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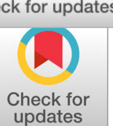

Coupling of both a transactivation module and a double-stranded DNA-binding domain boosts Cas12i3 variant-based cytosine and adenine editing in plants∞

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ABSTRACT

CRISPR/Cas12i3 belongs to the type V-I Cas system, characterized by its smaller protein size and less restricted canonical “TTN” protospacer adjacent motif. Developments of Cas12i3-mediated base editing systems for either C-to-T or A-to-G transitions will expand the editing scope and enrich the plant base editing toolkits for crop improvement. However, while the Cas12i3-based cytosine base editor (CBE) only shows very low editing efficiency in plants, its adenine base editor (ABE) has not been documented as yet. Here, we engineered a series of Cas12i3 (5M)-based CBEs (V0–V5) and ABEs (V0–V5) by fusing a deactivated dCas12i3 (5M) with a transactivation module VP64, a single-stranded DNA-binding domain Rad51, or a double-stranded DNA-binding domain HMG-D, or in combinations, and systemically evaluated their performance in rice protoplasts. Our results demonstrated that synergistic combinations of both VP64 and HMG-D outperformed other architectures and significantly boosted the efficiencies of Cas12i3 (5M)-based CBE and ABE for C-to-T and A-to-G base editing and expanded the editing window. In stable lines, in comparison to the non-fusion control, the optimized Cas12i3 (5M)-based CBE-V5 and ABE-V5 enabled up to 4.78- and 3.35-fold higher editing efficiencies, with the maximum C-to-T and A-to-G efficiencies reaching 32.35% and 38.24%, respectively, and a higher proportion of homozygous mutants in the T_0 generation. Furthermore, we generated herbicide-resistant rice germplasm by using CBE-V5 and ABE-V5, demonstrating their potential for precision breeding in crops. Together, here, we report novel Cas12i3 (5M)-based CBE and ABE that substantially enrich base editing toolkits for improvement of rice and potentially other crops.

Keywords: base editing, Cas12i3 (5M), double-stranded DNA-binding domain (HMG-D), rice (*Oryza sativa L.*), transactivation module VP64

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INTRODUCTION

Base editing facilitates precise single-nucleotide substitution in a sequence-specific manner, enabling the creation of targeted loss-of-function or gain-of-function mutations. This technology significantly accelerates research in functional genomics and applications in crop improvement, *de novo* domestication, and directed evolution. To date, a diverse range of base editors (BEs) have been reported: cytosine base editors (CBEs) for C-to-T transition (Komor et al., 2016), adenine base editors (ABEs) for A-to-G transition (Gaudelli et al., 2017), C-to-G base editors (CGBEs) for C-to-G transversion (Kurt et al., 2021; Zhao et al., 2021), adenine transversion base editor (AKBE) for A-to-K (G/T) transversion (Li et al., 2023; Wu et al., 2023), glycosylase-based guanine base editor (gGBE) for G-to-T transversion (Tian et al., 2022; Tong et al., 2023; Liu et al., 2024), and T-to-S (C/G) base editor (TSBE) for T-to-S (C/G) transition and transversion (He et al., 2024; Wu et al., 2025). So far, impressive progress has been achieved in improving the performance of CRISPR/Cas-mediated base editors. For example, a base editor engineered by fusing human cytidine deaminase APOBEC3A (hA3A) to Cas9 nickase, nCas9 (D10A), demonstrated enhanced efficiency and a consistently broad editing window of 17 nucleotides in both wheat and rice (Zong et al., 2018). Subsequently, a CRISPR-Cas12a base editing system composed of deactivated Cas12a (dCas12a) and hA3A with Y130F mutations (mhA3A) significantly improved C-to-T editing efficiency at the TTTV-PAM target sites in comparison with the original hA3A (Wang et al., 2020; Cheng et al., 2023). The incorporation of monomeric TadA8e deoxyadenosine deaminase, an engineered variant with eight key mutations, into ABEs, markedly boosted A-to-G base editing efficiency (Richter et al., 2020; Wei et al., 2021). Further improvement in the efficiencies of base editors can be achieved by rational optimization of several factors, such as incorporating surrogate reporter systems to enrich precisely edited events, changing the lengths of inter-protein linkers, and increasing the copy numbers of uracil DNA glycosylase inhibitor (UGI) and adding additional nuclear localization signals (NLSs) (Xu et al., 2020; Zeng et al., 2020).

CRISPR/Cas12i3, which belongs to the Class II Type V-I Cas system, has attracted considerable attention recently due to its smaller protein size and less restricted canonical "TTN" protospacer adjacent motif (PAM), compared to the widely used SpCas9 with PAM of "NGG" and Cas12a with PAM of "TTTV." Development of various Cas12i3-based genome editing tools will significantly expand the scope of genome editing and enrich the genome editing toolkits for crop improvement. However, the editing efficacy of wild-type Cas12i3 remains lower in both mammalian cells and plants (Bai et al., 2024; Duan et al., 2024; Lv et al., 2024; Wang et al., 2025a). To improve the performance of Cas12i3, five amino acids mutations (S7R, D233R, D267R, N369R, and S433R) in Cas12i3, hereafter referred to as Cas12i3 (5M), enabled significantly improved editing efficacies in both mammalian cells and plants (Duan et al., 2024). Subsequently, a robust Cas12i3 (5M)-mediated plant genome editing system, Opt-T5E-Cas12i3 (5M), was engineered through the fusion of a T5 exonuclease (T5E) in combination with an optimized crRNA expression strategy (Opt). This Opt-T5E-Cas12i3 (5M) system achieved a remarkable average editing efficiency of up to 88.99% across four endogenous target sites in stable lines of three elite Chinese wheat cultivars (Wang et al., 2025a). Furthermore, fusions of four 5′ exonucleases (Exo), including T5E, UL12, PapE, and ME15 to the N-terminal of Cas12i3 (5M), generated the Exo:Cas12i3 (5M) fusions and increased the gene editing efficiencies by up to 12.46-fold and 1.25-fold compared to Cas12i3 and Cas12i3 (5M), respectively. Although all the Exo:Cas12i5-5M fusions were capable of multiplex gene editing, UL12:Cas12i3 (5M) showed superior performance in the simultaneous editing of three, four, five, or six genes, with efficiencies of 82.76%, 61.36%, 52.94%, and 51.06% in rice stable lines (Wang et al., 2025b). Together, although impressive progress has been achieved in gene knockout and multiplex gene editing in wheat and rice, to date, only Cas12i3 (5M)-based CBE has been reported in soybean, with a very low editing efficiency of 2.16% (Niu et al., 2024). In particular, Cas12i3 (5M)-based ABE for plant base editing has not been documented as yet. Compared with Cas9 proteins, Cas12i3 (5M), like other Cas12 family members, lacks the HNH domain, which makes nCas9 (Cas9 nickase) more efficient and versatile for genome engineering such as base editing and prime editing. Thus, how to establish efficient Cas12i3 (5M)-based CBE and ABE systems in plants remains elusive.

