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Engineering a plant A-to-K base editor with improved performance by fusion with a transactivation module

Dear Editor,

Base editors (BEs) are promising tools that enable single-nucleotide substitutions in specific target genes, generating loss-of-function or gain-of-function mutations, which can greatly accelerate plant functional genomics and crop improvement (Ren et al., 2018; Zeng et al., 2020; Xu et al., 2021; Yan et al., 2021; Li et al., 2023). To date, three major classes of BEs have been engineered: cytosine BEs (CBEs) for C-to-T transitions (Komor et al., 2016), adenine BEs (ABEs) for A-to-G transitions (Gaudelli et al., 2017), and glycosylase BEs (CGBEs) for C-to-G transversions (Kurt et al., 2021; Zhao et al., 2021). ABE enabling A-to-K (K = G or T) editing remains to be exploited in both mammalian cells and plants. Recently, an adenine transversion BE (AYBE) was developed for A-to-Y (Y = C or T) transversion editing in human cells by fusing an ABE, ABE8e, with either the human hypoxanthine excision protein N-methylpurine DNA glycosylase (hMPG) or its mutant (mhMPG) (Tong et al., 2023) (Supplemental Figure 1). Development of a plant AYBE system for A-to-Y editing is highly desirable and will greatly expand the scope and potential for base editing in basic plant research and crop improvement. Nonetheless, such an BE has not been reported in plants to date. Here, we aimed to develop a series of plant AYBEs for A-to-Y base editing with improved performance. However, we only detected the occurrences of A-to-G, A-to-C, and A-to-T editing in rice protoplasts, and none of the stable lines showed A-to-C base transversions. Thus, we designated these BEs as plant A-to-K (K = G or T) base editors, or pAKBEs. Four pAKBEs were developed by fusion of pABE8e with an hMPG/mhMPG. Coupling with a transactivation module, VP64, improved the performance of pAKBEs in A-to-K base editing, and A-to-G and A-to-T editing efficiencies were as high as 17.24% and 82.22% in stable rice lines.

To establish a plant A-to-K base editing system, we first engineered two pAKBE versions, pAKBEv1–hMPG and pAKBEv2–mhMPG, by fusing a plant ABE, ABE8e, with either a rice codon-optimized hMPG or its mutant version mhMPG with 7 amino acid mutations (Tong et al., 2023) at the C terminus using a 13 aa linker (Figure 1A; Supplemental Figures 2 and 3; Supplemental Table 1). We then chose six endogenous rice targets (*OsDEP1*, *OsEPSPS*, *OsNRT1.1B*, *OsWaxy*-T1, *OsWaxy*-T2, and *OsWaxy*-T3) for evaluating the editing activities and editing windows of the two pAKBEs in rice protoplasts by deep amplicon sequencing (Supplemental Table 2). As expected, pABE8e induced A-to-G transition at average efficiencies of 1.31%–6.15%, with a typical ABE editing window of A4–A8, counting the PAM position as 21–23 (Figure 1B; Supplemental Figure 4). Of the six tested targets, the pAKBEv1–hMPG only induced A-to-C transversion editing at *OsNRT1.1B* at a very low average efficiency of 0.44% (Figure 1B; Supplemental Table 3). This A-to-C base transversion was located at position A6 in the protospacer region (Figure 1C). As expected, pAKBEv1–hMPG also induced A-to-G base transitions at positions A3–A8 in the protospacer region at average frequencies of 1.28%–6.35%, similar to those of pABE8e (Figure 1B and 1C; Supplemental Table 4). Similar to the AYBE with mhMPG in human cells (Tong et al., 2023), pAKBEv2-mhMPG significantly increased A-to-C base transversions by up to 3.2-fold (1.44%/0.44%) at *OsNRT1.1B*; enabled A-to-C and A-to-T base transversions at average frequencies of 0.63% and 0.63% at *OsDEP1*; and produced A-to-T base transversion frequencies of 0.48% at *OsWaxy*-T1, 0.20% at *OsWaxy*-T2, and 0.89% at *OsWaxy*-T3 (Figure 1B; Supplemental Table 3). However, unlike AYBE in human cells that typically produced A-to-Y base transversions at positions A7–A8 (Tong et al., 2023), pAKBEv2–mhMPG mainly produced A-to-C and A-to-T base transversions at positions A5–A6 (Figure 1C). This was probably due to the different lengths of linkers for fusion of hMPG or mhMPG to ABE8e. We also observed A-to-G transitions as the major editing outcomes, with an expanded window of A3–A9, accounting for 3.49%–19.39% of the total reads (Figure 1B and 1C; Supplemental Table 4). As editing byproducts, insertions or deletions (indels) occurred at *OsDEP1*, *OsNRT1.1B*, *OsEPSPS*, and *OsWaxy*-T2 with average frequencies from 0.38% to 2.24% (Figure 1B; Supplemental Figure 5; Supplemental Table 5).

