Review Article

CRISPR base editing and prime editing: DSB and template-free editing systems for bacteria and plants

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A B S T R A C T

CRISPR-Cas (Clustered Regularly Interspaced Short Palindromic Repeats-CRISPR associated) has been extensively exploited as a genetic tool for genome editing. The RNA guided Cas nucleases generate DNA double-strand break (DSB), triggering cellular repair systems mainly Non-homologous end-joining (NHEJ, imprecise repair) or Homology-directed repair (HDR, precise repair). However, DSB typically leads to unexpected DNA changes and lethality in some organisms. The establishment of bacteria and plants into major bio-production platforms require efficient and precise editing tools. Hence, in this review, we focus on the non-DSB and template-free genome editing, i.e., base editing (BE) and prime editing (PE) in bacteria and plants. We first highlight the development of base and prime editors and summarize their studies in bacteria and plants. We then discuss current and future applications of BE/PE in synthetic biology, crop improvement, evolutionary engineering, and metabolic engineering. Lastly, we critically consider the challenges and prospects of BE/PE in PAM specificity, editing efficiency, off-targeting, sequence specification, and editing window.

1. Introduction

Scientists have for long worked to develop a facile, precise, and efficient DNA editing tool. Though the efforts led to the development of some significant editing tools like zinc finger nuclease (ZFN) and transcription activator-like effector nucleases (TALEN), they are not simple enough to have widespread use [1]. The discovery of CRISPR-Cas [2–4] and its development into a genome editing tool [5] have revolutionized the field of biotechnology, therapeutics, DNA sequencing, and biology as a whole. CRISPR-Cas systems that naturally evolved as a prokaryotic adaptive immune system against phage infection and invasion of mobile genetic elements (MGEs), are found in around 45% bacterial and 90% archaeal genomes [6]. CRISPR-Cas systems generally consist of CRISPR array and Cas proteins. Upon the infection, CRISPR array is transcribed, producing precursor-CRISPR RNA (pre-crRNA), which is further processed to form CRISPR RNA (crRNA). Later, for simple use and design, crRNA was engineered to form a single-guide RNA (sgRNA or gRNA). crRNA complementary to the foreign nucleotide sequence guides the Cas proteins for DNA cleavage via recognition of Cas-specific protospacer adjacent motif (PAM) [7,8]. This strategy has been repurposed to develop editing tools by programming sgRNA to direct Cas proteins to the target site for DNA break, subsequently, initiating cellular repair machinery mainly either imprecise repair system NHEJ or precise repair system HDR. NHEJ has been used for gene disruption by the introduction of indels or translocations, while HDR has been used in combination with donor DNA templates for precise replacements, deletions, insertions or point mutations [9].

However, DSB can be lethal for some cells, especially for bacterial cells. While, in the case of eukaryotic cells, the DSB repair mechanisms compete with each other like NHEJ vs. HDR, the former being a preferred choice of repair [10]. The competition mostly leads to low HDR efficiency for precise editing. Especially in plants, DSB-induced HDR editing suffers low efficiency because of weak HDR in somatic plant cells. The requirement of donor DNA template makes matters worse due to the challenging delivery process and insufficient availability of donor DNA template during DSB repairing [11]. On the other hand, most bacteria lack NHEJ [12–14], leaving DSBs to be repaired mainly by HDR. However, HDR in most bacteria (except some bacteria such as *Streptomyces* [15]) is not effective enough and shows low editing efficiencies. Recombinases like RecET [16] and Lambda-Red [17] systems have been combined with DSB-induced HDR to increase editing

efficiency. Additionally, to increase editing efficiency, counter-selection techniques such as Cas induced DSB selection markers have been applied [18]. Still, the requirement of high dose of donor DNA template and low editing efficiencies between 10^{-6} to 10^{-1} in the absence of counter-selection, means that more efficient editing tools are required [19,20]. Development of base editor (BE) [21–23] and prime editors (PE) [24] answers the call for the need of precise and efficient non-DSB and template-free editing systems.

BE and PE use catalytically nuclease deficient Cas proteins (For example, dCas9(D10A and H840A) or nCas9(D10A or H840A), all generated from *Streptococcus pyogenes* Cas9 (SpCas9)), allowing DSB-free editing. In the case of BE, dCas/nCas(D10A) are fused to deaminase domains that allow C-to-T (by cytosine base editor, CBE) or A-to-G (by adenine base editor, ABE) substitution [21–23]. While PE consists of nCas9(H840A) fused to reverse transcriptase, which allows the insertions, deletions, and point mutations at specific loci [24]. BE and PE technologies have, to some extent, overcome the drawbacks of the DSB-dependent CRISPR editing systems. Plenty of groups have already adopted base editing systems for animal, plant and bacterial cells. CRISPR technologies and specifically base editing in animal cells have been extensively reviewed by Liu and Rees [25], Hees et al. [26], Molla and Yang [27], and Wu et al. [28]. Here, we comprehensively review the current developments in non-DSB and template-free editing systems in bacteria and plants.

Bacteria have for long been considered major platforms for bio-production and there has been an increasing interest in developing plants into key bio-production platforms. The development of a broad set of biological tools for bacteria and plants releases their potential to produce a wide array of valuable chemicals [29,30], secondary metabolites [31,32], recombinant proteins [33,34], biofuels [35,36] and food additives [37]. The regulation of interconnected metabolic pathways and synthetic multi-gene programmes require efficient, precise, and simple gene editing tools. Traditional CRISPR tools have been adopted in bacteria and plants for crop improvement [38], synthetic biology, and metabolic engineering to improve bio-production [39–42]. However, efforts to develop bacteria and plants into major cell factories for bio-production have been halted because of low editing efficiencies and the requirement of the donor DNA template. The adoption of the BE and PE systems should overcome the shortcomings, and these systems hold promise to further enhance microbial and plant bio-production abilities.

2. Development of base and prime editors

Base editors are chimeric complexes with the ability to deaminate cytosine and adenine bases. The catalytically inactive or nuclease deficient Cas proteins are utilized by BE to direct deaminase machinery to the target locus and carry out precise nucleotide substitution (Fig. 1) [21–23]. Apart from BE, the recently developed PE also allows non-DSB and template-free insertion, deletion or nucleotide substitution of arbitrary sequence (Fig. 2A) [24].

The first base editors were individually developed by Liu [22] and Kondo [23] groups for C-to-T substitution. The editors convert cytosine to uracil by deamination of exocyclic amine, which leads to U•G, a wobble base pair. U being an illegitimate base would generally be recognized by uracil-DNA glycosylase (UDG), which initiates nucleotide excision repair (NER) converting U back to the original base C [43,44]. However, the CBE uses uracil glycosylase inhibitor (UGI) to prevent the conversion of U back to C and activate mismatch repair system (MMR) which converts U•G-to-U•A base pair which is then converted to T•A base pair leading to C•G-to-T•A substitution [45]. The CBEs from both the groups (Liu and Kondo) are functionally similar but differ in the deaminase types and the arrangement of the domains. The BE system from Liu group uses rat apolipoprotein B mRNA editing enzyme (rAPOBEC1) fused via 16-residue XTEN linker to N-terminus, and the UGI fused via 4-amino acid linker to the C-terminus of the Cas9 (Fig. 1). Whereas the

Target-AID system from Kondo group uses activation induced cytosine deaminase (AID) ortholog PmCDA1 from sea lamprey. The PmCDA1 is fused to N-terminus of Cas9 via 100 amino acid linker, while UGI is fused to PmCDA1 (Fig. 1). Both the groups initially used dCas9 fused deaminase complex and reported humble editing efficiency of less than 5%. The low editing efficiency of the dCas9-dependent CBE systems is regarded to their ability to edit only a single DNA strand. The deaminated base can be corrected back to the original base by the cells; subsequently, only a handful of deamination results in the desired editing. Therefore, to yield higher editing efficiency, authors adopted nCas9(D10A) which nick's the unedited strand. Nick in the unedited strand favours the conversion of U•G-to-U•A base pair during the DNA repair process, ultimately increasing the editing efficiency (Fig. 1) [22,23].

Liu group was the first one to develop an adenine base editor [21]. There is no known naturally existing DNA adenosine deaminase. Therefore, the group evolved an *E. coli* TadA tRNA adenosine deaminase. TadA catalyzes the conversion of adenosine to inosine in the single-stranded anticodon loop of tRNA-Arg. The inosine is recognized by the polymerase as G and subsequently, G•C base pair is introduced [46]. The authors used *E. coli* to evolve TadA to accept ssDNA by initially making unbiased libraries with mutated wild-type ecTadA fused to dCas9. The complex was used along with target sgRNA to transfect *E. coli* containing chloramphenicol resistance gene H193Y mutant, which lost the resistance. The colonies that survived on chloramphenicol showed certain TadA mutations that were incorporated in future AB editors. The same strategy was repeated by applying stringent conditions and incorporation of observed mutations in the later series of ABEs. Finally, evolved TadA (TadA*) (W23R + H36L + P48A + R51L + L84F + A106V + D108N + H123Y + S146C + D147Y + R152P + E155V + I156F + K157 N) was developed and ABE7.10, wtTadA-TadA*-nCas9 fused together via two 32-amino acid linkers, was established (Fig. 1) [21].

