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RAPID COMMUNICATION

Expanding the targeting scope of FokI-dCas nuclease systems with SpRY and Mb2Cas12a

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Abstract

CRISPR-Cas9 and Cas12a are widely used sequence-specific nucleases (SSNs) for genome editing. The nuclease domains of Cas proteins can induce DNA double strand breaks upon RNA guided DNA targeting. Zinc finger nucleases (ZFNs) and Transcription Activator-Like Effector Nucleases (TALENs) have been popular SSNs prior to CRISPR. Both ZFNs and TALENs are based on reconstitution of two monomers with each consisting of a DNA binding domain and a FokI nuclease domain. Inspired by the configuration of ZFNs and TALENs, dimeric FokI-dCas9 systems were previously demonstrated in human cells. Such configuration, based on a pair of guide RNAs (gRNAs), offers great improvement on targeting specificity. To expand the targeting scope of dimeric FokI-dCas systems, the PAM (protospacer adjacent motif)-less SpRY Cas9 variant and the PAM-relaxed Mb2Cas12a system were explored. Rice cells showed that FokI-dSpRY had more robust editing efficiency than a paired SpRY nickase system. Furthermore, a dimeric FokI-dMb2Cas12a system was developed that displayed comparable editing activity to Mb2Cas12a nuclease in rice cells. Finally, a single-chain FokI-FokI-dMb2Cas12a system was developed that cuts DNA outside its targeting sequence, which could be useful for many versatile applications. Together, this work greatly expanded the FokI based CRISPR-Cas systems for genome editing.

KEYWORDS
FokI-dCas, genome editing, Mb2Cas12a, rice, SpRY

1 | INTRODUCTION

CRISPR-Cas based genome editing systems have revolutionized genetics, agriculture, biotechnology, and medicine. CRISPR-Cas systems are RNA-guided DNA nucleases that can induce target-specific DNA double-strand breaks (DSBs).[1,2] Repair of SSN-induced DSBs by the predominant nonhomologous end joining (NHEJ) pathway in most eukaryotic organisms and cell types could lead to insertion and deletion (indel) mutations. Other sequence-specific nucleases (SSNs) prior to CRISPR-Cas systems include zinc finger nuclease (ZFN) and TAL effector nuclease (TALEN).[3,4] Both ZFN and TALEN work as dimers, with each monomer consisting of a DNA binding domain and a FokI nuclease domain (hereafter, FokI).[5,6] This dimeric configuration confers high targeting specificity of ZFN and TALEN.[7] By contrast, DNA targeting of CRISPR-Cas systems such as Cas9, Cas12a, and Cas12b is guided by a guide RNA (gRNA), with the specificity defined by ~20 nt protospacer.[2,8,9] The intrinsic nuclease activity of aforementioned Cas proteins and the relatively short length of protospacer for

DNA targeting make the CRISPR-Cas system potentially more prone for inducing off-target mutations.

The Cas9 system, especially *Streptococcus pyogenes* Cas9 (SpCas9), is the most widely used CRISPR system for genome editing.[2,10] Different strategies have been applied to enhance targeting specificity of Cas9. One strategy is to engineer high fidelity Cas9 variants that are more specific.[11–13] A second strategy is based on a paired nickase system where a Cas9 nickase is guided by a pair of gRNAs to create simultaneous nicking at both strand of DNA, resulting in a DNA DSB.[14] Paired Cas9 nickase systems have been demonstrated in both human cells[15,16,14] and plants.[17] However, Cas9 nickases are still able to induce off-target mutations due to its single strand DNA cleavage activity.[15,16,14] To eliminate the potential off-target effects resulting from the nickase activity, a third strategy was developed, which is based on fusion of FokI to the deactivated Cas9 (dCas9) and DNA targeting through paired gRNAs.[18–20] Such dimeric FokI-dCas9 systems work fundamentally on the same principle of ZFN and TALEN, but bypass protein engineering of DNA binding domains as the DNA targeting is through paired gRNAs. Dimeric FokI-dCas9 systems were developed and evaluated for genome editing in human cells, demonstrating higher targeting specificity than Cas9 or paired Cas9 nickase systems.[18–20] The targeting scope of such dimeric FokI-dCas9 systems however is limited by the NGG protospacer adjacent motif (PAM) of SpCas9.

SpRY was recently engineered to confer near-PAM-less genome editing in human cells[21] and plants.[22–25] SpRY could greatly expand the targeting scope of the FokI-dCas9 system. Besides CRISPR-Cas9, CRISPR-Cas12a (formerly Cpf1) has emerged as a robust and highly specific CRISPR system for genome editing at TTTV PAM sites.[26–30,9] Interestingly, MbCas12a, a Cas12a ortholog, was shown to have a relaxed TTV PAM requirement.[31] In this study, we wanted to expand the FokI based CRISPR genome editing toolbox with new targeting capability and flexibility. First, we applied PAM-less SpRY to develop FokI-dSpRY for PAM-relaxed genome editing. We then harnessed the dimeric FokI strategy to develop FokI-dMb2Cas12a systems for genome editing at T-rich PAM sites. Finally, we developed a single-chain FokI-FokI-dMbCas12a system that rendered genome editing mediated by only a single gRNA. While we developed and tested these tools in rice cells, they should be widely applicable to genome editing in eukaryotic cells.

