Review

Base editing in crops: current advances, limitations and future implications

Rukmini Mishra1,†, Raj Kumar Joshi2,† and Kaijun Zhao1,*

1National Key Facility for Crop Gene Resources and Genetic Improvement (NFCRI), Institute of Crop Science, Chinese Academy of Agriculture Sciences (CAAS), Beijing, China
2Department of Biotechnology, Rama Devi Women’s University, Bhubaneswar, Odisha, India

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*Correspondence (Tel +86-10-82105852; fax 86-10-82108751; email: zhaoqiaojun@caas.cn)
†These authors contributed equally.

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Summary

Targeted mutagenesis via genome-editing technologies holds great promise in developing improved crop varieties to meet future demands. Point mutations or single nucleotide polymorphisms often determine important agronomic traits of crops. Genome-editing-based single-base changes could generate elite trait variants in crop plants which help in accelerating crop improvement. Among the genome-editing technologies, base editing has emerged as a novel and efficient genome-editing approach which enables direct and irreversible conversion of one target base into another in a programmable manner. A base editor is a fusion of catalytically inactive CRISPR–Cas9 domain (Cas9 variants) and cytosine or adenosine deaminase domain that introduces desired point mutations in the target region enabling precise editing of genomes. In the present review, we have summarized the development of different base-editing platforms. Then, we have focussed on the current advances and the potential applications of this precise technology in crop improvement. The review also sheds light on the limitations associated with this technology. Finally, the future perspectives of this emerging technology towards crop improvement have been highlighted.

Introduction

Genome editing via the CRISPR/Cas9 system has flourished as an efficient technology and has revolutionized the field of agriculture and plant science with its simplicity, versatility and high precision. In a CRISPR/Cas9 genome-editing system, the Cas9-sgRNA complex moves along the DNA strand and makes a double-stranded break (DSB) where the Cas9 encounters the appropriate protospacer adjacent motif (PAM) and the sgRNA matches the target DNA sequence (Jinek et al., 2014). These DSBs are subsequently repaired by the naturally occurring DNA repair pathways: nonhomologous end-joining (NHEJ) or homology-directed repair pathway (HDR). NHEJ is an error-prone repair pathway which results in random insertions and deletions whereas HDR is a high-fidelity repair method which results in gene insertion or gene replacements (Voytas and Gao, 2014) (Figure 1). CRISPR/Cas9 system is a highly efficient and robust system used for genome editing and has been successfully used for genome editing in crops and model plants due to its adaptability and high precision (Liang et al., 2017; Shao et al., 2017; Shi et al., 2017; Tomlinson et al., 2019; Zsögön et al., 2018). However, unintended mutations in the off-target regions and PAM specificity are still the major problems associated with this technology.

Many important agronomic traits are determined by point mutations or a few base changes in a gene (Doebley et al., 2006; Li et al., 2017; Ma et al., 2015). CRISPR/Cas9-mediated gene replacements via homology-directed repair (HDR) has been reported as a feasible approach to correct the point mutations in the target gene and has the potential for accelerating crop improvement (Li et al., 2018; Sun et al., 2016; Wang et al., 2017). However, infrequent occurrence of HDR and low efficiency of template DNA delivery have always been a challenging task in achieving success in plants (Ran et al., 2017). Moreover, CRISPR/Cas9 system is suitable for gene knockout or knock-in, but cannot convert one base into another. These limitations have highlighted the need for alternative approaches which can result in stable and precise genome editing in crops.

“Base editing” has emerged as a novel approach which enables precise nucleotide substitutions in a programmable manner, without disruption of a gene or requiring a donor template (Komor et al., 2016). A base editor is a fusion of catalytically inactive CRISPR–Cas9 domain (Cas9 variants, dCas9 or Cas9 nickase) and a cytosine or adenosine deaminase domain which converts one base to another (Figure 1). Single-base changes could generate elite trait variations in crop plants which help in accelerating crop improvement. The base-editing system can revert a single-base change or SNP without gene disruption, thereby minimizing the insertions and deletions. It is an efficient technology for engineering novel traits in agriculturally important crops and a key to food security (Eid et al., 2018).

In the last 3 years, the cytosine and adenine base editors (ABEs) have emerged as efficient tools for precise genome modification (C to T or A to G) in eukaryotic genomes (Hua et al., 2018; Liu et al., 2018; Qin et al., 2019; Zong et al., 2017). Base-editing approach has been efficiently optimized and demonstrated in several crops including rice, wheat, maize and tomato (Li et al., 2018; Lu and Zhu, 2017; Tang et al., 2019; Zong et al., 2017). Numerous articles and a huge accumulation of case studies on base-editing system and its application in crops have highlighted the need for an elaborate review which will be a valuable source of information for the scientific community. In the present review,
we have summarized the development of different base-editing platforms and their efficiencies in editing both DNA and RNA. The highlight of the review is the potential applications of base-editing technology in crop improvement using specific case studies. The review will also discuss the limitations and the future implications of this novel emerging technology.

**CRISPR-based base editors – overview**

**DNA base editors**

Base editors are chimeric proteins composed of a DNA targeting module and a catalytic domain which is capable of deaminating a cytosine or adenine base in the genome (Gaudelli et al., 2017; Komor et al., 2016). The DNA targeting module is either a catalytically dead Cas9 endonuclease (dCas9) or a Cas9 nickase guided by a sgRNA molecule. The dCas9 contains Asp10Ala and His840Ala mutations that inactivate its nuclease activity but retain the DNA binding ability. The binding of dCas9-sgRNA to the target DNA creates an ‘R-loop’ where a stretch of DNA gets unpaired. This small single-stranded domain of approximately 5–8 nucleotides acts as an editing or catalytic window for dCas9-tethered deaminase to modify the cytosines. The base editors are capable of making single-base changes or substitutions without creating a DSB in the DNA, thereby limiting the frequency of indels. There are two types of DNA base editors: cytosine base editors (CBEs) and ABEs. The characteristics, catalytic window and functions of CBEs and ABEs have been listed in Table 1.

