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IsDge10 is a hypercompact TnpB nuclease that confers efficient genome editing in rice

Dear Editor,

Cas9 and Cas12a have been widely applied in genome engineering in both plant and human cells (Tang and Zhang, 2023). However, the relatively large sizes restrict their delivery into cells via viral vectors. As hypothetical ancestors of Cas9 and Cas12a, IscB and TnpB have been reported as RNA-guided DNA endonucleases suitable for genome editing in human cells (Han et al., 2023; Xiang et al., 2023). More recently, a eukaryotic RNA-guided endonuclease named Fanzor has demonstrated genome editing capabilities in human cells (Saito et al., 2023). These nucleases, such as IsDge10, are significantly smaller (~390 amino acids for IsDge10) compared to Cas9 and Cas12a (e.g., ~1300 amino acids for SpCas9). However, the applicability of TnpB, IscB, or Fanzor for plant genome engineering remained unexplored. Here, we evaluated a series of nucleases from the TnpB, IscB, and Fanzor families and successfully developed a miniature plant genome editor using IsDge10, a TnpB nuclease from *Deinococcus geothermalis.*

First, we selected six different nucleases from these three small nuclease families, including IsDge10, IsAam1, enIscB, and SpuFz1 (Figure 1A) (Han et al., 2023; Saito et al., 2023; Xiang et al., 2023), and optimized their codons for expression in rice. Next, we used the ZmUbi1 (RNA polymerase II) promoter to drive the expression of the nuclease gene and the OsU6-2 promoter for their respective guide RNAs (Figure 1B). We also developed a dual-fluorescence reporter system that simultaneously expresses GFP and mCherry. In this system, the green fluorescence from GFP serves as a normalization standard, and the red fluorescence from mCherry can be perturbated by targeted mutagenesis by any of these seven nuclease systems (Supplemental Figure 1A). This reporter system was co-transfected with the nuclease system into rice protoplasts, enabling preliminary assessments of the editing capabilities of our constructed nuclease systems. In our design, the mCherry gene was targeted at one site with the transposon-associated motif (TAM) by each corresponding nuclease (Figure 1A; Supplemental Table 1). The results showed that IsDge10, IsAam1, enIscB, and SpuFz1 each exhibited detectable editing activity in rice protoplasts, as indicated by the reduction of mCherry-to-GFP ratios, although their editing activities appeared lower than that of Cas9 (Supplemental Figures 1B and 1C). We then selected these four nucleases for further experiments.

To evaluate the editing efficiency of these four systems at endogenous sites in rice, we selected seven sites per nuclease system and assessed the outcomes of targeted mutagenesis in rice protoplasts using next-generation sequencing of PCR amplicons (Figure 1A; Supplemental Table 1). Our data showed that IsDge10 exhibited an editing efficiency ranging from 2.20% to 15.04% across the seven target sites. In contrast, enIscB achieved 2.05%–8.27% editing efficiency at five out of seven sites, IsAam1 exhibited 2.36%–4.65% editing efficiency at two out of seven sites, and SpuFz1 showed no detectable editing activity (Figure 1C and Supplemental Figure 2). These results suggest that IsDge10 is superior to the enIscB, IsAam1, and SpuFz1 systems for genome editing in rice. Further analysis showed that IsDge10 primarily generated deletions ranging from 6 to 10 bp in size, occurring 13 to –23 bp away from the TAM (Figures 1D and 1E and Supplemental Figures 3–5). This cleavage pattern of IsDge10 is similar to those observed with Cas12 nucleases, but differs from that of enIscB, which cleaves proximally to the TAM-a characteristic consistent with enIscB's evolutionary relationship to the Cas9 group (Supplemental Figures 6 and 7). This suggests that IsDge10, like Cas12 nucleases, produces off-set DNA double-strand breaks distal to the TAM sites.

To assess the specificity of IsDge10, we focused on a high-activity target, site 01, and designed a series of protospacers with two adjacent mutations at various positions (Supplemental Table 1; Supplemental Figure 2A). Analysis of rice protoplasts showed that permutations of every two nucleotides from positions 1–14 bp within the protospacer completely abolished the editing activity of IsDge10, whereas mutations at positions 15–16 bp within the protospacer led to ~50% reduction in editing frequency (Figure 1F). In contrast, mutations at positions 17–20 bp of the protospacer did not significantly affect the editing activity of IsDge10 (Figure 1F). These findings delineate the core functional length of a spacer for IsDge10 and confirmed its high specificity as a nuclease.

We then tested whether IsDge10 could generate edits in stable rice lines. The same transfer DNA constructs targeting the seven sites were used for the stable transformation of rice. The analysis of T0 generation plants revealed successful editing at all seven sites, with mutation efficiencies ranging from 4.2% to 25% (Figure 1G). The mutations predominantly consisted of deletions of 5–10 bp or longer (Supplemental Figure 8), consistent with the editing profile observed in rice protoplasts (Figure 1D). Notably, only monoallelic mutations were detected in these rice lines (Supplemental Figure 9). Assuming that biallelic knockout of these genes is non-lethal, these findings indicate the potential for enhancing IsDge10 to achieve biallelic editing in rice.

To develop a multiplexed IsDge10 genome editing system, we adopted the dual RNA polymerase II promoter expression

Published by the Plant Communications Shanghai Editorial Office in association with Cell Press, an imprint of Elsevier Inc., on behalf of CSPB and CEMPS, CAS.

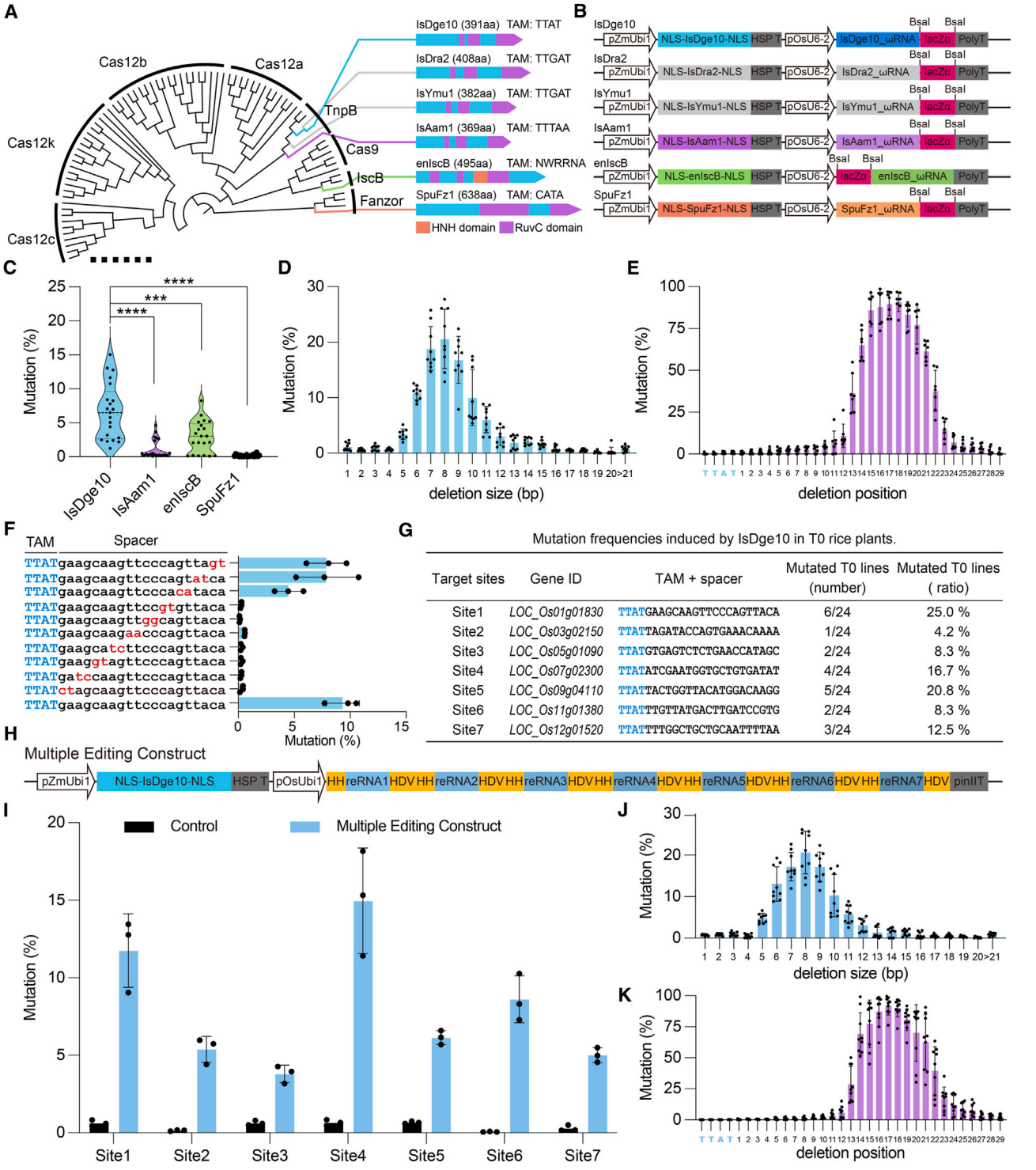

Figure 1. Development of the IsDge10 genome editing system in rice.

(A) Phylogenetic diagram illustrating the evolutionary relationships among TnpB, Cas9, Cas12, IscB, and Fanzor. The selected nucleases IsDge10, IsDra2, IsYmu1, IsAam1, enIscB, and SpuFz1 are highlighted and connected to their corresponding structural diagrams, showing their sizes and domains (HNH and RuvC).

(B) Schematics of the IsDge10, IsDra2, IsYmu1, IsAam1, enIscB, and SpuFz1 constructs used for genome editing in rice.

(C) Comparison of the mutation rates of the IsDge10, IaAam1, enIscB, and SpuFz1 systems in rice protoplasts.

(D) Deletion size profiles for three representative target sites in rice.

(E) Deletion position profiles for three representative target sites in rice.

(F) Assessment of targeting specificity using mismatched guide RNAs at a representative target site in rice protoplasts.