Apart from CBE and ABE, Liu group recently developed the search and replace editing, i.e., prime editing [24]. Contrary to BE that use deaminase, PE works on a completely different principle, which involves reverse transcription. PE consists of nCas9(H840A)-reverse transcriptase fusion complex which uses prime guide RNA (pegRNA, consisting of primer binding site (PBS) which is complementary to the sequence upstream of PAM site of the nicked strand and reverse transcriptase template (RT template)) to form a primer with the 3′ flap of the nicked strand for reverse transcription to encode desired edits (Fig. 2A). PE then uses the structure-specific flap endonucleases found in the cell that prefer 5′ flap as a substrate to its advantages by letting them digest the unedited 5′ flap while 3′ flap with edited DNA is ligated, forming a heteroduplex of DNA containing edited and unedited strand (Fig. 2B). This heteroduplex is then resolved by the cell repair machinery as it copies the edited strand to the complementary unedited strand leading to permanent incorporation of the edit. However, the mechanism of the 5′ flap digestion and the heteroduplex resolution are not thoroughly understood. PE initially used mouse-murine leukemia virus (M-MLV RT). The group increased the PE editing efficiency by several folds by developing an engineered M-MLV RT (D200 N + L603W + T330P + T306K + W313F), thus developing the PE2 system. The authors further improved the editing efficiency by 1.5–4.2 folds compared to PE2 by developing the PE3 system, which involves the induction of a second nick in the unedited strand 14 to 116 nt away from original pegRNA nick (Fig. 2C) [24]. The induction of the second nick increases the chances of the unedited strand, rather than the edited strand, to be repaired by MMR machinery, thus, increasing the possibility of getting the duplex DNA with the desired edits. To reduce the indel formation by PE3, PE3b strategy was developed in which the second nick is carried out after the flap resolution and the successful editing of the initial editing strand. This was achieved by using sgRNA containing mismatches between the spacer and the unedited allele, and making it complementary to the edited strand and not the original allele (Fig. 2C). PE3b results in 13-folds
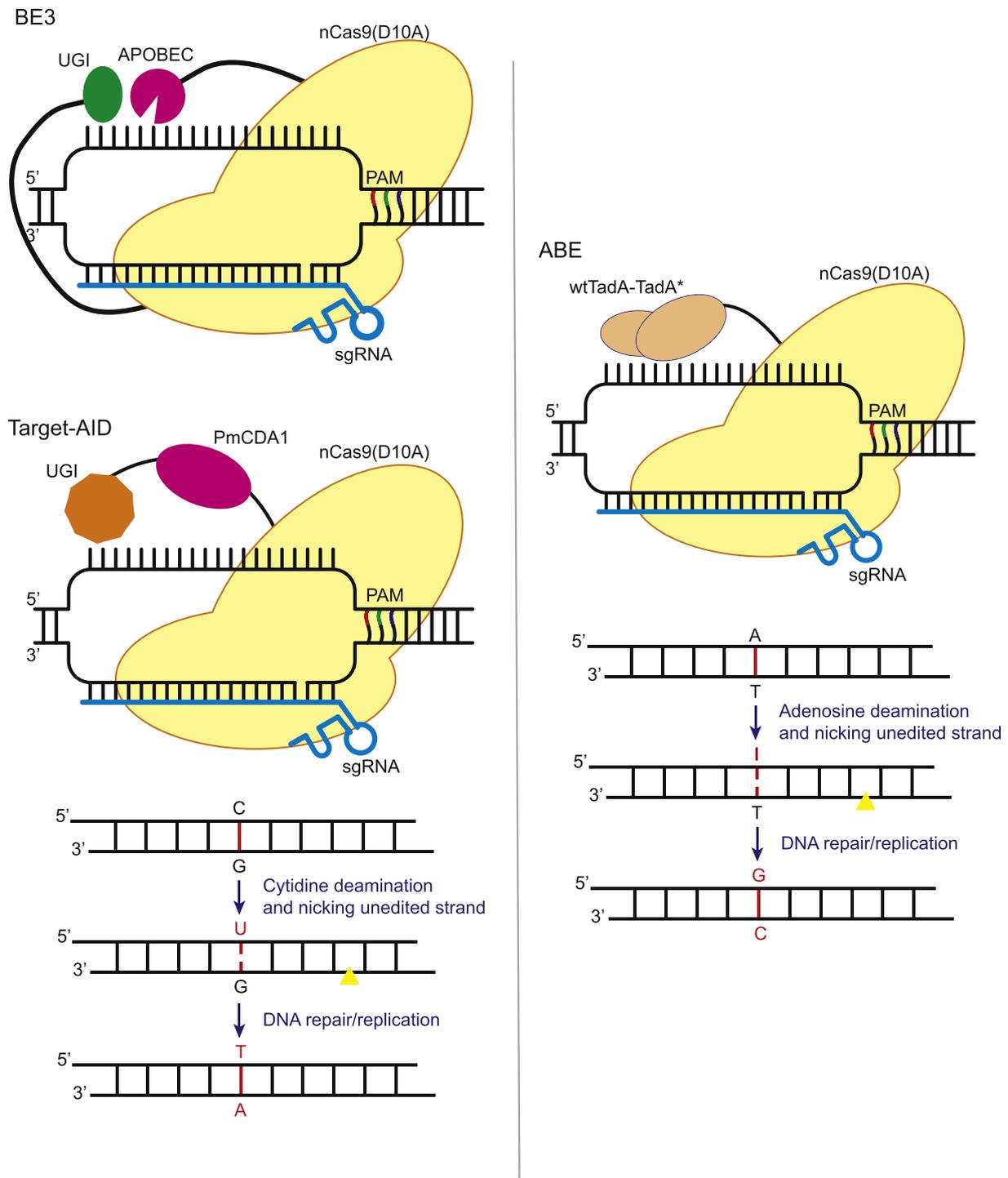
Fig. 1. Deaminase based Cas9 base editing. A schematic model of deaminase based Cas9 base editing systems: cytosine base editors (CBE, for example, BE3 & Target-AID) and adenine base editor (ABE). **CBE** consists of rat apolipoprotein B mRNA editing enzyme (APOBEC), and uracil glycosylase inhibitor (UGI) fused to N and C terminus of nCas9(D10A), respectively. Target-AID consists of activation-induced cytosine deaminase ortholog PmCDA1 and UGI fused to N terminus of nCas9(D10A). CBE involves deamination of cytosine by the deaminase which converts it into uracil, making U•G wobble base. UGI prevents its conversion back to C. Mismatch repair machinery (MMR) recognizes it forming U•A which is then converted to T•A by replication machinery leading C•G-to-T•A substitution. **ABE** consists of heterodimeric wtTadA-TadA* fused to nCas9(D10A). ABE performs deamination converting T•A-to-T•I which is then recognized by DNA repair and replication machinery and converted to C•G base pair (Yellow triangle: nick site).

lower indels compared to PE3. PE's strength to insert or delete arbitrary DNA sequence and allow all possible point mutations at target locus with high precision makes it a highly attractive editing system [24]. Although BE and PE have developed and applied broadly in animal cells, their adoption for bacteria and plants has provided a new avenue to advance their genome editing.

