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Supplemental information

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Running title: Hypercompact IsDge10 enables genome editing in rice

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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.

Reference

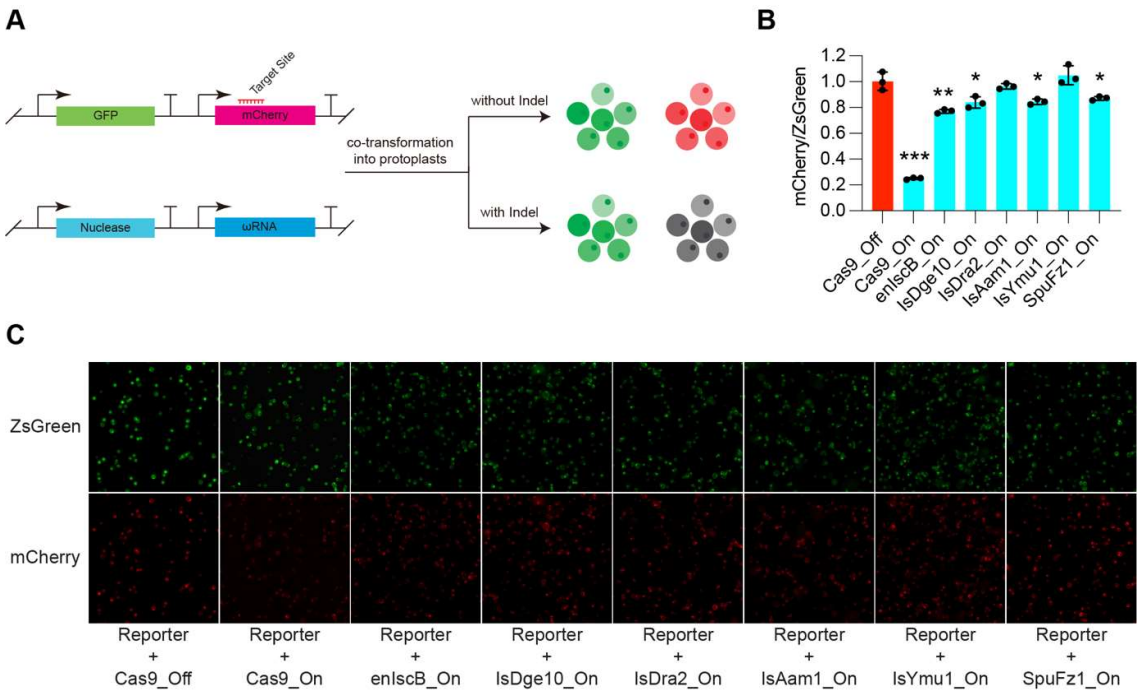
Han, Y., Liu, G., Wu, Y., Bao, Y., Zhang, Y., and Zhang, T. (2024). CrisprStitch: Fast evaluation of the efficiency of CRISPR editing systems. Plant Commun **5**:100783. 10.1016/j.xplc.2023.100783.

Stewart, C.N., Jr., and Via, L.E. (1993). A rapid CTAB DNA isolation technique useful for RAPD fingerprinting and other PCR applications. Biotechniques **14**:748-750.

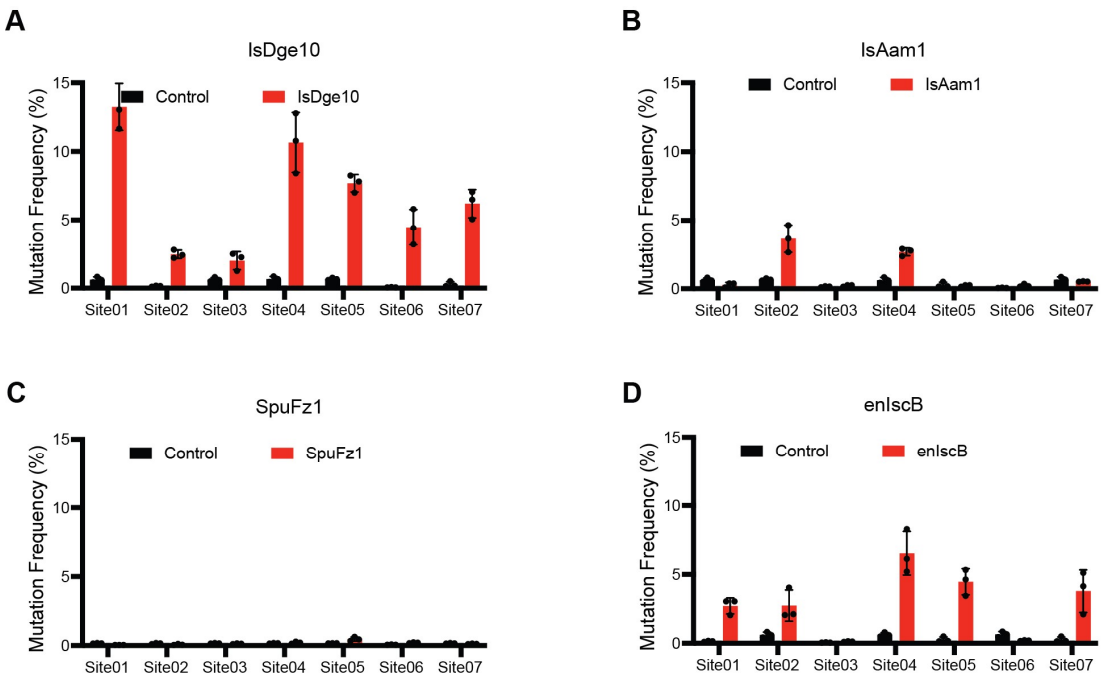
Tang, X., Ren, Q., Yang, L., Bao, Y., Zhong, Z., He, Y., Liu, S., Qi, C., Liu, B., Wang, Y., et al. (2019). Single transcript unit CRISPR 2.0 systems for robust Cas9 and Cas12a mediated plant genome editing. Plant Biotechnol J **17**:1431-1445. 10.1111/pbi.13068.