(legend continued on next page)

systems previously used for Cas12a, Cas12b, and Cas12j2, as well as their guide RNAs (Tang et al., 2017, 2019; Ming et al., 2020; Liu et al., 2022; Zheng et al., 2023; Zhou et al., 2023). The IsDge10 protein was expressed under the ZmUbi1 promoter, and the seven guide RNAs were expressed under the OsUbi1 promoter and processed by the HH (hammer head)-HDV (hepatitis delta virus) dual ribozyme system to form mature "guide RNA–ωRNA" complexes (Figure 1H). Interestingly, this multiplexed construct exhibited higher editing efficiencies at all seven target sites compared to those achieved using the OsU6-2 promoter for guide RNA expression (Figure 1C and Supplemental Figure 2A), with efficiencies ranging from 4.3% to 18.2% in rice protoplasts (Figure 1I). The deletions were typically 6–10 bp in length and occurred 13–23 bp away from the TAM (Figures 1J and 1K and Supplemental Figures 10 and 11). These results confirm that IsDge10 can edit multiple sites simultaneously using this robust dual polymerase II promoter system.

In summary, our study establishes IsDge10 is a novel and compact transposon-associated TnpB nuclease suitable for genome editing in rice. Compared to other compact nucleases tested, IsDge10 exhibits robust genome editing activity in rice and requires only a simple TTAT TAM. Although the current IsDge10 system does not yet match the efficiency of the widely used Cas9 and Cas12a systems, this study paves the way for further enhancements through protein engineering and evolutionary approaches. As one of the smallest nucleases functional in plants, IsDge10 holds great potential for various applications, including multi-nuclease combination editing, integration with diverse effectors to develop tools for transcriptional and epigenetic regulation, and incorporation into viral vectors for plant genome engineering.

FUNDING

This research was supported by the Biological Breeding-Major Projects (2023ZD04076) awarded to X.T. and Y.Z., and the National Natural Science Foundation of China (award nos. 32270433, 32101205, and 32072045) to Y.Z., X.Z., and X.T. It was also supported by the NSF Plant Genome Research Program (IOS-2029889 and IOS-2132693) to Y.Q.

ACKNOWLEDGMENTS

No conflict of interest is declared.

AUTHOR CONTRIBUTIONS

Y.Z. conceived the project and designed the experiments. R.Z., X.T., and Y.H. generated all the constructs. R.Z. performed the rice protoplast transformation and analyzed the mutation frequencies in protoplasts. R.Z., W.W., Y.W., D.W., and X.Z. conducted rice stable transformations. Y.L. revised the manuscript. Y.Z., Y.Q., and R.Z. analyzed the data and wrote the manuscript with input from all authors. All authors have read and approved the final version of the manuscript.

SUPPLEMENTAL INFORMATION

Supplemental information is available at *Plant Communications Online*.

Received: June 9, 2024
Revised: July 15, 2024
Accepted: August 19, 2024
Published: August 21, 2024

Rui Zhang[1,2], Xu Tang[2,3], Yao He[2], Yangcun Li[2], Wei Wang[1], Yawei Wang[1], Danning Wang[1], Xuelian Zheng[2], Yiping Qi[4,5,] and Yong Zhang[2,*]*

[1]Department of Biotechnology, School of Life Sciences and Technology, Center for Informational Biology, University of Electronic Science and Technology of China, Chengdu 610054, China
[2]Integrative Science Center of Germplasm Creation in Western China (Chongqing) Science City, Chongqing Key Laboratory of Tree Germplasm Innovation and Utilization, School of Life Sciences, Southwest University, Chongqing 400715, China
[3]State Key Lab of Rice Biology, China National Rice Research Institute, Hangzhou 311400 Zhejiang, China
[4]Department of Plant Science and Landscape Architecture, University of Maryland, College Park, MD 20742, USA
[5]Institute for Bioscience and Biotechnology Research, University of Maryland, Rockville, MD 20850, USA
***Correspondence: Yiping Qi (yiping@umd.edu), Yong Zhang (zhangyong916@swu.edu.cn)**
https://doi.org/10.1016/j.xplc.2024.101068

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(G) Genome editing efficiency of IsDge10 in stable rice lines at seven target sites.
(H) Schematics of the dual RNA polymerase II promoter-based and multiplexed IsDge10 system for genome editing in rice.
(I) Multiplexed editing of seven target sites in rice protoplasts.
(J) Deletion size profiles for three representative multiplexed target sites in rice.
(K) Deletion position profile for three representative multiplexed target sites in rice. Each dot represents a biological replicate. Data are presented as mean values ± SD. Data were analyzed using a two-tailed unpaired *t*-test. $^{***}P < 0.001$ and $^{****}P < 0.0001$. Solid line, median; dashed line, quartiles.

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Plant Communications, Volume *5*

Supplemental information

IsDge10 is a hypercompact TnpB nuclease that confers efficient genome editing in rice

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Rui Zhang[1, 2], Xu Tang[2, 3], Yao He[2], Yangcun Li[2], Wei Wang[1], Yawei Wang[1], Danning Wang[1], Xuelian Zheng[2], Yiping Qi[4, 5*], Yong Zhang[2*]

[1]Department of Biotechnology, School of Life Sciences and Technology, Center for Informational Biology, University of Electronic Science and Technology of China, Chengdu 610054, China; [2]Integrative Science Center of Germplasm Creation in Western China (Chongqing) Science City, Chongqing Key Laboratory of Tree Germplasm Innovation and Utilization, School of Life Sciences, Southwest University, 400715 Chongqing, China; [3]State Key Lab of Rice Biology, China National Rice Research Institute, Hangzhou 311400, Zhejiang, China; [4]Department of Plant Science and Landscape Architecture, University of Maryland, College Park, Maryland 20742, USA; [5]Institute for Bioscience and Biotechnology Research, University of Maryland, Rockville, Maryland 20850;

Running title: Hypercompact IsDge10 enables genome editing in rice

***Corresponding authors:**

Yong Zhang, Integrative Science Center of Germplasm Creation in Western China (Chongqing) Science City, Chongqing Key Laboratory of Tree Germplasm Innovation and Utilization, School of Life Sciences, Southwest University, Chongqing 400715, China; Email: zhangyong916@swu.edu.cn

Yiping Qi, Department of Plant Science and Landscape Architecture, University of Maryland, College Park, MD 20742, USA; Email: yiping@umd.edu

SUPPLEMENTAL INFORMATION

Supplemental Materials and Methods.

Supplementary Tables

Supplemental Materials and Methods

Vector construction

To construct the rice IsDge10 vector, the rice codon-optimized IsDge10 was synthesized by Genscript (Nanjing, China). and assembled with the Zea mays ubiquitin 1 (ZmUbi1) promoter and A. thaliana heat shock protein (AtHSP) terminator using Golden Gate assembly using BsaI-HFv2 from New England Biolabs, resulting in the IsDge10 entry vector (pZR406). Subsequently, the ωRNA of IsDge10 was synthesized through overlap extension polymerase chain reaction (PCR). PCR fragments containing the OsU6-2 promoter, IsDge10 ωRNA and lacZ-poly T, were amplified and then inserted into the BsaI-linearized pTSWB vector using Gibson assembly with the NEBuilder HiFi DNA Assembly Cloning Kit from New England Biolabs, yielding the ωRNA entry vectors pOsU6-2-IsDge10 ωRNA (pZR389). To generate the final IsDge10 backbone, the IsDge10 entry vector, ωRNA entry vector and pMOD_C0000a were assembled into the T-DNA backbone pTRANS_210d (Addgene Plasmid #91109) to generate rice IsDge10 backbone pGEL1011 using Golden Gate assembly. The backbones of IsAam1, IsDra2, IsYmu1, enIscB and SpuFz1 were generated by same way. In order to construct IsDge10 multiplex editing vector, PCR fragments containing the ZmUbi1 promoter-Hammerhead ribozyme, IsDge10 ωRNA-lacZ and hepatitis delta virus (HDV) ribozyme-pinII terminator were amplified and inserted into the BsaI-linearized pTSWB vector, yielding the ωRNA entry vector pOsUbi1- IsDge10 ωRNA (pZR527). To generate the final IsDge10 multiplex editing backbone, the IsDge10 entry vector, the ωRNA entry (pOsUbi1-IsDge10 ωRNA) and pMOD_C0000a were assembled into the T-DNA backbone pTRANS_210d to generate rice IsDge10 multiplex editing backbone pGEL1012 using Golden Gate assembly. Nucleotide sequences of IsDge10, IsAam1, enIscB and SpuFz1 codon-optimized for rice were shown in **Supplementary Figure 12**.

Rice protoplast transformation

The Japonica cultivar Nipponbare rice was used in this study. Rice protoplast isolation and PEG-mediated transformation were performed as previously described (Tang et al., 2019). Briefly, the rice plants were grown in the dark at 28 °C. Then healthy rice seedlings were cut in about 1.0 mm strips, and immediately transferred into the 10ml enzyme solution, followed by vacuum-infiltration for 30 min and incubation at 80 rpm for 6 hours at 25°C in the dark. Next, a 40 μm cell strainer was used to filter the digested products on a 90mm petri dish and further transferred into a sterile 50ml

Falcon tube. The protoplasts were collected by centrifugation at 100 g for 5 min and suspended in 10 ml W5 solution for washing. Then, the W5 solution was removed by centrifugation at 100 g for 2 min and the protoplasts were suspended at a concentration of 2×10^6 ml^{-1} in MMG buffer. For transformation, 30 μl plasmids (containing 30 μg DNA) were transformed into 200 μl protoplasts. The mixture was gently mixed with 230 μl PEG transformation buffer for 20 min. The transfection reaction was stopped by adding 1 ml W5 buffer. The protoplasts were collected by centrifugation at 250 g for 5 min, gently suspended in 600 μl W5 buffer, and then transferred to a 12-well culture plate. The plate was placed in the dark at 32°C for 48 hours before monitoring under a microscope or extracting DNA.

For fluorescence reporter system test in rice protoplasts, 30 μl plasmids (containing 15 μg DNA of reporter and 15 μg DNA of editing vector) were transformed into 200 μl protoplasts. After 48 hours of dark incubation, we used an Olympus IX73 Inverted Microscope to photograph the transformed rice cells. The microscope was configured with an excitation light intensity set to 25% and an exposure time of 200 ms for image capture. For each biological replicate, we randomly selected a field of view for image capture and separately recorded the raw grayscale values of the mCherry signal and the ZsGreen signal provided by the system. We then calculated the decrease in mCherry by dividing the mCherry signal by the ZsGreen signal. Each experiment was performed with three biological replicates.

Rice stable transformation

As with our previous study (Zhou et al., 2017), the cultivar Japonica Nipponbare was used for stable *Agrobacterium*-mediated transformation of rice. Briefly, the sterilized rice seeds were placed on solid N6-D medium. Precultured rice calli were transformed by inoculating *Agrobacterium* EHA105 carrying the recombinant expression vector. The inoculated calli were co-cultured with *Agrobacterium* for 3 d on 2N6-AS solid medium. Then the calli were washed and transferred to N6D-S solid medium containing 400 mg/l timentin and 50 mg/l hygromycin for 2 weeks. Resistant calli were then transferred to RE-III medium for obtain regenerated plants.