3. DSB & template-free editing in bacteria

CBE and ABE have been applied in several bacteria such as *Escherichia coli* [21,47–52], *Brucella melitensis* [48], *Clostridium beijerinckii* [53], *Corynebacterium glutamicum* [54], *Klebsiella pneumoniae* [55], *Pseudomonas* [56], *Staphylococcus* [52,57] and *Streptomyces*
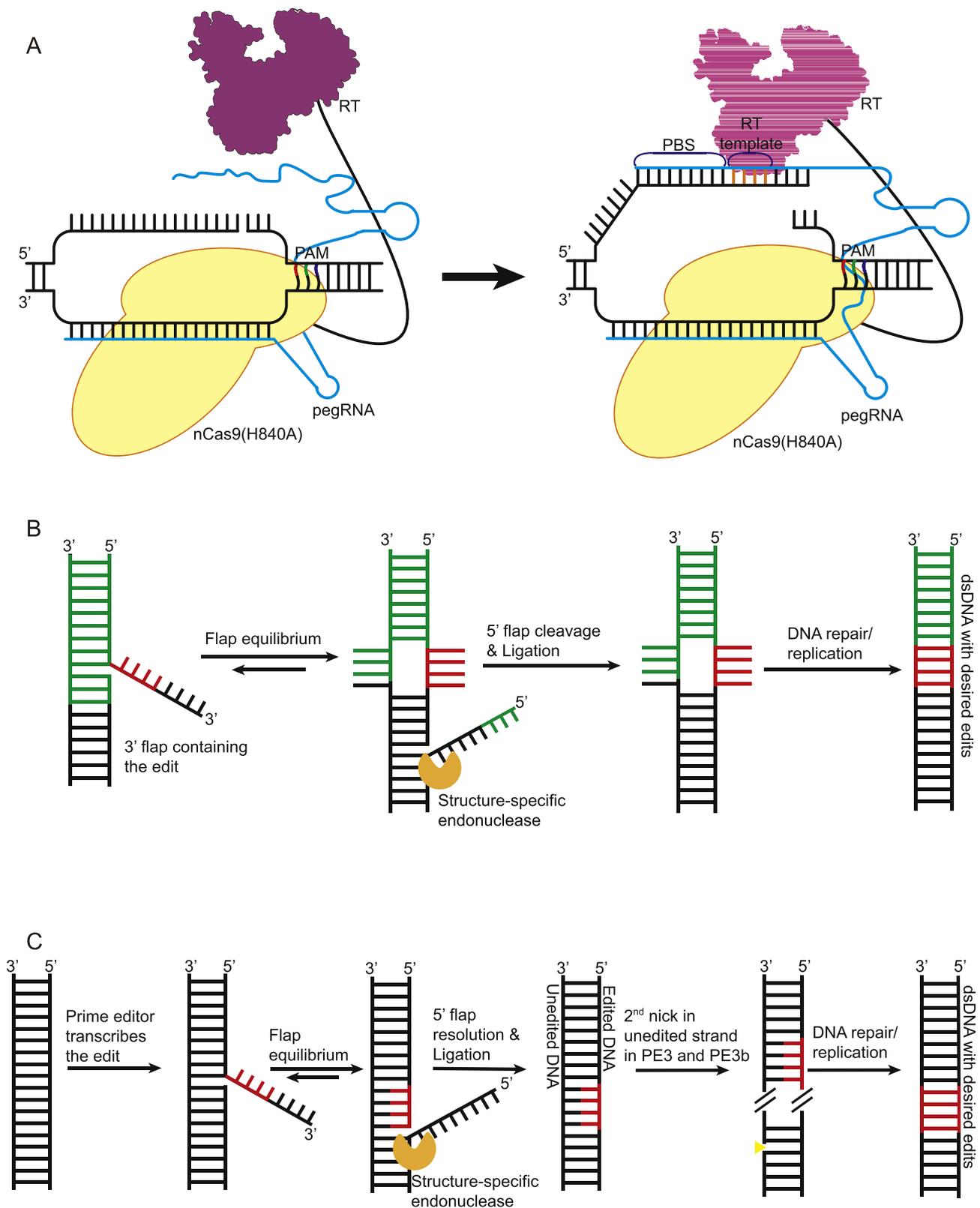
Fig. 2. Schematic representation of prime editing system. **(A)** The basic construct of PE consists of nCas9(H840A) fused to reverse transcriptase (RT) via a linker. The prime guide RNA (pegRNA) in PE consists of gRNA, primer binding site (PBS) and RT template. Once nCas9(H840A) nicks the strand, RT uses PBS fused to 3′ flap as a primer to encode RT DNA template. **(B)** After reverse transcription, PE1 and PE2 systems involve flap equilibrium of edited 3′ and unedited 5′ flap. The unedited 5′ flap is then cleaved off by structure-specific endonucleases followed by ligation and DNA repair/replication leading to permanent editing (Green bases: pegRNA binding regions, Red bases: desired edits). **(C)** After 5′ flap cleavage, a heteroduplex of the edited and unedited strand is formed. PE3 and PE3b involve a 2nd nick in the unedited strand 14-116 nt away from the initial pegRNA nick. This increases the chances of generation of dsDNA with the desired edit by favouring the repair of unedited strand by repair/replication machinery (Black strand: original DNA, Red strand: edited strand, Yellow triangle: nick site).

Table 1
Base editing systems in bacteria.

Organism	Editor type	Base editing system[a]	Target genes	Editing efficiency	Editing window (upstream of PAM)	Ref.
Escherichia coli						
E. coli	BE3	pEcBE3	*tetA, lacZ, rppH*	100%	−12 to −16	[48]
E. coli	Target-AID	dCas9-CDA-UL	*galK, rpoB, xylB, manA, pta, tipaA*	61–95%	−16 to −20 (extended sgRNA = −18 to −24)	[49]
E. coli	Cpf1-based BE	dLB-Cpf1-BE	*SupF* in shuttle vector	45–80%	−7 to −13 (downstream of PAM)	[47]
E. coli	ABE	pABE	*lacZ, yagl, murR*	66–100%	−13 to −17	[52]
E. coli	ABE	pnCas9-TadA pdCas9-TadA pnCas9-TadA + Cas9	*lacZ, poxb*	9.3–65.4% 1.3–8.3% 12.7–99%	−12 to −17	[50]
Streptomyces						
S. coelicolor *S. rapamycinicus*	Target-AID	dCas9-CDA-UL_str	*redw, redL, redX,*	50–100%, 60%[b] 20%[c]	−16 to −20	[59]
S. coelicolor *S. griseofuscus*	BE3	CRISPR-cBEST	actinorhodin gene cluster, CDA gene cluster, RED gene cluster	30–100%	−11 to −17	[58]
S. coelicolor	BE2	BE2	*red, actl-ORF2*	43–70% 43%[b]	−13 to −17	[60]
S. coelicolor	BE3	BE3	*red, actl-ORF2*	100% 100%[b]	−13 to −17	[60]
S. avermitilis			*ave*	0–100% multiplex editing		
S. avermitilis	HF-BE3	HF-BE3	*ave*	60–80%	−13 to −17	[60]
S. coelicolor	ABE	CRISPR-aBEST	*SCO5087*	0–40%	−12 to −17	[58]
S. coelicolor	ABE	ABEd	*actVB*	–	−14 to −17	[60]
S. coelicolor	ABE	ABEn	*actvB*	100%	−14 to −17	[60]
S. hygroscopicus	BE3	pWHU77-BE	*hygD, hygL, hygJ, hygY, hygM*	–	–	[151]
Staphylococcus						
S. aureus	BE3	pnCasSA-BEC	*agrA, cntA, esaD*	100%	−13 to −17	[57]
S. aureus	ABE	pABE	*agrA, murR*	50–100%	−13 to −17	[52]
Pseudomonas						
P. aeruginosa	BE3	pnCasPA-BEC	*rhlR, rhlB*	> 90%	−13 to −18	[56]
P. putida			*cadR, ompR*	> 90%		
P. fluorescens			*per, aspC*	> 84%		
P. syringae			*gacA, hrpL*	> 90%		
Klebsiella pneumoniae						
K. pneumonia	BE3	pBECKP	*fosA, dhak, bla_KPC-2, bla_CTX-M-65*	25–100%	−13 to −18	[55]
Clostridium beijerinckii						
C. beijerinckii	BE3	pCBEclos-opt	*pyre, xylR, spo0A, araR*	40–100%	–	[53]
Brucella melitensis						
B. melitensis	BE3	BE3	*virB10*	100%	–	[48]
Corynebacterium glutamicum						
C. glutamicum	Target-AID	CRISPR/dCas9-AID	*upp*	11.2%	–	[54]
C. glutamicum		CRISPR/nCas9(D10A)-AID	*Upp, rfp, ald, csp, ldhA, adhA, odhA,* 94 transcription factor genes	> 48%, 41–100%[b] 23.3%[c]	−16 to −20	

[a] Base editing system refers to the specific name of the editor used in the specific papers.
[b] Dual loci targeting efficiency.
[c] Triple loci targeting efficiency.

[58–60] (Table 1). However, PE has not been set up in bacteria yet. The development of PEs for bacterium would be a crucial milestone in the bacterial gene-editing toolbox. Here we review the base editing systems in bacteria.

3.1. Escherichia coli

Escherichia coli is an extensively studied gram-negative bacterium with well-established genetic literature and an ever-expanding toolbox that includes expression vectors, fermentation techniques, biochemical and biofuels production strains, and CRISPR tools [41,61–64]. It is the preferred bacteria for large scale production of chemicals such as fatty acids and 1,4-butanediol, and about 30% of approved therapeutics proteins are industrially produced using *E. coli* [63–67]. To enhance productivity, several gene-editing tools have been developed, however, none of the developed tools allow precise single base editing except BEs [68].

Zheng et al. used the BE3 system to develop pEcBE3 cytosine base editing system for *E. coli* [48]. The pEcBE3 consists of nCas9(D10A) fused to rAPOBEC1 and UGI. The editing system was used to induce a stop codon (TAA) by C-to-T conversion of Gln codon (CAA) with 100% efficiency in *tetA* in *E. coli* strain XL1-Blue. pEcBE3 system showed near 100% editing efficiency as it introduced base conversion at *lacZ* and *rppH* in *E. coli* [48]. Banno et al. [49] adopted another version of CBE, i.e., Target-AID which has a more distal activity window at −16 to −20 bases upstream of PAM compared to BE3, which shows activity at −12 to −16 bases upstream of PAM [22,23]. The authors initially used nCas9(D10A) but observed poor transformation efficiency and speculated growth defect or cell death similar to Cas9. Therefore, they adopted dCas9. The dCas9 was fused to PmCDA1, UGI, and protein degradation tag (LVA) developing dCas9-CDA-UL. The editing system successfully performed multiplex editing as six out of eight sequenced clones showed simultaneous editing of *galK, xylB, manA, pta, adhE,* and *tpiA*. dCas9-CDA-UL also successfully edited 41 loci simultaneously

depicting the multiplex editing strength of the system [49]. However, increased Cas9 tolerance for nonseed mismatches increases the occurrence of unwanted C-to-T base substitutions [69,70], and it's G/C-rich PAM sequence requirement limits the BE applications [71]. Therefore, Chen and colleagues developed a Cas12a based CBE system (dLB-Cpf1-BE) by fusing catalytically inactive *L. bacterium* Cpf1 (dLb-Cpf1) with rAPOBEC1 [47]. They successfully achieved 44%–74% C-to-T substitution efficiency in *E. coli* [47]. This system widens the genome target area by CBE as Cas12a requires a T-rich PAM sequence (For example, TTTV) [72,73].