**Cytosine base editors**

Cytosine base editors are the vectors that catalyse the conversion of cytosines to thymines. The cytidine deaminase enzyme removes an amino group from cytosine converting it to uracil, resulting in a U-G mismatch which gets resolved via DNA repair pathways to form U-A base pairs. Subsequently, a T gets incorporated in the newly synthesized strand forming T-A base pairs. This results in C-G to T-A conversion in a programmable manner. The first-generation base editor (BE1) was developed by David Liu and co-workers of Harvard University, USA, in 2016. It was composed of a cytidine deaminase enzyme APOBEC1 (from rats) linked to a dCas9 by a 16 amino acid XTEN linker (Komor et al., 2016). The XTEN is a peptide which links them and maintains a balance between the two proteins. The apolipoprotein B mRNA editing enzymes, catalytic polypeptide-like (APOBEC) family are a group of naturally occurring cytidine deaminases in vertebrates which protect them from invading viruses (Chiu and Greene, 2006). These enzymes act on single-stranded DNA/RNA as substrates.

The major limitation in BE1 was the frequent removal of uracil by uracil DNA glycosylase (UDG), resulting in low editing efficiency. Keeping in view the low editing efficiency and limitations of BE1, a series of improved base editors were developed further. The second-generation base editor BE2 (APOBEC-XTEN-dCas9-UGI) was developed by adding a uracil DNA glycosylase inhibitor (UGI) to the C terminus of the DNA targeting module (Komor et al., 2016). The addition of UGI inhibits the activity of UDG that catalyses the removal of U from DNA in cells and initiates base excision repair (BER) pathway. UGI is an 83-residue protein from *Bacillus subtilis* bacteriophage PBS1 which blocks UDG activity in human cells. This inhibition of BER increases the editing efficiency by threefold in human cells. Subsequently, BE3 base editor was developed, which was composed of rAPOBEC1 fused to the N terminus of nickase cas9 D10A through a 16-amino acid XTEN linker and a UGI fused to the C terminus by a 4-amino acid linker (Komor et al., 2016; Figure 2a). The major improvement in BE3 was the replacement of dCas9 with Cas9 nickase (nCas9), which nicks the strand.
opposite to the deaminated cytidine. dCas9 is converted to nCas9 either by replacing amino acid aspartate (D) by alanine (A) at position 10 (D10A) or by replacing histidine (H) by alanine at position 840 (H840). The nick initiates a long-patch BER, where the deaminated strand is used as a template to produce U-A base pair, further converted to T-A during DNA replication. Thus, the editing efficiency was further increased by sixfold in BE3 over BE2. The use of nCas9 also exhibited an increase in indel frequency of 1.1 % as compared to 0.1 % in BE2.

Cytosine base editors enable C to T conversion in a programmable manner (Figure 3a). However, an increase of more than one cytosines (Cs) within the catalytic window may result in off-target activity and conversion of non-target C to U. To overcome this limitation, several BE3 variants were generated with different Cas9 variants (using noncanonical PAM). The SpCas9 variants like VQR-BE3, EQR-BE3, VRER-BE3 and SaKKH-BE3 which target NGAN, NGAG, NGCG and NNNRRT PAMs, respectively, have increased the editing efficiency by 2.5-folds (Kim et al., 2017). Besides SpCas9 variants, SaCas9 (from Staphylococcus aureus) with NNGRT PAMs, has been used in several studies with enhanced efficiency. Several cytidine deaminase mutants like YEE-BE2 and YEE-BE3 with varying editing window widths were generated to enhance DNA specificity and reduce off-target editing. The triple mutant W90Y+R126E+R132E (YEE-BE3) exhibited maximal editing efficiencies within a narrow editing window width of approximately 2 nucleotides (Kim et al., 2017) (Figure 2b).

Another base-editing system, Target-AID (activation-induced cytidine deaminase), was developed which was composed of a nickase Cas9D10A and a cytidine deaminase pmCDA1 (from sea lamprey) (Nishida et al., 2016) (Figure 2c). AID causes deamination of cytidine and protects the vertebrate cells from foreign invaders by altering their genomes, facilitating somatic hypermutation and class switch recombination in vertebrates (Nishida et al., 2016). It targets the immunoglobulin (Ig) locus and generates diverse mutations that are selected through antigen binding. AID deficiency causes hyper-IgM syndrome which generates low-affinity antibodies (Revy et al., 2000; Xu et al., 2012). Thus, the target-AID system was used to perform targeted mutagenesis with improved efficiency in mouse and human cells. The use of nickase and UGI has enhanced the editing efficiency by twofold to threefold in BE3 and Target-AID.

To further expand and increase the base-editing efficiency, fourth-generation base editors BE4 (S. pyogenes Cas9-derived base editor) and SaBE4 (S. aureus Cas9-derived BE4) were developed by linking rAPOBE1C to Cas9D10A through a 32-aa linker and fusing two UGI molecules to both C and N terminal of Cas9 nickase by a 9-aa linker. The use of nCas9 and UGI has enhanced the editing efficiency by fourfold to fivefold in BE3 and Target-AID.