Mutagenesis analysis

The Next-Generation Sequencing (NGS) of PCR amplicons was used for evaluating

editing efficiency in rice protoplasts. Genomic DNA was extracted from the protoplasts using the CTAB method (Stewart and Via, 1993). The amplicons of the editing regions were amplified by *2 x Rapid Ta*q Master Mix (Vazyme, China). Amplicons were sent to Novogene (China, Tianjin) for deep-sequencing by the Novaseq6000 platform which produced 150 bp paired-end reads. The editing frequency was analyzed by the CRISPRMatch and CrisprStitch (Han et al., 2024; You et al., 2018). For stable rice T0 lines, DNA was extracted from the T0 generation using the CTAB method (Stewart and Via, 1993). Then the target sites were amplified by *2 x Rapid Ta*q Master Mix (Vazyme, China) and the products were sent to Sangon Biotech (Shanghai, China) for direct PCR product sanger sequencing. Sanger sequencing data were analyzed by Snapgene software (www.snapgene.com).

Statistical analysis

For all bar graphs, the mean and standard deviation (SD) were calculated and plotted using GraphPad Prism 8.0 software, with SD provided only for samples with n > 2. The data are presented as mean ± SD. Statistical significance was analyzed using an unpaired two-tailed t-test with equal variance in Microsoft Excel version 2212. Asterisks indicate significant differences according to Student's t-test (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001). The figures were further processed using Adobe Photoshop and Adobe Illustrator software.

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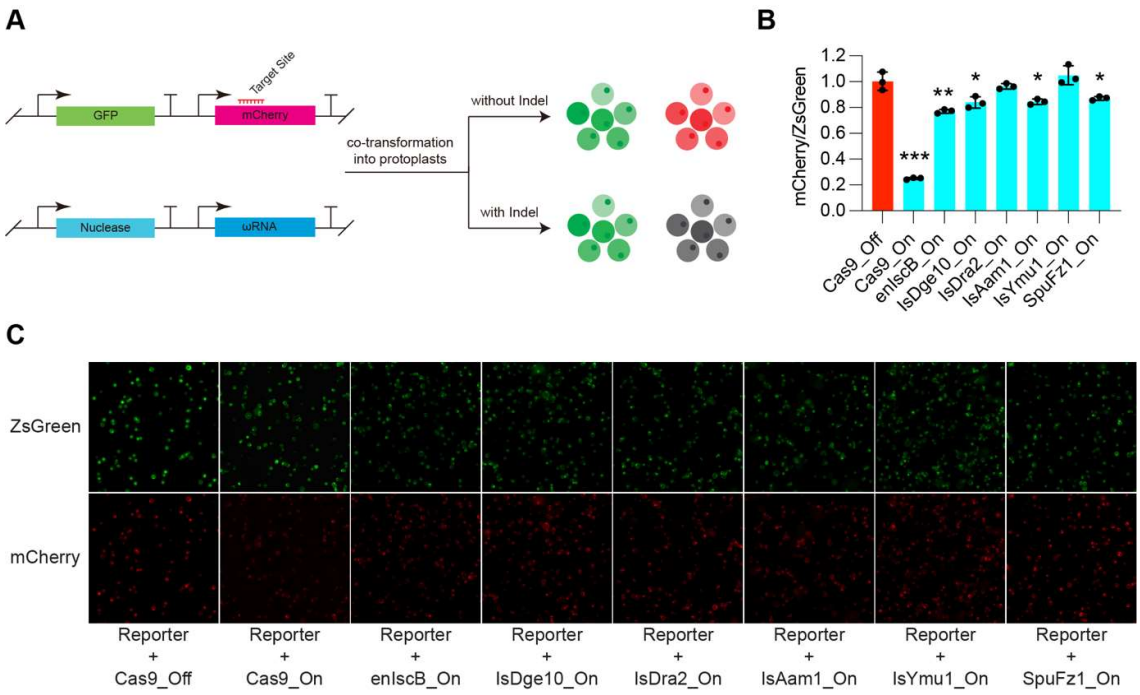
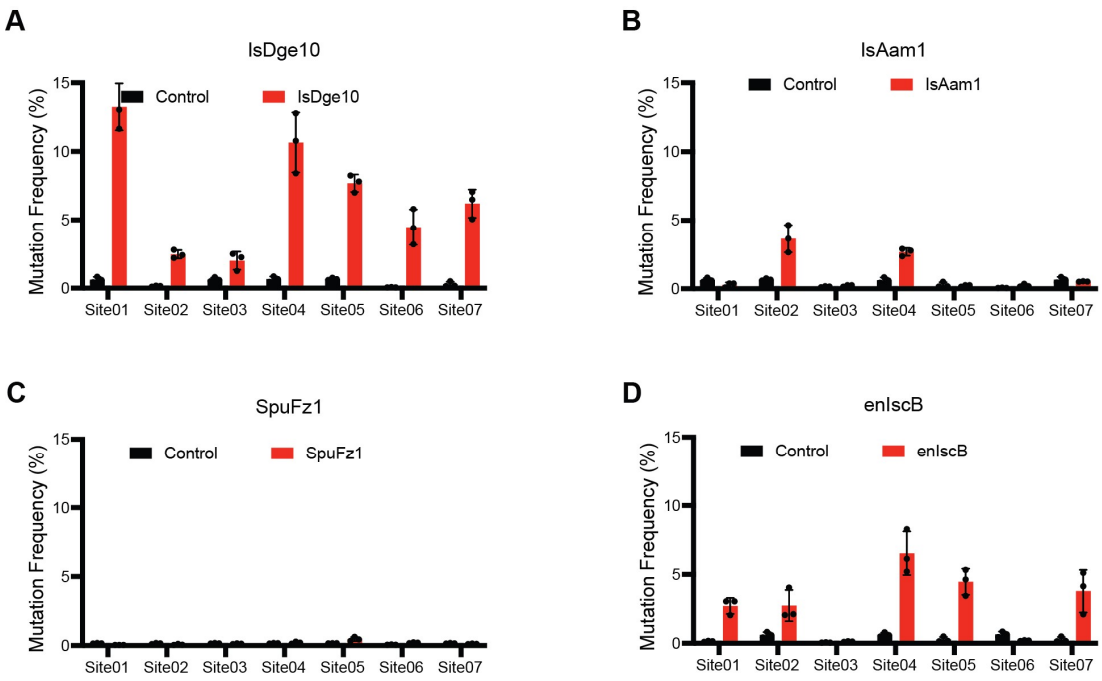
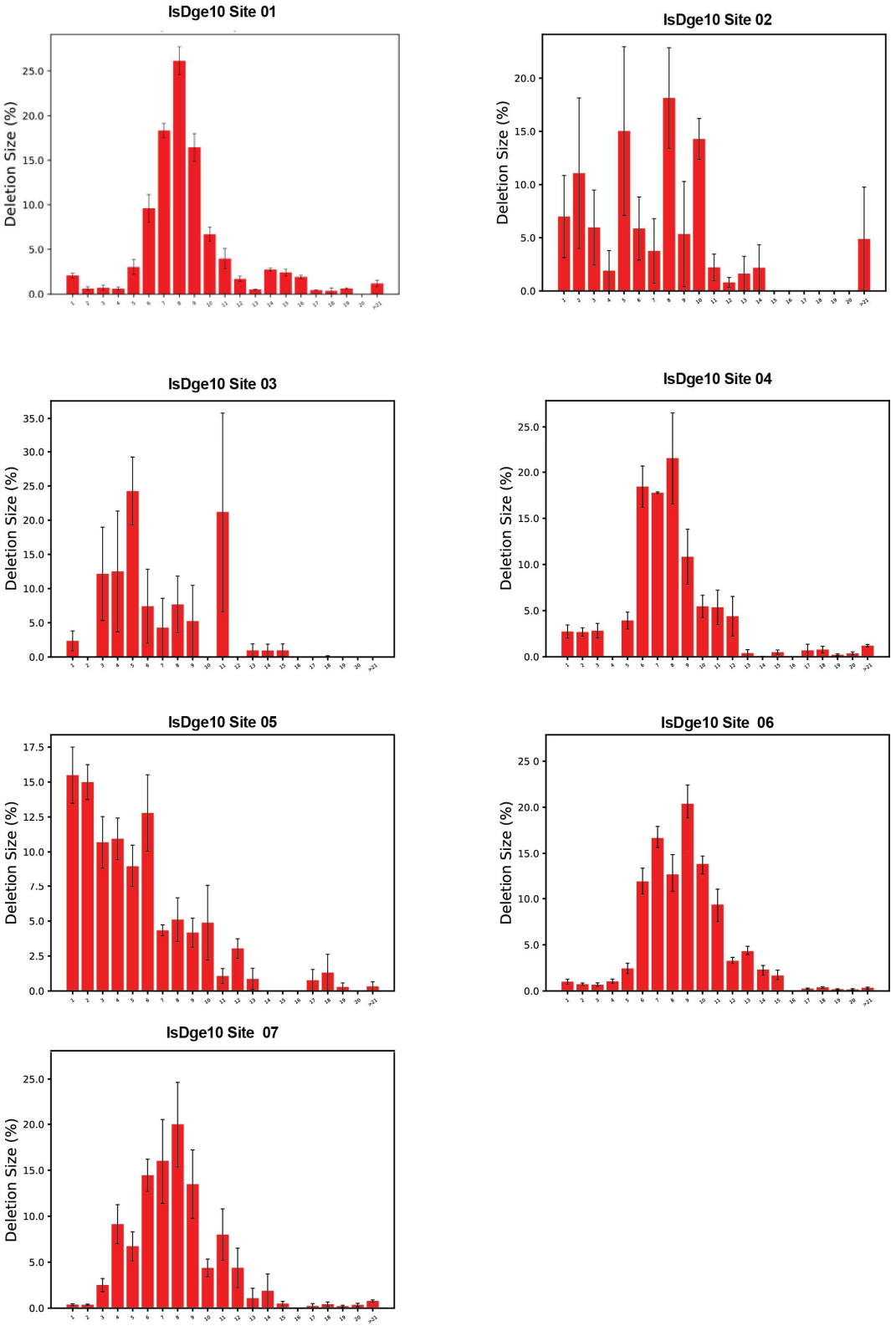


