV + a single desired mutation in RTT) | 8–14, 14–23 | *OsGS3, OsALS, OsACC, OsChalk5, OsDEP1, OsWaxy* | 1 bp Subs | 0.00–22.70 | 0.00–15.00 | *Agrobacterium* | Hygromycin | - | Xu et al. (2022) |
| PE-P2-RT-M (N-terminal M-MLV + multiple synonymous base mutations in RTT) | 8–14, 14–23 | *OsGS3, OsALS, OsACC, OsChalk5, OsDEP1, OsWaxy* | 3 bp Subs | 0.00–26.00 | 0.00–8.00 | *Agrobacterium* | Hygromycin | - | Xu et al. (2022) |
| PE3-HS (hygromycinY46*-based) | 9–13, 9–28; 13, 19 | *OsSPL14+mhptII, OsDHDPS+mhptII, OsNR2+mhptII* | 2–3 bp Subs, 1 bp Subs | 1.30–2.10 | NR | Bombardment | Hygromycin | - | Li et al. (2022a) |
| PE3-AS (OsALS-S627I-based) | 9–13, 9–28; 13, 12 | *OsSPL14+OsALS, OsDHDPS+OsALS, OsNR2+OsALS, OsSPL14+OsALS +OsDHDPS* | 2–3 bp Subs, 3 bp Subs | 2.40–14.30 | NR | Bombardment | Hygromycin, Bispyribac sodium | - | Li et al. (2022a) |
| PE3-DS (PE3-HS +PE3-AS) | 9–13, 9–28; 13, 12; 13, 19 | *OsSPL14+OsALS +mhptII, OsDHDPS +OsALS+mhptII, OsNR2+OsALS+ mhptII, OsSPL14 +OsALS+ OsDHDPS+mhptII, OsSPL14+OsALS+ OsEPSPS+mhptII* | 2–7 bp Subs, 3 bp Subs, 1 bp Subs | 3.20–54.20 | NR | Bombardment | Hygromycin, Bispyribac sodium | - | Li et al. (2022a) |
| PE4 | | | | | | | | | |
| PE4 | 13,16; 13, 14–23; | *OsALS, OsACC* | 1–3 bp Subs | 5.20–27.10 | 0.00–2.10 | *Agrobacterium* | Hygromycin | Herbicide resistance | Jiang et al. (2022b) |
| PE5 | | | | | | | | | |
| PE5 | 13,16; 13, 14–23; | *OsALS, OsACC, OsEPSPS* | 1–3 bp Subs | 1.60–64.10 | 6.40–18.30 | *Agrobacterium* | Hygromycin | Herbicide resistance | Jiang et al. (2022b) |
| PEmax | | | | | | | | | |
| ePE3max (the PEmax protein +an engineered pegRNA with evopreQ$_1$ ) | 13, 22 | *OsEPSPS* | 3 bp Subs | 37.20–39.80 | 18.40–21.00 | *Agrobacterium* | Hygromycin | Herbicide resistance | Jiang et al. (2022b) |

Continued

**Table 2. Continued**

| PEs | PE features (PBS length (nt) and RT template length (nt)) | Target gene | Mutation type | Editing effiency | | Transformation | Selection | Trait improvement | References |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Desired (%) | Undesired (%) | | | | |
| PEmax | | | | | | | | | |
| ePE5max (ePE3max+ OsMLH1dn) | 13, 22 | *OsEPSPS* | 3 bp Subs | 38.20–39.50 | 20.80–20.90 | *Agrobacterium* | Hygromycin | Herbicide resistance | Jiang et al. (2022b) |
| pPE2max-evopreQ$_1$ (PEmax+an engineered pegRNA with evopreQ$_1$) | 10–13, 11–18 | *OsPDS*, *OsACC*, *OsALS*, *OsCDC48* | 1 bp Ins, 1–2 bp Subs | 14.58–66.67 | NR | *Agrobacterium* | Hygromycin | Herbicide resistance | Li et al. (2022b) |
| enpPE2 (pPE2max-evopreQ$_1$+U6 composite) | 10–13, 11–18 | *OsPDS*, *OsACC*, *OsALS*, *OsCDC48* | 1 bp Ins, 1–2 bp Subs | 64.58–77.08 | NR | *Agrobacterium* | Hygromycin | Herbicide resistance | Li et al. (2022b) |

Aberrations: Del, deletion; HT, heat treatment condition; Ins, insertion; M-MLV, Moloney murine leukemia virus; Normal, normal condition; NR, not reported; PBS, primer binding site; RT, reverse transcriptase; RTT, reverse transcription template; Subs, substitution.