Eukaryotic genomic DNA is wrapped around histones and further compacted to form higher-order chromatin structures (Campos and Reinberg, 2009), which may hinder binding of the CRISPR complex to its targets. VP64, a transactivation module composed of four copies of the VP16 transactivation domain, can facilitate remodeling and unfolding of condensed chromatin, increasing the accessibility of CGBE to target sites and improving the efficiency of C-to-G base editing (Tumbar et al., 1999; Dong et al., 2022). We hypothesized that fusion of VP64 to pAKBE could increase its access to target sites and thus improve A-to-K base-editing efficiency. To test this hypothesis, we fused VP64 to the N terminus of TadA8e with a 33-aa linker in pAKBEv1–hMPG and pAKBEv2–mhMPG, generating pAKBEv3–hMPG–VP64 and pAKBEv4–mhMPG–VP64, respectively (Figure 1A). We then evaluated the performance and editing outcomes of the two updated pAKBEs in rice protoplasts by deep amplicon sequencing. As shown in Figure 1B and Supplemental Table 3, pAKBEv3–hMPG–VP64 enabled A-to-C and A-to-T base transversions at average frequencies of 1.99% and 0.47% at *OsNRT1.1B*, 0.44% and 0.10%

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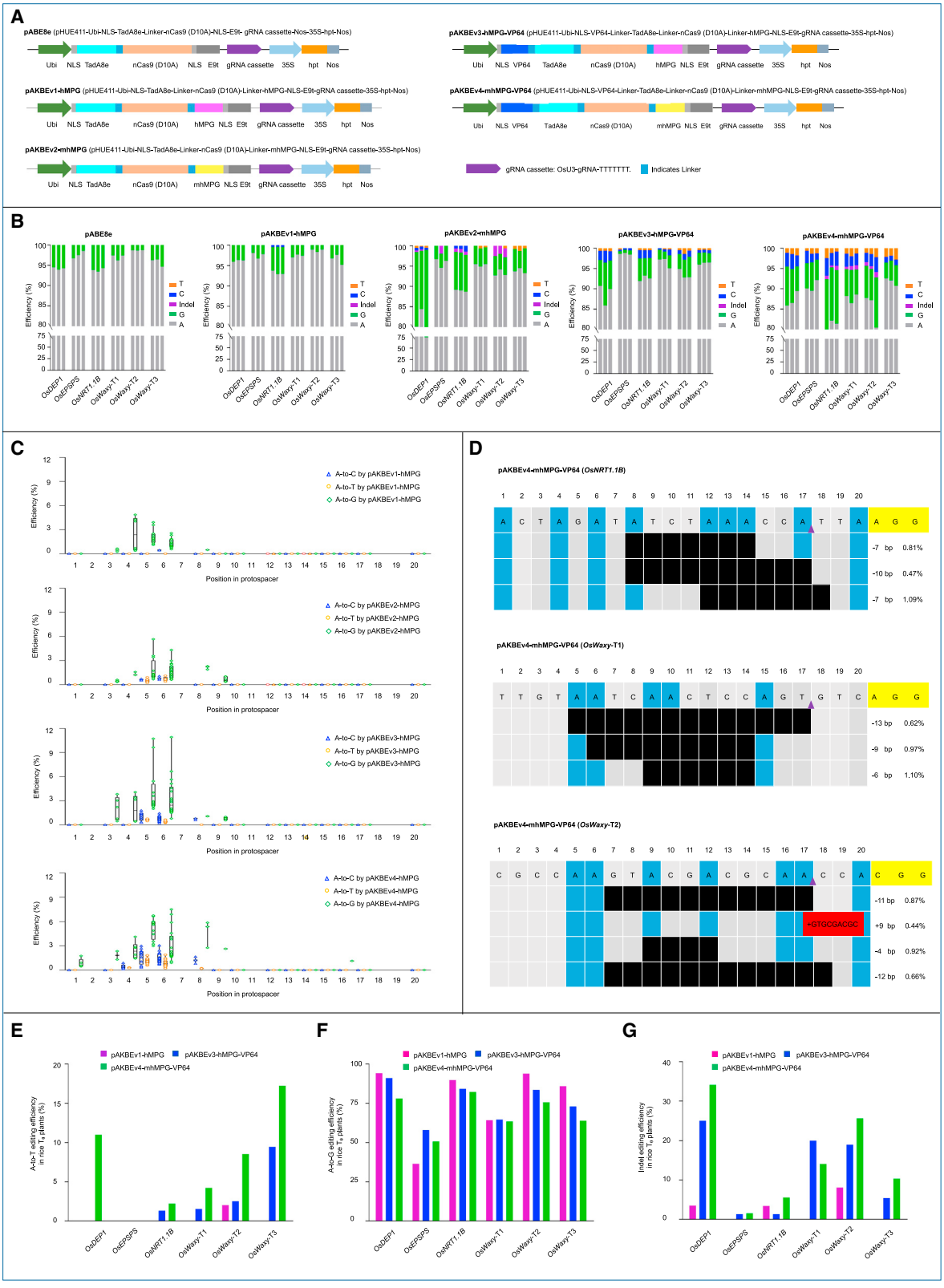