Besides being used to introduce point mutation in a precise and programmable manner, deaminases are also used to create a diverse library of point mutations localized to a targeted region of the genome. Targeted AID-mediated mutagenesis (TAM) and CRISPR-X are the two DNA base-editing platforms which have been used to generate localized sequence diversity through base editing (Hess et al., 2016; Ma et al., 2016) (Figure 2e,f). In the TAM system, dCas9 is fused to human AID which enables efficient genetic diversification in mammalian cells (Ma et al., 2016). When co-expressed with UGI, the mutation frequencies of

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**Table 1: List of base editors, characteristics, catalytic window and functions**

| Base editors | Characteristics | Type of base substitutions | Catalytic window | References |
|--------------|-----------------|---------------------------|------------------|-----------|
| DNA base editors | | | | |
| BE1 | (APOBEC1–XTEN–dCas9): Composed of a cytidine deaminase enzyme APOBEC1 (from rats) linked to a catalytically dead Cas9 (dCas9) by a 16 amino acid XTEN linker | C to T | −17 to −13 | Komor et al. (2016) |
| BE2 | (APOBE1C–XTEN–dCas9–UGI): UGI is fused to the C terminus of BE1. | C to T | −17 to −13 | Komor et al. (2016) |
| BE3 | (APOBE1C–XTEN–Cas9m–GI): rAPOBEC1 fused to the N terminus of nickase cas9 D10A through a 16-amino acid XTEN linker and a UGI fused to the C terminus by a 4-amino acid linker | C to T | −16 to −12 | Komor et al. (2016) |
| YEE-BE3 | (W90Y+R126E+R132E): triple mutant | C to T | −15 to −13 | Kim et al. (2017) |
| BE4 | Composed of rAPOBEC1 fused to Cas9D10A through a 32-aa linker and two UGI molecules are linked to both C and N terminal of Cas9 nickase by a 9-aa linker. | C to T | −17 to −13 | Komor et al. (2017) |
| SaBE4-GAM | Gam protein fused to Staphylococcus aureus Cas9-derived BE4 | C to T | −19 to −9 | Komor et al. (2017) |
| Target-AID | Composed of nickase Cas9D10A and a cytidine deaminase pmCDA1 (from sea lamprey) | C to T | −19 to −15 | Nishida et al. (2016) |
| TAM | dCas9 is fused to human AID, co-expressed with UGI | C to T | −16 to −12 | Ma et al. (2016) |
| CRISPR-X | dCas9 is used to target a hyperactive AID variant to induce localized, diverse point mutations. The sgRNA backbone contains two MS2 RNA hairpins that each recruit two M2S proteins fused to AID | C to T | −50 to +50 | Hess et al. (2016) |
| ABE | TadA is fused to a catalytically impaired CRISPR/Cas9 mutant | A to G | −17 to −14 | Gaudelli et al. (2017) |
| RNA base editor | | | | |
| ADAR | Catalytically inactive Cas13 (dCas13) is fused to a naturally occurring ADAR (adenosine deaminase acting on RNA) | A to 1 | −50 to +50 | Cox et al. (2017) |
dCas9-AIDx were increased by five times and restricted the mutations strictly to C to T or G to A substitutions. Cas9-AIDx is used to create a diverse mutation spectrum beyond the C to T or G to A substitutions and its independence of the AID hotspot motifs. TAM is established as an efficient genetic diversification strategy in mammalian cells to facilitate protein evolution which was not feasible earlier. The dCas9-AID was stably expressed in K562 cells, a chronic myelogenous leukaemia (CML) line that contains the BCR-ABL oncogene and is sensitive to imatinib. The TAM system is used to target BCR-ABL to identify known and unknown mutations.

Figure 2  Structural representation of base-editing platforms: (a) BE3 employs Cas9 nickase (nCas9D10A) along with a cytidine deaminase rAPOBEC1 (orange) and an uracil DNA glycosylase inhibitor (UGI) (Green). (b) YEE-BE3 employs YEE-rAPOBEC1. (c) Target-AID employ PmCDA1 (d) SaBE4-gam employs SaCas9D10A, Z x UGI and has a Gam protein (red) fused to its terminus. (e) and (f) The TAM and CRISPR-X systems used dCas9 to recruit variants of the deaminase AID (AIDx or MS2-AID*D). (g) ABE is composed of ecTadA (WT)–ecTadA* (7.10) heterodimer fused to Cas9n. (h) Catalytically inactive Cas13 (dCas13) is fused to a naturally occurring ADAR2 (adenosine deaminase acting on RNA).

Figure 3  A comparison of three different approaches of base editing. (a) CBE — mediated base-editing strategy results in C-T conversions. (b) ABE — mediated base-editing strategy results in A-G conversions. (c) ADAR — mediated RNA base-editing results in A-1 conversion.
novel mutations conferring imatinib resistance in chronic myeloid leukaemia cells. Imatinib (Gleevec) inhibits ABL and other tyrosine kinases by binding to their catalytic domains and has become a standard therapy against BCR-ABL + CML. TAM is an ideal platform to understand protein function and enables the identification of drug targets and new mechanisms of drug resistance.

In CRISPR-X, the dCas9 is used to target a hyperactive AID variant to induce localized, diverse point mutations (Hess et al., 2016). In this system, the sgRNA backbone contains two MS2 RNA hairpins that each recruit two MS2 proteins fused to AID. The AID exhibited a larger window of catalytic activities between −50 to +50 from the PAM sequence and induced a twofold to sixfold increase in mutation frequency. The CRISPR-X system has been successfully used to induce mutagenesis of the target of the chemotherapeutic bortezomib (PSMB5), identifying novel drug-resistant mutants that may reveal new properties of PSMB5 and its interaction with bortezomib (Hess et al., 2016).

The dCas9 has been successfully used as a DNA targeting module for gene editing purposes. However, the requirement of G/C-rich PAM sequences is still a limitation. In order to expand the scope of base editing, Li and colleagues have generated the first Cpf1-based cytidine deaminase base editor (Li et al., 2018). The Cpf1 is a type V class 2 CRISPR endonuclease. It favours T-rich, −TTTN− PAMs, generates cohesive end with 5-bp (Zetsche et al., 2015) and can process its sgRNA which enhances its use in multiplex genome targeting (Zetsche et al., 2017). The base editor, dLbCpf1-BE0, is composed of a rat APOBEC1 domain fused to a catalytically inactive Lachnospiraceae bacterium Cpf1 (dLbCpf1) and UGI. The editing window of this base editor ranges from positions 8 to 13 bp preceding the PAM and exhibits an editing efficiency of 20–22%. Besides dLbCpf1-BE0, Li et al. (2018) have generated other fusions dCpf1-8E-YE, dCpf1-8E6 and dCpf1-8E6-YE based on the Cas9-BEs generated by Komor et al. (2016). Thus, the use of Cpf1 based base editors could extend the scope of base editing by providing various choices of PAMs in the target gene.