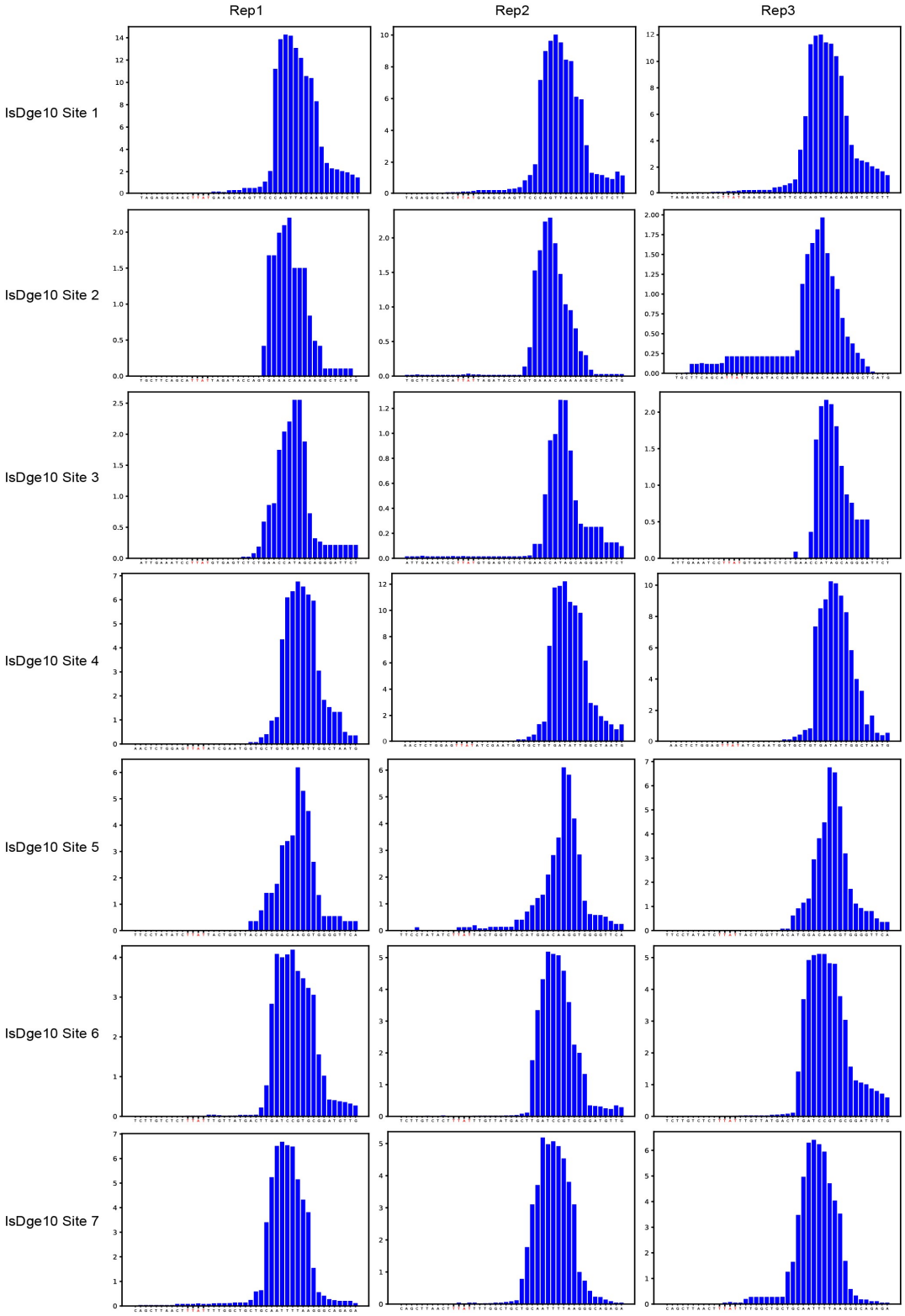
Supplementary Figure 1. Detection of genome editing activity with a florescent report assay in rice protoplasts. (A) A diagram of a dual-fluorescence reporter system. (B) The decrease of mCherry fluorescence induced by targeted mutagenesis of different editing systems. (C) Decrease of mCherry fluorescence expression after co-transfection. Each dot represents a biological replicate. Data are presented as mean values +/- SD. Data were analyzed using two-tailed unpaired t-Test.



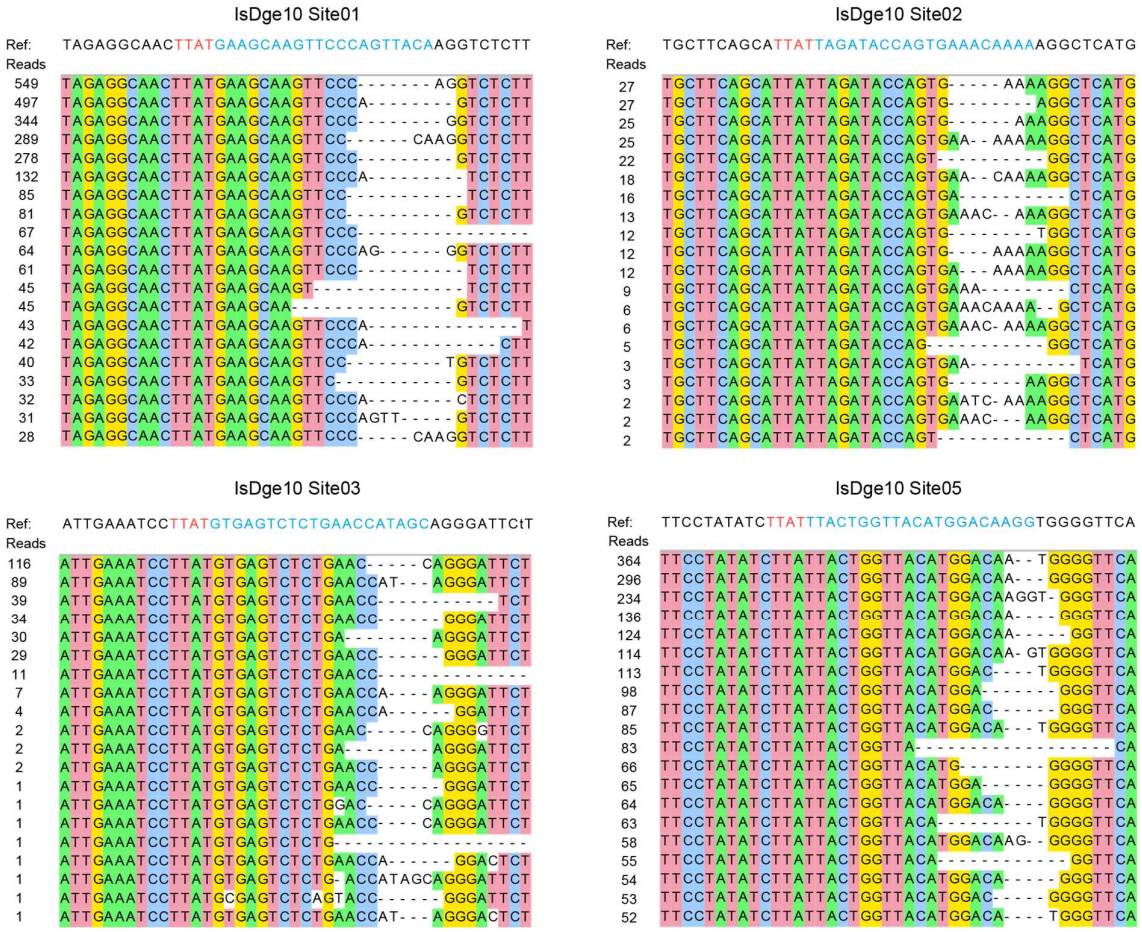
Supplementary Figure 2. Comparison of different nucleases at rice endogenous sites in rice protoplasts. (A) Editing efficiency of the IsDge10 system at seven endogenous sites in rice. (B) Editing efficiency of the IsAam1 system at seven endogenous sites in rice. (C) Editing efficiency of the SpuFz system at seven endogenous sites in rice. (D) Editing efficiency of the enIscB system at seven endogenous sites in rice. Each dot represents a biological replicate. Data are presented as mean values +/- SD. Data were analyzed using two-tailed unpaired t-Test.



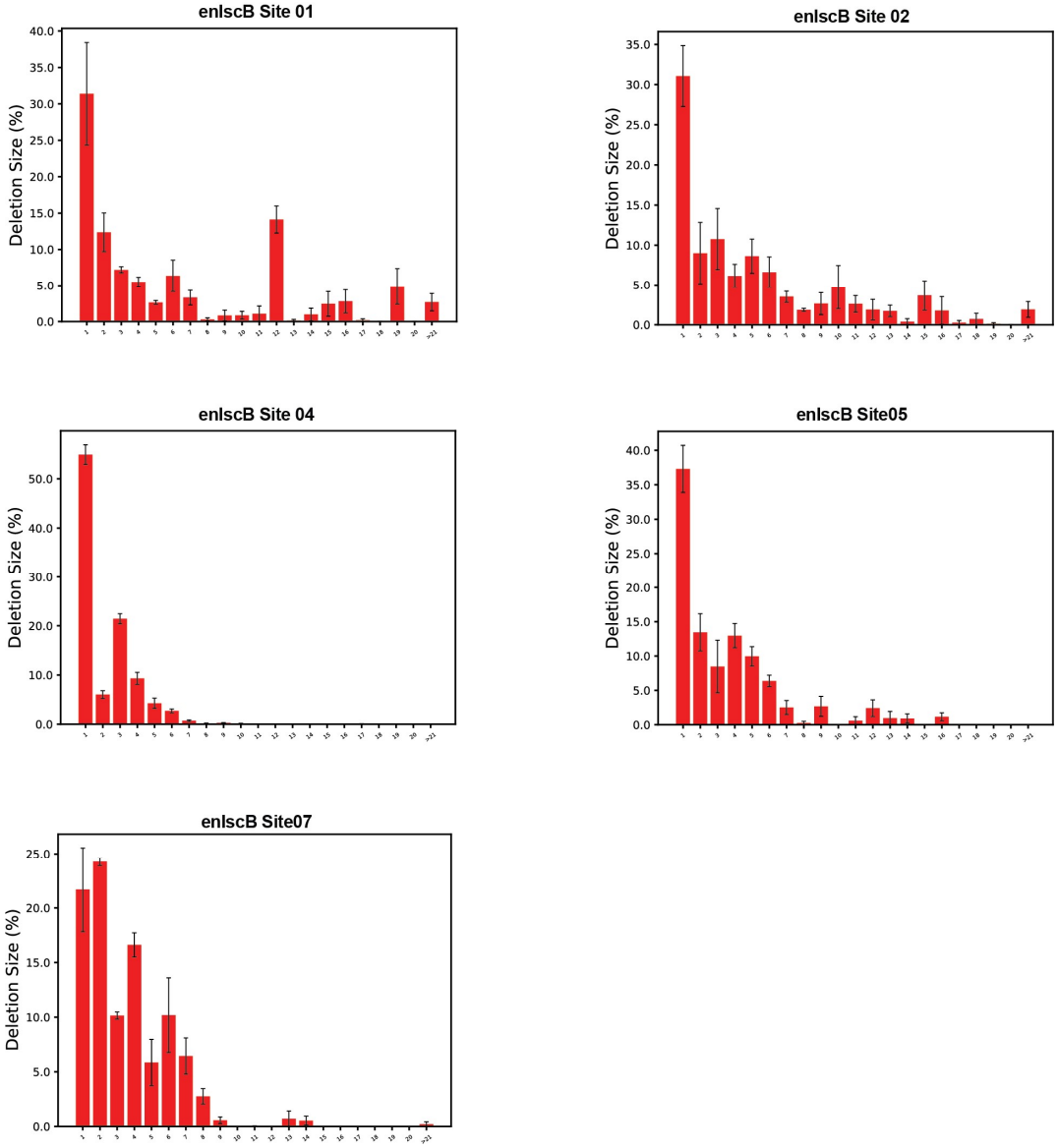
Supplementary Figure 3. Deletion size profile of IsDge10 at 7 target sites in rice protoplasts. Each assay contains three independent experiments (n=3). Data are presented as mean values +/- SD.