2 | EXPERIMENTAL SECTION

2.1 | Vector construction

The maize codon-optimized pYPQ166-SpRY (Addgene #161520) and rice codon-optimized pYPQ284 (Addgene #138116) were used to generate attL1-attR5 Cas entry clones in this study. Mutations were introduced to pYPQ166-SpRY harboring engineered Cas9-SpRY and pYPQ284 harboring Mb2Cas12a to generate dCas9-SpRY (D10A, H840A), Cas9-SpRY-D10A, and dMb2Cas12a (D864A) using the Q5 Site-Directed Mutagenesis Kit (New England Biolabs). The rice codon-optimized FokI with linkers was synthesized as synthetic DNA-gBlock by IDT. The FokI and linker fragments were cloned into the N terminal end of dCas9-SpRY and dMb2Cas12a at BsrGI and NcoI sites by T4 ligation to generate plasmids pYPQ-NF1-L1-dSpRY (Addgene #177681), pYPQ-NF1-L2-dSpRY (Addgene #177682), pYPQ-NF2-L1-dSpRY, pYPQ-NF2-L2-dSpRY, pYPQ-NF-L1-dMb2Cas12a (Addgene #177683), and pYPQ-NF-L2-dMb2Cas12a (Addgene #177684). To generate plasmids pYPQ-dMb2Cas12a-L1-CF and pYPQ-dMb2Cas12a-L2-CF, L1-CF and L2-CF fragments were PCR-amplified with primers CF-for and CF-rev from GBK-L1-CF and GBK-L2-CF by Q5 High-Fidelity DNA Polymerase (New England Biolabs) and inserted into the C terminal end of dMb2Cas12a at SpeI and NotI sites using NEBuilder HiFi DNA Assembly kit (New England Biolabs). For the single-chain Fok1-dMb2Cas12a systems, the two FokI domains were amplified from GBK-NF-L1 and GBK-NF-L2, and digested by SpeI, NcoI and BsrGI, AgeI, respectively. The linkers between two FokI domains were synthesized as gBlocks by IDT and digested by AgeI and SpeI. The three fragments were inserted to N terminal end of dMb2Cas12a at BsrGI and NcoI sites by T4 ligation. All primers used in this research are listed in Supplementary Table S1. All gBlocks used in this study are listed in Supplementary Table S2.

All target sites (Supplementary Table S3) were synthesized as single-strand oligonucleotides by IDT. The forward and reverse primers were phosphorylated with T4 polynucleotide kinase (New England Biolabs), annealed, and ligated with Instant Sticky-end Ligase Master Mix (New England Biolabs) into a gRNA expression vector at the BsmBI site. Successful insertion was verified using Sanger sequencing (Genewiz). For the dimeric Cas9-SpRY system, the left sgRNA was ligated into pYPQ131D (Addgene #69287) and the right sgRNA was ligated into pYPQ131C (Addgene #69284). Left and right sgRNA cassettes were assembled into the attL5-attL2 vector pYPQ142 (Addgene #69294) using Golden Gate assembly. To generate attL5-attL2 vectors for crRNA expression in the Cas12a system, two tandem HH-crRNA-HDV were used to express two crRNAs based on our previous method.[31]

The T-DNA expression vectors were assembled from a three-way Gateway LR reaction with an attL1-attR5 Cas entry clone, an attL5-attL2 gRNA entry clone and the attR1-attR2 destination vector pYPQ203 (Addgene # 86207). Final T-DNA vectors were confirmed by restriction digestion pattern with EcoRI and are listed in Supplementary Table S4.

2.2 | Rice protoplast transformation

Rice protoplasts were isolated and transfected as previously described[32] with some modifications. Briefly, 12–14 days old Japonica cultivar *Kitaake* shoots were isolated and incubated in the enzyme solution at 28°C in dark for 6–8 h. The cells were resuspended by 0.55 M sucrose after centrifugation and washed with W5 buffer. Finally, the cells were resuspended in MMG for transformation. 30 μg (1000 ng μl^{-1}) of plasmid DNA was mixed with 200 μl of protoplasts (1×10^6 ml) and 230 μl of 40% PEG solution. The mixture was incubated for 30 min at room temperature without shaking. Then, 900 μl

of W5 buffer was added to stop the reactions. Protoplasts were transferred into 12-well culture plates and incubated at 32°C in the dark for 48 h. Protoplasts transfected with water were used as the control.

2.3 | Sample preparation for high-throughput sequencing and mutagenesis analysis

After 48 h of incubation after rice protoplast transformation, the genomic DNA was isolated with dilution buffer (Thermo Scientific Phire plant Direct PCR kit). The target regions were PCR-amplified using barcode primers. PCR products of 6–10 samples were column-purified and pooled together for one sequencing sample. The sample concentration was normalized to 20 ng μl^{-1} and total of 1000 ng DNA for each sample. Then, the samples were sent to GENEWIZ and sequenced with an Illumina HiseqX platform. CRISPRMatch[33] was used for analyzing the next-generation sequencing data.