You, Q., Zhong, Z., Ren, Q., Hassan, F., Zhang, Y., and Zhang, T. (2018). CRISPRMatch: an automatic calculation and visualization tool for high-throughput CRISPR genome-editing data analysis. Int J Biol Sci **14**:858-862. 10.7150/ijbs.24581.

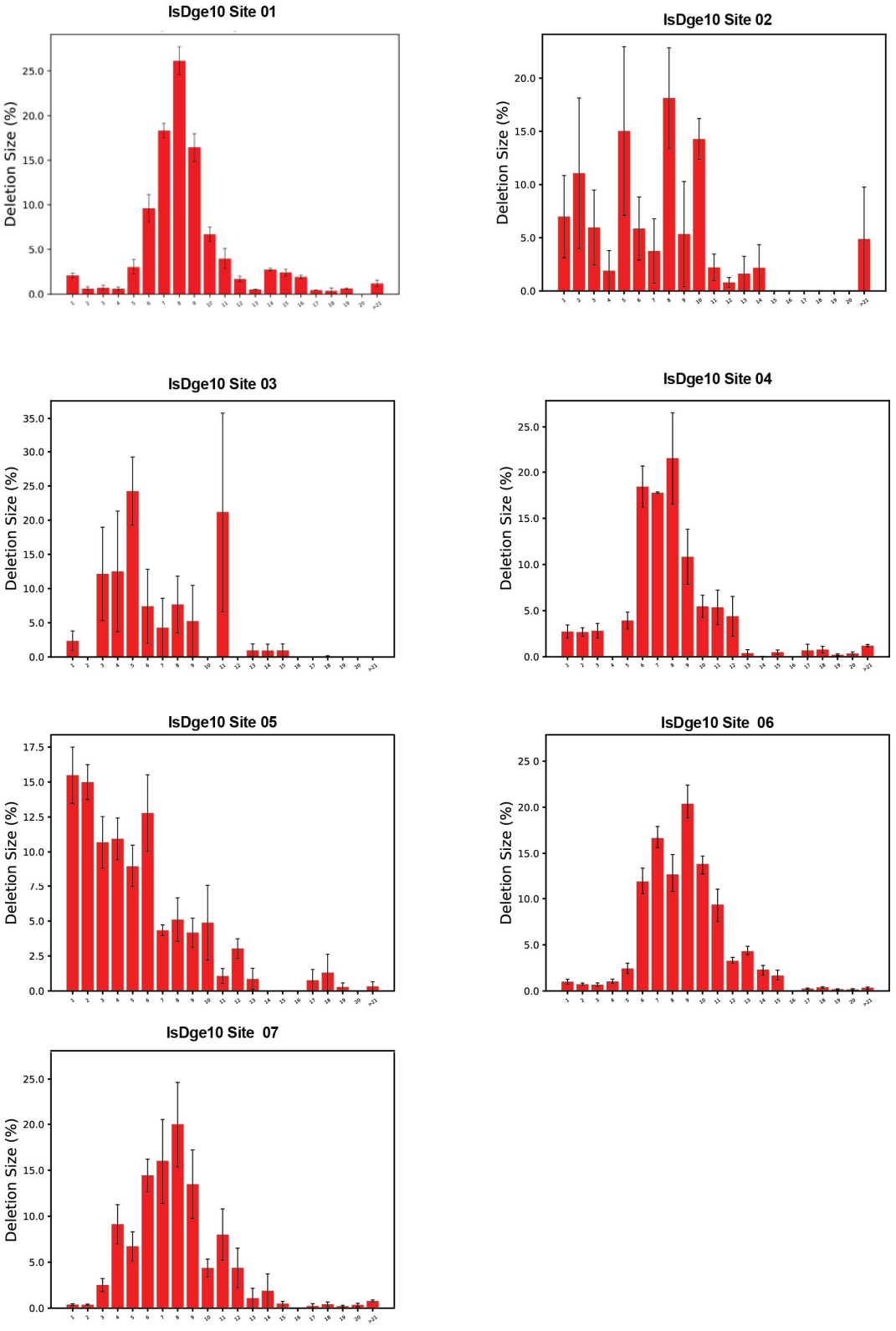
Zhou, J., Deng, K., Cheng, Y., Zhong, Z., Tian, L., Tang, X., Tang, A., Zheng, X., Zhang, T., Qi, Y., et al. (2017). CRISPR-Cas9 based genome editing reveals new insights into MicroRNA function and regulation in rice. Front Plant Sci **8**:1598. 10.3389/fpls.2017.01598.