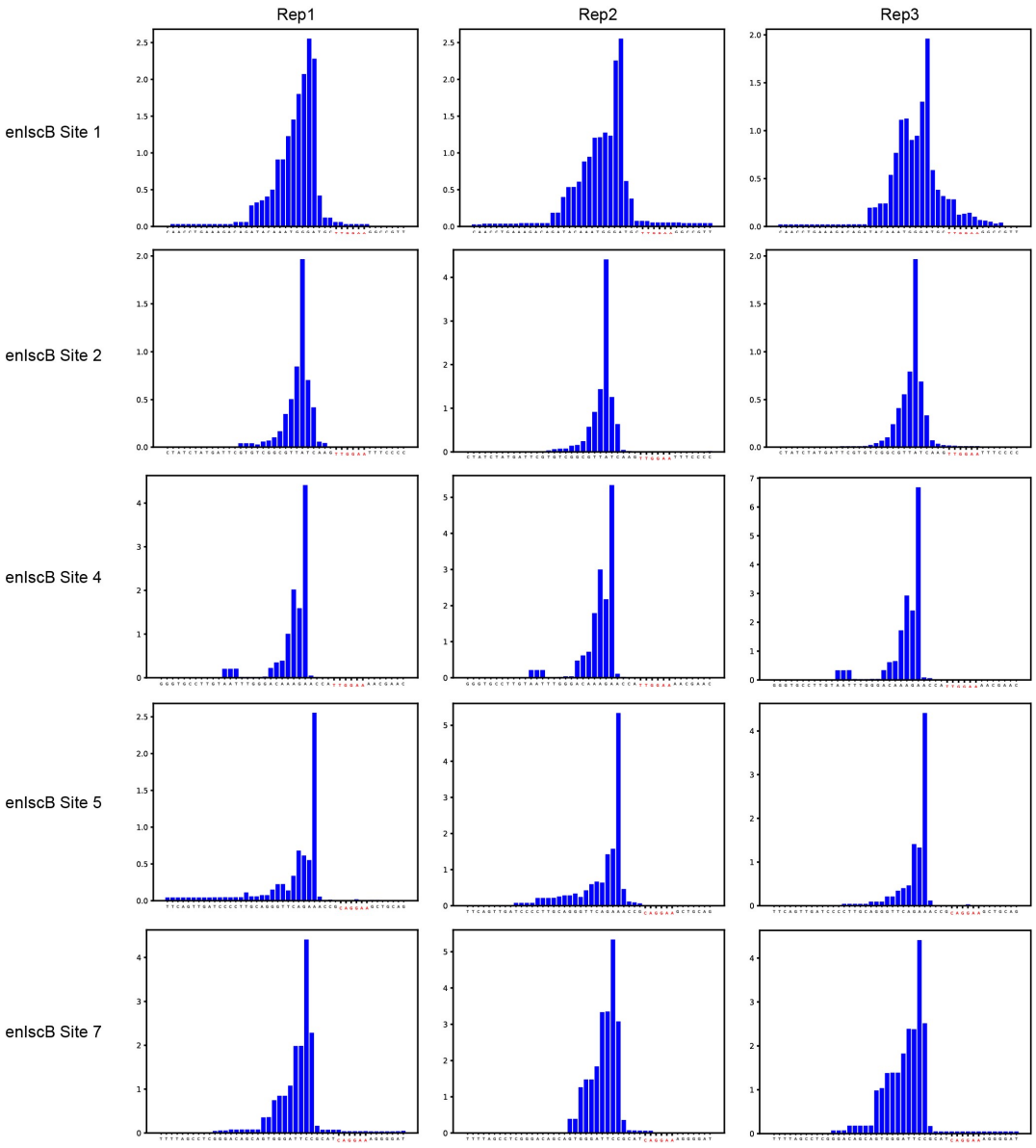
Supplementary Figure 4. Deletion position profile of IsDge10 at 7 target sites in rice protoplasts. Editing at each site was biologically replicated three times, designated as Rep1, Rep2, and Rep3, respectively. Sequence in red indicated the TAM of IsDge10.



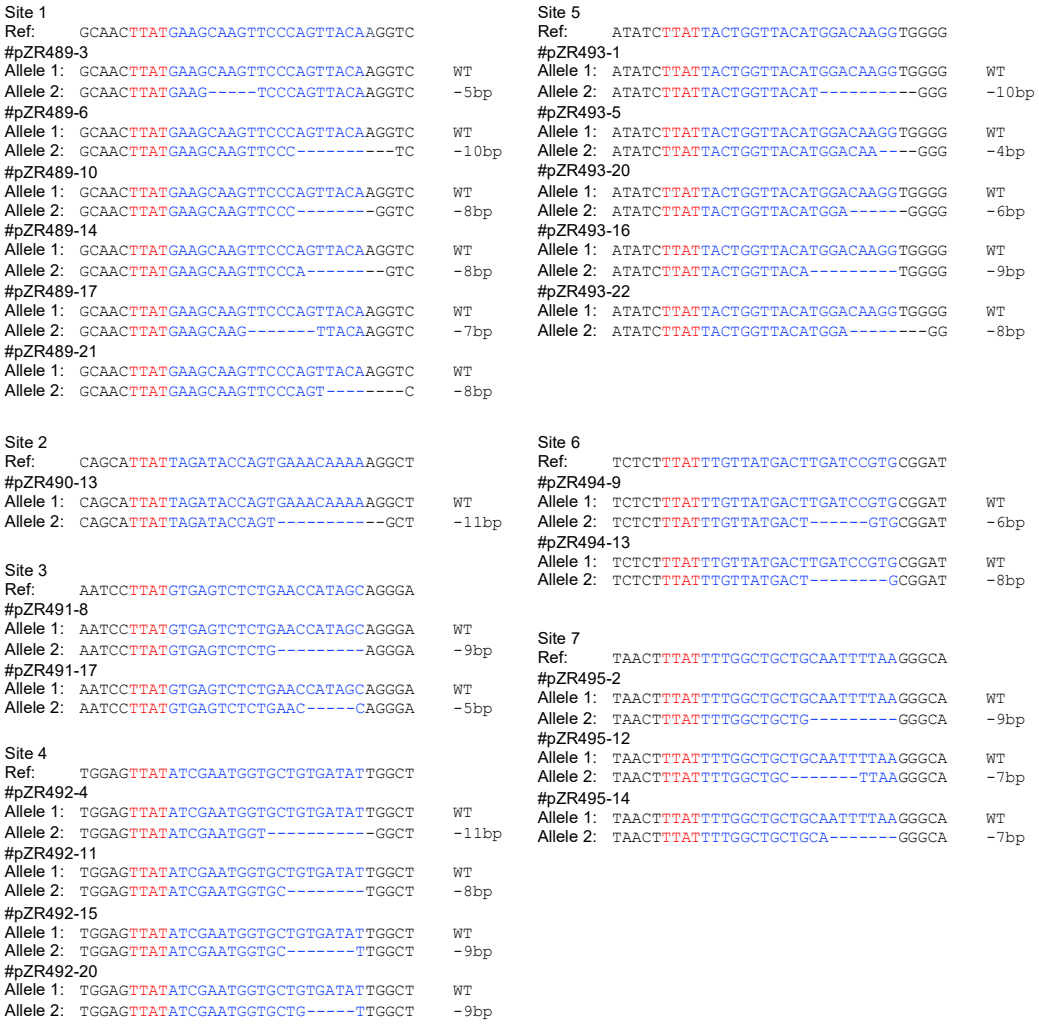
Supplementary Figure 5. Sequence alignment results of editing events of IsDge10 at 4 target sites in rice protoplasts. Sequences in blue indicate the guide RNA, and sequences in red indicate the TAM of IsDge10.



