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• LETTER TO THE EDITOR •

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A CRISPR/Cas9 toolkit for efficient targeted base editing to induce genetic variations in rice

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Dear Editor,

Gene-targeting technologies using sequence specific nucleases have been widely adopted to induce genetic modifications in both plant molecular biology research and crop improvement, of which generating targeted point mutation for gain-of-function phenotype is of great value. Although tremendous efforts have been made in developing geneinsertion or replacement approaches by harnessing DNA repair pathways through either homologous recombination (HR) or non-homologous end joining (NHEJ) mechanisms (Ma et al., 2016), introducing specific DNA sequence changes into genome at the desired site remains challenging. Recently, a simple base editing method for specific base changes has been developed in mammalian cells (Komor et al., 2016). The base editor includes a Cas9n nickase, cytosine deaminase (human AID, rat APOBEC1, or lamprey CDA1) and Uracil DNA glycosylase inhibitor (UGI). The sgRNA directs the Cas9n fusion protein to the target site, cytosine deaminase converts any cytosine within reach into uracil, which is then repaired by error-prone mechanisms and leads to various point mutations, notably thymines when UGI exists (Komor et al., 2016). Here we present a tool kit comprising rBE3 and rBE4 (rice Base Editor), a highly efficient tool for generating customized gain-of-function alleles at defined lociincultivated rice.

To explore the potential of the Cas9n-fused cytosine deaminase in inducing nucleotide changes in plant genome, we first codon-optimized rat *APOBEC1* gene and *UGI* gene of *Bacillus subtilis* bacteriophage PBS1, and attached them to Cas9n gene at both ends with XTEN linker sequence and nuclear localization signal (NLS) sequence, respectively (Figure 1A and B and Figure S1A and B in Supporting Information). The *APOBEC1-XTEN-Cas9n-UGI-NLS* chimeric gene, named *rBE3*, was expressed under the control of the CaMV35S promoter in rice leaf sheath protoplasts together with *OsCERK1*-targeting sgRNA transcribed from a rice U6 promoter as reported previously (Zhou et al., 2014). Genomic DNA was extracted from pooled protoplasts 2 days post transfection and treated with *Bam*HI, which recognizes and cleavesthe target site to enrich mutated alleles with successful nucleotide changes (Figure 1C). We then cloned and Sanger-sequenced PCR amplicons of the target region with the *Bam*HI-resistant genomic DNA as templates (Figure S2 in Supporting Information). Two different alleles of