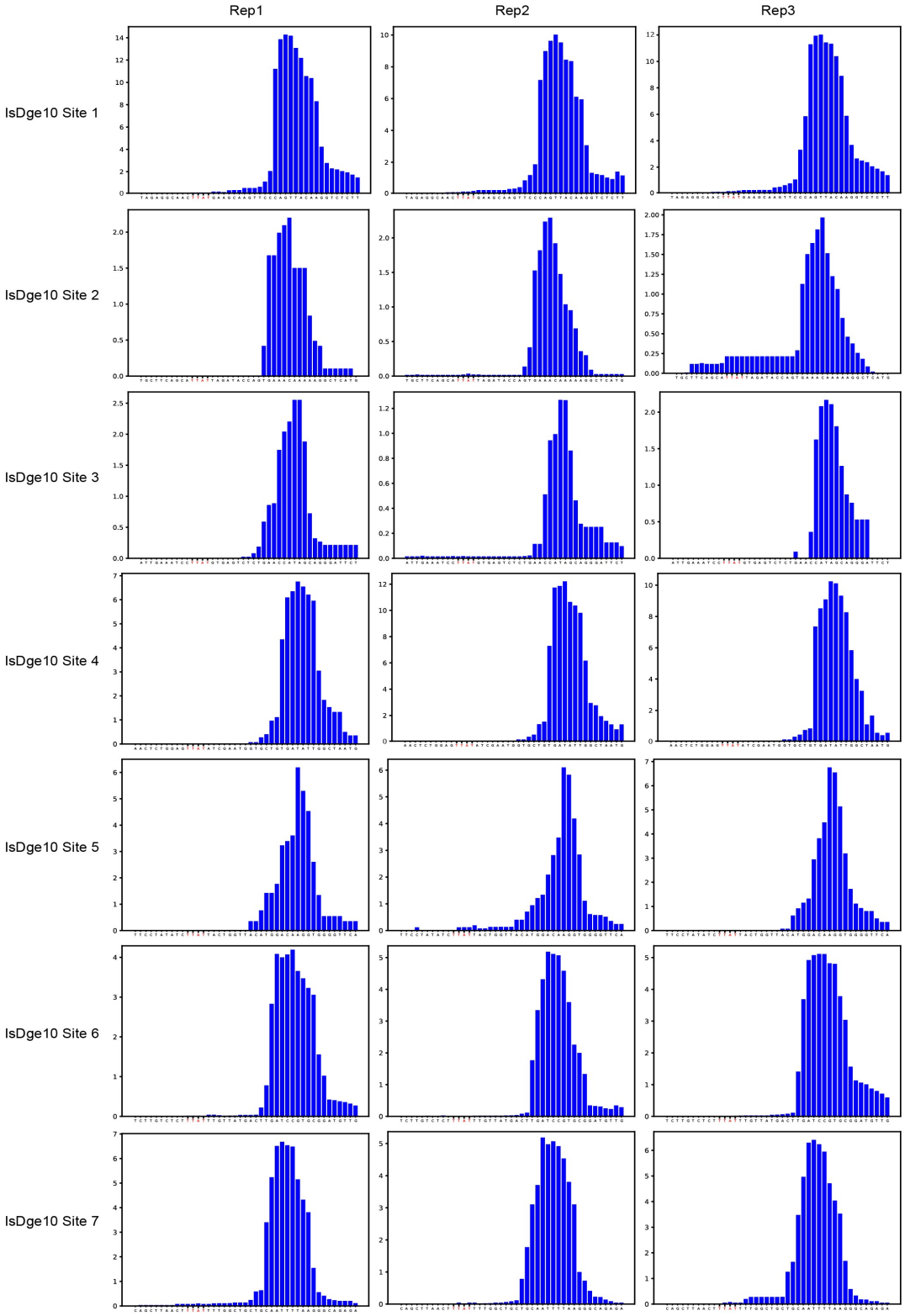
Supplementary Figure 1. Detection of genome editing activity with a florescent report assay in rice protoplasts. (A) A diagram of a dual-fluorescence reporter system. (B) The decrease of mCherry fluorescence induced by targeted mutagenesis of different editing systems. (C) Decrease of mCherry fluorescence 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.