Apart from CBE, ABE has also been adopted for *E. coli* [52]. ABE7.10 system successfully edited *yagl, murR,* and *lacZ* in MG1655 and DH5α strains, achieving high editing efficiency between 66% and 100% [52]. Additionally, Zhang and colleagues developed a double-check base editing system, which uses the DSB capability of Cas9 to eliminate unedited cells [50]. They were able to increase editing efficiency by 3–6 folds at specific loci, compared to ABE [50]. This provides an efficient way to enhance ABE editing capabilities in *E. coli.*

3.2. Streptomyces

Streptomyces is a gram-positive GC-rich Actinobacteria consisting of over 500 species. It is known for its industrial applications, as it produces 39% of all microbial metabolites [74,75] and 70% of the current antibiotics [76]. Applying metabolic engineering to enhance biosynthesis requires the development of novel *Streptomyces* editing tools. However, like other Actinomycetes, very GC-rich (~70%) and instable genome make genome editing difficult [77,78]. Development of BE for *Streptomyces* expands its genome editing toolbox, helping the advancement of efforts to increase its utility.

Zhao et al. developed a Target-AID CBE system, i.e., dCas9-CDA-UL$_{str}$ for *Streptomyces* [59]. The system consists of dCas9 controlled by *tipAp* leaky promoter fused with cytosine deaminase (CDA), UGI (U), and protein degradation tag (L). The editing system achieved 100%, 60%, and 20% editing efficiency with single, double, and triple target locus editing, respectively [59]. To expand the genetic toolbox of *Streptomyces*, Lee group developed two CRISPR-Base Editing SysTems, i.e., CRISPR-cBEST (CBE system) and CRISPR-aBEST (ABE system) consisting of nCas9(D10A) fused with either rAPOBEC1-UGI or TadA*, respectively [58]. The CRISPR-cBEST achieved 30%–100% editing efficiency in *S. griseofuscus* and *S. coelicolor*, while 0%–40% editing efficiency was reported by CRISPR-aBEST in *S. coelicolor*. Interestingly, the group reported varying editing efficiency of CRISPR-BEST depending on the adjoining base to the target base, showing 5′-TC > CC > AC > GC substrate priority [58]. In order to facilitate sgRNA designing for CRISPR-BEST, Weber group put forward an updated version of guide RNA design tool, i.e., CRISPy-web single guide RNA design tool [79].

The above described *Streptomyces* base editing systems are similar in the aspect that they efficiently perform C-to-T or A-to-G substitutions. However, they differ in the editing efficiencies which are described above, and the editing window. The Target-AID based, dCas9-CDA-UL$_{str}$ performs C-to-T substitutions further upstream of the PAM site at −16 to −20, whereas CRISPR-cBEST performs CB editing at −11 to −17 upstream of PAM site [58–60] (Table 1). The variance in activity spectra of editing systems can be useful depending on specific experimental requirements.

3.3. Staphylococcus

Staphylococcus is a gram-positive facultative anaerobic, highly common human pathogen. It colonizes 30% of the human population and is a leading cause of bacteremia [80]. The major public health crisis is unfolding with the emergence of antibiotic-resistant *Staphylococcus* strains, such as methicillin-resistant strains (MRSA) [81]. Countering the crisis requires studying virulence factors, chronicity, and pathogenicity, but these studies have been hindered due to a lack of

availability of genetic tools [82]. The existing genetic tools rely heavily on HDR, which makes them inefficient [83]. Enhancement in CRISPR tools and the development of BE have helped to engineer non-DSB and HDR-free genetic manipulation tools for *Staphylococcus*.

Ji group developed CBE [57] and ABE [52] systems for *Staphylococcus*. For CBE, pnCasSA-BEC was constructed by fusing nCas9(D10A) to rAPOBEC1 via XTEN linker [57]. pnCasSA-BEC was used in *S. aureus* to introduce premature stop codon by C-to-T substitution in *agrA, cntA,* and *esaD* with 100% editing efficiency between −13 and −17 bases upstream of PAM. The editing system was then tested in clinically isolated *S. aureus* strains such as MRSA, Newman, and USA300, achieving 100% editing efficiency. The group also performed bioinformatics analysis to calculate total editable sites in MRSA and Newman strains. The results showed that at least 95% of the genes possessed editable C(s), while ~70% of the genes contained editable stop codon in both the strains [57]. Moreover, an ABE system, pABE, was developed by the fusion of nCas9(D10A) with ABE7.10 [52]. pABE based A-to-G substitution resulted in 50%–100% editing efficiency between −13 and −17 bases upstream of PAM. The group used whole-genome sequencing of edited *S. aureus* RN4220 strains to study the off-target effect and reported no off-targeting at any of the potential off-target sites.

3.4. Pseudomonas

Pseudomonas belongs to the genus of gram-negative bacteria with 144 different species, largest among gram-negative bacteria [84]. Research in *Pseudomonas* has been expanding due to its biomedical and ecological importance, and biotechnological applications. For instance, *P. aeruginosa*, an opportunistic human pathogen, is the leading cause of morbidity in patients with cystic fibrosis, acquired immune deficiency syndrome, and cancer [85,86]. Whereas *P. putida*, a soil microbiome, has been engineered for the production of high-value chemicals and used for bioremediation of polluted sites [75,87–89]. While *P. fluorescens*, which inhabits the surface of plants, is engineered for the production of recombinant proteins [90]. CRISPR editing tools have been developed for *Pseudomonas* species to enhance their productivity [85,91–93]. However, the lack of effective DNA repair systems and high GC content (~62%) in some *Pseudomonas* species make it hard to efficiently use DSB-induced HDR-CRISPR tools [94].

Chan et al. developed pnCasPA-BEC, a CBE system for *Pseudomonas* species [56]. It was constructed by fusing nCas9(D10A) with rAPOBEC1 with XTEN linker. pnCasPA-BEC system was used to introduce stop codons in *rhlR* and *rhlB* with high efficiency (> 90%) in *P. aeruginosa* strains. pnCasPA-BEC was then used to edit genes in *P. putida, P. fluorescens,* and *P. syringae*, demonstrating the system's ability to be applied in a wide range of *Pseudomonas* species. The authors reported the editing window to be −13 to −18 bases upstream of PAM and substrate preference to be TC ≥ CC > AC > GC similar to previously described CBE systems [56].

3.5. Corynebacterium glutamicum

Corynebacteria are gram-positive industrially important soil bacteria. They are widely used for the production of bioproducts. Especially, *C. glutamicum* whose ability to produce amino acids from sugar and ammonia has been utilized for industrial-scale production of several amino acids such as glutamate, lysine, isoleucine, tryptophan and threonine [95,96]. *C. glutamicum* has also been engineered to produce a wide variety of biochemicals, such as polymer subunits and biofuels [97,98]. The engineering of *C. glutamicum* is laborious and challenging as the editing tools show low recombination efficiency and lack of positive selection for mutations. Therefore, an efficient editing tool that allows robust and easy genetic engineering is needed.

Wang et al. adopted Target-AID to develop an automated base editing method, i.e., MACBETH for *C. glutamicum* [54]. The editing system consists of nCas9(D10A) fused to AID under *tac* promoter and a

sgRNA cassette under a strong constitutive P_{cspB} promoter. The editing process was automated by using an integrated robotics system for plasmid construction and PCR amplification, and automated high-throughput colony picker for plating and colony picking. The editing system successfully performed single, dual, and triple locus editing with editing efficiencies between 23% and 100%. Additionally, the authors carried out genome-wide bioinformatics analysis for *C. glutamicum* ATCC 13032. They reported that the editing system could introduce an early stop codon in 88% of the total 3099 genes.

3.6. Other species

Base editing has been developed for a few more bacterial species like *Klebsiella pneumonia* [55] and *Clostridium beijerinckii* [53].

K. pneumonia is a human pathogen and an industrially relevant bacterium. Research in *K. pneumonia* has become increasingly significant because of the increase in its pathogenicity due to the emergence of multi-drug resistant strains. *K. pneumonia* strains resistant to carbapenems and extended-spectrum β-lactams (ESBL) have emerged in various parts of the world [99,100]. Ji group developed a CBE system for *K. pneumonia*, pBECKP, a single-plasmid editing system consisting of nCas9(D10A) fused with rAPOBEC1 via XTEN linker, and sgRNA under the control of strong constitutive promoter (J23119) [55]. pBECKP system was used to introduce a premature stop codon in *fosA* in a clinically isolated strain of *K. pneumonia* with 100% editing efficiency. The group successfully tested the ability of the editing system to edit a GC-rich spacer in *dhak*. Moreover, the group enhanced the carbapenems susceptibility of carbapenem-resistant *K. pneumoniae* strain's KP-CRE23 by effectively using pBECKP system to inactivate carbapenemase gene, *bla_KPC-2* [55].

C. beijerinckii is an industrially important gram-positive solventogenic bacterium. It produces biochemicals such as acetone, butanol, ethanol (ABE chemicals) utilizing various carbon sources [101–103]. Interestingly, during the fermentation of pentose sugar, *C. beijerinckii* does not show glucose repression effect in the presence of glucose, which makes it more attractive than other *Clostridium* species [104]. Li et al. developed a CBE system, pCBEclos-opt for *C. beijerinckii* [53]. pCBEclos-opt is a single plasmid editing system consisting of nCas9(D10A) fused with rAPOBEC1 and UGI, and sgRNA expression cassette. pCBEclos-opt was used to disrupt *pyrE* in *C. beijerinckii* leading to uracil analog 5-FOA resistance, which was used as a selective marker. Half of the colonies were edited in *pyrE* and other gene sites *araR*, *xylR,* and *spo0A* [53].