Figure 1. Development of pAKBE for A-to-K base editing in rice and its optimization by coupling with a transactivation module VP64.
(A) Diagrams of linearized pABE8e, original pAKBE (pAKBEv1–hMPG), and three optimized pAKBEs for A-to-K base editing. pAKBEv1–hMPG, version 1 of pAKBE with wild-type hMPG; pAKBEv2–mhMPG, pAKBE with mutated hMPG (mhMPG); pAKBEv3–hMPG–VP64, pAKBE with wild-type hMPG and VP64; pAKBEv4–mhMPG–VP64, pAKBE with mhMPG and VP64. In these pAKBEs, the AKBEs are driven by a maize *Ubiquitin* promotor, whereas the

(legend continued on next page)

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at *OsEPSPS*, 2.49% and 0.66% at *OsDEP1*, 0.78% and 0.27% at *OsWaxy*-T1, 1.30% and 0.82% at *OsWaxy*-T2, and 0.69% and 0.44% at *OsWaxy*-T3. The average A-to-Y transversion efficiencies were 3.15% at *OsDEP1*, 0.54% at *OsEPSPS*, 2.46% at *OsNRT1.1B*, 0.96% at *OsWaxy*-T1, 2.12% at *OsWaxy*-T2, and 1.13% at *OsWaxy*-T3. Compared with pAKBEv2–mhMPG, pAKBEv3–hMPG–VP64 not only increased A-to-Y transversion efficiencies by 1.26-fold (1.13%/0.89% at *OsWaxy*-T3) to 2.62-fold (2.46%/0.94% at *OsNRT1.1B*) but also enabled A-to-C transversion at non-editable sites such as *OsEPSPS*, *OsWaxy*-T1, *OsWaxy*-T2, and *OsWaxy*-T3 (Figure 1B; Supplemental Table 3). A-to-C and A-to-T transversions occurred mainly at positions A5–A6, with detectable A-to-C transversion also occurring at position A8 (Figure 1C). This result indicated that the presence of VP64 might facilitate chromosome unfolding and access of pAKBE to nearby "A" bases in the protospacer region. We also observed A-to-G transitions, mainly at positions A3–A9 in the protospacer region, at average frequencies of 0.82% to 8.00% (Figure 1B and 1C; Supplemental Table 4). Like pAKBEv1–hMPG, pAKBEv3–hMPG–VP64 did not induce any detectable indels (Figure 1B; Supplemental Table 5), indicating that although mhMPG enhances A-to-Y transversion editing activity, it may also increase hypoxanthine excision activity, leading to the production of indels (Supplemental Figure 1).