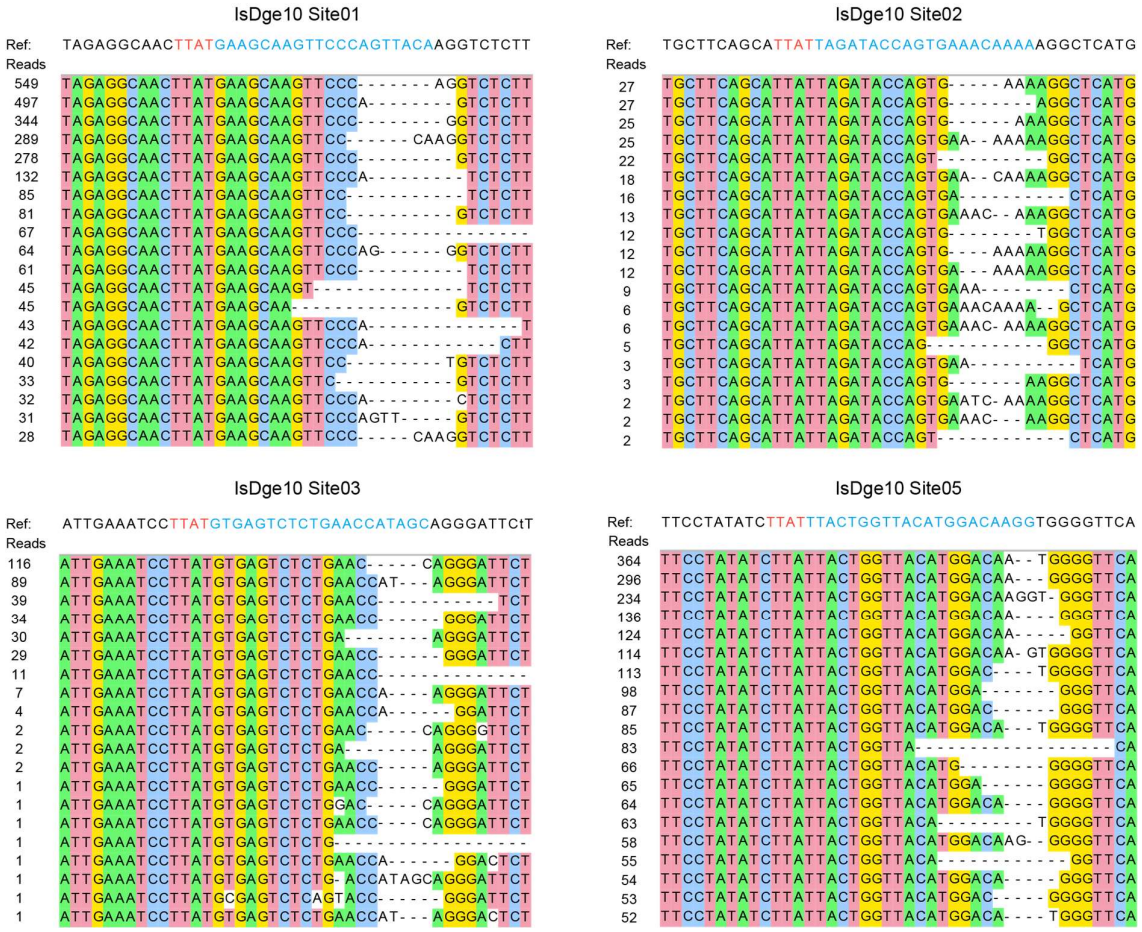
Supplementary Figure 2. Comparison of different nucleases at rice endogenous sites in rice protoplasts. (A) Editing efficiency of the IsDge10 system at seven endogenous sites in rice. (B) Editing efficiency of the IsAam1 system at seven endogenous sites in rice. (C) Editing efficiency of the SpuFz system 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.



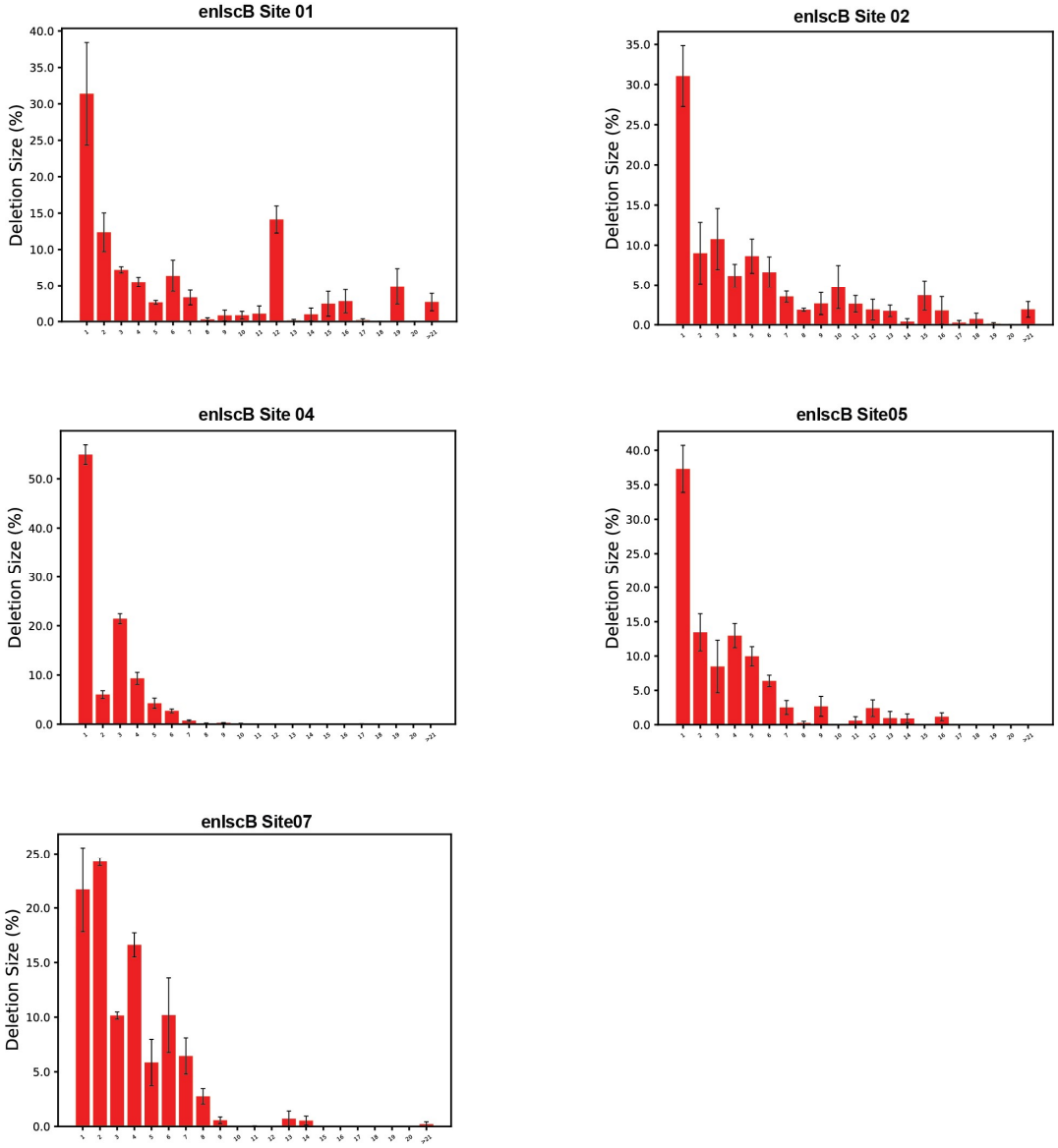
Supplementary Figure 3. Deletion size profile of IsDge10 at 7 target sites in rice protoplasts. Each assay contains three independent experiments (n=3). Data are presented as mean values +/- SD.