4. DSB & template-free editing in plants

Apart from bacteria, BE has been adopted for several plant species and more recently prime editors have been developed for plants to carry out efficient and precise editing. The DSB-induced HDR editing techniques have been applied in plants to improve the crops. However, its applications have been limited as most acceleration instances in agriculture are conferred by single-nucleotide mutations. The low frequency of HDR in plants due to their gene-repair system [105] and the installation of undesired by-products by the DSB-induced Cas9 system [106] render the editing technique an unfeasible approach. The adoption of BEs and PEs in the primary DNA sequences has enabled the precise engineering of crops with novel characteristics (Table 2).

4.1. Rice, wheat and maize

Rice, wheat, and maize are major monocot cereal crops that collectively account for about 50% of the total world's calorie consumption. This makes it critical to carry out research in them. CRISPR tools have been widely developed for these crops for the enhancement of bioproduction and trait improvement [107]. However, BE and PE are more reliable, facile, and efficient tools compared to DSB-dependent CRISPR

tools, and they have been effectively applied in rice, wheat, and maize (Table 2).

Gao group developed a CBE system composed of rAPOBEC1 and nCas9(D10A) for rice, wheat, and maize, and reported prominent editing efficiency [108]. The editing system showed 0.39–7.07% C-to-T substitutions in which the highest frequency appeared at or near −13 upstream of PAM with very few indels (0.01–0.22%). They attempted to use dCas9 instead of nCas9 in CBE; however, it led to decreased editing efficiency (0.00–1.29%). In the base-edited plants, 40 out of 92 transformed plants (43.48%) revealed at least one substitution in the editing window. At the same time, Kondo group also published a study in which they successfully applied Target-AID for point mutagenesis in rice [109]. They used the *ALS A96* target to develop herbicide imazamox (IMZ) resistant rice by the C-to-T substitution and two other target sites (G590 and W483) of *FTIP1e* (LOC_Os07g30020) of rice to test the CBE system. Finally, 8 of 66 clones were obtained, which carried resistance to IMZ and had both point mutations in *FTIP1e*. Similarly, BE3 was reported by Li et al. that could introduce precise point mutations in rice with minimal indels [110]. Lu et al. gained a frequency of 1.4%–11.5% in which the highest editing efficiency location was at or near −13 upstream of PAM, targeting *NRT1.1B* and *SLR1* in rice [111]. Moreover, human AID (hAID) was used to enhance CBE editing efficiency at GC-rich regions in rice [112]. The authors chose the hAID variant (K10E + T82I + E156G) to construct their hAID*Δ-XTEN-Cas9n-NLS chimeric complex, which showed high editing efficiency in GC-rich regions. However, the hAID*Δ-XTEN-Cas9n-NLS system led to more undesired indels compared to rBE3 and rBE4 [113].

Apart from CBE, Gao and colleagues constructed the ABE system, i.e., PABE system consisting of the wtTadA-TadA* adenosine deaminase, nCas9, three NLSs and esgRNA (sgRNA with improved expression) to generate A•T-to-G•C conversions in rice and wheat [114]. They achieved up to 7.5% A-to-G substitution frequency in protoplasts and 15.8%–59.1% editing efficiency in regenerated plants. Yan et al. reported that ABE could gain 16.67% efficiency at *OsMPK6* site in rice [115]. Furthermore, Wei and his group also reported an ABE system but observed the point mutation frequency of < 10% [116]. They increased the ABE editing efficiency by double selection (selection of edited cells using hygromycin and herbicide) [117], in which > 50% plants were edited.

Besides BE, the PE system has also been introduced in rice and wheat. Lin et al. used a maize Ubiquitin-1 (Ubi-1) promoter to develop the fusions of Cas9(H840) and M-MLV RT, CaMV RT, or RT-retron [118]. They reported that PPE3 and PPE3b had the same efficiency as PPE2, and obtained up to 21.8% regenerated prime-edited rice plants. Xu et al. [119] and Li et al. [120] adopted PE3 in rice T0 lines and rice calli, respectively, with the former achieving 26% frequency compared to 9% for the latter. Meanwhile, Wei group reported that PE2 could generate 0%–31.3% editing frequency in T0 plants HPT$^{−ATG}$ reporter in rice while the PE3 systems did not show preferential editing efficiency [121]. Apart from this, Zhu and his group replaced SpCas9(H840A) with SaCas9(N580A) to enhance PE3 efficiency, but Sa-PE3 exhibited a lower editing activity compared to Sp-PE3 [122].

4.2. Arabidopsis

Arabidopsis is the most thoroughly studied model flowering plant. Since the realization of the requirement of an efficient plant model organism, *Arabidopsis* has been seen as a go-to plant. Its fast life cycle, ease of handling, and limited minerals requirement make it a flawless model organism. Several decades of research have led to the development of a range of genetic tools. Since the first reported CRISPR-Cas based editing in *Arabidopsis* [123], several groups have developed CRISPR tools for it [107]. Plenty of groups have expanded *Arabidopsis* genetic toolbox using BE.

Jiang group developed the CBE system (BE3) in *Arabidopsis* [124]. They used *Agrobacterium*-mediated transformation to introduce the

Table 2
Base editing and prime editing systems in plants.

Organism	Editor type	Base editing system[a]	Target genes	Editing frequency[b]	Editing window (upstream of PAM)	Ref.
Rice	BE3	pnCas9-PBE	OsCDC48, OsNRT1.1B, OsSPL14	43.48[c] 0.39–7.07%[f]	−18 to −12	[108]
Rice	BE2	APOBEC1-XTEN-Cas9(D10A)	NRT1.1B, SLR1	≤13.3%[c]	−17 to −14	[111]
Rice	BE3	APOBEC1-XTEN-Cas9n-UGI-NLS	OsCERK1, OsSERK1, OsSERK2, ipa1	10–38.9%[c]	−19 to −13	[113]
Rice	BE4	APOBEC1-XTEN-Cas9n (VQR)-UGI-NLS	pi-ta	18.2%[c]	−19 to −13	
Rice	Target-AID	nCas9[Os]-PmCDA1[At]	ALS, FTIP1e	6–89%[c]	−19 to −17	[109]
Rice	ABE	PABE	OsALS, OsCDC48, OsAAT, OsDEP1, OsACC, OsNRT1.1B, OsEV, OsOD	3.2–59.1%[c]	−17 to −13	[114]
Rice	ABE	TadA-TadA7.10-Cas9n	OsMPK6, OsMPK13, OsSERK2, OsWRKY45	0–62.26%[c]	−17 to −14	[115]
Rice	ABE	pRABEsp-OsU6	OsSPL14, SLR1, OsSPL16, OsSPL18, LOC_Os02g24720	> 4.8%[c]	−17 to −14	[161]
Rice	ABE	pRABESA-OsU6sa	OsSPL14, OsSPL17, OsSPL16, OsSPL18	> 17%[c]	–	
Rice	ABE	pHUN411-ABE	Wx, GL2/OsGRF4, OsGRF3	< 10%[f]	−16 to −12	[116]
Rice	APOBEC3A	A3A-PBE	OsAAT, OsCDC48, OsDEP1, OsNRT1.1B, OsOD, OsEV	44–83%[c]	−20 to −4	[142]
Rice	hAID∗Δ	hAID∗Δ-XTEN-Cas9n-NLS	OsRLCK185, OsCERK1, Pi-d2, OsFLS2, OsAOS1, OsJAR1, OsJAR2, OsCOI2	–	−18 to −14	[112]
Rice	ABE	VQR-Cas9 (D10A)/VRER-Cas9 (D10A)	OsSPL14, OsSPL17, OsSPL16, OsSPL18, OsIDS1, OsTOE1	≤74.3%[c]	−19 to −11	[162]
Rice	ABE	SaKKH-Cas9 (D10A)	SNB	6.5%[c]	–	
Rice	BE	VQR-Cas9 (D10A)	PMS3	≤61.1%[c]	–	
Rice	BE3	xCas9(D10A)-rAPOBEC1	OsDEP1	≤30%[f]	−19 to −16	[175]
Rice	Target-AID	xCas9(D10A)-PmCDA1	OsDEP1	< 20%[c]	−19 to −16	
Rice	Target-AID	Cas9-NG (D10A)-PmCDA-UGI	OsDEP1, OsCDC48, OsPDS	0–56.3%[c]	−20 to −7	
Rice	Target-AID	NGv1 (D10A)	EPSPS, ALS, DL	5–95.5%[c] 4.2–86.3%[f]	−20 to −9	[176]
Rice	BE	NGv1 (D10A)	EPSPS, ALS, DL	4.3–21.8%[f]	−19 to −14	
Rice	BE3	pCXUN-BE3	OsPDS, OsSBEIIb	0.1–20%[c]	−17 to −13	[110]
Rice	BE3 Target-AID ABE	eBE3, eCDA, eABE	OsACC	–	–	[149]
Rice	BE3 ABE	Base-Editing-mediated Gene Evolution (BEMGE)	OsALS1, OsALS2, OsALS3	–	–	[150]
Rice	BE3 Target-AID	xCas9-epBE	OsMPK2, OsMPK5, OsMPK5, OsALS, OsNRT1.1B	5–64.3%[c]	−20 to −10/-7	[163]
Rice	BE4 ABE	xCas9n-CBE, Cas9n-NG-CBE, eCas9n-NG-CBE xCas9n-ABE, Cas9n-NG-ABE, eCas9n-NG-ABE	OsWaxy, OsEUI1, OsCKX2 OsWaxy, OsEUI1, OsCKX2	9.1–45.5%[c] 2–6.5%[c]	−18 to −13	[177]
Rice	ecTadA∗7.10-nCas9	ABE-P1S	OsSPL14, SLR1, OsSERK2, Tms9-1, OsNRT1.1B, OsACC1, OsDEP1	11.4–96.3%[c]	−20 to −9	[168]
Rice	ecTadA∗7.10-nSaCas9,	ABE-P2S	SPX-MSF2, OsSPL14, OsSPL17, OsSPL14, OsSPL17, OsSPL16, OsSPL18	15.9–61.1%[c]	−20 to −18	
Rice	ecTadA∗7.10-nSaKKH-Cas9	ABE-P5S	OsSPL13, SNB	6.1–33.9%[c]	−17 to −10	
Rice	BE3	nCas9-PBE	GL1-1, NAL1	58–68%[c]	−18 to −12	[125]
Rice	ABE	ABE7.10-nSpCas9-NGv1	sgOs-siteG1, sgOs-site2, sgOs-site3, sgOs-site4	29.2–45.8%[c]	−16 to −13	[178]
Rice	ABE	pPUN411-HABE, pPUN411-ABEH	Pid3, WX	> 97.9%[c]	–	[117]
Rice	PE	PPE2, PPE3, PPE3b	OsCDC48, OsALS, OsDEP1, OsEPSPS, OsLDMAR, OsGAPDH, OsAAT, TaUbi10, TaGW2, TaGASR7, TaLOX2, TaMLO, TaDME1	≤21.8%[c]	–	[118]
Rice	PE	pPE2, pPE3, pPE3b	OsPDS, OsACC1, OsWx	0–31.3%[c]	–	[121]
Rice	PE	PE-P1, PE-P2	OsALS, OsACC, OsDEP1	≤26%[c]	–	[119]
Rice	PE	pCXUN-Ubi-NLS-nCas9(H840A)-Linker1 (33aa)-M-MLV-RT-Linker2 (14aa)-NLS-PolyA-E9-Actin-Nos	hptll, OsEPSPS	2.22–9.38%[c]	–	[120]
Rice	PE	Sp-PE2, Sp-PE3, Sa-PE3	ALS, APO1, SLR1, OsSPL14, APO2	0–17.1%[c]	–	[122]
Wheat	BE3	pnCas9-PBE	TaLOX2	1.25%[d]	−18 to −12	[108]
Wheat	ABE	PABE	TaDEP1, TaGW2	0.4–1.1%[c]	−17 to −13	[114]
Wheat	APOBEC3A	A3A-PBE	TaALS, TaMTL, TaLOX2	16.7–22.5%[d]	−20 to −4	[142]
Wheat	BE3	PBE	TaALS-P174	33–75%[d]	−18 to −12	[141]
Maize	BE3	pnCas9-PBE	ZmCENH3	10%[c]	−18 to −12	[108]
Tomato	Target-AID	nCas9[At]-PmCDA1[Hs]/nCas9[At]-PmCDA1[At]	DELLA, ETR1	41–92%[c]	−20 to −18	[109]
Tomato	Target-AID	pDeSpnCas9-NG_PmCDA1_UGI	SlALS1	32%[c]	−20 to −13	[179]
Potato	Target-AID	pDeSpnCas9-NG_PmCDA1_UGI	StDMR6-1, StGBSSI	9–64%[c]	−18 to −16	
Potato	APOBEC3A	pDeSpnCas9-NG_hAPOBEC3A_PmCDA1_UGI	StDMR6-1, StGBSSI	8–42%[c]	−18 to −11	
Potato	APOBEC3A	A3A-PBE	StALS, StGBSS	6.5%[c]	−20 to −4	[142]
Potato	Target-AID	–	SlALS1	100%[c]	–	[128]
Arabidopsis	ABE	pcABEs	AtALS, AtPDS, AtFT, AtLFY	0–85%[c]	−16 to −12	[127]
Arabidopsis	BE3	BE3	ALS	2.7–40%[c]	−17 to −12	[124]
Arabidopsis	BE3	nCas9-PBE	MTA	39.3%[c]	−18 to −12	[125]