To develop Cas12i3 (5M)-based CBEs and ABEs in plants, we hypothesized that fusions of additional factors such as DNA-binding proteins/domains may make the Cas12i3 (5M)-based CBEs and ABEs in plants feasible and further enhance their performance. Among these DNA-binding proteins/domains, VP64, which is a transactivation module composed of four copies of VP16 transactivation domain, can facilitate remodeling and unfolding of condensed chromatin, and thus the accessibility of CRISPR reagents to target sites, resulting in improved C-to-G and A-to-K base editing efficiencies (Tumbar et al., 1999; Dong et al., 2022; Li et al., 2023; Li et al., 2024). Furthermore, a single-stranded DNA (ssDNA)-binding protein, Rad51, contains an ssDNA-binding domain (ssDBD) to increase affinity for the DNA substrate, and can indirectly improve the efficiency and fidelity of gene editing by stabilizing and protecting gRNA/pegRNA templates from degradation (Tan et al., 2022; Zheng et al., 2024). Moreover, the HMG-D domain (112 aa), deriving from the high-mobility group family of chromosomal proteins from *Drosophila melanogaster*, is characterized by its ability to effectively bind to double-stranded DNA (dsDNA) non-specifically; fusion of HMG-D to a miniature enIscB could significantly improve its base editing efficacy (Xue et al., 2024). Given the functional characteristics of these DNA-binding domains, fusions of these domains either alone or in combinations will possibly facilitate the establishments of either Cas12i3 variant-based

plant CBE or ABE, or both, as well as enhance their base editing performance.

In this study, we developed a series of Cas12i3 (5M)-based plant CBEs and ABEs through fusions of either cytidine deaminase human APOBEC3A (hA3A-Y130F) or adenosine deaminase TadA8e to deactivated Cas12i3 (5M), and also incorporated the transactivation module and DNA-binding domains either alone or synergistically. Through systemic evaluations in rice protoplasts and stable lines, we demonstrated that synergistic combinations of both VP64 and HMG-D with either Cas12i3 (5M)-based CBE or ABE yielded superior performance and significantly boosted the efficiencies of Cas12i3 (5M)-based CBE and ABE by up to 4.78- and 3.35-fold, with the maximum C-to-T and A-to-G efficiencies reaching 32.35% and 38.24% in rice stable lines, respectively, along with a higher proportion of homozygous mutants in the T_0 generation. Overall, our study presents novel Cas12i3 (5M)-based CBE and ABE that enable efficient base editing in rice, highlighting their potential for a wide range of applications in rice as well as other agriculturally important crop plants either for biological research or precision breeding for crop improvement.

RESULTS

Engineering a series of Cas12i3 (5M)-based CBEs and ABEs for cytosine and adenine base editing in rice

To engineer Cas12i3 (5M)-based CBEs, we first fused cytidine deaminase mhA3A (hA3A with Y130F mutations) to the N-terminus of dCas12i3 (5M), followed by two copies of UGI at the C-terminus of dCas12i3 (5M) to generate CBE-dCas12i3 (5M) (CBE-V0). We then incorporated either the ssDBD domain of Rad51 (hereafter referred to as Rad51) or HMG-D, or VP64 alone, or both VP64 and Rad51, or both VP64 and HMG-D synergistically to generate a series of CBEs in an architecture of CBE-Rad51-dCas12i3 (5M) (CBE-V1), CBE-HMG-D-dCas12i3 (5M) (CBE-V2), VP64-CBE-dCas12i3 (5M) (CBE-V3), VP64-CBE-Rad51-dCas12i3 (5M) (CBE-V4), and VP64-CBE-HMG-D-dCas12i3 (5M) (CBE-V5) (Figure 1A). Alternatively, to develop Cas12i3 (5M)-mediated ABEs, we first fused TadA8e to the N-terminus of dCas12i3 (5M) to generate ABE-dCas12i3 (5M) (ABE-V0). We then incorporated either Rad51 or HMG-D, or VP64, or in combinations to the N-terminus of ABE-V0 to obtain a series of ABEs with a structure of ABE-Rad51-dCas12i3 (5M) (ABE-V1), ABE-HMG-D-dCas12i3 (5M) (ABE-V2), VP64-ABE-dCas12i3 (5M) (ABE-V3), VP64-ABE-Rad51-dCas12i3 (5M) (ABE-V4), and VP64-ABE-HMG-D-dCas12i3 (5M) (ABE-V5) (Figure 1B).

To assess the base editing efficiencies and editing windows of these Cas12i3 (5M)-based BEs in rice, we designed 11 crRNAs targeting the coding regions of different endogenous genes (Table S1). These crRNAs were individually cloned into the OsU3–crRNA expression cassettes within these base editing vectors and transiently expressed in rice protoplasts. The targeted genomic fragments encompassing the selected gene/coding regions were amplified by PCR and subjected to Hi-tom high-throughput sequencing (Liu et al., 2019). Our sequencing results demonstrated that Rad51, HMG-D, and VP64 enhanced the editing efficiency of the Cas12i3 (5M)-mediated base editors to varying degrees. Notably, fusion proteins combining both VP64 and HMG-D with either hA3A-Y130F or TadA8e significantly increased C-to-T and A-to-G base editing efficiencies, respectively (Figures 2A, B, S1A), while CBE-V0 achieved a maximum C-to-T editing efficiency of 4.59% within an editing window spanning positions C8 to C15 (counting the base nearest to the PAM as position 1) of the protospacer sequences (Figure 2A, C). The incorporation of Rad51, HMG-D, or VP64 alone into CBE-V0 yielded a maximum editing efficiency of 7.57% (1.65-fold vs CBE-V0), 8.60% (1.87-fold vs CBE-V0), and 7.15% (1.58-fold vs CBE-V0) for CBE-V1, CBE-V2, and CBE-V3, respectively. The editing windows of these three systems showed varying degrees of expansions. Specifically, the editing windows of both CBE-V1 and CBE-V3 systems spanned from C7 to C15, whereas that of the CBE-V2 system extended from C7 to C16 (Figure 2A, C). Notably, compared to CBE-V0, whereas the CBE-V4 system showed a maximum C-to-T efficiency of 10.32%, an approximately 2.25-fold increase over CBE-V0, within an expanded window of C7 to C15 (Figure 2A, C), the CBE-V5 system outperformed other architectures and demonstrated a maximum C-to-T efficiency of 12.80%, representing an approximately 2.79-fold increase over CBE-V0 and a 1.24-fold increase over CBE-V4, within a wider editing window that encompassed C7 to C16 of the respective protospacer sequences (Figure 2A, C).