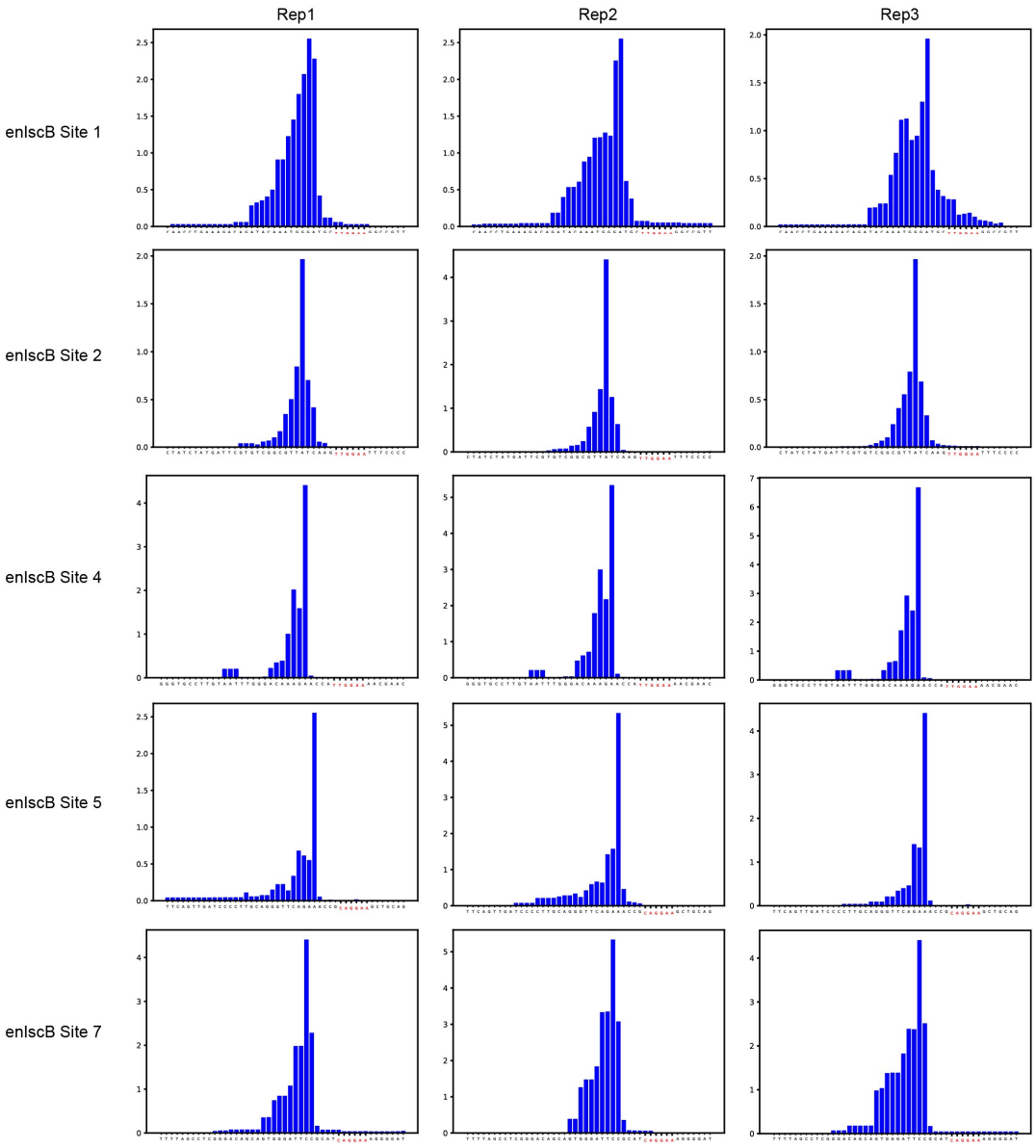
Supplementary Figure 4. Deletion position profile of IsDge10 at 7 target sites in rice protoplasts. Editing at each site was biologically replicated three times, designated as Rep1, Rep2, and Rep3, respectively. Sequence in red indicated the TAM of IsDge10.