Compared with pAKBEv2–mhMPG and pAKBEv3–hMPG–VP64, pAKBEv4–mhMPG–VP64 produced further significant improvements in A-to-Y editing efficiency in rice protoplasts, indicating that mhMPG and VP64 may have a synergistic positive effect on A-to-Y editing activity. As indicated in Figure 1B and Supplemental Table 3, pAKBEv4–mhMPG–VP64 induced A-to-C and A-to-T transversions at average frequencies of 3.66% and 1.47% at *OsNRT1.1B*, 3.28% and 1.44% at *OsDEP1*, 2.26% and 1.09% at *OsEPSPS*, 2.84% and 1.53% at *OsWaxy*-T1, 3.00% and 1.58% at *OsWaxy*-T2, and 1.34% and 2.25% at *OsWaxy*-T3 (Figure 1B; Supplemental Table 3). Average A-to-Y transversion efficiencies were 5.13% at *OsNRT1.1B*, 4.72% at *OsDEP1*, 3.35% at *OsEPSPS*, 4.37% at *OsWaxy*-T1, 4.58% at *OsWaxy*-T2, and 3.59% at *OsWaxy*-T3. Compared with pAKBEv1–hMPG, pAKBEv2–mhMPG, and pAKBEv3–hMPG–VP64, pAKBEv4–mhMPG–VP64 enhanced A-to-Y transversion efficiencies by up to 11.66-fold (5.13%/0.44% at *OsNRT1.1B*), 9.10-fold (4.37%/0.48% at *OsWaxy*-T1), and 6.20-fold (3.35%/0.54% at *OsEPSPS*), respectively, with the highest efficiency of A-to-Y transversion reaching 6.82% (at *OsNRT1.1B*) in rice protoplasts (Figure 1B; Supplemental Table 3). The A-to-C and A-to-T base transversions induced by pAKBEv4–mhMPG–VP64 occurred mainly at positions A5–A6, and detectable A-to-Y transversions were also found at positions A4 and A8 (Figure 1C). We also observed improved A-to-G transition editing activity at five of the six endogenous targets with average frequencies from 4.62% to 13.83% (Figure 1B; Supplemental Table 4) and an expanded editing window at positions A1–A9 (Figure 1C). The editing byproducts, indels, occurred at *OsNRT1.1B*, *OsWaxy*-T1, and *OsWaxy*-T2, accounting for 0.79%, 0.90%, and 0.96% of the total reads, respectively (Supplemental Table 5). The fact that both pAKBEv2–mhMPG and pAKBEv4–mhMPG–VP64 generated indels (Supplemental Table 5) suggests that mhMPG, rather than VP64, induces the indels. Interestingly, most indels occurred precisely between the nicks produced by nCas9 (D10A) and the "A" base or between two "A" bases in the protospacer region (Figure 1D; Supplemental Figure 5). For example, defined deletions such as a 7-bp deletion between positions A8 and A14 and a 10-bp deletion between the nick of nCas9 (D10A) and A8 were induced by pAKBEv4–mhMPG–VP64 at *OsNRT1.1B* at frequencies of 0.81% and 0.47%, respectively (Figure 1D).

On the basis of their performance in rice protoplasts, we next investigated the A-to-K editing activities of pAKBEv1–hMPG, pAKBEv3–hMPG–VP64, and pAKBEv4–mhMPG–VP64 in stable rice lines using the same six endogenous targets. With these three pAKBEs, we detected only A-to-T and A-to-G base substitutions in the recovered stable lines. For pAKBEv1–hMPG, we obtained A-to-T base transversions only at the *OsWaxy*-T2 site with an efficiency of 2.02% (2/99) (Figure 1E; Supplemental Table 6), together with predominant occurrences of A-to-G transition editing with a frequency of 93.94% (93/99) and on-target indels at a frequency of 8.08% (8/99) (Figure 1F and 1G; Supplemental Table 6). The genotypes of these independent lines with A-to-T base transversion are listed in Supplemental Table 7 and Supplemental Figure 6. For the remaining five targets, no lines with A-to-T transversions were detected, although A-to-G transition editing occurred at frequencies of 36.54%–94.19% and on-target indels at frequencies of 3.49%

