Plant Biotechnology Journal
Open Access

Plant Biotechnology Journal (2025) **23**, pp. 2358–2360 doi: 10.1111/pbi.70063

Brief Communication

Developing glycosylase-based T-to-G and C-to-K base editors in rice

Yongjie Kuang[1,†], Xuemei Wu[1,†], Meijie Liu[1,2], Fang Yan[1,3], Dongfang Ma[2], Xueping Zhou[1,4], Huanbin Zhou[1,3] and Bin Ren[1,*]

[1]*State Key Laboratory for Biology of Plant Diseases and Insect Pests, Institute of Plant Protection, Chinese Academy of Agricultural Sciences, Beijing, China*
[2]*Key Laboratory of Sustainable Crop Production in the Middle Reaches of the Yangtze River, College of Agriculture, Yangtze University, Jingzhou, Hubei, China*
[3]*Ministry of Agriculture and Rural Affairs Key Laboratory of Gene Editing Technologies (Hainan), National Nanfan Research Institute, Chinese Academy of Agricultural Sciences, Sanya, Hainan, China*
[4]*State Key Laboratory of Rice Biology, Institute of Biotechnology, Zhejiang University, Hangzhou, Zhejiang, China*

Received 7 January 2025;
revised 13 February 2025;
accepted 6 March 2025.
**Correspondence* (Tel +86-10-82108751; fax +86-10-82105819; email renbin@caas.cn)
†These authors contributed equally to this work.

Keywords: CRISPR/Cas9, thymine DNA glycosylase, cytosine DNA glycosylase, base editing, rice.

The advancement of CRISPR/Cas-mediated base editors holds significant promise for installing nucleotide variations to correct defective alleles or create elite alleles through artificial evolution, thereby accelerating crop genetic improvement. The cytosine and adenine base editors have been widely applied in many plant species by utilizing various DNA deaminases to deaminate cytosine (C) and adenine (A) as a crucial step to produce uridine (U) and inosine (I) intermediates, which are in turn transformed into thymine (T) and guanine (G) (Li *et al*., 2024). In addition, the rice deaminase-free guanine base editor was also developed by integrating the engineered human N-methylpurine DNA glycosylase (MPGv6.3) with SpCas9 (D10A) nickase (SpCas9n). This editor converted G to T via glycosylating G and initiating the base excision repair (BER) pathway to generate an apurinic/apyrimidinic (AP) site (Liu *et al*., 2024). However, no direct thymine base editing in plants has been reported so far. Recently, several engineered thymine DNA glycosylases (TDGs) and cytosine DNA glycosylases (CDGs), artificially evolved from human uracil-N-glycosylases hUNG1 and hUNG2, have exhibited efficient T-to-S (S = G/C) and C-to-G editing activities in human cells (He *et al*., 2024; Tong *et al*., 2024; Ye *et al*., 2024). The applicability of these variants for deaminase-free base editing in plants remains unexplored. Here, we employed the evolved UNG variants to develop glycosylase-mediated thymine and cytosine base editors, which are capable of performing efficient direct T and C editing, respectively, as well as enabling nucleotide insertions and deletions (indels) in rice.