For Cas12i3 (5M)-based ABEs, the ABE-V0 system yielded a maximum A-to-G editing efficiency of 4.62% within the editing window of A8 to A13 (Figures 2B, D, S1B). Upon coupling with Rad51, or HMG-D, or VP64 individually, the maximal editing efficiencies attained were 6.92% (1.50-fold over ABE-V0) for the ABE-V1 system, 8.69% (1.88-fold over ABE-V0) for ABE-V2, and 6.29% (1.36-fold over ABE-V0) for ABE-V3. In parallel, all three engineered ABEs showed broadened editing windows. For example, while the editing windows of ABE-V1 and ABE-V2 encompassed positions A7 to A15, that of ABE-V3 was defined as nucleotides of A8 to A14 (Figure 2B, D). Furthermore, consistent with the performance of CBEs, the synergistic combination of both VP64 and Rad51 enabled ABE-V4 to achieve a maximum A-to-G efficiency of 10.92%, an approximately 2.36-fold increase over ABE-V0, within an expanded window A7 to A15 (Figure 2B, D). Notably, ABE-V5 with both VP64 and HMG-D achieved a maximum A-to-G efficiency of 12.20%, an approximately 2.64-fold increase over ABE-V0 and 1.12-fold increase over ABE-V4, respectively, within a wider editing window of A7 to A15 (Figure 2B, D). Despite the significant enhancement in base editing efficiency achieved by CBE-V5 and ABE-V5, editing byproducts (other unexpected types of mutations within or outside the editing window) were undetectable. Thus, the CBE-V5 and ABE-V5 systems not only

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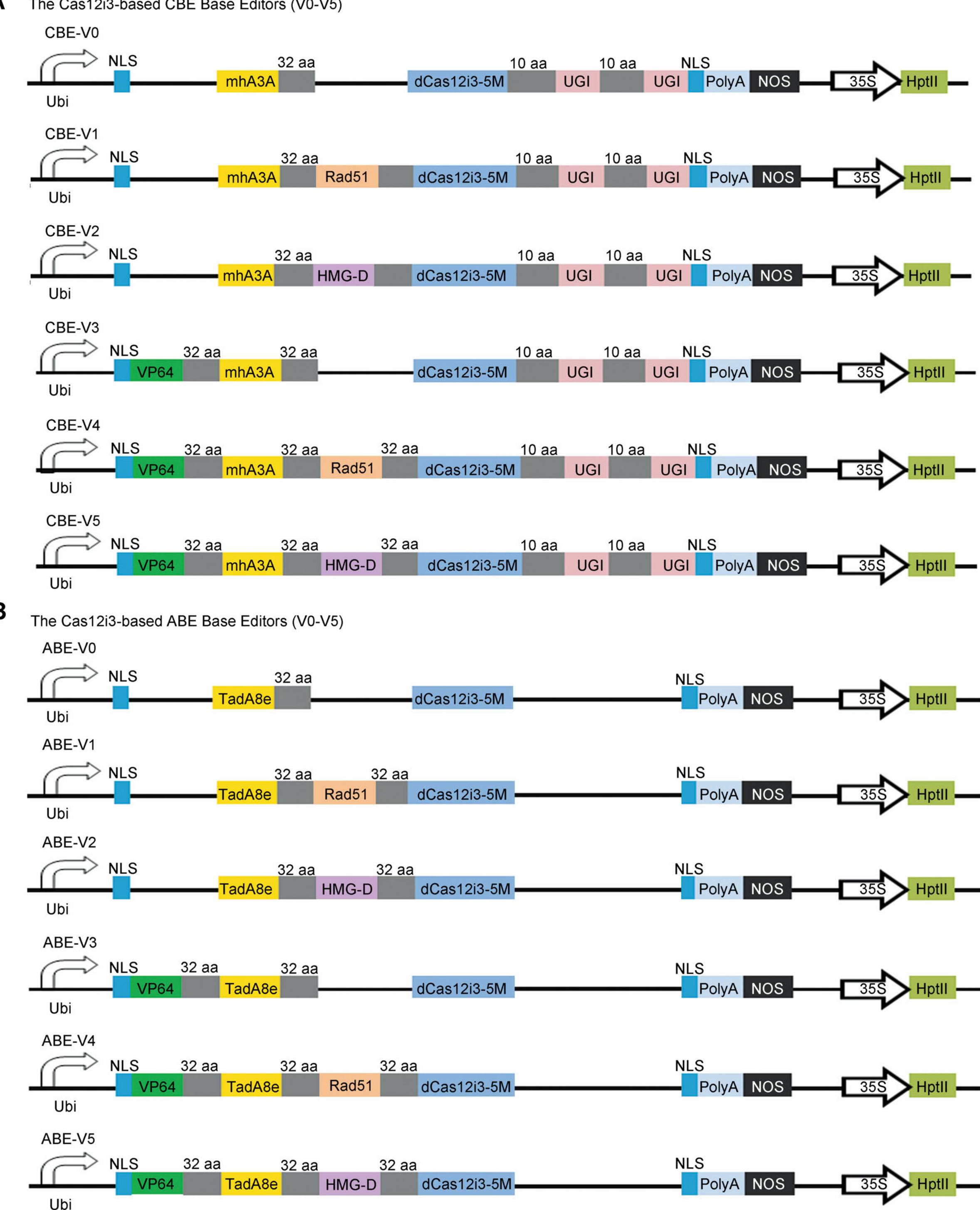

Figure 1. Development of a series of Cas12i3 (5M)-based cytosine base editors (CBEs) and adenine base editors (ABEs) for cytosine and adenine base editing

(A) Schematic diagram of the architectures of CBE-dCas12i3 (5M) (CBE-V0), CBE-Rad51-dCas12i3 (5M) (CBE-V1), CBE-HMG-D-dCas12i3 (5M) (CBE-V2), VP64-CBE-dCas12i3 (5M) (CBE-V3), VP64-CBE-Rad51-dCas12i3 (5M) (CBE-V4), and VP64-CBE-HMG-D-dCas12i3 (5M) (CBE-V5). **(B)** Schematic diagram of the architectures of ABE-dCas12i3 (5M) (ABE-V0), ABE-Rad51-dCas12i3 (5M) (ABE-V1), ABE-HMG-D-dCas12i3 (5M) (ABE-V2), VP64-ABE-dCas12i3 (5M) (ABE-V3), VP64-ABE-Rad51-dCas12i3 (5M) (ABE-V4), and VP64-ABE-HMG-D-dCas12i3 (5M) (ABE-V5). From left to right, Ubi, ubiquitin promoter; NLS, nuclear localization signal; VP64, tetramer of herpes simplex virus protein 16; mhA3A, a mutated human deaminase APOBEC3A; TadA8e, a deaminase variant containing eight amino acids mutations in deoxyadenosine deaminase TadA7.10; Rad51, a single-stranded DNA-binding domain (ssDBD) from RADIATION SENSITIVE 51; HMG-D, a double-stranded DNA-binding domain from *Drosophila melanogaster*; UGI, a uracil DNA glycosylase inhibitor; PolyA, polyadenylation signal; NOS, NOS terminator; and 35S, cauliflower mosaic virus 35S promoter. The boxes in gray indicate the different lengths of amino acid linkers.

