Optimizing base editors for improved efficiency and expanded editing scope in rice

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Dear Editor,

Base editors, presently including cytidine base editors (CBEs) and adenine base editors (ABEs), enable precise base alterations in the genome without inducing DNA double-stranded breaks (DSBs). Base editors are valuable tools for precision plant molecular breeding since many agronomic traits are controlled by variations in one or few DNA bases. The early developed CBE and ABE systems, consisting of the rat cytidine deaminase APOBEC1 (rAPOBEC1) or activation-induced cytidine deaminase (AID) PmCDA1, and the evolved tRNA adenine deaminase TadA, respectively, have been applied to many plant species. To improve the base editing efficiency, more effective cytidine deaminases such as the human APOBEC3A have been tested (Zong et al., 2018). On the other hand, for expanding the base editing scope in plants, several SpCas9 and SaCas9 variants such as VQR-Cas9, VRER-Cas9 and SaKKH-Cas9 that recognize PAMs other than the canonical NGG motif were introduced into the CBE and ABE toolbox (Hua et al., 2018b; Qin et al., 2018). However, relative to the widely used CRISPR/Cas gene editing technologies for inducing DSBs and subsequent repair-caused mutations, the efficiency of base editing is still low. In addition, base editors reported thus far are constrained by recognition of only a few kinds of PAM sequences.

We have previously reported the initial adoption of CBEs and ABEs in rice (Hua et al., 2018b; Lu and Zhu, 2017). In our CBE system, we fused rAPOBEC1 to the N-terminus of SpCas9 nickase (Cas9n, D10A) using the unstructured 16-residue peptide XTEN as linker. A traditional nuclear localization signal, SV40 NLS peptide, was added to the C-terminus of the Cas9n. Two agronomically important genes of rice, NRT1.1B and SLR1, were selected for editing by this CBE system. However, the base substitution efficiencies were low, with only 2.7% for NRT1.1B and 13.3% for SLR1, respectively (Lu and Zhu, 2017). In our ABE system, we synthesized wild-type ecTadA and its mutant form ecTadA*7.10 and linked them together using a 32-amino acid (aa) linker; the resulting recombinant protein was fused to the N-terminus of the SpCas9 or SaCas9 nickase with the same linker. Testing at different targets showed that the base substitution efficiencies ranged from 5% to 60%, with most of the target sites having efficiencies lower than 30% as reported by other groups (Hua et al., 2018b).

Recently, Koblan et al. (2018) found that the expression levels of base editors are major bottlenecks for base editing efficiency. They improved BE4 and ABEmax base editors by adopting bipartite nuclear localization signals (bpNLS), optimizing codon usage and ancestral reconstruction of the deaminase component. The resulting BE4max, AncBE4max and ABEmax editors showed increased editing efficiencies in a variety of settings, especially under suboptimal conditions or at sites previously edited with low efficiencies (Koblan et al., 2018). To improve the base editing efficiency in plants, we directly adopted the above GenScript codon-optimized nucleotide sequences of bpNLS-Anc689 APOBEC-32 aa Linker and bpNLS-adenine deaminase of ABEmax 7.10-32 aa Linker into our previous CBE and ABE editors, resulting in Anc689BE4max and ABEmax, respectively (Figure 1a, b).

To directly compare the performance of Anc689BE4max with our previous CBE, the NRT1.1B and SLR1 were selected for editing using the previously tested sgRNA. As shown in Figure 1c, 72.4% of the transgenic rice lines harboured the target C to T replacement at NRT1.1B target site, and 76.2% of these lines (55.2% of total transgenic lines) are homozygous (Figure 1d). Most of the regenerated plantlets transformed with Anc689BE4max-sgRNA$$\text{SLR1}$$ displayed an obvious dwarf phenotype...
Figure 1 Optimizing base editors for improved efficiency and expanded editing scope in rice. (a and b) Constructs of the Anc689BE4max (a) and ABEmax (b) base editors. Optimized ABE7.10 refers to the GenScript codon-optimized sequence of adenine deaminase of ABE7.10; this sequence and Anc689APOBEC were directly derived from Koblan et al. (2018). (c,g) Frequencies of base substitutions at the target sites of NRT1.1B (c) and SLR1 (g); the PAM motif is marked in box. (d,h,l) Distribution of the genotypes from transgenic rice plantlets edited at the NRT1.1B (d), SLR1 (h) and ALS (l) target sites. Ho: homozygous, Bi: biallelic, He: heterozygous, WT: wild type, Other: other base substitutions but not C>T. N: the total number of identified plantlets. (e,f) Phenotype of the regenerated rice plantlets from base editing at SLR1. Scale bar equal to 1 cm. (i,o,q) Representative Sanger sequencing chromatograms at the SLR1 (i), ALS (o) and EPSPS (q) target sites. The plant ID (#), genotype and its base substitution status are shown above each chromatogram. The superscript indicates the base position within protospacer. The substituted bases are also marked by red arrows, and their positions in the gene are indicated in number. The PAM motif is marked in box. Ho: homozygous, Bi: biallelic, He: heterozygous, WT: wild type. (j) The target sites designed for base editing at the ALS5627TN of rice. The sgRNA-PAM sequences designed for Anc689BE4max-nCas9 and Anc689BE4max-nCas9NG are underlined in blue and red, respectively, and the PAM motif is marked in bold. The intended base and amino acid for substitution are marked in pink. (k,m) Frequencies of base substitutions at the target sites of ALS-sg1 (k) and ALS-sg2 (m). The PAM motif is marked in box, and the red triangles indicate the intended base for conversion. (n) Phenotype of the transgenic rice plantlets treated by herbicide. 0.03% Imazethapyr (Shandong CYNDA) was sprayed on the plantlets, and the photograph was taken 25 days after treatment. Scale bar equal to 1 cm. (p) Wild type and the mutated sequences of EPSPS. The designed sgRNA-PAM sequences are underlined, and the PAM motif is further marked in bold. The intended base and amino acid before and after editing are marked in blue and pink, respectively. The quantity of each genotype from transgenic plantlets is indicated by 9. (s) Summary of editing efficiencies for different base editors. Base editing efficiency was calculated by scoring the number of plantlets with anticipated base substitution within the target site relative to the total number of identified transgenic plantlets. The designed sgRNA-PAM sequences for EPSPS-sg2 and ALS-sg4 are 5'-GAGAGAGATGCCAAGAGGAGGAT and 5'-TACAAAAGAGAGGAGGAGTGAGTCCGT, respectively.
To further evaluate the efficiency of Anc689BE4max, we designed an sgRNA (ALS-sg1) for modifying the acetolactate synthase gene (ALS) in rice. It is known that a mutated form of ALS, ALS5627N (G1880 to A in Nipponbare DNA sequence), confers tolerance to imidazolinone herbicides (Piao et al., 2018) (Figure 1). Similar to the results from the base editing of NR7T1.1B and SLR1, 71.4% of the transgenic lines contained C to T substitution at their target site in ALS, and most of them were homozygous or biallelic C to T replacement from 29 transgenic lines (Figure 1p,q,s). The glyphosate tolerance will be tested in the T1 generation since there was no homozygous mutant in the T0 plants.