deletion and repair) method (Jiang et al., 2022a). These prime editing systems were developed by employing similar strategies such as using a pair of designed pegRNAs that target the opposite DNA strands, and the RTTs from the two respective pegRNAs were nonhomologous to the target sites but partially complementary to each other (Anzalone et al., 2022; Choi et al., 2022; Jiang et al., 2022a; Wang et al., 2022c). Although the above systems for replacement or knock-in of large DNA fragments through prime editing had been successfully applied in mammalian cells, the feasibilities of twinPE, GRAND editing, PRIME-Del, and PEDAR in plant prime editing remain to be investigated in the near future.

## Applications of diverse PEs in plants

Prime editing system substantially expands the scope and capabilities of precision genome editing and holds great promise to introduce precise genome modifications such as SNP and/or small indels into plant genomes to improve agriculturally important traits in crops (Li et al., 2020d). Since the first report of prime editing in mammalian cells in 2019 (Anzalone et al., 2019), the feasibilities and efficacies of PE2 and PE3 for precise genome editing had soon been investigated in rice (Butt et al., 2020; Hua et al., 2020a; Jiang et al., 2020; Li et al., 2020c; Lin et al., 2020; Tang et al., 2020; Xu et al., 2020a, 2020c), other plant species (Jiang et al., 2020; Lin et al., 2020; Lu et al., 2021), and followed by further optimization to improve their prime editing efficiencies thereafter (Jiang et al., 2020; Li et al., 2020c; Lu et al., 2021; Xu et al., 2022; Li et al., 2022a; Jiang et al., 2022b; Xu et al., 2020a, 2020c) (Table 2). Applications of diverse PEs in plants, their features and editing efficiencies and so forth, are summarized in Table 2.

The feasibility and efficacy of a series of plant codons optimized from PE2 and PE3 were first validated and investigated in rice and wheat protoplasts (Lin et al., 2020; Tang et al., 2020) or stable rice plants (Li et al., 2020c; Xu et al., 2020a, 2020c) almost simultaneously in five laboratories. Except for the intrinsic nature of target genes, various parameters such as PBS length, RT template length, and the position of nicking sgRNA significantly affected the precise editing efficiency of PE2 and PE3 in rice and wheat protoplasts (Lin et al., 2020). The PE2 system could also induce programmable editing at different genome sites at a frequency of 0% to 31.3% in rice stable lines, suggesting that the efficiency of pPE2 varied greatly at different genomic sites and with pegRNAs of diverse structures (Xu et al., 2020a). By using the *polII* promoter *Actin* to drive the expression of the tandem repeats of polycistronic transfer RNAs to simultaneously produce pegRNA and nicking sgRNA in a PE3, 28 bp and a 59 bp fragments with desired edits were precisely installed into an exogenous inactive hygromycin phosphotransferase (HPT) gene *hptII* to restore its function, and an endogenous gene *OsEPSPS*, which encodes a 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), to generate a novel allele with TAP-IVS mutations (T173I, A174V, and P177S) which confers rice glyphosate resistance in rice stable lines, respectively (Li et al., 2020c).

Furthermore, development of a plant PE by fusion of HPT to the C-terminus of nCas9-M-MLV with a self-cleaving 2A peptide (P2A) linker and paired with an enhanced sgRNA (esgRNA) improved the prime editing efficiency and achieved versatile nucleotide substitutions in rice stable lines (Xu et al., 2020c). Compared with normal PE, this strategy could increase the editing efficiency up to 22-fold at the *OsALS-1* site (from 1.20% to 26.00%) (Xu et al., 2020c). Later on, a PE2-based plant PE with a pegRNA of 13-nt PBS and 15-nt RTT was transformed into rice to alter the target codon TGG for Trp548 of the ALS gene generated herbicide resistant rice plants (Butt et al., 2020). At the same time, prime editing of *OsIPA1* in rice reduced the number of unproductive tillers and improved rice yield (Butt et al., 2020). An inactive *eGFP* gene was used as a transgenic reporter. After prime editing, it was restored into a wild-type *EGFP* sequence with two precise base conversions (T-G and G-C) at efficiencies of 15.60% and 17.10% for PE2 and PE3, respectively (Hua et al., 2020a). Simultaneously, by using a pegRNA designed to introduce a S627N mutation in *OsALS*, PE3 was also employed to generate rice plants resistant to imidazolinone herbicides at an efficiency of 9.10% (Hua et al., 2020a) (Table 2).