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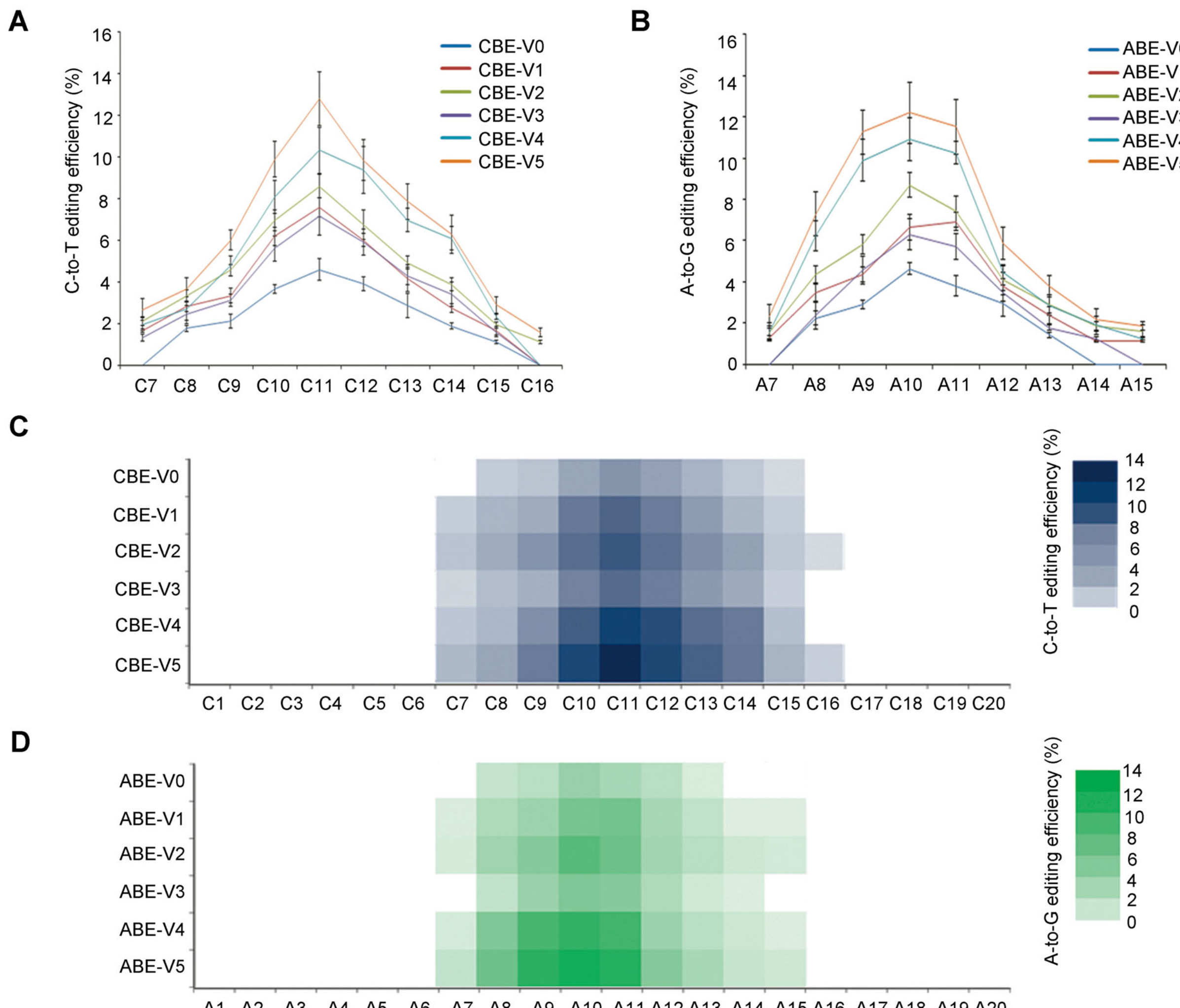

Figure 2. Editing efficiencies and windows of a series of Cas12i3 (5M)-based cytosine base editors (CBEs) and adenine base editors (ABEs)

(A) Editing efficiencies and editing windows of different Cas12i3 (5M)-mediated cytosine base editing systems in rice protoplasts. Error bars indicate the standard error of three independent biological replicates. **(B)** Editing efficiencies and editing windows of different Cas12i3 (5M)-mediated adenine base editing systems in rice protoplasts. Error bars indicate the standard error of three independent biological replicates. **(C)** Heatmap showing the distribution profiles of C-to-T editing efficiency of different Cas12i3 (5M)-mediated cytosine base editing systems. The darker the color, the higher the editing efficiency. **(D)** Heatmap showing the distribution profiles of A-to-G editing efficiency of different Cas12i3 (5M)-mediated adenine base editing systems. The darker the color, the higher the editing efficiency.

showed significantly enhanced base editing efficiencies but also displayed broadened editing windows, enabling effective cytosine and adenine editing of bases distal to the PAM site.

Synergistic combination of VP64 and HMG-D significantly enhanced the efficiencies of Cas12i3 (5M)-based CBE and ABE in rice stable lines

Given that CBE-V5 and ABE-V5 showed substantially higher base editing efficiencies in rice protoplasts, we next chose them to evaluate their performance in base editing in rice stable lines by using six endogenous genes, three for CBE and three for ABE, which had been tested in rice protoplasts. We first evaluated the performance of CBE-V0 and CBE-V5 in C-to-T base editing at three endogenous genes, *OsALS, OsARF4*, and *OsSBEIIb*, in rice stable lines (Figure 3A). For CBE-V0, the editing efficiencies were 5.71% (2/35) at *OsALS*, 9.38% (3/32) at *OsARF4*, and 11.76% (4/34) at *OsSBEIIb*, with an average editing efficiency of 8.95% (Figures 3B, S2A, C). Compared with CBE-V0, CBE-V5 showed substantially higher editing efficiencies of 27.27% at *OsALS* (9/33), 32.35% (11/34) at *OsARF4*, and 22.58% (7/31) at *OsSBEIIb*, with an average editing efficiency of 27.40% (Figure 3B). The editing efficiencies of CBE-V5 were 1.92- to 4.78-fold higher than that of CBE-V0 at the above three endogenous genes tested in stable lines. Notably, among these edited independent lines generated by CBE-V5, 5 out of 9 (55.56%) mutants at *OsALS*, 7 out of 11 (63.63%)