gRNA is expressed under the control of an *OsU3* promoter and terminated with "TTTTTTT." mhMPG, hMPG with G163R/N169S/S198A/K202A/G203A/S206A/K210A mutations; VP64, four copies of the VP16 transactivation domain.

(B) Bar plots showing on-target DNA base-editing frequencies of six endogenous targets achieved with pABE8e and four pAKBEs in rice protoplasts. The frequencies of targeted A-to-T and A-to-C edits are highlighted in orange and blue, respectively. The frequencies of targeted A-to-G edits are highlighted in green. The frequencies of indels in the protospacer region are shown in purple. The editing outcomes of each target by different pAKBEs are calculated based on three independent biological replicates (each replicate was performed with three repeats) and are displayed side by side.

(C) Editing windows of pAKBEs. The x axis represents protospacer positions 1–20 in the target region, with the PAM at positions 21–23. Single dots represent individual biological replicates (each replicate with three repeats). In total, three independent biological replicates were performed per site. Horizontal lines indicate the median; whiskers extend to minimum and maximum efficiencies. All values are presented as mean ± SEM of three repeats.

(D) Small indels generated from the 5′-deaminated "A" base to the nCas9 nicking site or between two "A" bases in the protospacer region by pAKBEs. The proportions of indels versus total reads are shown below the PAMs. PAMs are highlighted in yellow, and black bars represent the deleted nucleotide fragment. "−", deletion; "+", insertion.

(E) A-to-T editing efficiencies of three pAKBEs at six endogenous targets in stable rice lines. On-target A-to-T editing efficiencies of pAKBEv1–hMPG, pAKBEv3–hMPG–VP64, and pAKBEv4–mhMPG–VP64 at six endogenous targets in stable rice lines are shown in purple, blue, and green, respectively.

(F) A-to-G editing efficiencies of three pAKBEs at six endogenous targets in rice stable lines. On-target A-to-G editing efficiencies with pAKBEv1–hMPG, pAKBEv3–hMPG–VP64, and pAKBEv4–mhMPG–VP64 at six endogenous targets in stable rice lines are shown in purple, blue, and green, respectively.

(G) Occurrences of indels induced by three pAKBEs at six endogenous targets in stable rice lines. On-target indel editing efficiencies with pAKBEv1–hMPG, pAKBEv3–hMPG–VP64, and pAKBEv4–mhMPG–VP64 at six endogenous targets in stable rice lines are shown in purple, blue, and green, respectively.