Adenine base editors

Base-editing capabilities and study of genetic diseases were further expanded by the development of a new class of ABES that could modify adenine bases (Gaudelli et al., 2017) (Figure 2g). Unlike cytidine deaminases, adenine DNA deaminases do not occur in nature. In 2017, David Liu and group developed ABES by using Escherichia coli TadA (E. coli TadA) through extensive protein engineering and directed evolution. E. coli TadA is a tRNA adenine deaminase that converts adenine to inosine in the single-stranded anticodon loop of tRNA Arg (Figure 3b). It shares homology with the APOBEC enzyme. The first-generation ABES were developed by fusing a TadA with a catalytically impaired CRISPR/Cas9 mutant (Gaudelli et al., 2017). Among the series of ABES developed, ABET7.7, ABET7.8 and ABET7.9 are considered to be the most active ABES with a broader sequence compatibility. The seventh-generation ABES (ABET7.10) were recommended for conversion of A.T to G.C in a wide range of targets with increased efficiency and product purity. ABES introduce point mutations with higher efficiency and have greatly expanded the scope of base editing by enabling all four transitions (C to T, A to G, T to C and G to A) in a programmable manner.

RNA base editors (ADAR)

Feng Zhang and his group were the first to develop RNA base editors by using a catalytically inactive Cas13 (dCas13) and a naturally occurring ADAR (adenosine deaminase acting on RNA) to direct adenosine to inosine conversion in mammalian cells (Cox et al., 2017) (Figure 2h). Cas13 is a type VI CRISPR-associated RNA-guided RNase with RNA binding abilities. Among a set of Cas13 enzymes assayed for RNA knockdown activity, Cas13b ortholog from Prevotella sp. (PsCas13b) was found to be more efficient and specific in RNA binding and knockout applications. The adenosine deaminase acting on RNA (ADAR) family of enzymes mediates endogenous editing of transcripts via hydrolytic deamination of adenosine to inosine (Nishikura, 2010) (Figure 3c). These enzymes are capable of precise base editing in RNA. This system used to edit RNA transcripts was referred to as RNA Editing for Programmable A to I Replacement (REPAIR). REPAIRv2 was further produced with higher specificity than other RNA editing platforms used previously (Stafforst and Schneider, 2012). REPAIR system is effectively used to mimic protective alleles that protect against several autoimmune diseases (Ferreira et al., 2010). REPAIR presents a promising RNA editing platform with broad applicability for research, therapeutics and biotechnology.

Application of base editors in crop improvement

Several agriculturally important traits are conferred by SNPs in the genome, and base editing has played a critical role in correcting those point mutations and accelerating crop improvement. Cytosine and adenine base editors have been successfully used in a wide range of major crops and model plants to edit specific genes conferred by single nucleotide polymorphisms (Hua et al., 2018; Li et al., 2018; Lu and Zhu, 2017; Ren et al., 2018) (Table 2).

CBEs in crop improvement

Several studies have demonstrated the successful applications of cytidine base editors in wide range of plants including rice, maize, tomato, wheat, cotton and watermelon (Lu and Zhu, 2017; Qin et al., 2019; Tian et al., 2018; Zong et al., 2017). A “base editing” system was developed by using rat cytidine deaminase enzyme (APOBEC1) fused to the N terminus of Cas9 (D10A) using the unstructured 16-residue peptide XTEN as a linker (Lu and Zhu, 2017). The APOBEC1-XTEN-Cas9 (D10A) fusion sequence was constructed into a binary vector, under the control of the maize ubiquitin promoter (UBI). This CRISPR/Cas9-xyr5APOBEC1 base-editing system was then used to induce point mutations in two rice genes NRT1.1B and SLR1 with agronomic importance (Lu and Zhu, 2017). NRT1.1B gene encodes a nitrogen transporter and SLR1 gene encodes a DELLA protein. Earlier studies showed that nitrogen use efficiency in rice was enhanced with a C to T substitution (Thr327Met) in NRT1.1B (Hu et al., 2015) and reduced plant height with an amino acid substitution in or near its TVHYNP motif (Asano et al., 2009; Hu et al., 2015). The base-editing system was used to target one site each from these two genes and C to T substitution was achieved at a frequency of 1.4%–11.5% while 1.6%–3.9% of the edited plants accounted for C to G substitution. Besides base substitutions, indel mutations were also observed in sequencing results and it may be caused by the Cas9 (D10A) that nicks the nonedited strand. Although UGI increases the efficiency of base editing, it was not used in the above study.

Multiple herbicide resistance point mutations have been introduced into rice plants through multiplex base editing (Shimatan et al., 2017). A target-activation-induced cytidine deaminase (Target-AID) system along with a construct comprising...
of either dCas9 or nCas9 fused to Petromyzon marinus cytidine deaminase (PmCDAT1) and sgRNAs was used to target the desired gene. A point mutation in Acetolactate synthase (ALS) gene results in herbicide resistance in plants (Yu and Powles, 2014). In rice, the C287T mutation of ALS homolog gene results in an A96V amino acid substitution in the encoded protein that confers resistance to the herbicide imazamox (IMZ). The researchers using the Target-AID based base editing to introduce similar point mutation in the ALS gene. As expected, spontaneous resistance mutations were observed regardless of Target-AID treatment at a frequency of 1.56%, but the resistant lines obtained from nCas9Ps-PmCDAT1Al transformatiads induced 3.41% IMZ tolerance. While no off-targets were detected, seven out of the 14 edited lines showed the ALS-A96V mutation.