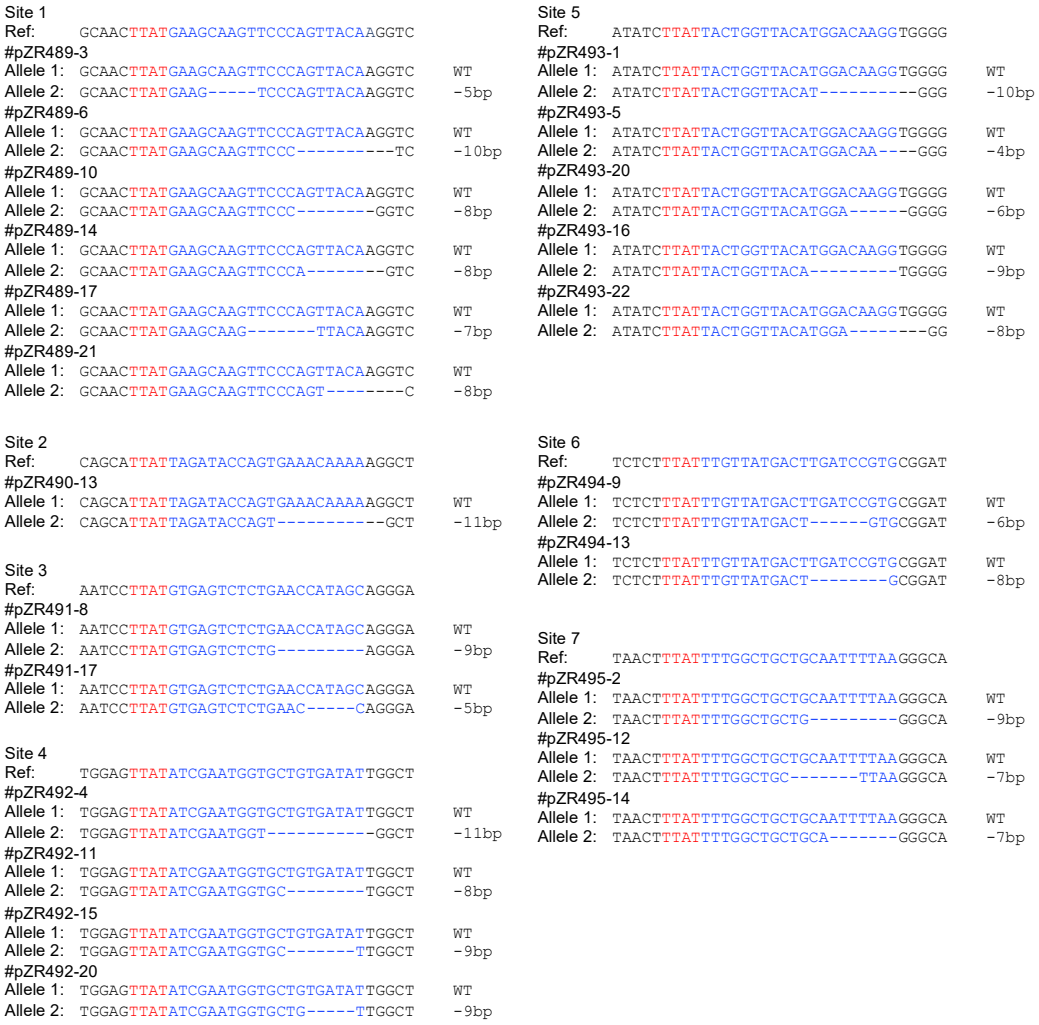
Supplementary Figure 5. Sequence alignment results of editing events of IsDge10 at 4 target sites in rice protoplasts. Sequences in blue indicate the guide RNA, and sequences in red indicate the TAM of IsDge10.