and 3.41% at *OsDEP1* and *OsNRT1.1B*, respectively (Figure 1F and 1G; Supplemental Table 6). pAKBEv3–hMPG–VP64 induced A-to-T transversion editing of *OsNRT1.1B*, *OsWaxy*-T1, *OsWaxy*-T2, and *OsWaxy*-T3 at efficiencies of 1.32%, 1.54%, 2.53%, and 9.46%, respectively (Figure 1E; Supplemental Table 6). Compared with pAKBEv1–hMPG, pAKBEv3–hMPG–VP64 not only increased the A-to-T transversion editing at *OsWaxy*-T2 by 1.25-fold (2.53%/2.02%) (Figure 1E; Supplemental Table 6) but also enabled A-to-T transversion editing at *OsNRT1.1B*, *OsWaxy*-T1, and *OsWaxy*-T3. The genotypes of the stable rice lines are indicated in Supplemental Table 7 and Supplemental Figure 7. Similarly, we observed A-to-G transitions at efficiencies from 57.89% to 91.07%, and indels were detected at all six targets in the tested independent stable rice lines with frequencies of 1.32%–25% (Figure 1F and 1G; Supplemental Table 6). As in rice protoplasts, pAKBEv4–mhMPG–VP64 outperformed other pAKBEs in A-to-T transversion editing in rice stable lines (Figure 1E). With the exception of *OsEPSPS*, we achieved A-to-T transversion editing efficiencies of 10.98% (9/82), 2.22% (2/90), 4.23% (3/71), 8.54% (7/82), and 17.24% (10/58) at *OsDEP1*, *OsNRT1.1B*, *OsWaxy*-T1, *OsWaxy*-T2, and *OsWaxy*-T3, respectively, in stable rice lines. The highest A-to-T transversion editing efficiency was 17.24% (at *OsWaxy*-T3) (Figure 1E; Supplemental Table 6). Compared with pAKBEv3–hMPG–VP64, pAKBEv4–mhMPG–VP64 enabled A-to-T base editing at non-editable targets such as *OsDEP1* and increased A-to-T transversion editing efficiencies in rice stable lines by 1.68-, 2.75-, 3.38-, and 1.82-fold at *OsNRT1.1B* (2.22%/1.32%), *OsWaxy*-T1(4.23%/1.54%), *OsWaxy*-T2 (8.54%/2.53%), and *OsWaxy*-T3 (17.24%/9.46%), respectively (Figure 1E; Supplemental Table 6). The genotypes of each independent line are shown in Supplemental Table 7 and Supplemental Figure 8. Some of these lines carried pure A-to-T base transversions. For example, we obtained seven independent lines with A-to-T transversions at *OsWaxy*-T2. Three of these lines, which contained three, four, and three individual plants, respectively, were biallelic, with one allele showing pure A-to-T transversion (Supplemental Table 7). Again, A-to-G transitions predominated at all six target sites at frequencies ranging from 50.77% to 82.22% in the tested stable lines (Figure 1F; Supplemental Table 6). On-target indels occurred at frequencies of 1.54%–34.15% in these edited stable lines (Figure 1G; Supplemental Table 6). As observed in rice protoplasts, some of these indels in tested stable lines occurred precisely between nicks produced by nCas9 (D10A) and "A" bases or between two "A" bases in the protospacer region (Figure 1G; Supplemental Figure 9).

To evaluate the specificity of pAKBEv1–hMPG, pAKBEv3–hMPG–VP64, and pAKBEv4–mhMPG–VP64 in stable rice lines with A-to-K editing, we examined the off-target possibilities for each on-target site. No off-target effects were found at potential off-target sites (CRISPR–GE, http://skl.scau.edu.cn/) in the tested lines (Supplemental Table 8).

In summary, we successfully engineered four pAKBEs for A-to-K transversion editing in rice. Tests of their editing activities in rice protoplasts and stable lines demonstrated that pAKBEv4–mhMPG–VP64 outperformed the other pAKBEs in A-to-K base editing, enabling A-to-C and A-to-T transversions at some target loci that were otherwise non-editable and producing A-to-T and A-to-G editing efficiencies as high as 17.24% and 82.22% in stable rice lines. Although we did not achieve A-to-C transversion in stable rice lines, further optimization of pAKBEs by artificial design of mhMPG, or by exploring the MPGs from other resources as described in a very recent publication (Chen et al., 2023), will improve their A-to-C editing activity. Nevertheless, given that pAKBEs induced diverse editing outcomes such as A-to-G and A-to-T, as well as on-target indels with defined lengths when a single sgRNA was used (Figure 1), we propose that pAKBEs will be a useful tool for saturated mutagenesis of agriculturally important genes (Song et al., 2022; Zhou et al., 2023). These engineered pAKBEs, in combination with previously developed ABEs, CBEs and CGBEs, will allow more types of base conversions, including transitions and transversions, in plants, thus greatly expanding the plant base-editing toolbox and possibly extending it to other crops for both basic research and genetic improvement.

SUPPLEMENTAL INFORMATION

Supplemental information is available at *Plant Communications Online*.

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AUTHOR CONTRIBUTIONS

L.X. and Y.L. conceived the project. Y.L., S.L., C.L., C.Z., J.L., and Y.H. performed the experiments. S.L. and Y.L. wrote the manuscript. L.X. revised the manuscript. All authors read the final version of the manuscript.

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