Genetic variations were efficiently induced in rice crop by using a CRISPR/Cas9 toolkit comprised of rBE3 and rBE4 (rice base editors) (Ren et al., 2017). In this study, the researchers fused a codon-optimized rat APOBEC1 gene and UGI gene of B. subtilis bacteriophage PBS1 to Cas9n gene at both ends. The resulted base editor rBE3 (APOBEC1-XTEN-Cas9n-UGI-NLS) was expressed under the control of the CaMV35S promoter in the rice leaf sheath protoplasts together with OsCERK1-targeting sgRNA transcribed from a rice U6 promoter. Subsequently, the researchers further optimized the rBE system with human AID (hAID) mutant version termed hAID*Δ for introducing point mutations in rice, thereby extending the base-editing efficiency (Ren et al., 2018). rBE5 (hAID*Δ-XTEN-Cas9n-UGI-NLS) base editor was first tested in rice leaf sheath protoplasts, targeting two important genes, that is OsRLCK185 and OsCERK1. Sequencing results revealed distinct mutations with a high frequency of C to T substitution suggesting that rBE5 base editor functions well on GC, AC, TC as well as CC sequence contexts in rice cells. Subsequently, rBE5 was used to target Ph-d2, an agriculturally important rice gene that harbour a point mutation modulating defence response to blast fungus (Chen et al., 2006). G to A conversion was detected in eight heterozygous lines with 30.8% mutation efficiency.

Zhou and his group further expanded the toolkit by fusing a UGI gene to the 3’ terminal of rBE5 resulting in pUb: rBE9 vectors. rBE9 vectors were used to target four different chromosomal sites (OsAOS1, OsJAR1, OsJAR2 and OsCOI2) in rice transgenic calli and evaluated its editing efficiency in different sequence contexts (Ren et al., 2018). Sequencing results revealed that rBE9 functioned more efficiently on GC context and more efficiently on multiple target C in the editing window of sgRNA than rBE3, resulting in more genetic variation at the target loci. Overall, the study indicated that the hAID*Δ-based rBE5 and rBE9 vectors favour GC and function on AC, TC and CC as well. Considering the high GC content of the rice genome, the pUb: rBE5 and pUb:rBE9 vector systems could be suitably used for generation of both gain-of-function and loss-of-function mutants of rice with respect to several agronomically important traits. Further, the usage of these tool kits could be expanded into other monocot and dicot plants for molecular breeding in crops.

More recently, a new plant base editor, A3A-PBE, was developed by using human APOBEC3A, fused to Cas9 nickase to further enhance the base-editing efficiency in plants (Zong et al., 2018). The third-generation base editors, BE3, are successfully used to create C to T substitutions in various organisms. However, the editing window was limited to 5 nucleotide (nt) sequence and the editing activity was low in GC contexts. Thus, the previous base editor nCas9-PBE (Zong et al., 2017) was improved to create A3A-PBE, where the rat APOBEC1 was replaced with human APOBEC3A whose codons were optimized for cereals. The efficiency of A3A-PBE was tested in wheat and rice genes and C-to-T conversion was observed with increased efficiency (13.1%) than nCas9-PBE. The editing window spanned a larger editing space of 17 nt and had a low

Table 2 List of genes targeted by cytidine and adenine base editors in different crops

| Crop name | Targeted genes | Type of base editor used | Functions | References |
|-----------|----------------|--------------------------|-----------|------------|
| Oryza sativa | NRT1.1B and SLR1 | CBE | Enhance nitrogen use efficiency | Lu and Zhu (2017) |
| | C287 | CBE | Herbicide resistant | Shimatani et al. (2017) |
| | OsPDS, OsSBE1ib | CBE | Nutritional improvement | Li et al. (2017) |
| | OsCDC48 | CBE | Regulate senescence and death | Zong et al. (2017) |
| | OsSPL14 | CBE | Herbicide resistance | Tian et al. (2018) |
| | OsMPK6 | ABE | Pathogen-responsive gene | Yan et al. (2018) |
| | OsACC-T1 | ABE | Herbicide resistance | Li et al. (2018) |
| | SLR1 | ABE | Della protein for plant height | Hua et al. (2018) |
| | OsSPL14 | ABE | Plant architecture and grain yield | Hua et al. (2018) |
| | OsRLCK185, OsCERK1 | CBE | Defence response | Ren et al. (2018) |
| | Pd2 | ABE | Blast resistance | Ren et al. (2018) |
| | Wx | ABE | Rice amylose synthesis | Hao et al. (2019) |
| | GL2/OsGRF4, OsGRF3 | ABE | Grain size and yield | Hao et al. (2019) |
| | ALS | CBE | Herbicide resistance | Veillet et al. (2019) |
| Thanostestesum | TaOX2 | CBE | Lipid metabolism | Zong et al. (2017) |
| Zea mays | TaDEP1, TaGW2 | ABE | Panicle length and grain weight | Li et al. (2018) |
| Solanum tuberosum | zmCENH3 | CBE | Chromosomal segregation | Zong et al. (2017) |
| Solanum lycopersicum | SALS, SLGBSS | CBE | Herbicide resistance, Starch synthesis | Zong et al. (2018) |
| Solanum lycopersicum | SLALS1 | CBE | Herbicide resistance | Veillet et al. (2019) |
| Citrullus lanatus | ALS | CBE | Herbicide resistance | Tian et al. (2018) |

ABE, adenine base editor; CBE, cytidine base editor.
frequency of undesired on-target indels. The potato genes, StALS and StGBS5, were also targeted by A3A-PBE. C-to-T conversion was observed in potato protoplasts with 11-fold higher efficiency than nCas9-PBE. The efficiency of A3A-PBE was tested in different contexts, and it was observed that unlike nCas9-PBE, A3A-PBE edited cytosines equally well irrespective of any context. The study also indicated that A3A-PBE fused with different Cas9 variants could potentially target 90% of the cytidines and guanidines in the rice genome.