3 | RESULTS

3.1 | Development of dimeric FokI-dSpRY systems for PAM-less genome editing in rice

We reasoned that the use of PAM-less SpRY could make paired FokI-dCas9 systems more practical for genome editing due to the unconstrained PAM requirement (Figure 1A). To develop this SpRY-based system, we adopted a similar configuration with N-terminal fusion of FokI to dCas9[18–20] and developed four FokI-dSpRY systems based on two different rice codon optimizations of FokI (NF1 and NF2) and two different linkers (L1 for the GGGGSx5 linker of 25 Aa and L2 for the XTEN linker of 16 Aa) that connect FokI and dSpRY. Two target sites (Q2-TAA and Q32-CGC) were chosen for initial testing in the rice and each target was defined by a pair of gRNAs with relaxed NNN (N = A,C,G,T) PAMs (Figure 1B). The spacer length between the two gRNA target sites is either 15 or 16 bp (Figure 1B). The four FokI-dSpRY systems were closely compared in rice protoplasts by plasmid-based transfection, followed by next generation sequencing (NGS) of PCR amplicons. Editing activities were detected at both target sites, and the NF1-L1-dSpRY construct showed the most robust editing at both sites, with deletion frequencies of 0.4% and 0.3%, respectively (Figure 1C). Such editing frequencies are on par with those achieved at different target sites by the SpRY nuclease in rice cells.[23] It has been shown that SpRY is less efficient at editing PAM-less sites than SpCas9 at editing NGG PAM sites.[22–25]

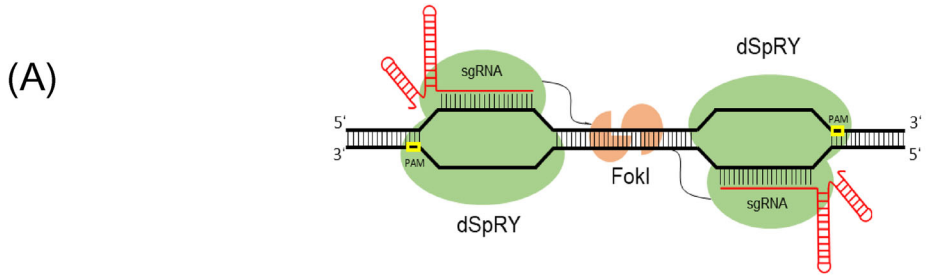
We compared the dimeric NF1-L1-dSpRY with the paired SpRY-D10A nickase system at three independent target sites (Q2-TAA, Q12-CAA, and Q36-CAT) (Figure 1B). Interestingly, NF1-L1-dSpRY showed comparable or higher editing efficiency across all sites (Figure 1D). The data suggests that the SpRY-based dimeric FokI system is more robust than the SpRY-based nickase system. We further compared editing profiles of these two systems. The results showed that the paired FokI-dSpRY system (NF-L1-dSpRY) resulted in high-frequency deletions at the spacer region in between the two SpRY gRNA target sites (Figure 1E). By contrast, the paired SpRY-D10A nickase system generated deletions that are defined by either or both gRNAs, leaving the spacer regions with low-frequency deletions (Figure 1F). Hence, these data suggest that the paired FokI-dSpRY system is more desirable as it mutates the targeted spacers at a more predictable way with high robustness.

3.2 | Assessment of paired crRNAs for Mb2Cas12a-mediated simultaneous editing

Inspired by the successful development of the FokI-dSpRY system, we sought to develop dimeric FokI systems based on Cas12a. We chose to work on Mb2Cas12a as it was shown to recognize relaxed TTV PAMs and further relaxed DNA targeting can be achieved with the Mb2Cas12a-RVRR variant.[31] As a first step, we applied NGS to screen multiple paired gRNAs with the Mb2Cas12a nuclease in both PAM-out and PAM-in orientations in rice protoplasts. The dual Pol II promoter and double ribozyme system were used to express Mb2Cas12a with two gRNAs (Supplementary Figure S1A).[31] A total of 14 target sites were assessed with the PAM-out orientation, which generated ~2%–6% editing efficiencies across these sites in rice protoplasts analyzed by NGS (Supplementary Figure S1B). Analysis of editing profile of two chosen sites (OsDep1-F11 and OsEPFL9-F17) showed deletion positions covering in between the midpoints of both protospacers, with higher deletion frequencies at the individual Mb2Cas12a cleavage sites (Supplementary Figure S1C). Similarly, the PAM-in orientation was assessed at 10 target sites, which generated ~3%–12% editing frequencies in rice protoplasts (Supplementary Figure S1D). Interestingly, the PAM-in orientation resulted in much larger deletion coverage than the PAM-out orientation, spanning in between the distal ends of the paired protospacers (Supplementary Figure S1E). These data suggest nearly all tested gRNAs worked and the use of paired gRNAs helped generate different deletion profiles by Mb2Cas12a. The high success rate of gRNAs for Mb2Cas12a paved the road to the development of dimeric Fok1 nuclease systems based on Mb2Cas12a.