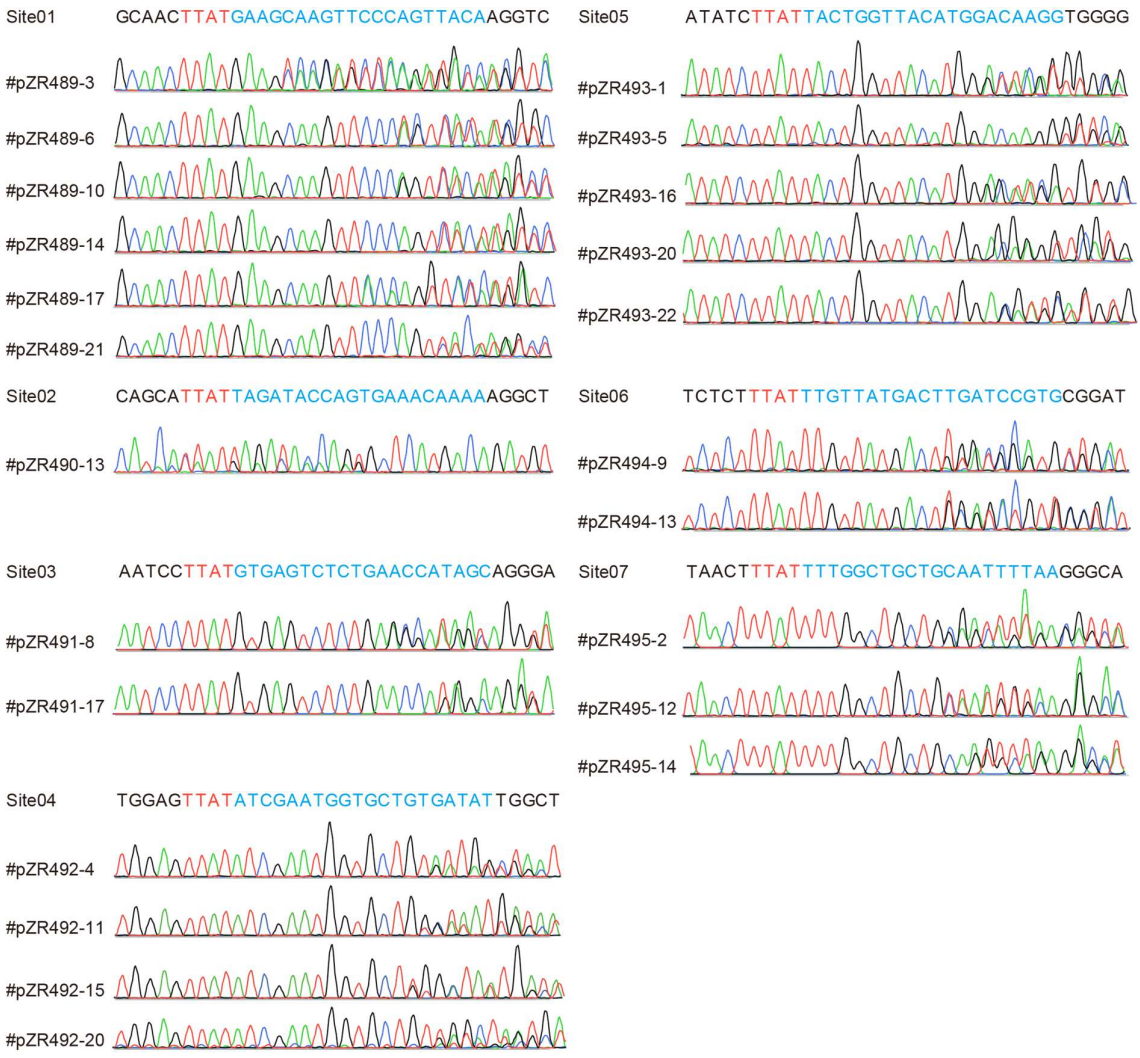
Supplementary Figure 6. Deletion size profile of enIscB at 5 target sites in rice protoplasts. Each assay contains three independent experiments (n=3). Data are presented as mean values +/- SD.



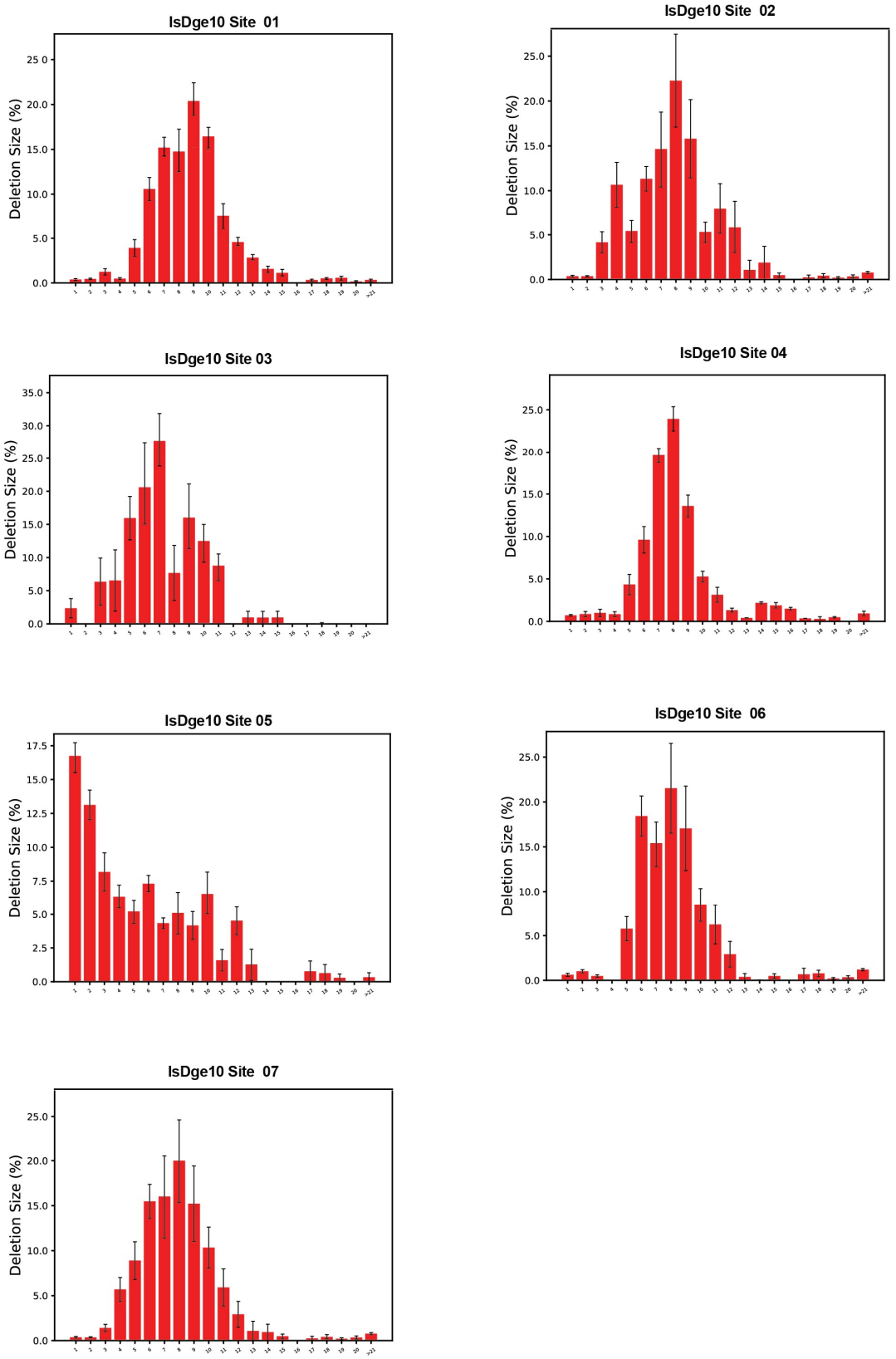
Supplementary Figure 7. Deletion position profile of enIscB at 5 target sites in rice protoplasts. Editing at each site was biologically replicated three times, designated as Rep1, Rep2, and Rep3, respectively. Sequence in red indicated the TAM of enIscB.



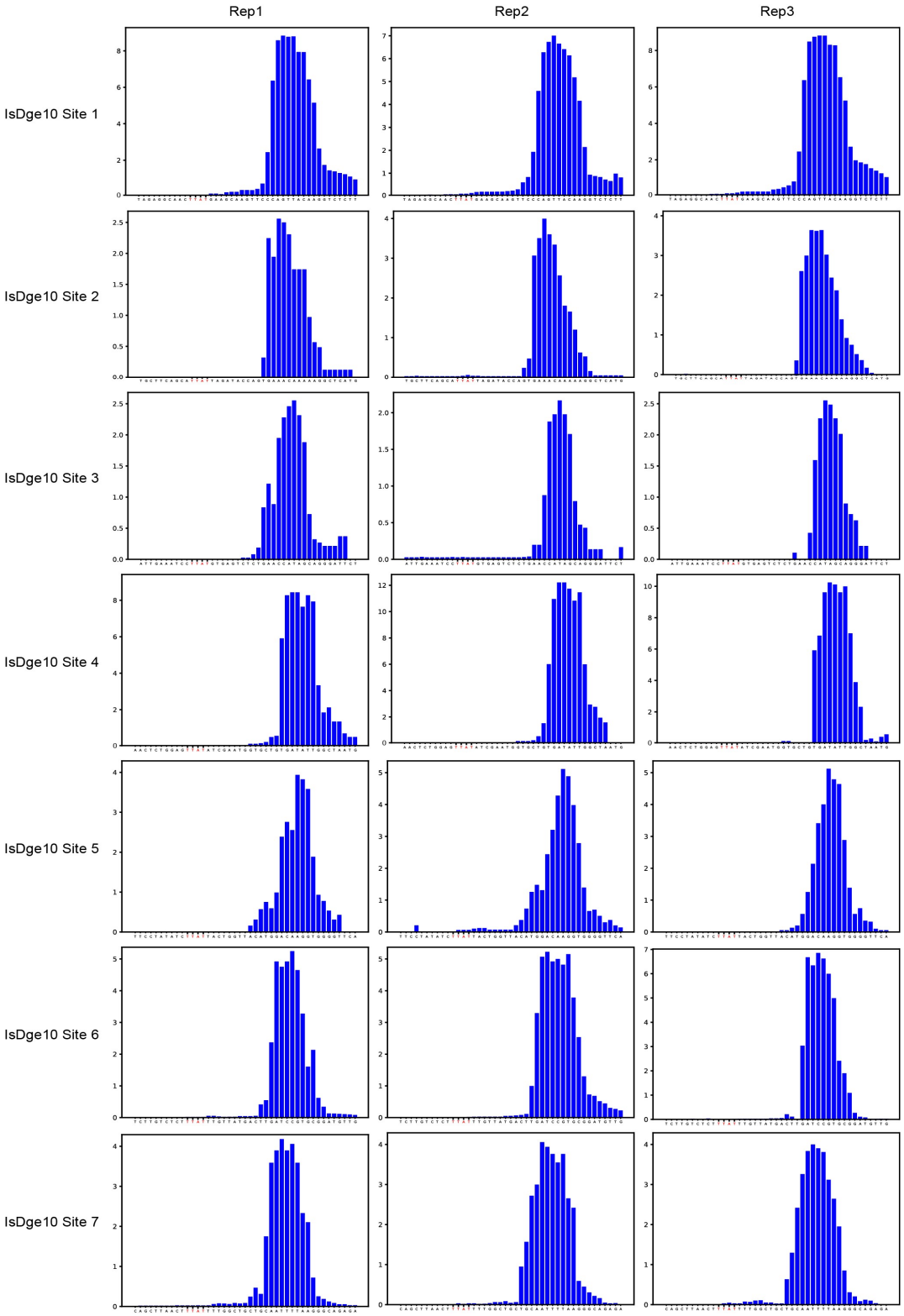
Supplementary Figure 8. Genotype of IsDge10 induced genome editing in rice T0 lines. Sequences in blue indicate the guide RNA, and sequences in red indicate the TAM.