In watermelon (Citrullus lanatus), transgene-free herbicide-resistant varieties were generated by using CRISPR/Cas9-mediated base-editing system (Tian et al., 2018). The ALS gene encodes the enzyme that catalyses the initial step of the biosynthetic pathway for branched-chain amino acids. Single-precise base editing. The ABE7-10 is a highly efficient ABE that is used to convert A-T to G-C in a programmable manner in mammalian cells (Gaudelli et al., 2017). ABE-P1 (ABE plant version 1), the modified version of ABE7-10, was used for precise A,T to G,C conversion in rice plants (Hua et al., 2018). The editing efficiency of ABE-P1 was tested in rice by targeting IPA1 (OsSPL14), an important gene for plant architecture in rice for the base editing. Previous reports say that a point mutation in the Osmr156 binding site of OsSPL14 perturbs Osmr156-mediated cleavage of OsSPL14 transcripts, resulting in rice plants with an ideal architecture and enhanced grain yield (Jiao et al., 2010). In this study, a sgRNA was designed to target the Osmr156 binding sequence in OsSPL14. Out of 23 transgenic lines, 6 showed expected T,C substitutions at the target region with an editing efficiency of 26%. Nine predicted off-target sites did not have any base-editing events. The base-editing window of ABE-P1 (4-7) in rice was broader than ABE7-10 in mammalian cells, which has a 4 nucleotides base-editing window. The results indicate the specificity and efficiency of ABEs in rice.

Furthermore, the efficiency of this adenine base-editing system, ABE-P1, was tested in rice by targeting the SLR1 gene in rice, which encodes a DELLA protein. Previous reports say, point mutations in the DELLA and TVHYNP domains of SLR1 could block its GA-dependent degradation, thereby reducing the plant’s height (Asano et al., 2009). The researchers designed a second sgRNA (sgRNA2) targeting the TVHYNP domain of SLR1. Out of 40 mutated lines, 5 had an expected T,C substitution at position 6 in the protospacer. A third sgRNA (sgRNA3) was designed to target the Osmr156 binding sites of OsSPL16 and OsSPL18 rice genes simultaneously. Interestingly, two lines (SG3-11 and SG3-12) were simultaneously edited at OsSPL16 and OsSPL18, demonstrating multiplex editing in rice. To further expand the scope of adenine base editing, the researchers replaced the SpCas9 (D10A) nickase and its sgRNA scaffold with the SaCas9 (D10A) nickase and a sgRNA scaffold matching SaCas9 in the prRABEp-OsU6 vector. The resulting base editor, ABE-P2 could recognize a different PAM sequence, NNGRRT. To test the efficacy of prRABEp-OsU6 vector, a fourth sgRNA (sgRNA4) was designed that simultaneously targets the Osmr156 binding sites of OsSPL14 and OsSPL17 genes. Out of 31 transgenic rice lines, 14 lines harbourd T-C substitutions in the target site in OsSPL14 and 19 lines had T-C substitutions in the target site in OsSPL17. The base-editing efficiencies were observed to be 45.2% and 61.3% at the OsSPL14 and OsSPL17 target sites, respectively, which are higher than those of prRABEp-OsU6 with sgRNA1. In summary, several sgRNAs were designed to test the efficiency and specificity of the adenine base-
editing system in rice. These ABEs have the ability to efficiently convert A:T to G:C in rice in a programmable manner. Also, the lack of indels or any form of mutations in both target and potential off-targets witness the specificity of these base editors in rice. Overall, the study has broadened the scope of genome editing in rice and advanced precision molecular breeding of crops.

Similarly, ABE7.10 (base editors used in human cells) was adapted and optimized to an adenine base-editing system in plants to create point mutations at multiple endogenous loci in rice and wheat (Li et al., 2018). To develop an ABE system in plants, seven ABE fusion proteins, named PABE-1 to PABE-7, were created which varied in the position of the adenosine deaminase and the number and locations of nuclear localization sequences. Among them, PABE-7 base-editing construct, together with the sgRNA, was found to be efficient in inducing A to G substitutions with high fidelity at multiple loci in rice and wheat. The plant ABE system was further used to develop herbicide resistance in rice (Li et al., 2018). A point mutation (C208BR) at the acetyl-coenzyme A carboxylase (ACC) gene in *Lolium rigidum* provide broad-spectrum resistance to herbicides (Yu et al., 2007). Therefore, the plant ABE system was used to target the OsACC-T1 gene at C2186R position that corresponds to C208BR from *L. rigidum*. Out of 160 transformed lines, 33 harboured at least one T to C substitution in the target region with 20.6% mutation efficiency. The plant ABE system was also used to generate base-edited plants in wheat by targeting TaDEP1 and TaGW2 genes. PABE-7 and pTaU6-egRNA constructs were delivered into immature wheat embryos by particle bombardment and plants were generated. For TaDEP1 site, 5 heterozygous TaDEP1 mutant plants were identified harbouring an A to G substitution with four mutants heterozygous for TaDEP1-A (tadep1-AaBBDD) and one mutant heterozygous for TaDEP1-B (tagw2-AABBDD). For TaGW2 target site, 2 heterozygous mutants were identified with an A to G substitution at position 5 for TaGW2-B (tagw2-AABBDD). This is the first report of achieving A to G base-edited plants in wheat and herbicide-resistant rice plants. The expanded deamination window (4–8 nt) of the protospacer and high-fidelity substitutions at the targeted loci with low indels make this plant ABE system a reliable tool for achieving targeted base editing in crop plants.