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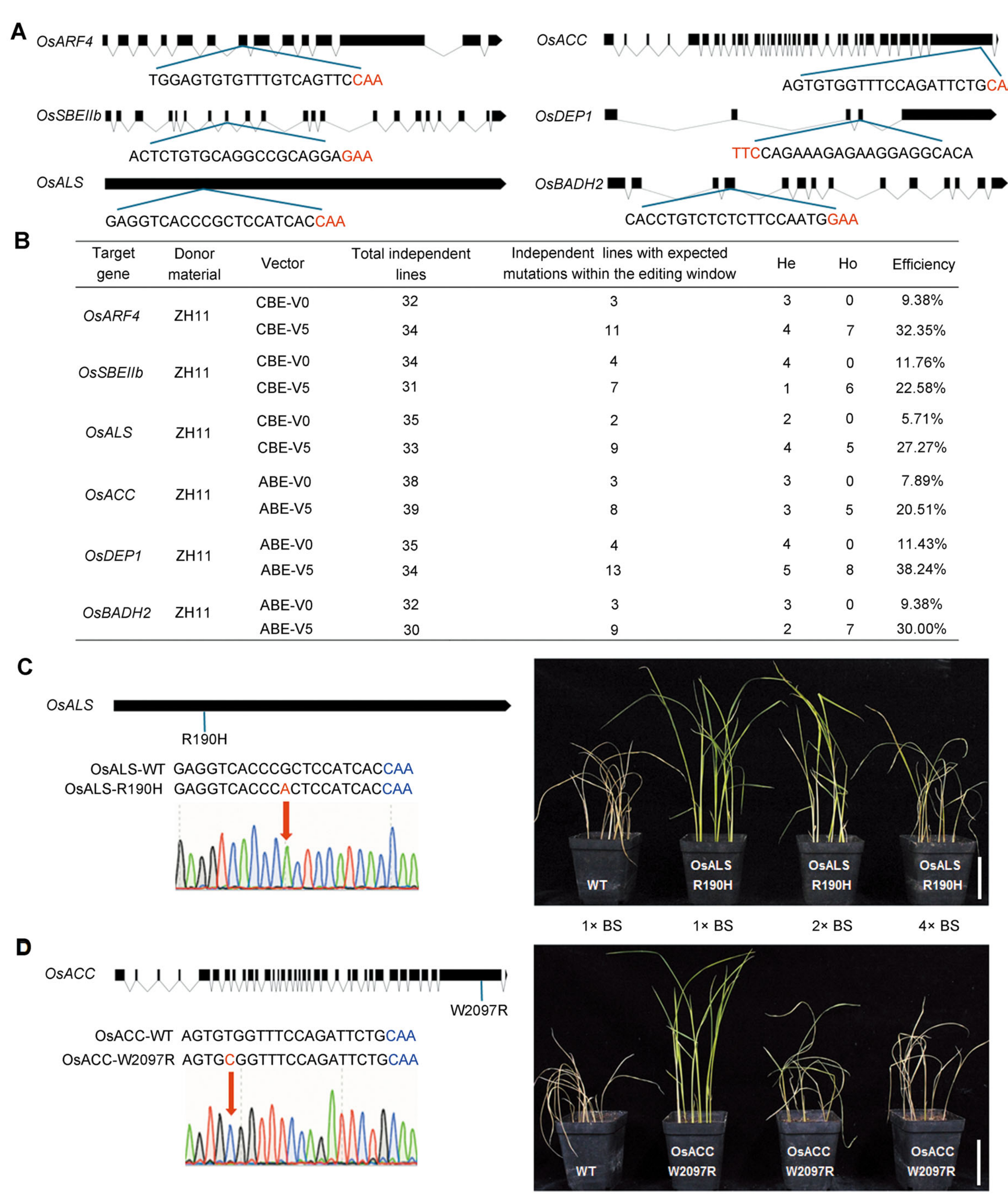

Target gene	Donor material	Vector	Total independent lines	Independent lines with expected mutations within the editing window	He	Ho	Efficiency
OsARF4	ZH11	CBE-V0	32	3	3	0	9.38%
		CBE-V5	34	11	4	7	32.35%
OsSBEIIb	ZH11	CBE-V0	34	4	4	0	11.76%
		CBE-V5	31	7	1	6	22.58%
OsALS	ZH11	CBE-V0	35	2	2	0	5.71%
		CBE-V5	33	9	4	5	27.27%
OsACC	ZH11	ABE-V0	38	3	3	0	7.89%
		ABE-V5	39	8	3	5	20.51%
OsDEP1	ZH11	ABE-V0	35	4	4	0	11.43%
		ABE-V5	34	13	5	8	38.24%
OsBADH2	ZH11	ABE-V0	32	3	3	0	9.38%
		ABE-V5	30	9	2	7	30.00%

Figure 3. Cas12i3 (5M)-based CBE-V5 and ABE-V5 enable more robust C-to-T and A-to-G editing of endogenous target genes in rice stable lines

(A) Gene structures of target genes including *OsARF4, OsSBEIIb, OsALS, OsACC, OsDEP1*, and *OsBADH2* used in rice stable transformation. Exon regions are shown as blank boxes, and the PAM sites (5′-TTN-3′) are highlighted in red. **(B)** Comparison of cytosine and adenine editing efficiencies of Cas12i3 (5M)-based CBE and ABE systems at TTN PAMs in target genomic sites in T_0 transgenic rice. Transgenic rice with heterozygous and homologous mutations are abbreviated He and Ho. **(C)** Bispyribac-sodium (BS) resistance of transgene-free homozygous lines with the *OsALS-R190H* allele. The *OsALS-R190H* seedlings showed normal growth at two weeks after treatment with 1× and 2× BS, whereas complete mortality was observed in the *OsALS-R190H* seedlings after 4× BS treatment and the wild-type seedlings after 1× BS treatment. The PAM sequences and target bases in the editing window are highlighted in blue and red, respectively. The mutant site is marked by a red arrow. Bar = 7 cm. (D) Haloxyfop resistance of transgene-free homozygous lines with the OsACC-W2097R allele. The *OsACC-W2097R* mutants survived two weeks after 1× Haloxyfop treatment, while complete mortality was observed in the wild-type seedlings after 1× Haloxyfop treatment and the *OsACC-W2097R* mutants after 2× and 4× Haloxyfop treatment. The PAM sequences and target bases in the editing window are highlighted in blue and red, respectively. The mutant site is marked by a red arrow. Bar = 7 cm.

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mutants at *OsARF4*, and 6 out of 7 (85.71%) mutants at *OsSBEIIb* had homozygous mutations, and the remaining mutants were heterozygous (Figure 3B). Thus, CBE-V5 not only significantly boosted the C-to-T base editing efficacy but also increased the proportion of homozygous lines obtained in the T_0 generation. In contrast, CBE-V0 did not produce any homozygous individuals in the T_0 generation, with all the obtained independent lines with precise edits showing heterozygous genotypes.

We also investigated the A-to-G base editing efficiencies of ABE-V0 and ABE-V5 at three endogenous target genes, *OsACC, OsDEP1*, and *OsBADH2*, in rice stable lines (Figure 3A). ABE-V5 achieved an editing efficiency of 20.51% (8/39) for *OsACC*, which is approximately 2.60-fold higher than that obtained using ABE-V0 (7.89%, 3/38); a base editing efficiency of 38.24% (13/34) was observed for *OsDEP1*, which is approximately 3.35-fold higher than that obtained using ABE-V0 (11.43%, 4/35); and a base editing efficiency of 30.00% (9/30) was observed for *OsBADH2*, which is approximately 3.20-fold higher than that obtained using ABE-V0 (9.38%, 3/32) (Figures 3B, S2B, C). Notably, among these edited independent lines generated by ABE-V5, 5 out of 8 (62.50%) mutants at *OsACC*, 8 out of 13 (61.54%) mutants at *OsDEP1,* and 7 out of 9 (77.78%) mutants at *OsBADH2* had homozygous mutations, and the remaining lines were heterozygous (Figure 3B). Similar to CBE-V5, an increased proportion of homozygous lines was observed with ABE-V5 in the T_0 generation. In contrast, no homozygous lines were produced by ABE-V0, as all the T_0 lines with desired edits were heterozygous. It is worth noting that, among these edited independent lines generated by CBE-V5 and ABE-V5, no detectable byproducts were identified, indicating the high fidelity of the CBE-V5 and ABE-V5 base editing systems.