(*continued on next page*)

Table 2 (*continued*)

Organism	Editor type	Base editing system[a]	Target genes	Editing frequency[b]	Editing window (upstream of PAM)	Ref.
Arabidopsis	BE3	BE3	*eIF4E1*	50%[c]	–	[126]
Rapeseed	ABE	pcABEs	*BnALS, BnPDS*	8.8% [e]	− 16 to − 12	[127]
Watermelon	BE3	CBE3	*ALS*	23%[c]	− 14 to − 13	[130]
Cotton	BE3	GhBE3	*GhCLA, GhPEBP*[c]	26–58%[c] ≤18.63%[f]	− 17 to − 12	[129]
Soybean	BE3	pTF101.1-sgRNA-BE	*GmFT2a, GmFT4*	≤18.2%[c]	–	[180]
Oilseed rape	BE3	CBE	*BnALS1*	1.8%[c]	–	[181]

[a] Base editing system refers to the specific name of the editor in the specific papers.
[b] Editing frequency refers to the ratio of edited to unedited plants.
[c] *Agrobacterium* mediated system.
[d] Particle bombardment.
[e] Protoplast transformation.
[f] Efficiency in targeted sequence.

editing system in *Arabidopsis*. BE3 was used to confer herbicide resistance by C-to-T conversion in the *ALS* at Pro197. A low editing efficiency (1.7%) was achieved in the T1 generation, and the resistance was successfully passed onto the next generation. In another study, BE3 was used for gene inactivation by mRNA mis-splicing [125]. Maize *Ubiquitin*-1 (*ZmUbi*) promoter-driven nCas9-PBE system was used to introduce C-to-T mutation at the donor site of *Arabidopsis MTA* intron 1 which led to the development of dwarfism (heterozygous mutant) and embryo-lethality (homozygous mutant) phenotype. The study reported successful mutation in 39.3% of transgenic T1 plants [125]. CBE typically leads to C-to-T mutations; however, at times, it can also produce C-to-G or C-to-A mutations. Bastet et al. used this ability to develop *Arabidopsis* with resistance against the potyvirus family by editing *elF4E* [126].

Moreover, Kang et al. were first to report that ABE (ABE7.10) in *Arabidopsis* [127]. They initially directly introduced the editing system (pcABE7.10) into the *Arabidopsis* protoplast and achieved editing efficiency of up to 4.1% and reported insignificant off-targeting at the potential off-target sites. They later developed phenotypically variant *Arabidopsis* by targeting *FT* to develop late-flowering phenotype. They reported that as a promoter, RPS5A led to more efficient A-to-G conversions compared to the 35 S promoter-ABE system [127].

4.3. Other species

Base editing has been applied to some other agriculturally and economically significant plant species (Table 2). Shimatani et al. applied codon-optimized Target-AID in tomato and demonstrated the nucleotide substitutions in T1 plants, with rare off-target mutations (0.14–0.38%) [109]. They tested the on-target frequency by introducing C-to-T or C-to-G substitutions in *DELLA* and reported editing frequency to be 26.2%–53.8%. A CBE system (fusion of PmCAD1 and nSpCas9) was developed in tomato and potato and produced 12.9%, and 10% C-to-T mutated transgene-free plants in the first generation, respectively [128].

BE3 system has been adopted for the cotton crop by Qin et al. [129]. They developed a GhBE3 editing system consisting of nCas9(D10A) fused to cytosine deaminase and UGI domain. It was used to edit *GhCLA* and *GhPEBP* with mutation frequencies between 26.67% and 57.78% in T0 plants, and off-targeting as low as 0.1%. Xu and his team engineered the ability of herbicide resistance in watermelon (up to 23% in T0), using CBE3 by converting C-to-T in the codon of Pro190 (CCG) of *ALS* gene [130].

5. Applications: current & future

CRISPR-Cas editing has evolved exponentially since its discovery as

an adaptive immune system of bacteria. Development of BE and more recently PE in plants and bacteria (to date, PE is not reported in bacteria yet) have enhanced their genetic toolbox and provided the ability to perform precise and efficient nucleotide substitutions at target locus (Tables 1 and 2). These tools can be exploited to develop efficient strategies to enhance bio-production and advance the establishment of bacteria and plants as major bio-production platforms.

5.1. Synthetic biology

Technologies to incorporate and record biological information in living cells have started to emerge recently [131–133]. BEs can be exploited to develop synthetic devices for external stimulus, event, and memory recording (Fig. 3A). Liu and Tang demonstrated the use of BE in *E. coli* in an analog event recorder, CRISPR-mediated analog multi-event recording apparatus (CAMERA2) [134]. They incorporated the BE2 system in a writing plasmid in which BE or guide RNA (gRNA) was under stimulus-dependent promoter induced by an exogenous signal. Upon the introduction of the exogenous signal, the dCas9/gRNA are transcribed, leading to base editing in the recording plasmid. Detection of base editing at the target site in recording plasmid represents the presence of the specific stimulus. CAMERA2 showed the ability to respond and record multiple independent stimuli, as incorporating multiple small-molecule responsive guide RNAs into a single writing plasmid led to the detection of targeted editing corresponding to the relative stimulus. Apart from detecting the presence of small-molecule dependent stimuli, other external stimuli like light, antibiotics, nutrient, and phage infection can be detected via CAMERA2 [134].