To further stimulate the prime editing in plants, designing the PBS in a pegRNA with a melting temperature of 30°C led to optimal performance and substantially enhanced prime editing efficiency in rice protoplasts (Lin et al., 2021). Furthermore, in contrast to mammalian cells (Anzalone et al., 2019), N-terminal-M-MLV-RT-Cas9 nickase fusion performed better in rice than the commonly used C-terminal fusion. In addition, introduction of multiple-nucleotide substitutions in RTT stimulated prime editing with enhanced efficiency. By using these two methods synergistically, prime editing with an average editing frequency as high as 24.3% at 13 targets of 11 endogenous genes (including *OsGS3*, *OsALS*, *OsACC*, *OsChalk5*, *OsDEP1*, *OsWaxy*, *OsGRF4*, *OsSD1*, *OsEPSPS*, *OsCold1*, and *OsPSR1*) in rice transgenic plants is two- to three-fold higher than the canonical PE3 (Xu et al., 2022). Moreover, based on the reports in human cells (Chen et al., 2021; Nelson et al., 2022), adding an evopreQ$_1$ at the end of pegRNA alone or cooperating with appropriate heat treatment significantly improved the prime editing efficiency up to 54.8-fold at the site ROC5-3 in rice (Zou et al., 2022). An enhanced plant PE2 system, enpPE2, was developed by stacking various optimization strategies including updating the PE architecture to PEmax and expressing engineered pegRNA with a structured motif such as evopreQ$_1$ under the control of a composite promoter. In rice, enpPE2 exhibited editing frequencies of 64.58% to 77.08% at sites ACC-T, PDS-T, ALS-T and CDC48-T, which are much higher than the frequencies in comparison to the canonical PE2 (Li et al., 2022b). Indeed, coordination of the PEmax architecture and the epegRNA (pegRNA with evopreQ$_1$) greatly improved prime editing efficiency (Jiang et al., 2022b). In addition, four types of PEs including PE2, PE3, PE4 and PE5, had been systemically compared at the same four sites of two endogenous genes, *OsALS* and *OsACC* in rice. As listed in Table 2, whereas PE3 outperformed PE2 at most sites, PE4 outperformed PE2 (5.20%–27.10% vs 1.00%–7.60%). But PE5 did not increase the prime editing efficiency in comparison with PE3 (1.60%–64.10% vs 1.30%–70.30%) (Jiang et al., 2022b). In general, PE3 and PE5 outperformed PE2 and PE4, respectively (Jiang et al., 2022b), which seems to be inconsistent with previous reports in rice protoplast (Lin et al., 2020), but is coherent with the previous reports in mammalian cells (Anzalone et al., 2019; Chen et al., 2021). Using the above optimized PEs, homozygous and heterozygous *OsEPSPS* edited rice were successfully obtained, which provides valuable germplasm for breeding non-transgenic glyphosate-resistant rice varieties (Li et al., 2020c; Jiang et al., 2022b). Moreover, removing its ribonuclease H domain from M-MLV-RT and incorporating a viral nucleocapsid protein at the N-terminal of M-MLV-RT could improve the efficiency of prime editing by two to three times in plants, respectively (Zong et al., 2022). Combining these two strategies, the desired mutation efficiency of 11.30% was obtained at the site OsALS-T6 in the $T_0$ generation, which makes rice plants resistant to imidazolinone herbicides (Zong et al., 2022) (Table 2).

Development of a multiplex precision gene editing system is highly desirable for pyramiding beneficial alleles in crop improvement. Recently, a surrogate prime editing system for multiplexing has been developed in rice for accurate simultaneous editing of multiple endogenous genes. Three surrogate PEs including hygromycinY46*-based, OsALS-S627I-based, and a combined double surrogate system, respectively, were designed for prime editing of endogenous genes. While the hygromycinY46*-based and OsALS-S627I-based surrogate PEs could increase the editing efficiencies by ~2–14-fold, the double surrogate system could stimulate the prime editing efficiencies up to ~50-fold. Furthermore, a series of stable lines with several precisely edited endogenous genes were simultaneously successfully generated by using this double surrogate system (Li et al., 2022a) (Table 2). Pyramiding of multiple excellent alleles by prime editing in major crops such as rice, wheat and so forth, will greatly facilitate crop improvement and speed up the breeding process (Li et al., 2022a).