The N-terminal domain-truncated version with 1–88 amino acids truncation (Δ1–88) of hUNG2 increased the targeted pyrimidine base editing activities in human cells (Tong *et al*., 2024). Therefore, the truncated TDG-EKΔ (Δ1–79/G107E/Y147A/R260K), TDG3Δ (Δ1–79/F85L/G107E/L142V/Y147A/K175E/S238L/A255V/K259E/T266A/V274A/Y275S) and gTBEv3 (Δ1–79/Y156A/A214T/Q259A/Y284D), were selected for their relatively high T-to-G editing activities (He *et al*., 2024; Tong *et al*., 2024; Ye *et al*., 2024) and then fused at the N-terminus of SpCas9n, resulting in three rice thymine base editors, rBE127aΔ (TDG-EKΔ-SpCas9n), rBE127bΔ (TDG3Δ-SpCas9n) and rBE127cΔ (gTBEv3-SpCas9n) (Figure 1a, Figure S1 and Table S1). Five target sites were selected to evaluate their direct T editing capability in rice (Table S2). Sequencing of transgenic lines showed that all thymine base editors successfully achieved direct T editing at five target sites (Figure 1b,c). The average T-to-G editing efficiencies of rBE127aΔ (8.00%-29.41%), rBE127bΔ (5.00%-20.83%) and rBE127cΔ (12.50%-62.50%) were 13.68%, 11.63% and 34.82%, respectively (Figure 1c). T-to-C and T-to-A editing events were also introduced by rBE127bΔ and rBE127cΔ at some of targeted sites with the efficiency of <10.00% (Figure 1c). Overall, rBE127cΔ demonstrated the highest T editing efficiency (Figure 1c–e), and we named those thymine base editors collectively TGBEs as the predominant base editing events being T-to-G conversions (Figure 1c). Besides the base conversions, a significant number of indels were also induced by rBE127aΔ, rBE127bΔ and rBE127cΔ (Figure 1b,c, Figures S2–S6). Meanwhile, the T editing windows of rBE127bΔ (spanning from protospacer positions −1 to 8, counting the NGG as positions 21–23) and rBE127cΔ (positions 4–12) were broader than rBE127aΔ (positions 2–8) (Figure 1e). Moreover, rBE127cΔ generated more bi-allelic mutations with the pure base editing or the base editing/indel than rBE127aΔ and rBE127bΔ (Figure 1f). These data indicate that the glycosylase-mediated TGBEs enable highly efficient T-to-G editing in rice.

To evaluate CDGs' direct C editing features in rice, TGBEs were updated by replacing the TDGs with CDG4Δ (Δ1–79/G107E/K175E/D183G/N204D/R220Q/Y248H/T266A/V274A/Y275H/K302T) and gCBEv2 (Δ1–88/K184A/N213D/A214V) (Tong *et al*., 2024; Ye *et al*., 2024), resulting in glycosylase-based rice cytosine base editors rBE128aΔ (CDG4Δ-SpCas9n) and rBE128bΔ (gCBEv2-SpCas9n) (Figure 1a). The editing results of the eight targeted sites indicated that both rBE128aΔ and rBE128bΔ achieved efficient and comparable direct C editing activity (Figure 1g, Figure S7). Among the three types of C base editing outcomes, C-to-G editing events were the most dominant with average efficiencies of 29.41% and 29.77% for rBE128aΔ and rBE128bΔ respectively, and C-to-T editing events came second with average efficiencies of 14.43% and 20.16% for rBE128aΔ and rBE128bΔ, respectively. In contrast, C-to-A editing events exhibited the lowest efficiencies of 1.22% and 3.65% for rBE128aΔ and rBE128bΔ, respectively (Figure 1g, Figure S7d,e). Therefore, we named these glycosylase-based rice cytosine base editors as

© 2025 The Author(s). *Plant Biotechnology Journal* published by Society for Experimental Biology and The Association of Applied Biologists and John Wiley & Sons Ltd.
This is an open access article under the terms of the Creative Commons Attribution-NonCommercial License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited and is not used for commercial purposes.