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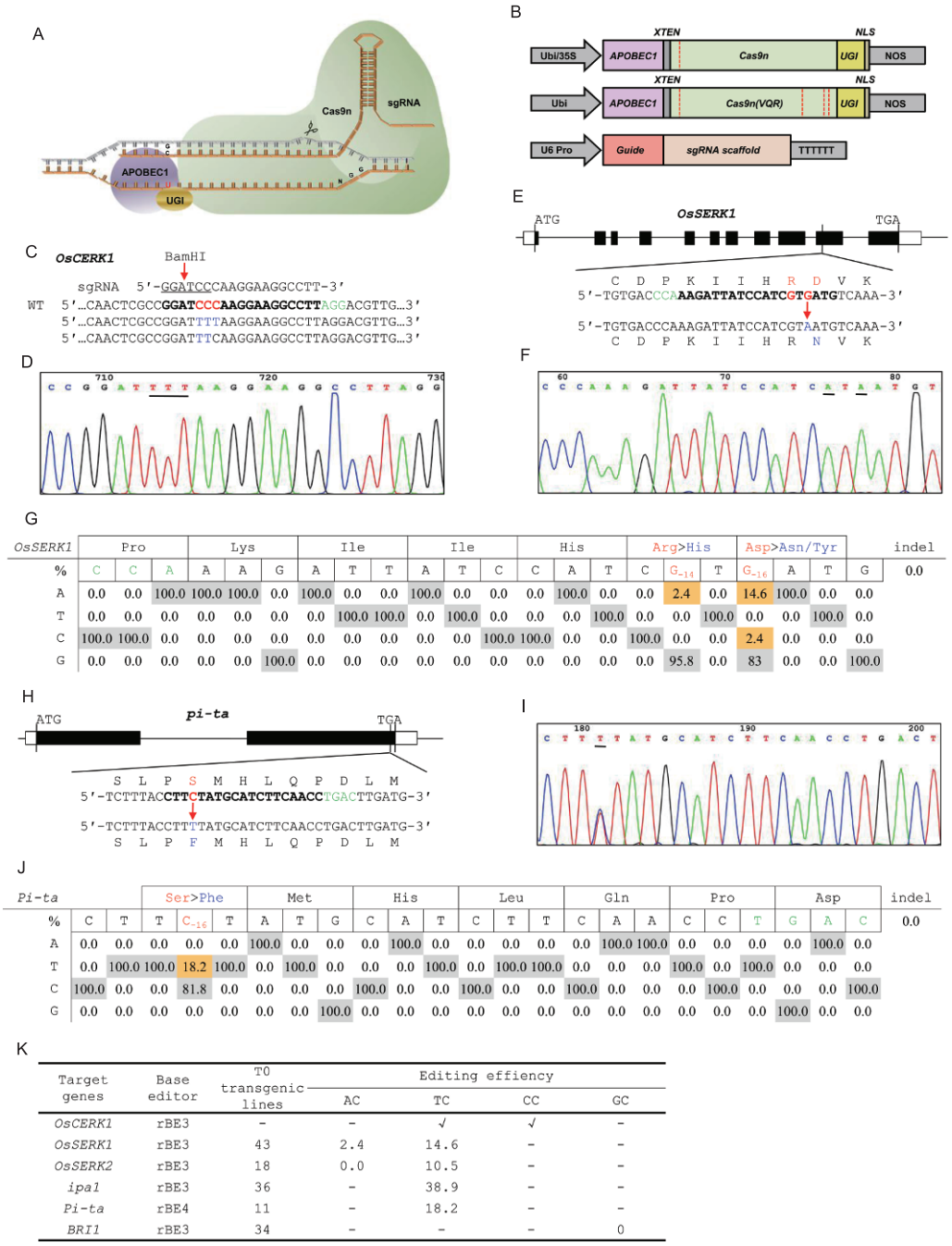

Target genes	Base editor	T0 transgenic lines	Editing effiency			
			AC	TC	CC	GC
OsCERK1	rBE3	-	-	√	√	-
OsSERK1	rBE3	43	2.4	14.6	-	-
OsSERK2	rBE3	18	0.0	10.5	-	-
ipa1	rBE3	36	-	38.9	-	-
Pi-ta	rBE4	11	-	18.2	-	-
BRI1	rBE3	34	-	-	-	0

Figure 1 Targeted base editing in rice using the rBE3 and rBE4 system. A, Diagram of the rBE3 system mediating specific, guide RNA-programmed C to U conversion. B, Gene constructs of the rBE3 and rBE4 system used to test targeted base editing activity in rice. C, rBE3 mutated cytosines to thymines at sgRNA-targeted locus of endogenous *OsCERK1* gene in rice protoplasts. D, Representative Sanger sequencing chromatogram of cloned *OsCERK1* allele with three Cytosines changed. E, rBE3 converted endogenous *OsSERK1* genes into kinase-dead versionin rice plants through *Agrobacterium*-mediated transformation. F, Representative Sanger sequencing chromatogram of cloned *OsSERK1* allele with two guanines changed. G, 17% of transgenic rice lines showed target base editing events in *OsSERK1* without any indels. H, rBE4 induces genetic variation of rice susceptible gene *pi-ta* at the key position of Serine 918 through *Agrobacterium*-mediated transformation. I, Representative Sanger sequencing chromatogram of rBE4-edited *pi-ta* gene in T0 rice line. J, 18.2% of transgenic rice lines showed target base editing events in *pi-ta* without any indels. K, Summary of base-editing results of rBE3 and rBE4 in T0 trangenic rice lines. −, not available; √, worked in protoplasts. For (C, E and H), the PAM sequences are in green, putative target bases in the activity window in red, and detected nucleotide mutations in blue. For (D, F and I), nucleotide mutations are underlined in sequencing chromatograms.