Except for rice, prime editing was also used in maize and dicotyledonous plants such as tomato. Enhancing pegRNA expression by using two promoter systems (the CaMV35S-CmYLCV-U6 composite promoter) can improve the efficiency of prime editing in maize (Jiang et al., 2020). Transgenic lines harboring homozygous S621I and chimeric W542L mutations in *ZmALS1* and *ZmALS2* were successfully obtained by pZ1WS systems (Jiang et al., 2020) (Table 2). In addition, an optimized PE3 system pCXPE03 was developed by replacing the 35S promoter with the ribosomal protein S5A (RPS5A) promoter of tomato to drive the nCas9(H840A)-RT fusion protein. A total of seven pegRNAs were designed for prime editing of three tomato genes, including *GAI* (Solyc11g011260), *ALS2* (Solyc03g044330) and *PDS1* (Solyc03g123760). Apart from *GAI*, the desired mutations in

*ALS2* and *PDS1* were obtained at the efficiencies of 6.70% and 3.40%, respectively (Lu et al., 2021) (Table 2).

Directed evolution (DE) is a technology of making random mutation(s) in a target gene to generate novel germplasms and enrich genetic diversity (Zhang and Qi, 2019). Currently, base editing can enable artificial evolution of agriculturally important genes in crops to explore novel gene resources and germplasms (Kuang et al., 2020; Li et al., 2020a; Liu et al., 2020; Xu et al., 2021a; Wang et al., 2022b). Compared with base editing, prime editing has greater potential for evolving plant genes, because it can install all types of small genetic modifications that can be harnessed for producing all possible substitutions for key amino acids with improved agronomic performance when combined with a well-designed pegRNA library. For example, a prime editing library-mediated saturation mutagenesis (PLSM) method had been developed to identify 16 types of herbicide resistance-conferring mutations at six different target residues in *OsACC1* using a pegRNA library with all possible combinations of substitutions (64 types), which enabled a more comprehensive screening than that achieved by base editing (Xu et al., 2021b). Among the 16 kinds of mutations, three types of mutations were first reported in plants. The PLSM system is an alternative approach to create novel germplasms for crop breeding.

## RECOMMENDATION OF PROPER BE AND PE FOR BOTH BASIC RESEARCH IN PLANTS AND CROP IMPROVEMENT

According to optimizations and applications of the current BEs and PEs in plants as described above, we recommend to select appropriate BEs and PEs for precise gene editing for both basic biological research in plants and crop improvement. For base editing, we recommend using evoFERNY for CBE (Zeng et al., 2020), and TadA9 or TadA8e-DBD (hyTadA8e) for ABE (Yan et al., 2021; Tan et al., 2022) in base editing. In addition, it would be good to select the appropriate Cas protein capable of targeting the region near the desired editing site due to the limitations of PAM sites and the editing windows of various BEs. At present, the suitable choice is to select the editors constructed from these three Cas proteins with broad PAM sites, including Cas9-NG (NGN PAM), ScCas9$^{++}$ (NNG PAM), and SpRY (NNN prefer NRN PAM) (Hua et al., 2019; Ren et al., 2019; Zhong et al., 2019; Wang et al., 2020a; Liu et al., 2021b). Moreover, using a surrogate system to restore the defective genes into the functional ones encoding antibiotics or herbicides could be more cost-effective and improve the base editing efficiency (Xu et al., 2020b).

For prime editing, we recommend using PEmax or a PE with M-MLV-RT fused to the N-terminus of nCas9(H840A) (Chen et al., 2021; Jiang et al., 2022b; Li et al., 2022b; Xu et al., 2022) (Figure 2). Further, except the intended base substitutions, introduction of additional multiple-nucleotide synonymous substitutions in RTT could stimulate prime editing efficiency (Chen et al., 2021; Li et al., 2022c; Xu et al., 2022). For example, synonymous substitutions could be introduced at +1 ~ +6 positions (counting 3′-base of RTT as position +1), in order to avoid the repeat nicking of edited targets (Xu et al., 2022) (Figure 3). Furthermore, additional structured RNA sequences, such as evopreQ$_1$ appended to the 3′-end of pegRNA will stabilize the pegRNAs and thus improve the prime editing efficacy (Li et al., 2022b; Nelson et al., 2022; Zou et al., 2022) (Figure 3). Moreover, a strong composite promoter, such as CaMV 35S enhancer+CmYLCV promoter+U6 promoter, could be used to enhance the expression of the pegRNA and thus improve the prime editing efficiency (Jiang et al., 2020). Lastly, using the reporter genes such as antibiotics or herbicides as surrogates to enrich the lines with desired edits improved the prime editing efficiency in a cost-effective and labor-saving way, especially for multiplex prime editing in plants (Li et al., 2022a) (Table 2).