3.3 | Development of dimeric FokI-dMb2Cas12a systems for genome editing

To develop dimeric FokI systems based on Mb2Cas12a, we first generated deactivated Mb2Cas12a (dMb2Cas12a) which lost its nuclease activity as assessed in rice cells (data not shown). FokI was fused to either N-terminus or C-terminus of dMb2Cas12a to be coexpressed with a pair of gRNAs (Figure 2A). Four target sites in the rice genome were chosen, with spacer lengths ranging from 15 to 18 bp, for testing both PAM-in and PAM-out orientations (Figure 2B). We tested four dMb2Cas12a-based dimeric FokI systems varying in the FokI fusion configurations and linkers (L1 and L2) at the target sites with either PAM-in or PAM-out orientation (Figure 2C). The NGS results



(B)

Target name	Left half -site with PAM	Spacer	Right half -site with PAM	Spacer length
Q2-TAA	TTACAGGAATACTATGATCCAAA	ccgttcaatgctgga	GTTGGTCTTTGCTCCTGCAGAGG	15
Q12-CAA	TTGGTTTAAGTTGGAGCTTATCC	caacatacagaactt	GTTTGGCGAGCTTGGTATTAATGA	15
Q32-CGC	GCGCCCCGCGGCGGCGGCGGCT	gcgtttttctctatag	GTTTCTCTTTCACACTCGCTCGC	16
Q36-CAT	ATGGAGGCGCCGAGGCCCAAGTC	gccgccgaggtacccg	GACCTGTGCGGCCGGCGGCGGAT	16

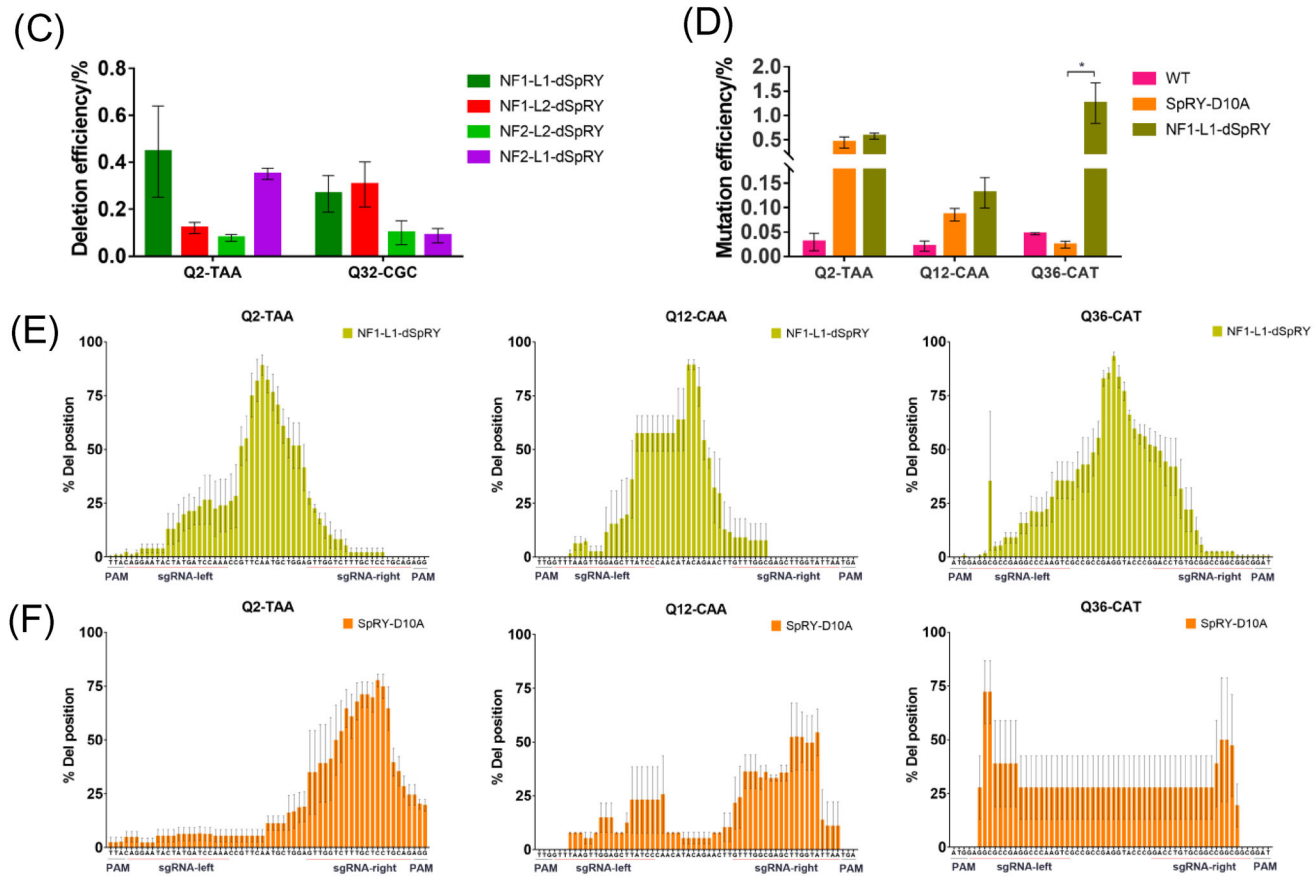
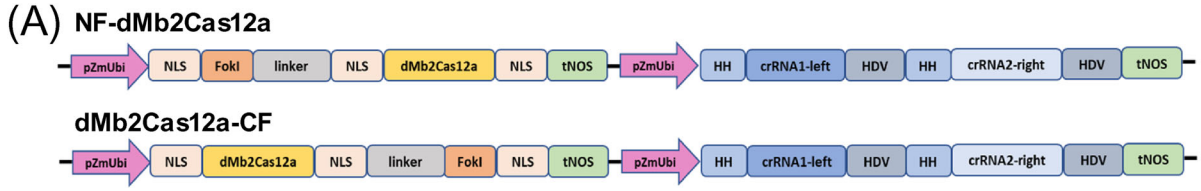