mutated *OsCERK1* were identified among 31 positive clones, of which 28 contained CCC>TTT conversions (Figure 1C and D), suggesting rBE3 might function in a 7-bp activity window spanning from −19 to −13 bp upstream of the PAM as

reported in human cells (Komor et al., 2016). To be noted, no any other mutations were detected beyond the target site in the amplicons (Data not shown). Therefore, the engineered Cas9n-based cytosine deaminase is fully functional inconverting cytosines strictly into thymines in the presence of UGI in rice cells.

We next investigated whether rBE3 can induce C to T (or G to A) conversion in rice plant by accommodating the rBE3 system to *Agrobacterium*-mediated plant transformation. The Gateway-based binary vector system reported previously (Zhou et al., 2014) was utilized by replacing the *Cas9* gene with the *rBE3* gene, in which *rBE3* was driven by the maize ubiquitin promoter and a "Gateway att" site was carried to enable directional cloning of 1–4*sgRNA* expression cassettes by Gateway reaction. *OsSERK1,* encoding a receptor-like kinase, is homologue of *Arabidopsis BAK1* in rice. Substitution of Asp by Asn at the conserved amino acid position 428 resulted in loss of kinase activity. To generate the rice mutant of *OsSERK1(D428N)* gene, we designed a sgRNA to target the corresponding regionof *OsSERK1* (Figure 1E). 41 independent transgenic lines of Kitaake were obtained and genotyped directly by Sanger sequencing. We identified sevenmutant plants at a frequency of 17% and all were heterozygous (Figure 1F and G). Among those, fivedesired mutants were obtained which carried single nucleotide change of G>A (D428N) at position −16 (Figure 1G and Figure S3A in Supporting Information), one mutant carried a G>C conversion, resulting in OsSERK1(D428Y) (Figure S3B in Supporting Information), and one mutant had two nucleotides at position −14 and −16 mutated (Figure 1F). Base editing efficiency was dependent on sequence context, following the order TC>CC≥AC>GC (the second nucleotide C is the target nucleotide) (Komor et al., 2016). Given that, the alteration of AC_{-14} is likely too little to be detected due to the low efficiency. Remarkably, we did not find potential indels introduced by Cas9n nickase (data not shown).

To further test the efficiency of rBE3 in targeted base editing in rice, we selected two additional genes for experiment. As *OsSERK2* is another homologue of *Arabidopsis BAK1*, we tried to generate the kinase-dead mutant *OsSERK2(D433N)* as well (Figure S4A in Supporting Information). *ipa1* encodes OsSPL14 and is regulated by OsmiR156. A point mutation at the OsmiR156 cleavage site damped the regulation, resulting in ideal plant architecture and enhanced grain yield (Jiao et al., 2010). Therefore, destroying the OsmiR156 cleavage site in *ipa1* without causing amino acid changes of OsSPL14 by using rBE3 is a perfect strategy for producing genetic resources in molecular crop breeding (Figure S5A in Supporting Information). As shown in Figure S4B and C in Supporting Information, out of 19 independent transgenic lines genotyped by Sanger sequencing, 2 lines were heterozygous for *OsSERK2*; all carried the expected $G_{-15}A$ to $A_{-15}A$ conversion, resulting in *OsSERK2(D433N)*. $G_{-18}T$ and $G_{-13}T$that located in the activity window remained intact (Figure S4C in Supporting Information). For *ipa1*, 14 mutants (38.9% efficiency) were isolated from a screen of 36 independent lines. Among which, 8 ideal plants carried a C>T conversion at position −15, which destroy the miRNA recognition site without changing amino acid Leucine (Figure S5C and D in Supporting Information). Surprisingly, substitution of C by G was found in another 6 mutants (Figure S5B and D in Supporting Information). It is not understood how the unintended nucleotide conversion happened with high frequency in the presence of *UGI*, it is likely that chromatin marks or the action of miRNA play roles in this case.more sgRNA should be tested in the future to answer this question. For TC_{-18}, no nucleotide change was defined. In addition, no possible point mutations caused by Cas9n nickase were detected in the samples for both *OsSERK2(D433N)* and *ipa1* targeting regions (Data not shown). Combined together, these data suggest rBE3 protein is effective in targeted base editing in rice plants and much more competitive compared with Cas9 used for gene replacement in terms of editing efficiency and cost of transformation methods.