A to G conversion in rice has been facilitated by a fluorescence-tracking ABE developed by using *E. coli* TadA variants and Cas9 variants (Yan et al., 2018). The wild-type *E. coli* TadA gene and the engineered TadA*7.10 were fused to Cas9n and dCas9 with two 32-amino acid XTEN2 linkers, resulting in rBE14 and rBE15, respectively. Similarly, A142N and P152R mutations were incorporated into TadA*7.10 to generate TadA*7.8 to create two more rice base editors rBE17 and rBE18. Later on, rBE14, rBE15, rBE17 and rBE18 vectors together with a sgRNA and an mGFP5-ER cassette were introduced to target the pathogen-responsive phosphorylation site in the endogenous *OsMPK6* gene into rice cells to investigate the efficiency of the ABEs. No mutants were identified for rBE15, rBE17 or rBE18 except for rBE14 with 16.67% efficiency. Sequencing results showed a pure A to G conversion at protospacer position –15 indicating that all mutant lines were heterozygous or monoallelic with one *OsMPK6* allele carrying the desired Y227P substitution. The study indicates that rBE14, together with the other rBE vectors, has the potential to facilitate generation of DNA variations in rice for both functional genomics and crop improvement. The study also suggests that the TadA variant TadA*7.10 is more suitable for base editing of A to G in the rice genome. Overall, these findings suggest that a fluorescence-tracking ABE along with the Cas9n-guided TadA: TadA7.10 heterodimer, not only introduce an A to G conversion in rice efficiently but also makes it more convenient to select the base-edited plants through detection of fluorescence.

The scope of base editing was expanded in rice by generating new adenine and CBEs with engineered SpCas9 and *S. aureus* Cas9 (SaCas9) variants (Hua et al., 2018). A number of rice genes like OsSPL14, OsSPL16, OsSPL17, OsSPL18, OsTOE1 and OsSNS1 were targeted by newly created ABEs like ABE-P2, ABE-P3, ABE-P4 and ABE-P5. The CBEs (CBE-P1 and CBE-P3) were used to target SNB and PMSS3 genes, respectively. It was also reported that adenine and CBEs can be simultaneously executed in rice. These new base editors with different Cas9 variants have increased the scope of base editing and could be useful in rice functional genomics research in rice and other crops in the future.

Most recently, a rice codon-optimized ABE-nCas9 tool was synthesized to introduce targeted A→T to G→C point mutation in the rice genome (Li et al., 2019). In this study, the rice codon-optimized ecTadA XTN-TadA*7.10 was cloned into pHUN411 binary vector under the control of a maize ubiquitin promoter. The rice amylase synthesis gene Wx was targeted by this vector. Wx-mq is a minor mutant allele that results in low amylose content in rice endosperm (Sato et al., 2002) and the Wx-mq allele contains a point mutation (T to C) at position 595, resulting in the replacement of tyrosine by histidine at residue 191. A sgRNA (Wx-sg) was designed, cloned into the pHUN411-ABE vector and transformed into rice plants via Agrobacteria. 16.67% clones had the desired T to C conversion at position 5, and approximately 27.78% clones harboured the substitution at position 6. Further the editing efficiency was increased by generating the pHUN411-ABE-sg2.0 vector using an extended version of sgRNA. The Wx-sg was fused into the ABE-sg2.0 binary vector, and base changes were observed in 5 out of the 33 transgenic lines (15.15%). The editing efficiency of ABE vectors was also tested by targeting GL2/OsGRF4 and OsGRF3 genes, responsible for grain size and yield in rice. Out of 35 transgenic lines of the pHUN411-ABE-GL2-sg, 4 harboured the targeted base mutations. The transgenic plants using both vectors were analysed for off-target activity, and none of them showed any off-target mutations. Thus, the study indicates that the plant ABE systems combined with the modified single-guide RNA variants have the ability to expand the application of CRISPR-Cas9 tools as well as advance precise molecular crop breeding.

The scope of base editing was expanded by development of novel ABEs using a Cas9 variant SpCas9-NGV1 that successfully induced A to G base substitutions in endogenous sites of the rice genome (Negishi et al., 2019). The SpCas9-NGV1 includes a 7-aa mutation in the PAM-interacting domain and recognizes NG as PAM (Endo et al., 2019, Nishimatsu et al., 2018). To study the function of ABEnSpCas9 in rice, the researchers constructed a binary vector harbouring a single-guide RNA (sgRNA) and the ABEnSpCas9 expression cassette which was then integrated into the rice genome via Agrobacterium-mediated transformation. Sequencing results revealed that ABEn7.10 nSpCas9 effectively induced A to G substitutions at NGG PAM target sites at the position 16 to 13 nt upstream from the PAM. Further, ABEn7.10 nSpCas9-NGV1, a new ABE system harbouring the nickase type of SpCas9-NGV1 instead of nSpCas9, was used to target the endogenous sites in rice. The sequencing analysis revealed that all the four sites with NGG, NGA, NGC and NGT, respectively, as PAM sequences showed A to G substitutions and these were also inherited to the next generation. The study indicates that these
new ABE systems developed with Cas9 variants can be used as valuable tools for precise genome engineering in crops.

Limitations of base editing

Targeting limitations

Successful base editing requires the presence of a specific PAM sequence (NGG PAM for SpCas9) and the target base must be within a narrow base-editing window (Gaudelli et al., 2017; Komor et al., 2016). This specific PAM requirement is a severe limitation which lowers the editing efficiency in plants. To broaden the PAM compatibility and expand the scope of base editing, several research groups have developed novel ABE and CBE base editors using Cas9 variants which recognize PAMs other than the NGG motif (Endo et al., 2019; Hua et al., 2018; Nishimasu et al., 2018; Qin et al., 2019; Wang et al., 2019). These optimized base editors can improve the base-editing efficiency and expand its scope in targeting different sites in crop plants.