FIGURE 1 Dimeric FokI-dSpRY systems for PAM-less genome editing in rice protoplasts. (A) Architectures of FokI-dSpRY fusion variants. The two FokI nuclease domains (orange) fused to the N terminus of dSpRY (green) dimerize upon paired dSpRY binding to the target DNA. (B) The paired target sites in the rice genome. The PAM is highlighted in red. (C) Comparison of deletion efficiency by NF1-L1-dSpRY, NF1-L2-dSpRY, NF2-L1-dSpRY and NF2-L2-dSpRY at four paired target sites by amplicon deep sequencing. L1 for linker GGGGSx5 and L2 for linker XTEN. (D) Comparison of deletion efficiency by NF1-L1-dSpRY and SpRY-D10A at three paired target sites by amplicon deep sequencing. (E, F) Deletion positions by NF1-L1-dSpRY (E) and SpRY-D10A (F) at three target sites. Data are presented as mean values ± SD of three biologically independent replicates. Significant differences were indicated using one asterisk ($p < 0.05$)

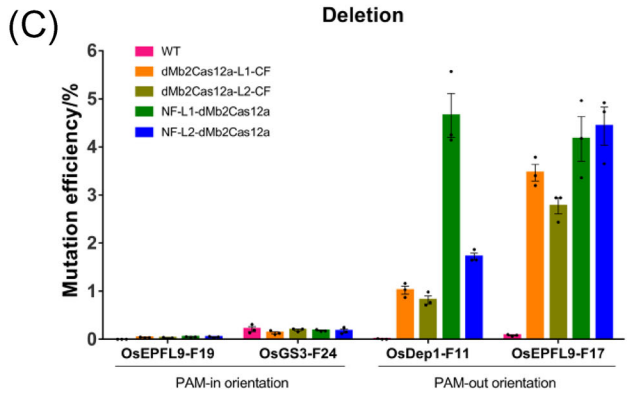
in rice protoplasts showed that the PAM-in orientation failed to edit the target sites, while the PAM-out orientation edited the two target sites (Figure 2C). At the PAM-out sites (OsDep1-F11 and OsEPFL9-F17), FokI-L1-dMb2Cas12a showed the most robust editing among all four configurations (Figure 2C). The data are consistent with previous reports on the development of dimeric FokI-dCas9 systems where the PAM-out orientation worked better than the PAM-in orientation.

We next analyzed the deletion profiles of the four dMb2Cas12a-based dimeric FokI systems. Regardless of the linker choice, the fusion of FokI to the C-terminus of dMb2Cas12a generated flatter deletion



(B)

Target name	Left half-site with PAM	Spacer	Right half-site with PAM	Space length
OsEPFL9-F19	GTACATATGCCATCTTGCTATTACAAA	gtatgtaattaattgagt	TTTGTGTTTTTCTGTTCCTCAAGTATG	18
OsGS3-F24	CTCTTCCACCTAGCTCCGCCATTCAAA	gcaaagcaccaagct	TTTGCCTCTCCCTCTACCATGCCTGCC	15
OsDep1-F11	TTTGAAATGCATATGTACTTGTACCAT	tacagaaattcttaaaa	CTGCTGTCTTGACAGCTACTTATCAAA	17
OsEPFL9-F17	TTTAGAAAAAGAAATAGACTGATGCAC	cgcagatcagagcttcca	TCAATTGCTTTCTGCAAGATATCCAAA	18