Supplementary Figure 9. Sanger sequencing results of IsDge10 induced genome editing in rice T0 lines. Sequences in blue indicate the guide RNA, and sequences in red indicate the TAM.



Supplementary Figure 10. Deletion size profile of IsDge10 at 7 target sites in rice protoplasts using a multiple-editing strategy. Each assay contains three independent experiments (n=3). Data are presented as mean values +/- SD.



Supplementary Figure 11. Deletion position profile of IsDge10 at 7 target sites in rice protoplasts using a multiple-editing strategy. Editing at each site was biologically replicated three times, designated as Rep1, Rep2, and Rep3, respectively. Sequence in red indicated the TAM of IsDge10.

>IsDge10
CCGAAGAAGAAGAGGAAGGTTGGCATCCACGGGGTGCCAGCTGCTATGACTACCCACCGGAAAGTTTATCGCTATCGCATAGAACCAACACCAGTGCAGGAATCTAAACTGTACATGT
TGGCCGGAAGCCGCCGTTTTGTGTTCAACTGGGCGCTTGCTAGGAGGAGGGAGCACTACGCGGAGACAGGAAAAACACTCGGGTACAATGCTCAAGCAGGCGAGCTGACGGCGCTT
AAAAATCAAGAGGAGACATCCTGGTTGAAGGAGTCCGACTCACAACTGCTGCAGCAGGCTTTGAAGGATGTCGAGAGGGCTTTTGTCAATTTCTTCGAGAAGCGTGCGCGCTTTCCTAG
ATTTAAATCCAAGAAAACCGACACGCCGCGGTTCCGTATACCTCAAAGGGTCAGGATAGAGGGGAGTAGAGTGTATGTACCCAAGGTCGGGTGGGTGAAGTTACGTAAAAGCCAGGAA
ATTGAAGGCAAAACCAAGTCCGCCACCTTCAAACGCGAGGCGGATGGTCACTGGTACGTCCTCCTCGTTTCTGAATTTGAGATGCCTGATGTTCCTCTTCCCCCGGTGCCGGAGTCGG
AGGTGGTGGGCATCGACCTAGGCCTCAAAGATTTCTACGTACTGTCTGATGGCGGCCGGAAGGAAGCTCCGAGGTTTGCACGGAAGGGACAGAGGAAACTTCGGAGAGCGGCGAGG
CGACATTCAAAATGCACTCGGGGTAGCAACCGCAAGGCCAAGGCCAAAAGGAAGCTCGCCCGCGTCCACCGCCAGATTGCCAACCAACGAAAGGACTTCGTGCACAAGGCAACATCA
GGACTCGTTCAGCAGTATCAAGGGTTCTGCATCGAGAACCTATCAATTAAGGGGATGGCAAAGACAAAGCTGTCAAAGTCCGTTCTGGATGCTGCATTAGGTGAGTTCCGGAGGCAGTT
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GAGTGGACCTGCGAGTGCGGCGCCGTCCATGATCGCGACCTCAATGCCGCCAGGAACATCAAGCGGGAAGGTTTGTCGCAAATTGTTGTGGCGGGCCATGCTGAAACTCTCAACGCC
AGAGGCGAAGGGGTAAGGCCAGCGATCGCCGGCAGTCCAAGAAAGCGGCCAGCGGCGACGAAGAAGGCGGGGCAGGCGAAGAAGAAGAAG

>IsDge10 scaffold
GTGGAGCGGTTCACGACCGCGACCTCAACGCCGCCCGGAACATCAAGCGGGAAGGGCTTTCGCAAATCGTCGTCGCGGGGCACGCGGAGACGTTAAACGCTCGGGGAGAGGGTGTCAGACCTGCGATAGCGGGCAG
CCCTCGAtgaAGCGAGAATCCAACGGCTTTAGCCGTTGGAGTGTCAA

>IsAam1 protein
CCGAAGAAGAAGAGGAAGGTTGGCATCCACGGGGTGCCAGCTGCTATGGTTAACAAATCCTACAAATTTAGACTCTACCCCACAAAAGAACAAGAACAGCTGCTCGCCAAGACCTTTG
GCTGCGTGAGGTTTGTGTACAACAAGATGTTGGAGGAGCGTATACAGATCTACGAGAAGTTCAAAGACGATAAGGAGGCTCTTAAGAAACAAACATTCCCTACGCCTGCAAAGTACAAA
AAGGAATTCCCATGGCTCAAAGAGGTTGATAGCCTGGCGCTAGCTAATGCACAACTAAACCTCCAGAAGGCGTTCCAGAATTTCTTCTCTGGTCGGGCAGGTTTTCCCAAGTTTAAGAA
CCGCAAGGCCAAGCAATCATACACTACAAATGTGGTTAATGGCAACATCCAGCTTTCCGACGGCTACATTAAGTTACCAAAATTAAAGTGGGTCAAATTCAAGCAGCATAGGGAGATCC
CGGCCCACCACATCATCAAAGCCTGCACCATAAAAAAAACTAAAACTGGAAAATATTATGTGTCGATTCTGACCGAGTATGAACACCAGCCGGTGCCGAAGGAGATTCAAACTGTTGTC
GGATTGGACTTCTCCATGAACGGGCTGTTCGTGGATTCAGAAGGAAAACGAGCGAACTATCCACGCTTCTACCGGCAGGCCCTCGAAAAGTTGGCCAAGGAGCAGCGCATACTGTCG
CGTCGCAAGAAGGGTAGTAACAGATGGCACAAGCAGAGACTGAAGGTGGCTAAGCTCCACGAGAAGATCGCAAATCAGAGGAAGGATTTTCTTCATAAAAAGAGTTATGAGCTGGCCA
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GGCGAAGAAGAAGAAG

>IsAam1 scaffold
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GAAGCCCCCACCTCTAAGCGAAGCGTAGGTGGTGGGAGCATGTCAC

>enIscB protein
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CAGAAAATCCTGCCGGTCGCCAAGGTTGTTCTGGAGCTGAACCGTTTCTCCTTCATGGCAATGAATAACCCCAAGGTTCAACGGTGGCAGTACCAGCGCGGCCGCTCTACGGCAAAG
GCAGCGTAGAGGAGGCCGTGTCCATGCAGCAGGACGGCCACTGCCTGTTCTGCAAGCATGGAATCGACCACTACCACCACGTCGTTCCACGACGCAAAAATGGAAGTGAAACTTTGG
AGAATAGAGTCGGCCTTTGTGAGGAGCACCACAGGCTCGTCCACACTGACAAAGAGTGGGAGGCGAACCTAGCCTCCAAGAAGTCCGGCATGAACAAGAAGTATCATGCTTTATCTGT
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TCATTATTTGGACGCCTACTGCATTGCTTGTTCTGCTCTCACCGACGCAAAAAGGTCAGCTCGCCCAAAGGTCGTCCTTATATGGTGAGGCAGTTTAGGAGGCACGATAGGCAAGCAT
GCCATAAAGCTAATCTTAACAGGAGATATTACATGGGCGGCAAGCTCGTGGCCACCAACCGGCATAAGGCCATGGATCAAAAGACTGACAGCCTCGAAGAGTACCGCGCCGCTCATTC
TGCGGCCGATGTGTCAAAGCTGACGGTGAAGCACCCGTCGGCACAGTACAAGGATATGTCAAGGATCATGCCGGGCTCCATACTTGTTAGTGGGGAGGGGAAACTTTTACCCTACGT
CGGTCGGAAGGTAGGAACAAAGGACAGGTGAACTACTTCGTGTCGACGGAGGGCATCAAGTACTGGGCGCGCAAGTGCCAGTATCTGCGGAATAATGGGGGTCTGCAGATATATGTT
AAGCGGCCAGCGGCGACGAAGAAGGCGGGGCAGGCGAAGAAGAAGAAG

>enIscB scaffold
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CAGGGGGAACAATCACCTCTCTGTATCAGAGAGAGTTTTACAAAAGGAGGAACGG

>SpuFz1 protein
CCGAAGAAGAAGAGGAAGGTTGGCATCCACGGGGTGCCAGCTGCTATGCCTCCCAAGAAAAAGCAGAAGCTGGAGCGGTTGAAGAAGCTCGACAAACCTACTTTGCACACGTGCAAT
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GGTGAATGTTCAGGAGCACTTCATGCAGATGCTGCTGAGGTATATCAATTTAAGATTTGACGTGAAGGGACAGAAACAAAGGCTCCCGCCCAAATCCGACGCCCGGAAGGCATTCTTTA
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CCATTTCTGCCGAATGACCCTCTTGCCTACGCGATAGTCGCCGATCCGATGTCTTTCTTTCCGGCTTATTGCAAGCTATCGGGCCTGTACGAGCAGTATGGTTTTCAAAGGTTTTCTGCC
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GGCGATGAAGGAGGAAGCCGGCGTCGATCTTATTGAAAGTCGAATACCGTCGCACAAGACGATGAACCTCATGGACTTCACTCGTTACCTGCTCGTCCGGCGGGCGGACTGGGACCG
CAGAAAGGAATTCTACTCGCACCCCGCGCACACAAGGTGGAAGTGGCACTCCTTCATCAACAGACAGAAGTCCGAGTCCAAGCTCATCAGCAACATGAGGAACAAATATGGACGGAAC
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TAGATGAGTACAAGACAAGTTCAGTCTGTCCAAGATGCTCTAGCTCCGAGTTCGTCGAAAAAAAGTTCAAGACCCGCCCTCACTCTAGGCCGTGGAGGCGGAGGGAGGGGAAAATTGA
GAAGGTTCACGGCCTACTCGGTTGTACCAACCCAAACTGCCTGCAACAGGCGTGGACATCAGGAATGCGGTACTGGAACAGGGACATGCTCTCAACATGTAATATGTTGCTCATTGTA
CGGAGCATGCTTGACGGGCATGGCCGGCCGGAAGTTTTCTCGCGCTCTGTGCCTGCAGTGGCGAAGCGGCCAGCGGCGACGAAGAAGGCGGGGCAGGCGAAGAAGAAGAAG

>SpuFz1 scaffold
GAGTTTGTGGAGAAAAAATTCAAAACACGACCTCATTCAAGACCTTGGCGCCGTCGTGAAGGCAAGATTGAAAAAGTCCACGGACTGCTGGGTTGTACCAACCCTAACTGTTTGCAGCAAGCCTGGACATCGGGAA
TGCGCTACTGGAATCGAGATATGCTGTCAACCTGCAACATGCTATTGATTGTGAGATCAATGTTGGATGGACACGGTAGACCAGAAGTGTTCAGCAGGAGTGTTCCAGCCGTAGCGTAGAACGTAGATTGGTTAGA
TTAGGTTTTCCGAGCCGGTTGTCGCGCGGTTCAATCCCTGGTGCGGGTGCTAGTGCCAATACCCACCGGCTCCGCACTA

Supplementary Figure 12. Nucleotide sequences encoding IsDge10, IsAam1, enIscB and SpuFz1 codon-optimized for rice. Sequences in blue indicate the nuclease-coding sequences, and sequences in red indicate the coding regions for nuclear localization signals (NLS).