Size of catalytic window

Cytosine deaminase base editors can potentially edit any C that is present in the wide activity window of approximately 4–5 nucleotides (or up to 9 nt). This is a severe limitation in base editors which result in low specificity and editing efficiency. Therefore, efforts have been made to generate high-precision base editors with narrow catalytic windows that can precisely edit a single cytidine residue within the catalytic window with high accuracy and efficiency (Tan et al., 2019). These are developed by removing nonessential sequences from the deaminase and testing different proline-rich linkers of specific lengths that can narrow down the catalytic window and improve accuracy. Thus, these highly precise base editors with high efficiency can be used as valuable tools for precision crop breeding.

Off-target editing

The CRISPR-mediated base-editing technology is a much more precise tool used for base conversions without any gene disruption. However, off-target editing is still a major concern. In the base-editing systems, off-targets occur when additional cytosines proximal to the target base get edited. The off-target activity has been greatly reduced in human cells by generating a high-fidelity base editor (HF-BE3), by installing mutations into BE3 base editor (Rees et al., 2017). However, in a recent study, it was observed that CBEs BE3 and high-fidelity BE3 (HF1-BE3) induce unexpected and unpredictable genomewide off-target mutations in rice crop (Jin et al., 2019). These mutations were usually the C to T type of single nucleotide variants (SNVs). The study also indicates that to minimize the off-target mutations, it is necessary to optimize the cytidine deaminase domain and/or UGI components. Furthermore, use of improved variants of CBEs, YEE-BE3, could also be employed to minimize the off-target edits in plants (Jin et al., 2019).

Future perspectives of the emerging technology

In the last two years, several research groups have engineered SpCas9s, SpCas9-NG variants and xCas9 variants to extend the Cas9 recognized sites and expand the scope of base editing in plants (Endo et al., 2019; Hua et al., 2018; Nishimasu et al., 2018). In a recent study, the optimized BE4max, AncBE4max and ABEmax editors (Koblan et al., 2018) were further upgraded by using codon-optimized bipartite nuclear localization signals (bpNLS) and were used to target rice genes (Wang et al., 2019). The base editors showed much higher editing efficiencies as compared to the previous known CBE and ABE editors. These optimized and improved base editors are valuable tools for molecular breeding of crops. Thus, new engineered variants need to be adopted to improve the existing CBE and ABE base editors and increase the editing efficiency and expand the scope of base editing in a wide range of crops in the future.

The plant ABE system has been well adapted and successfully used in a wide range of crops (Hua et al., 2018; Li et al., 2018; Yan et al., 2018). However, there are ample opportunities for improving and extending the plant ABE system by using engineered Cas9 variants recognizing different PAM sequences (Kim et al., 2017; Hu et al., 2018) or Cpf1 (Li et al., 2018). Furthermore, the sgRNAs could be ligated with different aptamers (MS2, PP7, COM and boxB (Ma et al., 2016; Zalatan et al., 2015) to facilitate simultaneous base conversions (C-T and A-G) and correct point mutations related to important agricultural traits (Li et al., 2018). Protein delivery of base editors results in the precise conversion of nucleotides with enhanced DNA specificity (Rees et al., 2017). Thus, high-fidelity plant base editors should be created and delivered through RNP delivery to establish DNA free strategy with enhanced specificity and reduced off-target editing.

Directed evolution employs multiple rounds of mutation followed by selection to engineer biomolecules with novel functions and protein variants with improved abilities (Soskin and Tawfik, 2010). CRISPR-X generates diverse libraries of localized point mutations in mammalian cells that can be applied to study and improve protein function (Hess et al., 2016). Most recently, a CRISPR/Cas-based-directed evolution platform (CDE) was developed for plants to evolve the ric (Oryza sativa) SF3B1 spliceosomal protein for resistance to splicing inhibitors (Butt et al., 2019). These mutant variants confer variable levels of resistance to splicing inhibitors. This directed evolution platform can be used to engineer crop traits for better performance and develop resistance to biotic and abiotic stresses. It offers possibilities for breeding climate resilient crops that can enhance global food security. Thus, base-editing diversification strategies for direction evolution need to be explored in the future that can increase genetic diversity in plants.

The use of DNA base editors in correcting point mutations related to agricultural traits has already been demonstrated in several crops. However, RNA editing has not been used in plants yet. Currently, the REPAIR system enables A to I conversion in RNA editing. In the future, additional fusions of dCas13 with other catalytic RNA editing domains such as APOBEC could also enable C to U conversions (Cox et al., 2017). Conversion of A to I may also be possible on DNA substrates by using catalytically inactive dCas9 or dCpf1, either through formation of DNA–RNA heteroduplex targets (Zheng et al., 2017) or mutagenesis of ADAR domain (Cox et al., 2017). The REPAIR system is used to correct disease-relevant mutations in human but its use in plants is still not explored. Use of RNA editing may not be highly desirable in crop bioengineering as it requires stable expression of CRISPR base editors. However, it would be good for functional gene analysis. Thus, researchers may explore the applications of this system in crop plants in the near future.

Conclusion

Base editing is a novel editing tool in the genome engineering toolboxes. It is an efficient genome-editing approach which enables nucleotide substitutions in a programmable manner.
without the requirement of a DSF or donor template. In the last three years, cytidine and adenosine deaminase-based base editors have been successfully developed and used for the base editing in plants as well as in animals. Narrowing down the catalytic window and adopting the Cas9 variants to improve the existing CBE and ABE base editors can expand the scope of base editing in crop plants. These upgraded base editors and mutations of cytidine deaminase can increase DNA specificity and lower the off-target activity. The highly precise base editors can be widely used in model plants and crops for precision breeding. The emerging base-editing technology is still in its infancy and a lot of efforts are to be made to optimize and expand the scope of editing and increase its efficiency. Nonetheless, it is a novel editing approach which has the potential to modify crops precisely and accelerate crop improvement in the future.

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Conflict of interest

The authors declare that there is no conflict of interest.

Authors’ contributions

RM and RKJ conceived and drafted the manuscript. KJ collaborated in the manuscript preparation and critically reviewed the manuscript. All authors revised and approved the final manuscript.

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