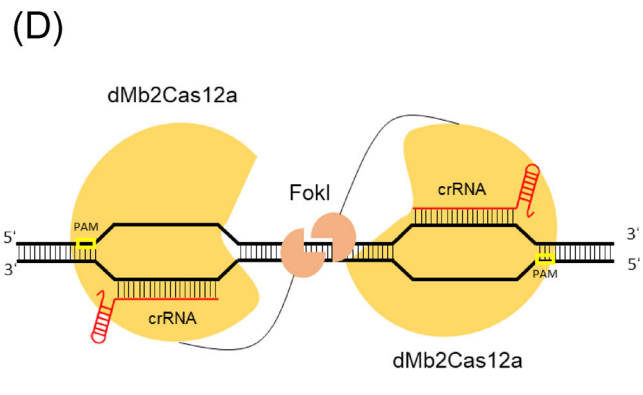


FIGURE 2 Mutagenesis frequencies of four dimeric Fok1 and dMb2Cas12a fusion systems. (A) Illustration of FokI-dMb2Cas12a fusion and dMb2Cas12a-FokI fusion. The crRNA is flanked by HH and HDV ribozymes for precise processing. (B) The paired target sites in the rice genome. The PAM is highlighted in red. (C) Comparison of deletion efficiency by dMb2Cas12a-L1-CF, dMb2Cas12a-L2-CF, NF-L1-dMb2Cas12a and NF-L2-dMb2Cas12a at two paired sites in the PAM-out orientation and two paired sites in the PAM-in orientation by amplicon deep sequencing. L1 for linker GGGGSx5 and L2 for linker XTEN. Data are presented as mean values ± SD of three biologically independent replicates. (D) Architectures of dimeric FokI-dMb2Cas12a in the PAM-out orientation. Two FokI nuclease domains (orange) fused to dMb2Cas12a (gold) bind to the target with 2 crRNAs (red)

position profiles (Figure 3A,B), compared to the fusion of FokI to the N-terminus of dMb2Cas12a (Figure 3C,D). Analysis of the proportion of deletion sizes showed drastic differences between N- and C-terminus fusion configurations. The C-terminus FokI fusion resulted in relatively higher deletion frequencies for 1 bp deletions or deletions over 20 bp (Figure 3E). The N-terminus FokI fusion generated deletions that enriched with 3–12 bp (for the OsDep1-F11 site) or 3–5 bp (for the OsEPFL9-F17 site) (Figure 3E). Hence, the deletion positions and deletion size profiles are corelated.

Our results showed that the 25 Aa GGGGSx5 linker (L1) seems better than the 16 Aa XTEN linker (L2) for targeted mutagenesis by FokI-dMb2Cas12a (Figure 2C), as with our earlier results with the FokI-dSpRY (Figure 1C). To further validate this observation, we compared the FokI-dMb2Cas12a systems with these two linkers at two additional target sites (OsPDS-F5 and OsDep1-F10) (Figure 4A). Editing activity of > 1% was detected at both sites, based on NGS analysis (Figure 4B). Although both linkers showed comparable editing frequencies at the OsPDS-F5 site, L1 linker outperformed L2 linker at the OsDep1-F10 sites (Figure 4B). These data again supports that FokI-dMb2Cas12a based on L1 linker is a more robust system. Analysis of the editing profiles showed that targeted deletions predominantly occurred at the center of the spacers of the target sites (Figure 4C,D) with relatively even distribution of deletion sizes up to 15 bps (Figure 4E), regardless of the linkers.

3.4 | Development of single chain Fok1-dMb2Cas12a systems for genome editing

While FokI nuclease activity is dependent on dimerization of the FokI monomers, it is appealing to establish the dimerization without using of two paired gRNAs. In fact, single-chain FokI systems have been previously described with ZFN[34] and TALEN.[35] To see whether the single-chain FokI system could be also adapted to a CRISPR-Cas system, we tried two different strategies to develop a single-chain FokI nuclease system based on Cas12a. In the first strategy, the two FokI monomers are linked by a flexible linker (95aa) and fused to the N-terminus of dMb2Cas12a, resulting in a single-chain FokI-FokI-dMb2Cas12a system (or NF-L3-NF-L1-dMb2Cas12a) (Figure 5A). In the second strategy, an independent FokI monomer