Genetic stability and generation of transgene-free rice mutant lines

To determine the heritability of base mutations generated by the Cas12i3 (5M)-based CBE-V5 and ABE-V5, we genotyped the T_1 progeny derived from the initial T_0 lines. The T_1 progenies of both homozygous and heterozygous T_0 plants were selected for segregation analysis. All mutations present in the parental T_0 lines were found to be heritable in the subsequent generation, indicating complete fidelity of transmission without any novel mutations (Table S2). Consistent with the expected mode of inheritance, the desired base mutations in the homozygous lines were inherited at a perfect 100% transmission rate in T_1 progeny. Meanwhile, mutations that were heterozygous in the T_0 lines segregated in a 1:2:1 (homozygous: heterozygous: wild type) Mendelian pattern in the subsequent generation. To assess for the presence of plasmid DNA in the mutant lines, PCR amplification was performed using the primer sets specific for the dCas12i3 (5M), crRNA cassette, and *hptII* sequences. We successfully recovered transgene-free homozygous lines in the T_1 progenies (Table S2).

Off-target analyses

To evaluate the specificity of the Cas12i3 (5M)-mediated base editing system in rice stable lines with targeted mutagenesis, we further assessed whether the potential off-target effects occurred in the base edited lines. Subsequently, several potential off-target sites were predicted using the web tools CRISPR-GE (http://skl.scau.edu.cn/). We identified the potential off-target sites of *OsARF4, OsSBEIIb, OsALS, OsACC, OsDEP1*, and *OsBADH2* targets with 1- to 4-nt mismatches (Table S3). We then used genome-specific PCR and Sanger sequencing to examine the potential off-target effects. Among the above six endogenous genes tested, no off-target effect was detected at the putative off-target loci in the tested lines (Table S3).

Evaluations of herbicide tolerance of rice lines with the generated *OsALS* and *OsACC* alleles

Both Acetolactate synthase (ALS) and Acetyl-coenzyme A carboxylase (ACC) represent key enzymatic targets for engineering herbicide resistance (Kuang et al., 2020; Li et al., 2020; Xu et al., 2021). Following segregation, we obtained *OsALS*-R190H and *OsACC*-W2097R transgene-free homozygous lines in the T_1 generation (Figure 3C, D). To further validate the herbicide tolerance of these rice lines, the *OsALS*-R190H and *OsACC*-W2097R seedlings at the three-leaf stage were sprayed with different concentrations of bispyribac-sodium (BS) and haloxyfop, respectively. We observed that the seedlings carrying the *OsALS-R190H* mutation remained viable and showed normal growth at two weeks after treatment with 1× and 2× BS, whereas complete mortality was observed in the *OsALS-R190H* seedlings after 4× BS treatment and the wild-type seedlings after 1× BS treatment (Figure 3C). These results indicate that these T_1 homozygous lines conferred medium herbicide resistance to BS, consistent with a previous report showing that the R190H allele is a moderate herbicide-resistance allele (Kuang et al., 2020). The *OsACC-W2097R* mutants survived for two weeks following 1× haloxyfop treatment, whereas complete mortality was observed in the wild-type seedlings the treatment. Furthermore, the mutant lines demonstrated dosage-dependent sensitivity, as evidenced by their mortality upon exposure to elevated herbicide concentrations (2× and 4×) (Figure 3D). Although the W2097 residue is a known target for ACCase-inhibiting herbicides, our results establish that the novel and specific W-to-R substitution generated here represents a weak allele compared to the documented ACCase mutations (Li et al., 2020; Xu et al., 2021). Collectively, our data demonstrate that Cas12i3 (5M)-mediated CBE-V5 and ABE-V5 systems enable the precise generation of herbicide-tolerant alleles in rice. These newly developed Cas12i3 (5M)-mediated base editing systems in our study not only serve as effective tools for targeted mutagenesis of agronomically important genes in rice but also hold potential for use in accelerating precision breeding in other agriculturally important crops.

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DISCUSSION

Here, we report a more robust Cas12i3 (5M)-based CBE and the first case of Cas12i3 (5M)-based ABE that substantially enriches base editing toolkits for improvement of rice and potentially other crops. We first engineered a series of Cas12i3 (5M)-based CBEs (V0-V5) and ABEs (V0-V5) by fusing Rad51, or HMG-D, or VP64, individually or in combinations (Figure 1). Through systemic evaluations of these Cas12i3 (5M)-based CBEs and ABEs in rice protoplast and stable lines, we demonstrated that synergistic combinations of both VP64 and HMG-D showed superior performance and significantly boosted the efficiencies of Cas12i3 (5M)-based CBE and ABE for either C-to-T or A-to-G base editing and expanded the editing window (Figure 2). In comparison to the non-fusion controls CBE-V0 and ABE-V0, optimized Cas12i3 (5M)-based CBE-V5 and ABE-V5 yielded up to 4.78-fold and 3.35-fold higher editing efficiencies, with the maximum C-to-T and A-to-G efficiencies reaching 32.35% and 38.24% in rice stable lines, respectively (Figure 3). Notably, the optimized systems CBE-V5 and ABE-V5 not only significantly boosted the base editing efficiencies and expanded the base editing window but also substantially increased the proportion of homozygous mutants in the T_0 generation (Figures 2C, D, 3B). Furthermore, we generated herbicide-resistant rice germplasm by using CBE-V5 and ABE-V5, demonstrating their potential for precision breeding in crops. Interestingly, no detectable off-target effect and editing byproducts of both CBE-V5 and ABE-V5 were found in this study. Despite this, deep whole-genome sequencing of CBE-V5 and ABE-V5 edited plants, with tissue-cultured wild-type plants as controls, will be helpful to definitively determine their genome-wide off-target effects and further elucidate their TTN PAM-related off-target patterns in the future (Tang et al., 2018).