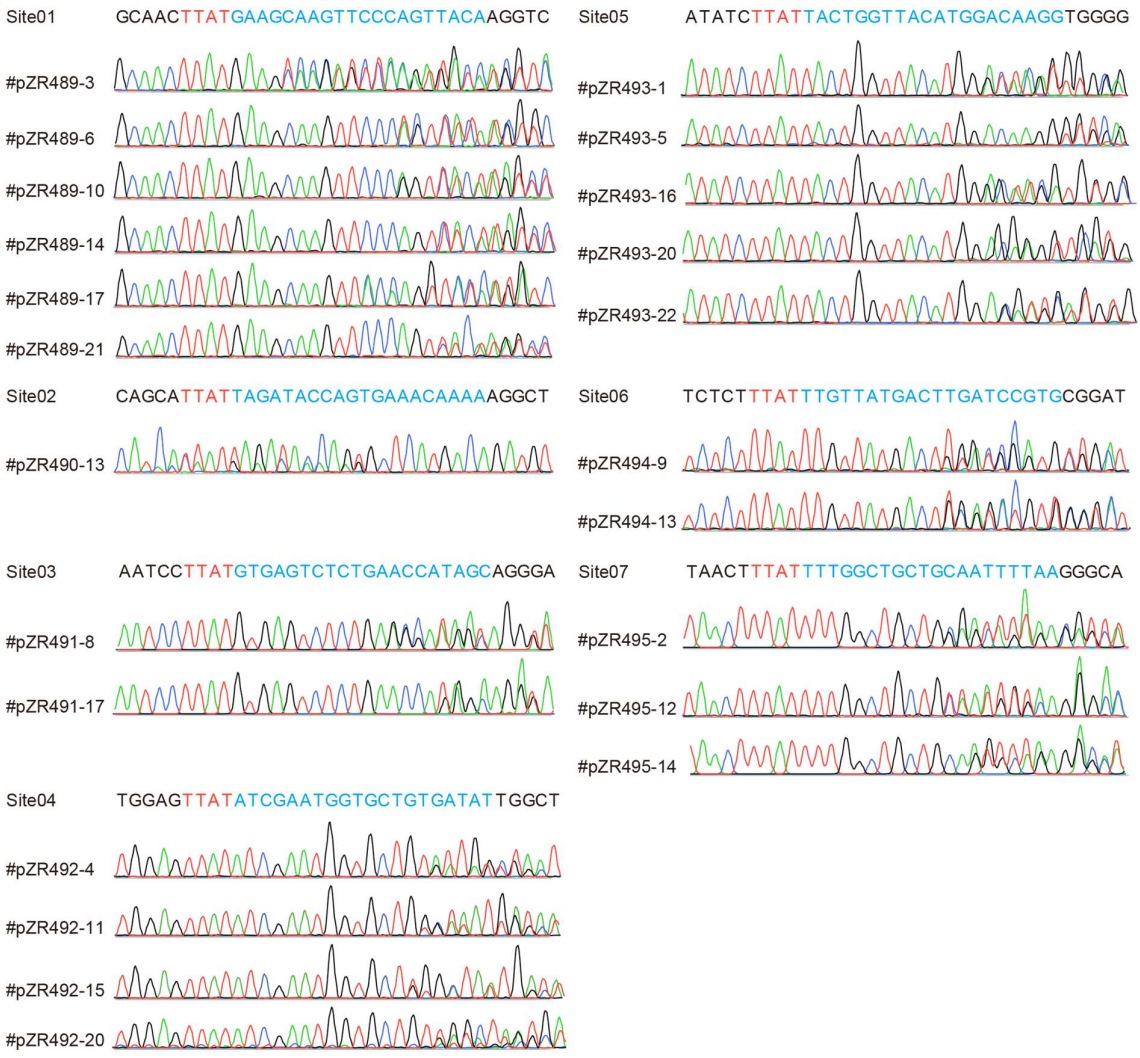
Supplementary Figure 6. Deletion size profile of enIscB at 5 target sites in rice protoplasts. Each assay contains three independent experiments (n=3). Data are presented as mean values +/- SD.



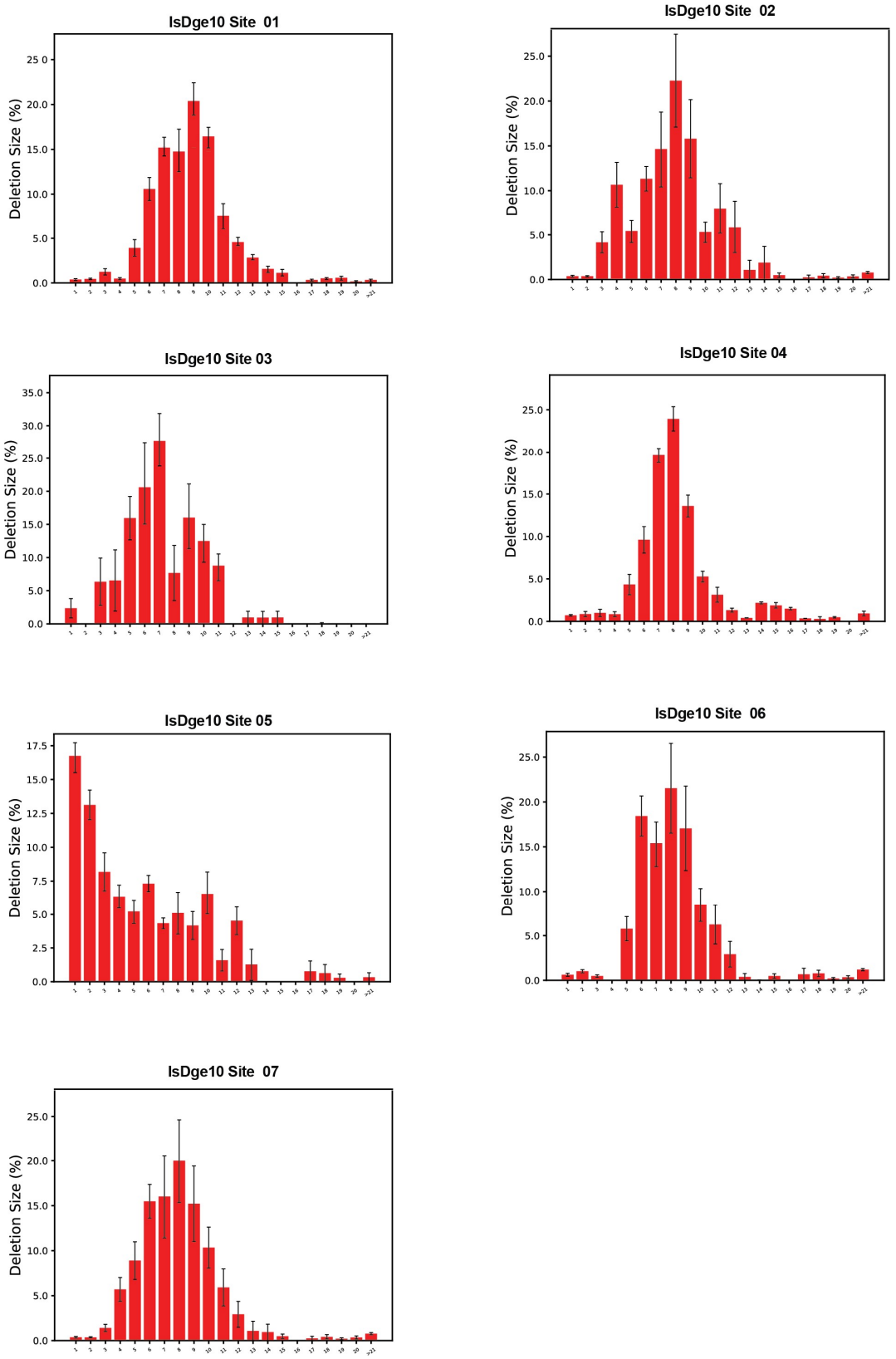
Supplementary Figure 7. Deletion position profile of enIscB at 5 target sites in rice protoplasts. Editing at each site was biologically replicated three times, designated as Rep1, Rep2, and Rep3, respectively. Sequence in red indicated the TAM of enIscB.