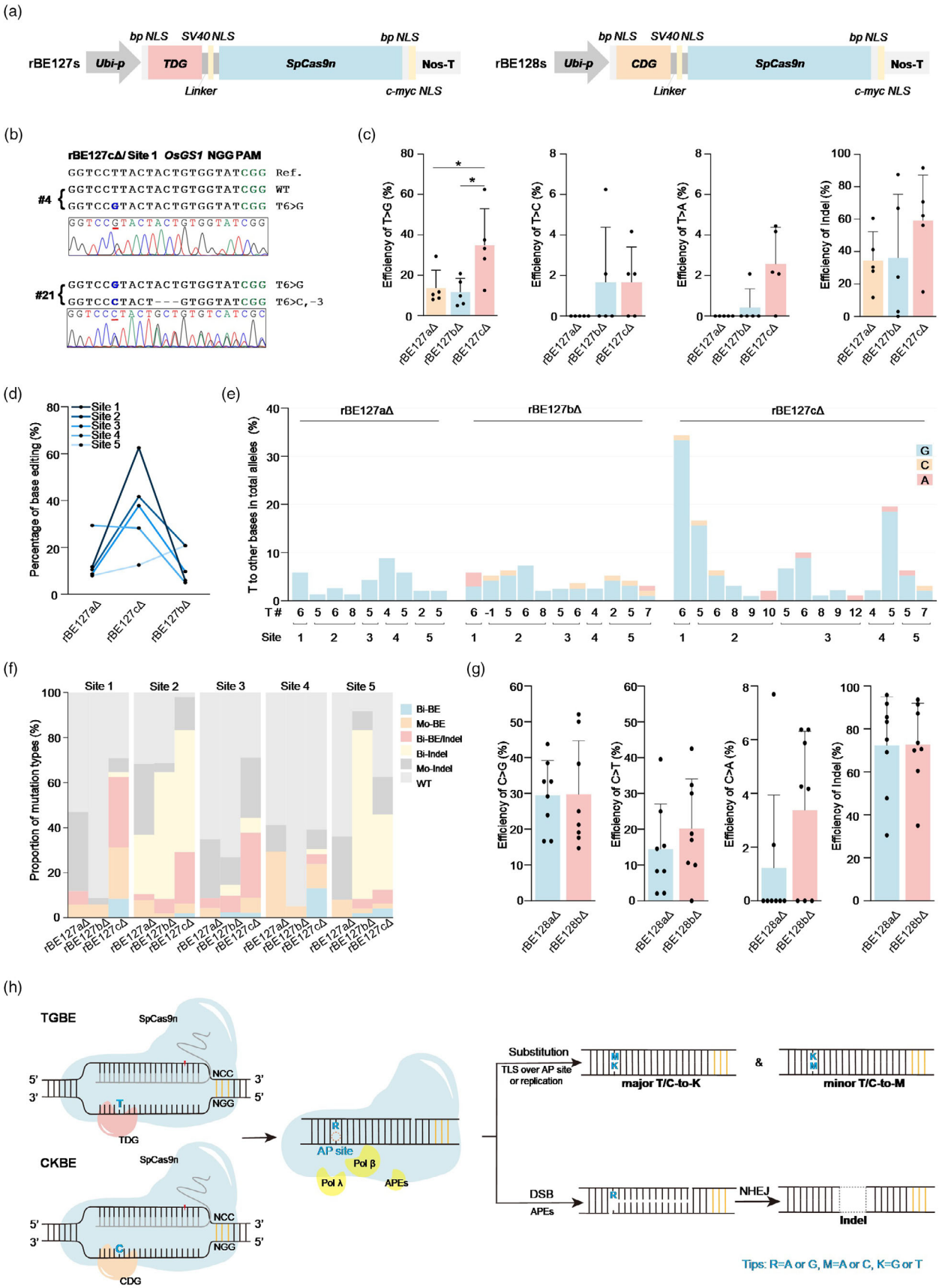
(a)
rBE127s
Ubi-p
bp NLS
TDG
SV40 NLS
SpCas9n
bp NLS
Nos-T
Linker
c-myc NLS
rBE128s
Ubi-p
bp NLS
CDG
SV40 NLS
SpCas9n
bp NLS
Nos-T
Linker
c-myc NLS
(b)
rBE127cΔ/ Site 1 OsGS1 NGG PAM
GGTCCTTACTACTGTGGTATCGG Ref.
#4
GGTCCTTACTACTGTGGTATCGG WT
GGTCCGTACTACTGTGGTATCGG T6>G
#21
GGTCCGTACTACTGTGGTATCGG T6>G
GGTCCCTACT---GTGGTATCGG T6>C,-3
(c)
Efficiency of T>G (%)
Efficiency of T>C (%)
Efficiency of T>A (%)
Efficiency of Indel (%)
rBE127aΔ
rBE127bΔ
rBE127cΔ
(d)
Site 1
Site 2
Site 3
Site 4
Site 5
Percentage of base editing (%)
rBE127aΔ
rBE127cΔ
rBE127bΔ
(e)
rBE127aΔ
rBE127bΔ
rBE127cΔ
T to other bases in total alleles (%)
G
C
A
T#
Site
(f)
Site 1
Site 2
Site 3
Site 4
Site 5
Proportion of mutation types (%)
Bi-BE
Mo-BE
Bi-BE/Indel
Bi-Indel
Mo-Indel
WT
(g)
Efficiency of C>G (%)
Efficiency of C>T (%)
Efficiency of C>A (%)
Efficiency of Indel (%)
rBE128aΔ
rBE128bΔ
(h)
TGBE
SpCas9n
NCC
NGG
TDG
CKBE
SpCas9n
NCC
NGG
CDG
AP site
Pol β
Pol λ
APEs
Substitution
TLS over AP site or replication
major T/C-to-K
&
minor T/C-to-M
DSB
APEs
NHEJ
Indel
Tips: R=A or G, M=A or C, K=G or T