To evaluate the activity of our ABEmax editor, we designed a third sgRNA (ALS-sg3) to edit the ALS gene by ABEmax and our previous ABE side by side. The results showed that ABEmax doubled the editing efficiency of ABE (Figure 1r,t). We further tested ABEmax at the ALS-sg1 target site, and the results showed that 48.3% of the transgenic lines harboured A to G substitution (Figure 1t). The general editing efficiencies of ABEmax seem lower than those of Anc689BE4max (Figure 1s,t). We further evaluated the ABEmax-nCas9NG system with non-canonical NGG PAMs. Testing at the EPSPS-sg2 target site harbouring the AGT PAM showed an editing efficiency of 41.2%. However, testing at another target site (ALS-sg4) bearing the CGT PAM showed the editing efficiency lower than 10% (Figure 1t).

In summary, our upgraded base editors not only show substantially increased editing efficiencies, but also have expanded editing scopes compared to previously reported CBes and ABes. These improved base editors are more powerful tools for molecular breeding of crops, although more plant species and more target sites need to be tested in the future.

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Conflict of interest
The authors declare no conflicts of interest with respect to this work.

References
Endo, M., Mikami, M., Endo, A., Kaya, H., Itoh, T., Nishimasu, H., Nureki, O. et al. (2018) Genomic editing in plants by engineered CRISPR-Cas9 recognizing NG PAM. Nature Plants, 4, 14–17.
Hua, K., Tao, X. and Zhu, J. (2018a) Expanding the base editing scope in rice by using Cas9 variants. Plant Biotechnol. J. 17, 499–504.
Hua, K., Tao, X., Yuan, F., Wang, D. and Zhu, J. (2018b) Precise A.T to G.C base editing in the rice genome. Mol. Plant, 11, 627–630.
Koblan, L.W., Doman, J.L., Wilson, C., Levy, J.M., Tay, T., Newby, G.A., Maianti, J.P. et al. (2018) Improving cytidine and adenine base editors by expression optimization and ancestral reconstruction. Nat. Biotechnol. 36, 843–846.
Lu, Y. and Zhu, J.K. (2017) Precise editing of a target base in the rice genome using a modified CRISPR/Cas9 system. Mol. Plant, 10, 523–525.
Nishimasu, H., Shu, X., Ishiguro, S., Gao, L., Hirano, S., Okazaki, S., Noda, T. et al. (2018) Engineered CRISPR-Cas9 nuclease with expanded targeting space. Science, 361, 1259–1262.
Piao, Z., Wang, W., Wei, Y., Zonta, F., Wan, C., Bai, J., Wu, S. et al. (2018) Characterization of an acetohydroxy acid synthase mutant conferring tolerance to imidazolinone herbicides in rice (Oryza sativa). Planta, 249, 693–703.
Qin, R., Li, J., Li, H., Zhang, Y., Liu, X., Mao, Y., Zhang, X. et al. (2018) Developing a highly efficient and widely adaptive CRISPR-SaCas9 toolset for plant genome editing. Plant Biotechnol. J. 17, 706–708.
Zhou, M., Xu, H., He, X., Ye, Z., Wei, L., Gong, W., Wang, Y. et al. (2006) Identification of a glyphosate-resistant mutant of rice 5-enolpyruvylshikimate 3-phosphate synthase using a directed evolution strategy. Plant Physiol. 140, 184–195.
Zong, Y., Song, Q., Li, C., Jin, S., Zhang, D., Wang, Y., Qiu, J. et al. (2018) Efficient C-to-T base editing in plants using a fusion of nCas9 and human APOBEC3A. Nat. Biotechnol. 36, 950–953.