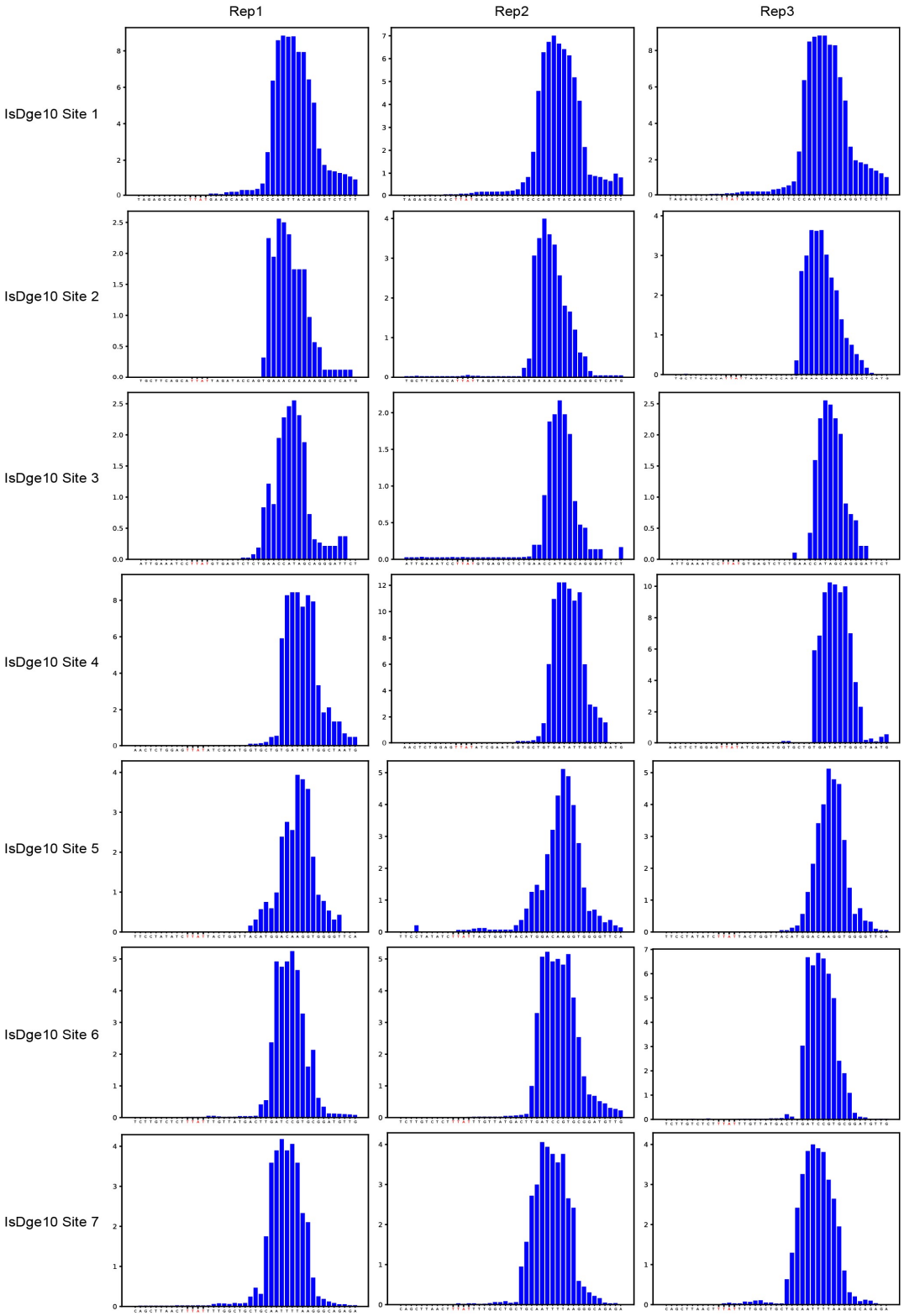
Supplementary Figure 8. Genotype of IsDge10 induced genome editing in rice T0 lines. Sequences in blue indicate the guide RNA, and sequences in red indicate the TAM.



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
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>IsDge10 scaffold
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>IsAam1 protein
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>IsAam1 scaffold
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>enIscB protein
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>enIscB scaffold
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>SpuFz1 protein
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>SpuFz1 scaffold
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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	