Figure 1 Characterization of DNA glycosylase-mediated thymine and cytosine base editors in rice. (a) Schematic illustrations of human UNG variants-mediated base editors with SpCas9n in rice. (b) Sanger sequencing chromatograms of T-to-G base editing and indel induced by rBE127cΔ. The nucleotide conversions and PAM are marked in blue and bold, respectively. (c) Summary editing efficiencies of the thymine base editors. Statistical analyses with asterisks denote significant differences (*t*-test, $*P \leq 0.05$). (d) Total T-to-other bases editing efficiencies of the thymine base editors. The three points connected by the line represent the same target site. (e) Frequencies of T base editing outcomes in the alleles caused by thymine base editors. T#: protospacer position number. (f) Ratio of different mutation types induced by thymine base editors. Bi, bi-allelic; Mo, mono-allelic; WT, wild type. (g) Summary editing efficiencies of the cytosine base editors. (h) Editing model of deaminase-free glycosylase-mediated pyrimidine base editors in rice.

CKBEs (K = G/T). Moreover, bi-allelic plants with base substitutions and indels were also frequently detected (Figures S7c, S8–S15). The CKBEs exhibited comparable base editing efficiency and slightly broader editing windows (positions 2–9) than previously reported plant deaminase-based CGBEs (positions 5–8 or positions 5–9) (Figures S7d,e and S16; Tian *et al.*, 2022; Wang *et al.*, 2023; Yu *et al.*, 2024). These data demonstrate that glycosylase-based CKBE enables efficient C-to-K editing and is a potential screening platform for direct evolution in rice.

The potential off-target effects of TGBE and CKBEs were evaluated in the transgenic lines by CRISPR-GE (http://skl.scau.edu.cn/). Only one off-target site (LOC_Os04g31960) with one nucleotide (nt) mismatch at site 6 was efficiently mutated by rBE128aΔ and rBE128bΔ (Table S3). However, other predicted off-target sites with 2 or 3 nucleotide mismatches targeted by TGBE and CKBEs revealed significantly lower off-target activity, with mutation frequencies ranging from 0% to 8.51% (Table S3).