Chromosomal DNA-binding proteins/domains have the ability to facilitate remodeling and unfolding of condensed chromatin, increasing the affinity for ssDNA or dsDNA substrates when fused to other proteins, and potentially modulate both the genome editing capability and outcomes upon coupling to the Cas reagents or the Cas–deaminase complex, and thus increase the efficiencies of genome editing either for gene knockout or base editing (Li et al., 2023; Xue et al., 2024; Zheng et al., 2024). In this study, we found that fusing Rad51, or HMG-D, or VP64 individually to CBE-V0 and ABE-V0 could increase the maximal editing efficiencies by up to 1.65- and 1.50-fold, 1.87- and 1.88-fold, 1.58- and 1.36-fold, in rice protoplasts, respectively (Figures 2A, 2B, S1). Our results are in agreement with previous studies reporting that incorporation of DNA-binding domains indeed enhances genome editing performance (Li et al., 2023; Xue et al., 2024; Zheng et al., 2024). Impressively, synergistic couplings of both Rad51 and VP64, or both HMG-D and VP64, to CBE-V0 and ABE-V0, significantly improved the base editing efficiencies by 2.25- to 2.79-fold and 2.36- to 2.64-fold in rice protoplasts, respectively. We further consolidated these observations in rice stable lines. We demonstrated that synergistic combinations of both VP64 and HMG-D with either Cas12i3 (5M)-based CBE or ABE yielded superior performance and significantly boosted the efficiencies of Cas12i3 (5M)-based CBE and ABE by up to 4.78- and 3.35-fold, with maximum C-to-T and A-to-G efficiencies reaching 32.35% and 38.24% in rice stable lines, respectively. The superior performance upon fusion of HMG-D with Cas12i3 (5M)-based CBE or ABE either alone or coupling with VP64 is primarily due to the function of HMG-D as a dsDNA-binding domain, which can directly bind to dsDNA non-specifically in the target region, inducing larger DNA loops and physically stabilizing the binding of the complex of the CRISPR reagent to the dsDNA at the initial stage, and thereby exerts a more profound enhancing effect and expands the editing window (Xue et al., 2024). In contrast, Rad51 has an ssDNA-binding domain and primarily functions in binding to the end of ssDNA, thus stabilizing transient ssDNA intermediates formed after deamination or protecting the crRNA (Mimitou and Symington, 2011; Tan et al., 2022; Zheng et al., 2024). Except for enlarging the dsDNA loops upon fusion to Cas12i3 (5M)-based CBEs and ABEs due to their enlarged sizes and configurations, the role of Rad51 in this study is somehow constrained due to the absence of induced DNA nicks, because the dead Cas12i3 (5M) does not induce nicks at all. Thus, although both Rad51 and HMG-D boosted the base editing efficacy, our results in this study further demonstrate that HMG-D is superior than and more compatible with Cas12i3 (5M)-based CBE and ABE editors, especially upon coupling with VP64 (Figures 2, 3B). Furthermore, the broadened editing windows of both CBE-V5 and ABE-V5 facilitate simultaneous editing of multiple adjacent sites within the target loci in the gene-encoding or -regulatory regions, which is conducive for screening a wider spectrum of allelic variants through directed evolution. Notably, a higher proportion of homozygous mutants was observed in the T_0 generation of the edited plants. Overall, our finding highlights the use of synergistic combinations of a transactivation module and DNA-binding proteins/domains as a powerful strategy to boost the efficiency of base editing, and may potentially be useful for engineering other genome editing toolkits to further improve their performance.

To date, the various base editing tools documented are mainly based on Cas9 and Cas12a nucleases. The development of Cas12i3 (5M)-mediated BEs would have significant value for expanding the applicability and scope of base editing technologies for crop improvement. Although both the developed CBE-V5 and ABE-V5 systems showed significantly enhanced base editing efficiencies compared to CBE-V0 and ABE-V0, respectively, when compared to Cas9- or Cas12a-mediated base editing system in plants (Ren et al., 2021; Wei et al., 2021; Tan et al., 2022; Cheng et al., 2023; Fan et al., 2024), there is room for further improvement. In the future, further improvements of Cas12i3 (5M)-mediated BEs could be achieved by rational optimization and engineering, such as internally embedding deaminases or DNA-binding proteins within the dCas12i3 (5M) protein

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(Hu et al., 2025), using a 35S-CmYLCV-U6 composite promoter to drive the crRNA expression (Jiang et al., 2020; Jiang et al., 2022; Wang et al., 2025a), changing the lengths of inter-protein linkers (Cheng et al., 2023), and optimizing the deaminase component (Contiliani et al., 2025). Furthermore, given the advantages of possessing distinct and less restricted PAM, use of Cas12i3 (5M) has the potential to develop a variety of alternative base editing tools such as Cas12i3 (5M)-based CGBE, AKBE, gGBE, etc. through fusions with distinct functional modules, such as uracil-DNA glycosylase (UNG) and human N-methylpurine DNA glycosylase (hMPG) or rice OsMPG (Kurt et al., 2021; Zhao et al., 2021; Tian et al., 2022; Tong et al., 2023; Wu et al., 2023; Li et al., 2023, 2024; Liu et al., 2024).

In summary, here, we successfully developed an efficient Cas12i3 (5M)-based CBE and established the first case of Cas12i3 (5M)-based ABE for base editing in plants through synergistic coupling of both a transactivation module VP64 and a double-stranded DNA-binding domain HMG-D to the dCas12i3 (5M)-deaminase fusion. Our finding establishes the use of synergistic combinations of a transactivation module and DNA-binding proteins/domains as a powerful strategy to boost the efficiency of base editing, which is also potentially useful for optimizing the performance of genome editing tools. Together, the engineered novel CBE-V5 and ABE-V5 systems presented here significantly broaden the editing scope and enrich the base editing toolkits for rice, thereby enabling broader applications in functional genomics and trait improvement in rice as well as other agriculturally important crop species.

MATERIALS AND METHODS

Construction of the base editing vectors

The pHUE411–dCas12i3 (5M) used in this study was constructed by replacing Cas9 in pHUE411-Cas9 with dCas12i3 (5M) (E844A). Then, we fused the rice codon-optimized VP64, HMG-D, Rad51, mhA3A, TadA8e, and UGI from different resources (Table S4). pHUE411-CBE–dCas12i3 (5M) was constructed by fusing the cytidine deaminase mhA3A (hA3A with the Y130F mutation) to the N-terminal of dCas12i3 (5M) and two copies of UGI to the C-terminal of dCas12i3 (5M) in vector pHUE411–dCas12i3 (5M). pHUE411-VP64-CBE–dCas12i3 (5M), pHUE411-CBE-Rad51–dCas12i3 (5M), pHUE411-CBE-HMG-D –dCas12i3 (5M), pHUE411-VP64-CBE-Rad51–dCas12i3 (5M), and pHUE411-VP64-CBE-HMG-D–dCas12i3 (5M) were constructed by fusing VP64, Rad51, and HMG-D or in combinations, respectively, to the N-terminal of dCas12i3 (5M) in vector pHUE411-CBE–dCas12i3 (5M). pHUE411-ABE–dCas12i3 (5M) was constructed by fusing the adenosine deaminase (TadA8e with the V106W mutation) to the N-terminal of dCas12i3 (5M) in vector pHUE411–dCas12i3 (5M). pHUE411-VP64-ABE–dCas12i3 (5M), pHUE411-ABE-Rad51–dCas12i3 (5M), pHUE411-ABE-HMG-D–dCas12i3 (5M), pHUE411-VP64-ABE-Rad51–dCas12i3 (5M), and pHUE411-VP64-ABE-HMG-D–dCas12i3 (5M) were constructed by fusing VP64, Rad51, and HMG-D or in combinations, respectively, to the N-terminal of dCas12i3 (5M) in vector pHUE411-ABE–dCas12i3 (5M). PCR was performed using high-fidelity DNA polymerase Phusion (New England BioLabs, NEB, Ipswich, Massachusetts, United States). These PCR products were assembled into the pHUE411–dCas12i3 (5M) vector by One Step Cloning (ClonExpress II One Step Cloning Kit, Vazyme). The double strands of protospacers targeting *OsACC, OsSBEIIb, OsALS, OsARF4, OsDEP1*, and *OsBADH2* loci were synthesized, annealed, and inserted into *Pme*I (NEB) digested pHUE411-BEs–dCas12i3 (5M), pHUE411-VP64-BEs–dCas12i3 (5M), pHUE411-BEs-Rad51–dCas12i3 (5M), pHUE411-BEs-HMG-D-dCas12i3 (5M), pHUE411-VP64-BEs -Rad51–dCas12i3 (5M), and pHUE411-VP64-BEs-HMG-D -dCas12i3 (5M) individually. All vectors were confirmed by Sanger sequencing (TSINGKE, Beijing, China). The primer sets used for the construction of these vectors are listed in Table S5.