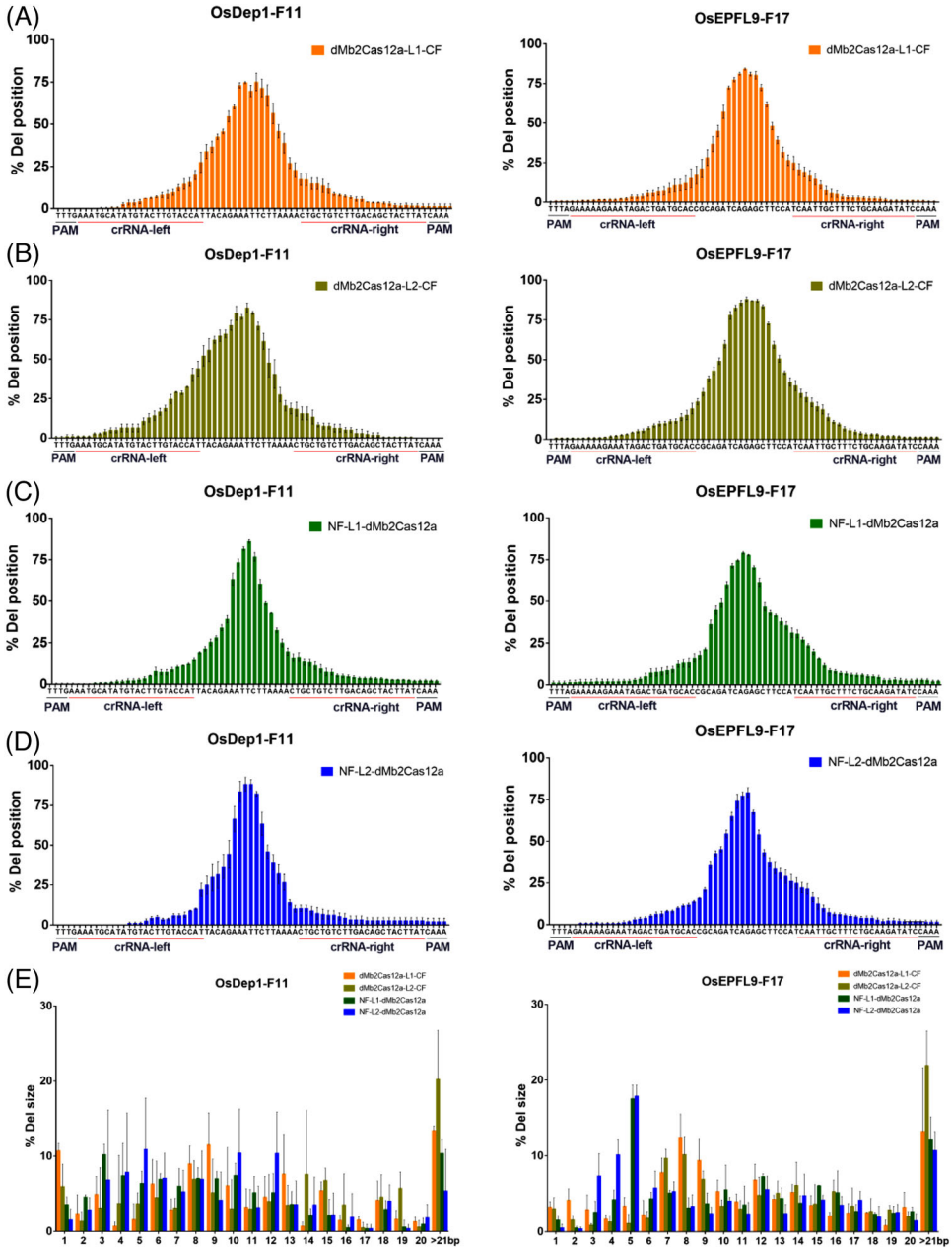


FIGURE 3 Analysis of mutation profiles of four dimeric Fok1 and dMb2Cas12a fusion systems. (A–D) Deletion positions at the OsDep1-F11 site and the OsEPFL9-F17 site by dMb2Cas12a-L1-CF (A), dMb2Cas12a-L2-CF (B), NF-L1-dMb2Cas12a (C), and NF-L1-dMb2Cas12a (D). (E) Comparison of deletion sizes by four dimeric Fok1 and dMb2Cas12a fusion at the OsDep1-F11 site and the OsEPFL9-F17 site. L1 for linker GGGGSx5. Data are presented as mean values ± SD of three biologically independent replicates

is coexpressed with the NF-L1-dMb2Cas12a through the T2A self-cleaving peptide,[36] generating a FokI-T2A-FokI-dMb2Cas12a system (or NF-L4-NF-L1-dMb2Cas12a) (Figure 5B). If autonomous dimerization could happen between the FokI in NF-L1-dMb2Cas12a and the free FokI, it would reconstitute FokI dimerization and exert nuclease activity for inducing DBSs (Figure 5B). To this end, we constructed these two single-chain FokI-dMb2Cas12a systems (Figure 5C) and tested them at targeting two different sites in the rice genome (Figure 5D). Based on NGS analysis of the target sites from transfected protoplasts, the single-chain FokI-FokI-dMb2Cas12a system generated relatively low but substantial targeted mutagenesis at the two target sites of OsDep1, 0.2% and 0.4% at OsDep1-crRNA7 and OsDep1-crRNA8, respectively (Figure 5E). Further analysis of editing profiles at OsDep1-crRNA7 and OsDep1-crRNA8 sites showed deletions largely occurred outside the protospacer or target site in the PAM-distal region (Figure 5F), generating deletions of variable sizes (Figure 5G). For the FokI-T2A-FokI-dMb2Cas12a system, no editing was detected at the two target sites (Figure 5E), suggesting DNA binding is a prerequisite for dimerization of FokI monomers.