Supplementary Table 1. Guide RNAs used in this study.

Targeted gene	Targeted site	Spacer sequence
mCherry	IsDge10-mCherry-gRNA	cctcctcgcccttgctcacc
mCherry	IsAam1-mCherry-gRNA	gtgggagcgcgtgatgaact
mCherry	IsDra2-mCherry-gRNA	gttgacgttgtaggcgccgg
mCherry	IsYmu1-mCherry-gRNA	gttgacgttgtaggcgccgg
mCherry	enIscB-mCherry-gRNA	ggtggccccctgcccttcgc
mCherry	SpuFz1-mCherry-gRNA	tggagggctccgtgaacggc
LOC_Os01g01830	IsDge10-Site1	gaagcaagttcccagttaca
LOC_Os03g02150	IsDge10-Site2	tagataccagtgaaacaaaa
LOC_Os05g01090	IsDge10-Site3	gtgagtctctgaaccatagc
LOC_Os07g02300	IsDge10-Site4	atcgaatggtgctgtgatat
LOC_Os09g04110	IsDge10-Site5	tactggttacatggacaagg
LOC_Os11g01380	IsDge10-Site6	ttgttatgacttgatccgtg
LOC_Os12g01520	IsDge10-Site7	tttggctgctgcaattttaa
LOC_Os01g04200	IsAam1-Site1	tgaaaggacaactctaggaa
LOC_Os03g01920	IsAam1-Site2	tatctctgcgttgaacacaa
LOC_Os05g02880	IsAam1-Site3	ctgtgggattgatggtcact
LOC_Os07g04160	IsAam1-Site4	atttaagattggatcaggac
LOC_Os09g01680	IsAam1-Site5	aaattaataaggaccctctc
LOC_Os11g03794	IsAam1-Site6	gcaaggaccattgctttctg
LOC_Os12g03899	IsAam1-Site7	tattaggttagcacagcatg
LOC_Os01g01830	SpuFz1-Site1	gcttatggcctaagtgaaag
LOC_Os03g01420	SpuFz1-Site2	gaggctgccaccgccaacgc
LOC_Os05g01520	SpuFz1-Site3	tcaccagggaacatcaattc
LOC_Os07g01890	SpuFz1-Site4	tatatgtgatagataatgga
LOC_Os09g02130	SpuFz1-Site5	cgtgacagaaagggttactg
LOC_Os11g01872	SpuFz1-Site6	tggagaaactatactcaaaa
LOC_Os12g02260	SpuFz1-Site7	attaaataacctggtggaca
LOC_Os06g02490	enIscB-Site1	GACAGATACAAATGGGATGC
LOC_Os02g03700	enIscB-Site2	TTCGTGTCGGCGTTATCAAG
LOC_Os07g12820	enIscB-Site3	TAGTGGCTGAATATATTTCT
LOC_Os03g11614	enIscB-Site4	TAATTTGGGACAAAGAACCA
LOC_Os06g30310	enIscB-Site5	CTTGCAGGGTTCAGAAACCT
LOC_Os02g18850	enIscB-Site6	GCCGGCGGTGGGGTGGGGTT
LOC_Os04g52479	enIscB-Site7	ACAGCAGTGGGATTCCGCAT
LOC_Os01g01830	IsDge10-Site1-mm01	ctagcaagttcccagttaca
LOC_Os01g01830	IsDge10-Site1-mm02	gatccaagttcccagttaca
LOC_Os01g01830	IsDge10-Site1-mm03	gaaggtagttcccagttaca
LOC_Os01g01830	IsDge10-Site1-mm04	gaagcatcttcccagttaca
LOC_Os01g01830	IsDge10-Site1-mm05	gaagcaagaacccagttaca
LOC_Os01g01830	IsDge10-Site1-mm06	gaagcaagttggcagttaca
LOC_Os01g01830	IsDge10-Site1-mm07	gaagcaagttccgtgttaca
LOC_Os01g01830	IsDge10-Site1-mm08	gaagcaagttcccacataca
LOC_Os01g01830	IsDge10-Site1-mm09	gaagcaagttcccagtatca
LOC_Os01g01830	IsDge10-Site1-mm10	gaagcaagttcccagttagt

Supplementary Table 2. Oligos used in this study.

Primer Name	Sequence (5'-3')	Purpose
lsDeg10-01-HTS-F	aagagatgtgctcgaatcagc	*Primer for HTS at lsDge10-Site1*
lsDeg10-01-HTS-R	tcttcatttctgggatcgca	
lsDeg10-02-HTS-F	atggctggtactagcagaataag	*Primer for HTS at lsDge10-Site2*
lsDeg10-02-HTS-R	gtgtattcttgtgggatatcttgg	
lsDeg10-03-HTS-F	tgcatgtttgttgacagaaaaga	*Primer for HTS at lsDge10-Site3*
lsDeg10-03-HTS-R	aaccttgcaatgcgattgtat	
lsDeg10-04-HTS-F	tttgcttttcttatctggctttgtt	*Primer for HTS at lsDge10-Site4*
lsDeg10-04-HTS-R	catcaccatgtgataccaaagttg	
lsDeg10-05-HTS-F	gcaagaggtagctgtccagc	*Primer for HTS at lsDge10-Site5*
lsDeg10-05-HTS-R	taccggttgggaatcgaggc	
lsDeg10-06-HTS-F	gaaacaagcaagctcacctg	*Primer for HTS at lsDge10-Site6*
lsDeg10-06-HTS-R	ctgttcgtaaatgaatagtcccaa	
lsDeg10-07-HTS-F	gtagccttctcatctgtaactatctt	*Primer for HTS at lsDge10-Site7*
lsDeg10-07-HTS-R	cctgaagatgcgatgaccag	
lsAam1-01-HTS-F	tgcaagttgtttttgctcct	*Primer for HTS at lsAam1-Site1*
lsAam1-01-HTS-R	gacataaatttgaactaccacaaaca	
lsAam1-02-HTS-F	aactacatagtgatagcctattgaca	*Primer for HTS at lsAam1-Site2*
lsAam1-02-HTS-R	tttgccatgtctatatggcac	
lsAam1-03-HTS-F	gatcaacttatcaatcaatacctgaga	*Primer for HTS at lsAam1-Site3*
lsAam1-03-HTS-R	tgttgactgatgctcttttcaac	
lsAam1-04-HTS-F	tcaataatattggaacgctttgca	*Primer for HTS at lsAam1-Site4*
lsAam1-04-HTS-R	gagcatttgtatacgaacaattgaat	
lsAam1-05-HTS-F	atgctcatatgctagcttcttt	*Primer for HTS at lsAam1-Site5*
lsAam1-05-HTS-R	acttaacctgcaattatacagcg	
lsAam1-06-HTS-F	caatgcgggcgaagtatgag	*Primer for HTS at lsAam1-Site6*
lsAam1-06-HTS-R	cattgtctgatgaagttccaatgt	
lsAam1-07-HTS-F	tctctgcaggctgccataaa	*Primer for HTS at lsAam1-Site7*
lsAam1-07-HTS-R	aacatgaagctcttgcattgt	
enIscB-01-HTS-F	AAATCATGACCTTTCAAGTTCCAA	*Primer for HTS at enIscB-Site1*
enIscB-01-HTS-R	TGGGAGTTTGCAGATATGAC	
enIscB-02-HTS-F	TTTCTGGGTCGGTATCGGGA	*Primer for HTS at enIscB-Site2*
enIscB-02-HTS-R	CCCCTGAAACCATACTCCTG	
enIscB-03-HTS-F	CGAAATCTGACCATATCCTGCC	*Primer for HTS at enIscB-Site3*
enIscB-03-HTS-R	CATTCATGAAGTAAGACAGGGTG	
enIscB-04-HTS-F	CTATGAATATGAAATTAGCATC	*Primer for HTS at enIscB-Site4*
enIscB-04-HTS-R	ATTTACTGTACGAGGTTAATGAAAG	
enIscB-05-HTS-F	CAGTTCCAGACACTTCCAGC	*Primer for HTS at enIscB-Site5*
enIscB-05-HTS-R	ATGATCCTGTAGGCCTTGGA	
enIscB-06-HTS-F	ATTACTGTTCTTGCTCGAGTTC	*Primer for HTS at enIscB-Site6*
enIscB-06-HTS-R	CTCGCTGTCCATCTCCGAGA	
enIscB-07-HTS-F	ATTGTTGCCAAGGCACCCTGG	*Primer for HTS at enIscB-Site7*
enIscB-07-HTS-R	GGAGATTAAGTTTCCGCACC	
SpuFz1-01-HTS-F	tgggacagttgctctttcga	*Primer for HTS at SpuFz1-Site1*

SpuFz1-01-HTS-R	cacctgcaccaataattgatgga	
SpuFz1-02-HTS-F	gttccatgggccctcaaaga	*Primer for HTS at SpuFz1-Site2*
SpuFz1-02-HTS-R	cacaccaggcgtatgttcct	
SpuFz1-03-HTS-F	gcatggcgcatgttttcctt	*Primer for HTS at SpuFz1-Site3*
SpuFz1-03-HTS-R	tccagtcctctgaagaaaggt	
SpuFz1-04-HTS-F	cgacatcataccaaatgtgccc	*Primer for HTS at SpuFz1-Site4*
SpuFz1-04-HTS-R	acagctcttggatcccatca	
SpuFz1-05-HTS-F	agggatggagggagaatagtt	*Primer for HTS at SpuFz1-Site5*
SpuFz1-05-HTS-R	ccttcagcttgagcctacca	
SpuFz1-06-HTS-F	gaccagcacttcgatagcct	*Primer for HTS at SpuFz1-Site6*
SpuFz1-06-HTS-R	cacaagttatctgacagaggcct	
SpuFz1-07-HTS-F	tatgtgccagttccacgagc	*Primer for HTS at SpuFz1-Site7*
SpuFz1-07-HTS-R	gctcaagggttccaccaaga	