Protoplast transfection

We used the *Japonica* rice cultivar (*Japonica cv* Zhonghua11) to prepare protoplasts. Protoplast isolation and transformation were performed as previously described (Ren et al., 2019). Rice protoplasts were isolated from 14 d-old seedlings grown on 1/2 MS medium. They were transfected by dCas12i3 (5M)-BEs–crRNA (*ARF4*), dCas12i3 (5M)-BEs–crRNA (*DEP1*), dCas12i3 (5M)-BEs–crRNA (*SBEIIb*), dCas12i3 (5M)-BEs–crRNA (*BADH2*), dCas12i3 (5M)-BEs–crRNA (*HRC*), dCas12i3 (5M)-BEs–crRNA (*EPSPS*), dCas12i3 (5M)-BEs–crRNA (*ALS*), dCas12i3 (5M)-BEs–crRNA (*ACC*), dCas12i3 (5M)-BEs–crRNA (*ROC5*), dCas12i3 (5M)-BEs–crRNA (*PDS*), dCas12i3 (5M)-BEs–crRNA (*miR528*), and dCas12i3 (5M)-BEs without crRNA as a control (Table S1). In each transfection, 20 μg of dCas12i3 (5M)-BEs–crRNA plasmid DNA was introduced into approximately 10^6 protoplasts by PEG-mediated transfection. The transfected protoplasts were incubated at 25°C, and at 36–48 h post-transfection, they were collected and genomic DNA was amplified by PCR, and then for Hi-Tom high-throughput sequencing (Liu et al., 2019).

Agrobacterium-mediated rice transformation

For rice transformation of the aforementioned vectors, the *Japonica* rice cultivar Zhonghua11 was used as the donor material in this study. The constructed vectors were introduced into *Agrobacterium tumefaciens* strain EHA105 using the freeze–thaw method. A colony of the *A. tumefaciens* strain was then grown in liquid LB medium (containing 100 mg/L kanamycin and 25 mg/L rifampicin) at 28°C with shaking. The calli of rice Zhonghua 11 were carefully placed in a sterile tube and incubated with the *Agrobacterium* strains for 20 min. After incubation, the embryos were transferred onto co-culture medium and co-cultivated for 3 d in the dark at 28°C. After the co-cultivation process, the embryos were placed in selection medium containing 30 mg/L

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and 50 mg/L hpt for two weeks in the dark at 28°C. Then, well-grown calli were transferred to regeneration media to generate green plants in light at 28°C–30°C (16 L: 8D).

Molecular characterization of the regenerated plants

The genomic DNA of the regenerated plant was extracted from approximately 0.2 g of leaf tissue using a DNA Quick Plant System (Tiangen Biotech, Beijing, China). PCR amplification and detailed genotyping were performed using rTaq polymerase (Vazyme, Beijing, China) with 200 ng of genomic DNA as a template. All plants were further genotyped individually by PCR and Sanger sequencing, and the Sanger sequencing chromatograms at target sites were analyzed using SnapGene software. Some PCR products were also cloned into the TA cloning vector P-easy (TransGen Biotech, Beijing, China), and 20 positive colonies for each sample were sequenced.

Off-target analysis

To investigate off-target effects, we selected several potential off-target sites for each target, based on the prediction of the CRISPR-GE (http://skl.scau.edu.cn/offtarget/) (Xie et al., 2017; Table S3). Site-specific genomic PCR and Sanger sequencing were used to determine the off-target effects. The primer sets are listed in Table S5.

Segregation and statistical analysis

Genomic DNA was isolated from leaf tissues of T_1 seedlings using a DNA Quick Plant System (Tiangen, China). PCR amplification was performed using EASY Taq polymerase (TransGen Biotech, China) and 200 ng of genomic DNA as a template. PCR products were directly sequenced to perform segregation analysis of mutation events in the T_1 generation. Furthermore, PCR amplification was used to detect the presence of *dCas12i3 (5M)*, the *crRNA* expression cassette, and the *hptII* gene in T_1 seedlings. A χ^2-test was performed to evaluate whether the segregation patterns of the mutation events were consistent with Mendelian inheritance or indicative of somatic editing.

Herbicide-tolerant test in rice plants

Rice seeds were germinated on 1/2 MS (Murashige and Skoog) medium in a growth chamber (28°C, 16 h light/8 h dark). After 14 d, plantlets with *OsALS-R190H* and *OsACC-W2097R* alleles, as well as wild-type plants were transferred to soil and grown in a greenhouse. Rice plants at the three-leaf stage were then sprayed with bispyribac-sodium (220 μM) or haloxyfop (230 μM) at a 1×, 2×, 4× field-recommended dose using a cabinet spray chamber. The phenotypes were then investigated after 14 d of bispyribac-sodium or haloxyfop applications, with wild-type plants included as the control.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest. A patent application has been filed on the Cas12i-mediated plant base editing system in plants.

AUTHOR CONTRIBUTIONS

C.Z. performed most of the research and drafted the manuscript. J.Y. and L.Y. performed construction of vectors. C.Z., Y.L., and C.Y. carried out molecular characterization of the edited plants. S.L. and Y.H. performed the off-target analyses. L.X. designed the experiments, supervised the study, and revised the manuscript. All the authors read and approved the final version of this manuscript.

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SUPPORTING INFORMATION

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Figure S1. Base editing efficiencies of different Cas12i3 (5M)-mediated base editing systems in rice protoplasts
Figure S2. Genotypes of the edited plants in the T_0 generation
Table S1. Protospacer sequences targeting different rice genes selected for testing the editing activities of Cas12i3 (5M)-mediated base editing systems in protoplasts in this study
Table S2. Transmission and segregation of target mutations and transgenes from the T_0 to T_1 generation
Table S3. Analysis of potential off-target effects
Table S4. Rice codon-optimized sequences of VP64, Rad51, HMG-D, mhA3A TadA8e, and UGI
Table S5. Primers used in this study

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