In general, recruiting Cas9n-based rBE3 to target locus requires both PAM recognition and sgRNA binding. A number of modified Cas9 with alternative PAM sequences has been reported to date. To expand the application of base editing in rice, we mutated Cas9n to Cas9n(VQR), shifting the corresponding PAM sequence from NGG, NAG to NGA, AGTG, AGCG *etc.* in rice in case that no PAM sequence is available for Cas9n recognition sometime. The *APOBEC1-XTEN-Cas9n(VQR)-UGI-NLS* chimeric gene, named *rBE4*, was constructed and introduced into the Gateway system for rice transformation.

The blast susceptible protein pi-ta in rice shares a single amino acid difference with the resistance protein Pi-ta: Serine instead of Alanine at position 918 (Bryan et al., 2000). To test the efficiency of rBE4, we designed a sgRNA targeting *pi-ta* in Kitaake to generate an allele *pi-ta(S918F)* since no PAM sequenceis available for rBE3 recognition (Figure 1H). Out of 11 independent transgenic lines screened, two mutants (18.2% efficiency) were identified with the expected TC_{-16} to TT_{-16} conversion (Figure 1I and J). Similar to the findings mentioned above, no other trace of nucleotide changes except T_{-16} were detected (Data not shown). Therefore, rBE4with altered PAM specificity is functional as well, whichbroadens the application of targeted base editing in rice.

Taking all the data together, rBE3 and rBE4 convert cytidines to all the other nucleotides, mostly thymineinthe presence of UGI, within a seven-base acitvity window between −19 and −13 bp upstream the PAM sequence. Efficiency of cytidine conversion is locus-dependent, and sensitive to the 5′-preceding nucleotide of the target base. Of which, TC is mostly favored by APOBEC1, AC and CC are preferred as well, but GC is obstinate (Figure 1K).

In summary, we have developed a CRISPR/Cas9 toolkit, extensively enabling efficient base editing at desired sites in the rice genome. Multiple sgRNAs can be easily cloned into entry vectors with digestion of restriction enzymes and integrated into the binary vector through Gateway reaction. The base editor is much more efficient and economical in gene correction in plants than gene replacement through HR or gene insertion via NHEJ, and plant resources with genetic variation of interests would be easily isolated through *Agrobacterium*-mediated plant transformation. In a word, the application of the rBE3/rBE4 toolkit would be promising in generating gain-of-function mutants for fundamental researches and materials with genetic variation for rice breeding (Tables S1 and S2 in Supporting Information).

Compliance and ethics *The author(s) declare that they have no conflict of interest.*

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SUPPORTING INFORMATION

Figure S1 Complete DNA sequences of the rice codon-optimized *APOBEC1* and *UGI* genes.

Figure S2 PCR/RE assay of base editing of *OsCERK1* using rBE3 in rice protoplasts.

Figure S3 Representative Sanger sequencing chromatograms of rBE3-edited *OsSERK1* gene in T0 rice lines.

Figure S4 Targeted base editing of *OsSERK2* in rice using the rBE3 system.

Figure S5 Targeted base editing of *ipa1* in rice using the rBE3 system.

Table S1 Rice genes selected for editing with the rBE3 and rBE4 systems

Table S2 List of oligonucleotides used in this study

The supporting information is available online at life.scichina.com and www.springerlink.com. The supporting materials are published as submitted, without typesetting or editing. The responsibility for scientific accuracy and content remains entirely with the authors.