Another molecular recorder was put forward by Farzadfard et al., a read and write DNA-state reporter genetic circuit, DNA-based Ordered Memory, and Iteration Network Operator (DOMINO) [135]. Similar to CAMERA2, DOMINO uses base editing, CDA-nCas9-UGI system, which results in C-to-T substitution at the target site for permanent DNA recording. In the circuit, CBE-nCas9-UGI expression is controlled by small-molecule induced promoter termed as operational signal while the small-molecule inducing gRNA is termed as the input signal. The introduction of an operation signal enables reading, while the introduction of the input signal enables writing. The device allows independent, sequential, and temporal operation of logic and memory operations. DOMINO system can also provide phenotypical readouts to the input signal as demonstrated by the use of GFP, where the sequential addition of operational and then input signal led to the exclusion of stop codon at target sites [135].

The readable, writeable, and recordable systems can play a vital role if developed for industrially significant bacterial strains where overtime accumulation of by-products can affect the yield. Such stimulus detecting systems can help enhance productivity by early detection of by-
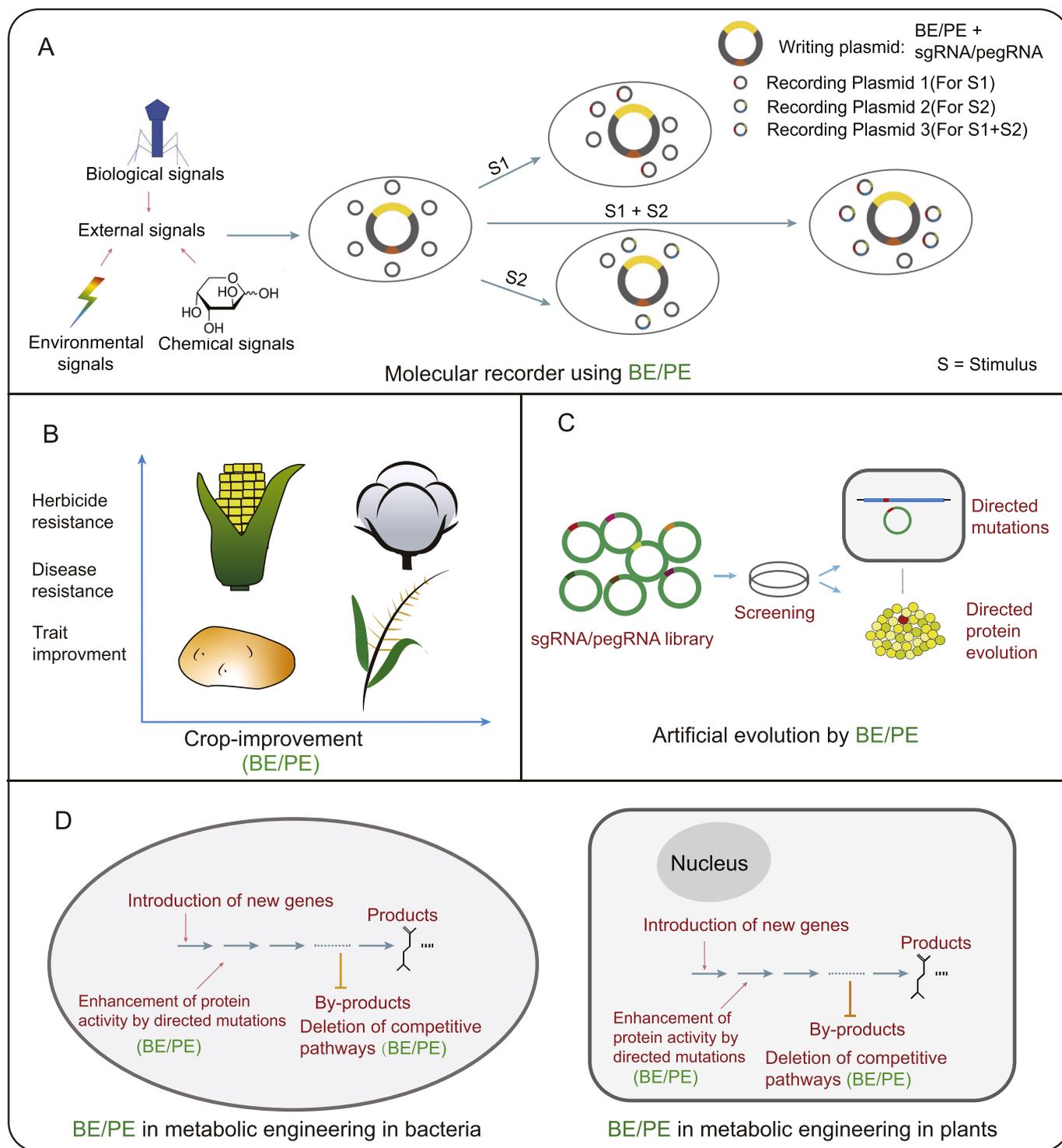
Fig. 3. Base editing and Prime editing applications (current & future). **(A)** Molecular recorders use BE to carry out nucleotide substitution relative to a specific stimulus. The base editing takes place in the presence of an external signal. The output signal either be phenotypic which can be recorded in the real-time or genotypic read via sequencing. DNA-writing technology can be expanded by the incorporation of PEs. **(B)** BE and PE can play a critical role in crop improvement. It has already been used for herbicide and disease resistance and trait improvement. **(C)** Artificial evolution can be achieved by BE/PE via the development of sgRNA/pegRNA libraries. UGI deprived CBE can lead to versatile mutations like C-to-G and C-to-A, this ability can be exploited for direct protein evolution. **(D)** Metabolic engineering in plants and bacteria can be improved by enhancing the enzymatic activity and deleting the competitive pathways by BE/PE tools.

products. For example, *E. coli* strains are widely used for the industrial-scale production of several therapeutic proteins; however, protein production is affected by the accumulation of acetate [136,137]. The CAMERA2 and DOMINO systems can be used for dose and time-dependent recording of acetate accumulation by using acetate induced promoters like *glnAP2* promoter [138]. Moreover, BE dependent

recording systems could also work as a bacterial synthetic population quality control device to record and detect unwanted variations and production of undesired molecules in real-time [139]. Such techniques may have broad industrial utility.

Besides industrial applications, BE or PE-based DNA writers and molecular recorders could be adopted for medical purposes. Their non-

DSB and template-free feature make them a perfect candidate for medical use. DNA recorders with the ability to detect disease-specific molecular stimuli may lead to the development of ingestible live biosensors [133]. These live biosensors could assist in the early diagnosis of major diseases like cancer and Parkinson's. The live biosensors could also be programmed to detect environmental signals like chemicals and toxins, thus offering a whole new direction to such technology. In the future, we expect to see more DNA-writing technology and especially information recording and storage systems, with the use of not just BE but also PE. PEs dependent analog and digital information storage systems may be a game-changer in the field for the fact that they can insert arbitrary sequences.

5.2. Crop improvement

BE systems have widely been used in agriculture, in which single-nucleotide mutations generally lead to the acceleration of the crops in most cases. With the functional SNPs, crops could have a significant improvement in their traits directly related to bioproduction, such as growth rates, yield, and sensitivity to environmental stress (Fig. 3B) [140]. BEs can be used to introduce such desired SNPs to introduce preferred traits without the concern of linkage drag. BEs can also be utilized to produce herbicide-resistant crops that have superiority in agriculture, as demonstrated in plenty of studies [108,109,114,124,130,141,142]. Besides herbicide-resistant, disease-resistant crops are another popular trend, for that the plants would have the ability to impart resistance if the allelic pseudogenes are corrected [27]. Specific disease resistance targets like *elF4E* (resistant alleles provide resistance to a wide range of single-stranded RNA viruses) [143] require several amino acid changes to develop resistance to a wide range of viruses [144]. Studies favor the use of resistant alleles that can still encode for functional translational initiation factors over loss-of-function alleles, which lead to a narrower resistance spectrum [144–146]. Achieving multiple amino acid changes using DSB-dependent genome editing tools and mutagenesis techniques can be challenging. Fortunately, BE provides a better technique to carry out such mutations. Therefore, Bastet et al. adopted CBE to develop enhanced resistance to potyviruses in *Arabidopsis* [126]. The study emphasizes the utilization of BE in developing disease-resistant crops. Despite some limitations, like targeting site, editing window, or off-target editing, BE/PE systems have powerful potential to facilitate crop bio-production or improvement through high precision and efficiency.

5.3. Evolutionary engineering

BE could play a vital role in the introduction of gene mutations and directed protein evolution. CBE lacking UGI has been shown to have the capacity to make diverse mutations other than C-to-T [22,147,148]. This ability has already been used in CRISPR-X and targeted AID-mediated mutagenesis (TAM) based studies to identify known and novel mutations in mammalian cells in cancer therapeutics targets PSMB5, and BCR-*ABL*, respectively [147,148]. The identified mutations produce PSMB5 and BCR-*ABL* variants that are resistant to widely used cancer treatments like bortezomid and imatinib, respectively. Similarly, BE systems have effectively been used in plants to enable protein evolution, e.g., screening of ACCase variants [149] and genomic diversification, e.g., accelerated evolution of *OsALS1* [150] with gRNA library. Development of bacterial or plant protein and genetic libraries using UGI absent CBE or PE could provide functionally diverse protein variants with industrial importance (Fig. 3C). A bioinformatics study into targeted point mutations using BE/PE that leads to variation in protein activity and, ultimately, metabolic pathways, may widely assist in bacterial and plant metabolic engineering.