## FUTURE PERSPECTIVES FOR FURTHER OPTIMIZATION OF BE AND PE IN PLANTS

Although impressive progresses have been made during the last several years, the following aspects such as optimization of the existing BEs, exploitation of novel BEs, and optimization of PEs to further improve their precise editing efficiencies as well as developing novel PEs capable of installation of larger indels in plants, would be highly desirable in the next few years.

### Optimization of the existing BEs and exploitation of novel BEs

To date, CBE and ABE for base transition have been well optimized in terms of improving editing efficiency, expanding the target scope and reducing off-targets. However, for base transversion, such as CGBE for C to G and C to A, the editing efficiency is relatively lower in comparison with other BEs in plants (Koblan et al., 2021; Tian et al., 2022). Thus, it is still necessary to increase the efficiency of CGBE. Most importantly, in order to increase the flexibility of BEs, exploitation of other types of BEs for transversion of A to C (T to G) or A to T (T to A) will certainly be very beneficial in substitution of any base pair into the desired one within the editing window in a target gene of interest in plants. In addition, concerning the base editing window, two aspects are worthy of further optimization. (i) Narrow the editing window of BE to a single base, reduce the by-products of unintended editing, for example, a more precise adenine base editor ABE9 (Figure 1L), which was developed recently by introducing two mutations L145T and N108Q in ABE8e, maintained the editing activity and minimized the editing window to position 5–6 in mammalian cells (Chen et al., 2022b). Furthermore, by combining with PAM-less Cas proteins, it will be possible to

achieve accurate single base editing at any target sites in the genome. (ii) Widen the width of the editing window of BE for saturation mutation studies such as de novo domestication or DE to generate novel gene resources or germplasm in plants. For example, fusion of T7 RNA polymerase with different deaminases (cytidine and adenosine deaminase), substantially widens the mutational spectrum in mammalian cells (Cravens et al., 2021). In addition, engineering BEs fused with additional chromatin modulating peptides, such as pioneer factor SOX2 (SRY-box transcription factor 2), to initiate chromatin unfolding and stimulate transcription, could be a promising strategy to further increase base editing efficacy (Yang et al., 2022).

### Optimization of PEs

A series of parameters such as stable and properly folded pegRNAs, effective assembly of the PE-pegRNA complex, and more active reverse transcriptase are essential for efficient prime editing. In PE, the canonical pegRNA consists of a sgRNA, a RTT and a PBS (Figure 2A). PBS and RTT at the 3′-terminal of pegRNA are easy to partially degrade by exoribonucleases inside the cells, resulting in truncated pegRNAs (Feng et al., 2022; Nelson et al., 2022). The truncated pegRNAs can still search and recognize the target sites, but not be able to complete the correct editing due to loss of the PBS or RTT-PBS (Nelson et al., 2022). Adding a special RNA structure such as evopreQ$_1$ to the 3′ end of pegRNA can reduce the degradation of pegRNA from the 3′ end and significantly increase the prime editing efficiency (Nelson et al., 2022; Li et al., 2022b). In addition, single-stranded DNA (ssDNA)-annealing protein (SSAP) is a kind of phage recombination protein RecT (Noirot and Kolodner, 1998; Court et al., 2002). SSAPs promote complementary strand annealing and promote strand exchange at homologous regions by directly binding to either ssDNA or dsDNA (Noirot and Kolodner, 1998; Muyrers et al., 2000; Court et al., 2002). SSAPs improve the efficiency of Cas9-initiated targeting in mammalian cells (Wang et al., 2021a). SSAPs are also useful for stimulating the cleavage-free knock-in of large DNA fragments upon fusion with dCas9 in mammalian cells (Wang et al., 2022a). Fusion of SSAP to either the N- or C- terminal of the optimized PE would probably be an alternative strategy to further boost the prime editing efficiency.