In conclusion, we have successfully developed glycosylase-mediated rice pyrimidine base editing toolkits (TGBEs and CKBEs). Different from the T/C-to-A outcomes in *Escherichia coli* and T/C-to-S outcomes in mammalian cells (He *et al.*, 2024; Tong *et al.*, 2024; Ye *et al.*, 2024), the glycosylase-mediated pyrimidine base editing predominantly generated the T/C-to-K conversions and indels in rice. Thus, to facilitate this understanding, we constructed a schematic diagram to elucidate the editing model of these pyrimidine base editors in rice (Figure 1h). The glycosylase-based base editors can induce diverse editing events, including base transitions, base transversions and indels (Figures S2–S6 and S8–S15), and are more profitable for the artificial evolution of crop endogenous genes. Overall, the DNA glycosylases can serve as admirable DNA-modifying proteins to greatly facilitate the development of base editors in various crops and accelerate functional genomics research and genetic improvement.

Acknowledgements

This work was supported by the Biological Breeding-National Science and Technology Major Project (2023ZD04074), the National Natural Science Foundation of China (32102294), the Nanfan special project of the Chinese Academy of Agricultural Sciences (YBXM2431), the Innovation Program of Chinese Academy of Agricultural Sciences (CAAS-CSIAF-202401), the China National Postdoctoral Program for Innovative Talents (BX20230423), the China Postdoctoral Science Foundation (2023M733826).

Conflict of interest

The authors have declared no conflict of interest.

Author contributions

B.R. and H.Z. designed the research; Y.K., X.W., M.L. and B.R. conducted the experiments; F.Y., D.M. and X.Z. supervised the research; B.R., H.Z., Y.K. and X.W. wrote the original draft; all authors participated in discussion and revision of the manuscript.

Data availability statement

The data that supports the findings of this study are available in the supplementary materials of this article.

References

He, Y., Zhou, X., Chang, C., Chen, G., Liu, W., Li, G., Fan, X. *et al.* (2024) Protein language models-assisted optimization of a uracil-N-glycosylase variant enables programmable T-to-G and T-to-C base editing. *Mol. Cell*, **84**, 1257–1270.e6.

Li, B., Sun, C., Li, J. and Gao, C. (2024) Targeted genome-modification tools and their advanced applications in crop breeding. *Nat. Rev. Genet.* **25**, 603–622.

Liu, L., Zhang, Z., Wang, C., Yan, F., Sun, W., Zhou, X., Miao, W. *et al.* (2024) Developing guanine base editors for G-to-T editing in rice. *J. Integr. Plant Biol.* **66**, 1557–1560.

Tian, Y., Shen, R., Li, Z., Yao, Q., Zhang, X., Zhong, D., Tan, X. *et al.* (2022) Efficient C-to-G editing in rice using an optimized base editor. *Plant Biotechnol. J.* **20**, 1238–1240.

Tong, H., Wang, H., Wang, X., Liu, N., Li, G., Wu, D., Li, Y. *et al.* (2024) Development of deaminase-free T-to-S base editor and C-to-G base editor by engineered human uracil DNA glycosylase. *Nat. Commun.* **15**, 4897.

Wang, D., Chen, Y., Zhu, T., Wang, J., Liu, M., Tian, S., Wang, J. *et al.* (2023) Developing a highly efficient CGBE base editor in watermelon. *Hortic. Res.* **10**, uhad155.

Ye, L., Zhao, D., Li, J., Wang, Y., Li, B., Yang, Y., Hou, X. *et al.* (2024) Glycosylase-based base editors for efficient T-to-G and C-to-G editing in mammalian cells. *Nat. Biotechnol.* **42**, 1538–1547.

Yu, M., Kuang, Y., Wang, C., Wu, X., Li, S., Zhang, D., Sun, W. *et al.* (2024) Diverse nucleotide substitutions in rice base editing mediated by novel TadA variants. *Plant Commun.* **5**, 100926.

Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Appendix S1 Supplemental materials and methods.
Figure S1–S16 Supplementary Figures.
Table S1–S4 Supplementary Tables.