5.4. Metabolic engineering

The precise editing ability of BEs has been exploited for metabolic engineering studies to understand the biosynthesis pathways in bacteria (Fig. 3D). Li et al. studied the Hygromycin B biosynthesis pathway in the industrially significant *S. hygroscopicus* subsp. *hygroscopicus* bacterium [151]. The group used BE for *in vivo* gene inactivation to enhance the understanding of the pathway. They studied *HygD*, *HygJ*, *HygL*, *HygY*, and *HygM*, and found their critical roles in the pathway [151]. This work demonstrates the significance of base editing tools in gene characterization of biosynthesis pathway studies. Additionally, Ji group used pABE system for *S. aureus* to screen for key residues of CntBC, an essential transporter that plays a role as a transporter for staphylopine/transition metal complex acquisition [52]. Using the pABE-based screening method, authors were able to identify four key residues of CntBC. This depicts the efficacy of BEs in studies related to the identification of functional protein residues. Similar studies into industrially relevant bacterial biosynthesis pathways using BE will enhance our understanding of bio-production networks and assist in improving the yield. Metabolic pathways in bacteria are vastly interconnected as the central and branched pathways form a complex network for synthesizing and breaking of the molecules. Such inter-related networks make it tough to tweak metabolic flux to achieve the highest yield of target products. The BE/PE could assist in a better understanding of how the network affects yield with high precision at a broader scale, eventually facilitating enhanced biosynthesis.

Plant as workhorse has a more complex cell system compared to the bacteria, facilitating more complicated biosynthesis [152]. Besides introducing new metabolic pathways and enhancing related enzyme activities, knock-out/down of competitive pathway branched genes [153] is critical in plant metabolic engineering (Fig. 3D). By destroying the competitive polyunsaturated fatty acid *FAD2* alleles using DSB-dependent CRISPR-Cas9, Jiang et al. increased the oleic acid content in Camelina seeds from 16% to > 50% of the fatty acid composition [154]. In their research, less desirable polyunsaturated fatty acids still occupied a certain percentage, probably because of the rudimental N-terminal FAD2 domains. Such an issue can be resolved by BE system, as demonstrated by the introduction of premature stop codons (iSTOP or CRISPR-STOP) [155,156] to generate catalytically inactive proteins. Hence, BE and PE have the capacity to play a leading role in plant metabolic engineering.

5.5. RNA base editing

Contrary to DNA editing, RNA editing, including insertion, deletion, or substitution of nucleotides, allows accurate and efficient editing of RNA molecules. The development of RNA base editors exponentially expands the available RNA editing tools by allowing precise single base substitution.

In mammals, the most prevalent post-transcription RNA editing case is catalyzed by the adenosine deaminase enzymes (ADARs). ADAR binds to dsRNA and catalyzes adenosine to inosine (A-to-I), and ultimately, I is read as G by the cellular machinery [157]. Recently, RNA-guided RNA-targeting CRISPR nuclease C2C2 (later named as Cas13a) from *Leptotrichia shahii* was illustrated [158]. Cas13 generally encompasses two higher eukaryotes and prokaryotes nucleotide-binding (HEPN) domains, which contribute to the RNA-targeted nucleolytic activity. Mutations of HEPNs abolish RNA cleavage activity while maintaining RNA targeting activity, which was used by Zhang group to create an RNA base editing tool, namely, RNA Editing for Programmable A-to-I Replacement (REPAIR) [159]. They combined a catalytically inactive dCas13 variant with a RNA deaminase ADAR2 (E488Q) to form a fusion protein dCas13-ADAR2DD, which can execute RNA editing for programmable A-to-I (G) replacement. To expand the RNA base editors, a new tool, RNA Editing for Specific C-to-U Exchange (RESCUE) was later developed [160].

Till now, neither REPAIR nor RESCUE systems have been adopted for plants or bacteria. The systems can be safely used in cells for the fact that they do not lead to permanent genomic DNA mutations, which may be used for research into understanding the metabolic pathways and crop improvement.

6. Concluding remarks, challenges & prospects

Availability of the tools that allow precise, efficient, and DSB-free editing ability to resolution as low as a single base was unthought-of until recently. Development of BE and more recently PE have handed scientists with powerful gene-editing tools. The formidable tools have been deployed in diverse fields, from synthetic metabolic engineering to agriculture and medicine. Today with ever-increasing research in life-sciences, these tools have quickly been adopted for animal, plant, and bacterial models. Exploitation of these tools is required to further advance the bacterial and plant bio-production. Rapid advancements in the fields make it hard to keep up with the studies; therefore, this review summarizes the developments in BE and PE technology in bacteria and plants.

Though BE and PE editing tools are seeing an ever-increasing interest, there remain challenges that require further research. One major challenge surrounding BE and PE is their constrained target sites due to the PAM specificity of Cas proteins. BE fused to different Cas proteins like SaCas9(D10A) (PAM recognized, NNGRRT) [161], VQR-Cas9(D10A) (PAM recognized, NGA, AGTG, AGCG) [162], VRER-Cas9(D10A) (PAM recognized, NGCG) [162], SaKKH-Cas9(D10A) (PAM recognized, NNNRRT) [162], xCas9 (PAM recognized, NG and more) [163] and Cas12a (PAM recognized, TTTV and more) [47] have been developed, however, they are still limited to narrow PAM accessibility. The recently developed Cas proteins, SpG (PAM recognized, NGN) [164], SpRY (PAM recognized, NRN (R being A or G) > NYN (Y being C or T)) [164], Sc^{++} (PAM recognized, NNG) [165] and HiFi-Sc^{++} (PAM recognized, NNG) [165] are reported to have significantly relaxed PAM dependence, providing the ability to target previously inaccessible locus. The fusion of SpG/SpRY/Sc^{++}/HiFi-Sc^{++} Cas proteins with either BE or PE will help advance the available editing tools.

The next limitation surrounding BE/PE is editing efficiency. Although BE has been fused to several Cas proteins, their efficiency remains lower than the original BE. Liu group has worked on developing advanced base editors with higher Cas compatibility and increased editing efficiencies like ABE8e, which is reported to have higher editing efficiency compared to the original ABE7.10 [166], and fourth-generation CBE BE4 [167]. Hua et al. enhanced the efficiency by simplifying the adenine deaminase in rice [168]. Contrarily, PEs are still very young and therefore have limited studies. Lin et al. adopted PEs for plants and used different RTs in the hope of increasing efficiency [118]. They used RT-CaMV and RT-retron but reported either comparable or lower editing efficiency. Further studies into engineered and evolved RTs will help in the development of advanced PEs with higher editing efficiencies.

Another challenge facing BE/PE is off-target editing. BE and PE allow precise editing with lower indels and off-target effects compared to DSB-dependent CRISPR editing. CBE and ABE lead to 1.1% and ≤0.1% indels, respectively, compared to much higher 4.3% indels from Cas9-HDR editing [21,22]. Research into the characterization of the observed undesired indels, bystander, and off-target editing as either because of BEs or the Cas protein is required. CBE shows more off-target mutations than ABE [161,169–171]. Therefore, Doman et al. studied the Cas9-independent off-targeting by CBE and engineered CBE variants, which show a considerably lower level of Cas9-independent off-targeting mutations (~10–100 folds lower) and comparable or lower indels [51]. To limit the Cas9 dependent off-targeting, high-fidelity Cas9 was fused to BE3 and BE2 to develop HF-BE3 and HF-BE2, respectively [172]. Both of the developed HF-BEs showed several folds lower off-targeting, with HF-BE3 showing 37-fold lower off-targeting

relative to BE3. Other efforts to reduce BE off-targeting include co-expression of free UGI with BE3 containing triple UGI [173] and fusion of bacteriophage Gam protein with BE3 and BE4 [167]. These studies depict that the uracil base excision repair system (BER) has an important role in BE induced off-target effect as multi-copy UGI based systems generally lead to lower off-targeting. Further studies into off-target characterization and understanding the mechanisms that lead to it are required. In the case of PE, the initial work shows significantly lower off-targeting compared to DSB-dependent Cas9 editing [24]. PE, on average showed ≤0.6% off-target changes at four major Cas9 off-target loci compared to an average 32% off-targeting by Cas9 + sgRNA at the same four loci [24]. However, further research in the genome-wide off-target analysis is required to better understand the PE off-targeting as it may show undesired mutations at previously unknown off-target sites.

Other challenges facing base editing include sequence specification and editing window. Studies have reported 5′-TC ≥ CC > AC > GC sequence preference by BE3 [22,47,58]. Whereas, the editing window of BEs typically limits the editing within −12 to −16 and −16 to −20 distal to the PAM. Efforts to increase the editing window include the use of extended guide RNA [49], the development of advanced BE systems like BE-PLUS [174], and using human APOBEC3A (hA3A) in CBE [142]. We believe that studies into developing engineered or evolved BEs with lower sequence specificity and the wider or narrower editing windows will be important for future BE applications. Whereas PE requires further work into understanding its working mechanism and in-depth study of edit range.

Base editing and prime editing are young editing tools poised to play a critical role in basic research, crop improvement, synthetic biology, metabolic engineering, and evolutionary engineering. Both the editing tools require extensive research to reach their full potential, especially PE, whose adoption in bacteria/plants will be crucial for advancement of their bio-production capabilities.

Declaration of competing interest

None.

Acknowledgments

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