In comparison to HDR, the so far developed PEs only enable the replacement of short indels in plants (Li et al., 2020d). It is conceivable that if a larger DNA fragment or indel is to be replaced or inserted into the genome, a longer RTT needs to be designed. The longer RTT in the pegRNA may affect the formation of the normal secondary structure of the sgRNA and thus destabilize the pegRNA. Recently, twinPE and GRAND editors were developed to precisely insert large DNA fragments in mammalian cells (Anzalone et al., 2022; Wang et al., 2022c) (Figure 2H). In contrast to the previous PEs which require RTT hybridizing with the target sequence, twinPE or GRAND editing employs a pair of pegRNAs with two RTTs nonhomologous to the target site but complementary to each other. The twinPE and GRAND editing strategies significantly expand the capacity of prime editing in enabling donor-free insertion of large DNA sequences. Next, it will be intriguing to test whether these strategies are feasible for targeted gene/allele replacement in plants. Moreover, if the RTT is split from sgRNA-RTT-PBS and transcribed separately from sgRNA, it can not only solve the problem of pegRNA self-circularization, but also eliminate the possible interference of RTT-PBS on the nicking ability of sgRNA-guided Cas9(H840A), which is essential for efficient prime editing (Nelson et al., 2022).

Some well-characterized RNA binding proteins (RBPs), such as MCP, PCP, N22p and Com, specifically recognize and bind to the respective cognate attachment RNA sequences *MS2* (Peabody, 1993; Lim and Peabody, 1994; Chao et al., 2008), *PP7* (Lim et al., 2001; Chao et al., 2008), *boxB* (Austin et al., 2002; Daigle and Ellenberg, 2007) and *com* (Hattman, 1999), respectively. It is expected that fusion of RBP to either the N- or C-terminal of nCas9-M-MLV and adding the RBP recognition sequence such as *MS2* at the 3′ end of pegRNA (Feng et al., 2022), or in combination with evopreQ$_1$, would not only prevent the degradation of pegRNA but also be possible to tether the 3′ end of pegRNA to the vicinity of nicks for repairing, so as to improve the prime editing efficacy in plants. For example, the split RTT-PBS can be tethered to the vicinity of DNA nicks by fusing the tandem MS2 coat protein (tdMCP) to the nCas-M-MLV-RT complex and appending the stem-loop aptamer MS2 at the end of RTT-PBS (Feng et al., 2022). Also, it would be interesting to combine this strategy with twinPE or GRAND editing to enable the insertion or replacement of larger DNA sequences into the plant genome in a user-defined manner for crop improvement.

## CONCLUSION

In the context of climate changes, decreased farmland resources and frequent outbreaks of natural disasters, global crop production is still facing unprecedented challenges. BEs and PEs for precision genome editing hold great potential in accelerating the breeding process to ensure global food security and sustainable agricultural development (Bharat et al., 2020; Li and Xia, 2020; Li et al., 2021d; Molla et al., 2021; Zhan et al., 2021; Hua et al., 2022). For example, BEs and PEs are capable of introducing 35% and 85% of 384 causative mutations for the currently exploited 225 important quantitative agronomic trait genes in rice (Hua et al., 2022). Further, for plant functional genomics, sometimes not only the knock-outs but also a gain of function of the respective target gene by BE and PE, are essential for deciphering the biological processes (Kuang et al., 2020; Li et al., 2020c; Liu et al., 2021a; Xu et al., 2021b; Li et al., 2022a). Although impressive progresses have been made since the development of first-generation BE and PE, some issues remain to be addressed in years to come. First, continuous efforts in engineering a novel generation of BE and PE as aforementioned will certainly enrich the precision genome editing tools in plants. Second, BEs and

PEs, especially PE, are not widely used or even impossible in the polyploid species and agriculturally important food crops such as common wheat due to its complex hexaploidy genome, gene redundancy, as well as relatively lower transformation efficiency (Li et al., 2021c). Third, for base editing and prime editing in different plant species, we suggest using the aforementioned optimized strategies in combination with a stronger promoter to drive the expression of both nCas-deaminase and the sgRNA for BE, or nCas-M-MLV-RT and pegRNA for PE, respectively (Li et al., 2022b). Finally, it is worth noting that the innate nature of target genes may affect the editing outcomes of both BEs and PEs in plants; for example, some genes or targets could only be edited at a very lower efficiency or even not be accessible (Hua et al., 2022). Understanding the potential mechanism underlying this phenomenon will certainly benefit the precision genome editing of any targets at will in a user-defined manner in plants. Nevertheless, following the continuous endeavors on optimization of BE and PE as well as engineering a novel generation of BE and PE, we envision that both BEs and PEs will become the routine and customized precise gene editing tools for both plant fundamental research and crop improvement in the near future.

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## CONFLICTS OF INTEREST

The authors declare no conflicts of interests.

## AUTHOR CONTRIBUTIONS

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