(A)

Target name	Left half-site with PAM	Spacer	Right half-site with PAM	Space length
OsPDS-F5	TTTGATTTTCCTGAAACATTGCCTGCA	cccttaaatggtgagatcat	ATGCAGCGCTGGAGTTGTTTAATTAAA	20
OsDep1-F10	TTTATTGCTGTCCTTATCTAATGTCTT	atgctctgttttataaaat	TTGGTTGCATTTTCTCCCCCAGGGAAA	19

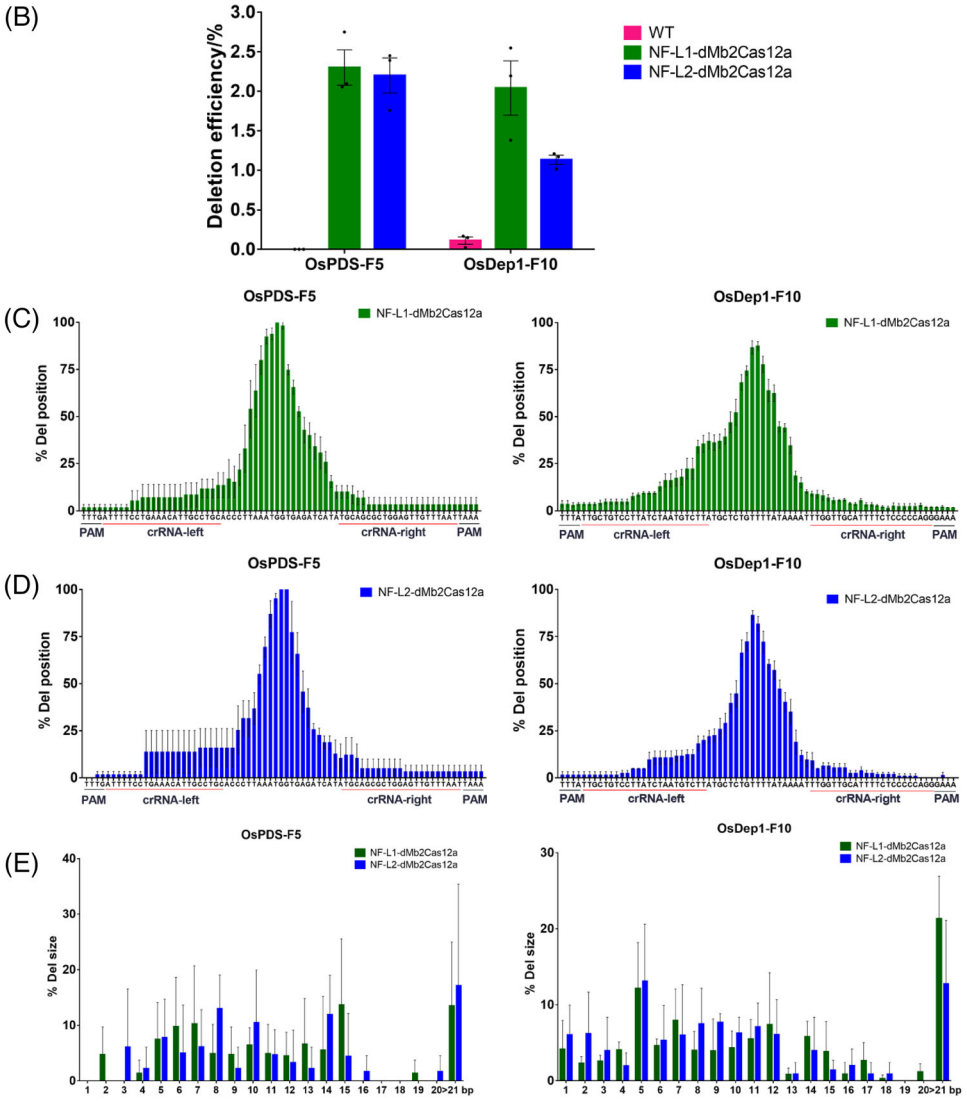


FIGURE 4 Assessment of Fok1-dMb2Cas12a systems with two different linkers at additional target sites. (A) The paired target sites in the rice genome. The PAM is highlighted in red. (B) Comparison of deletion efficiency by NF-L1-dMb2Cas12a and NF-L2-dMb2Cas12a at the OsPDS-F5 site and the OsDep1-F10 site by amplicon deep sequencing. (C, D) Deletion positions at the OsPDS-F5 site and the OsDep1-F10 site. (E) Deletion sizes at the OsPDS-F5 site and the OsDep1-F10 site. Data are presented as mean values ± SD of three biologically independent replicates

4 | DISCUSSION

In this study, we developed three FokI-dCas systems, which greatly expanded the FokI-based CRISPR genome editing toolbox. The first system is FokI-dSpRY, which showed more robust genome editing than the paired SpRY-D10A nickase (Figure 1D). Compared to previously established FokI-dSpCas9 systems,[18–20] the FokI-dSpRY system would allow for genome editing at sites which have previously been not accessible. However, the PAM-less feature of SpRY makes it very prone to off-targeting and self-cleavage (if delivered by DNA).[23,21] This limitation could be addressed with a paired SpRY nickase or a dimeric FokI-SpRY system. Remarkably, we found that the dimeric FokI-dSpRY system was more robust than the paired SpRY nickase system in rice cells (Figure 1D–F), suggesting that the dimeric FokI-dSpRY system is a promising tool for PAM-less genome editing with enhanced specificity. SpRY prefers editing at NR (R = A,G) PAM sites than NY (Y = C,T) PAM sites. We envision future improvement of the SpRY system for high DNA binding activity in a truly PAM-less manner will further contribute to the development of better dimeric FokI-dSpRY systems.

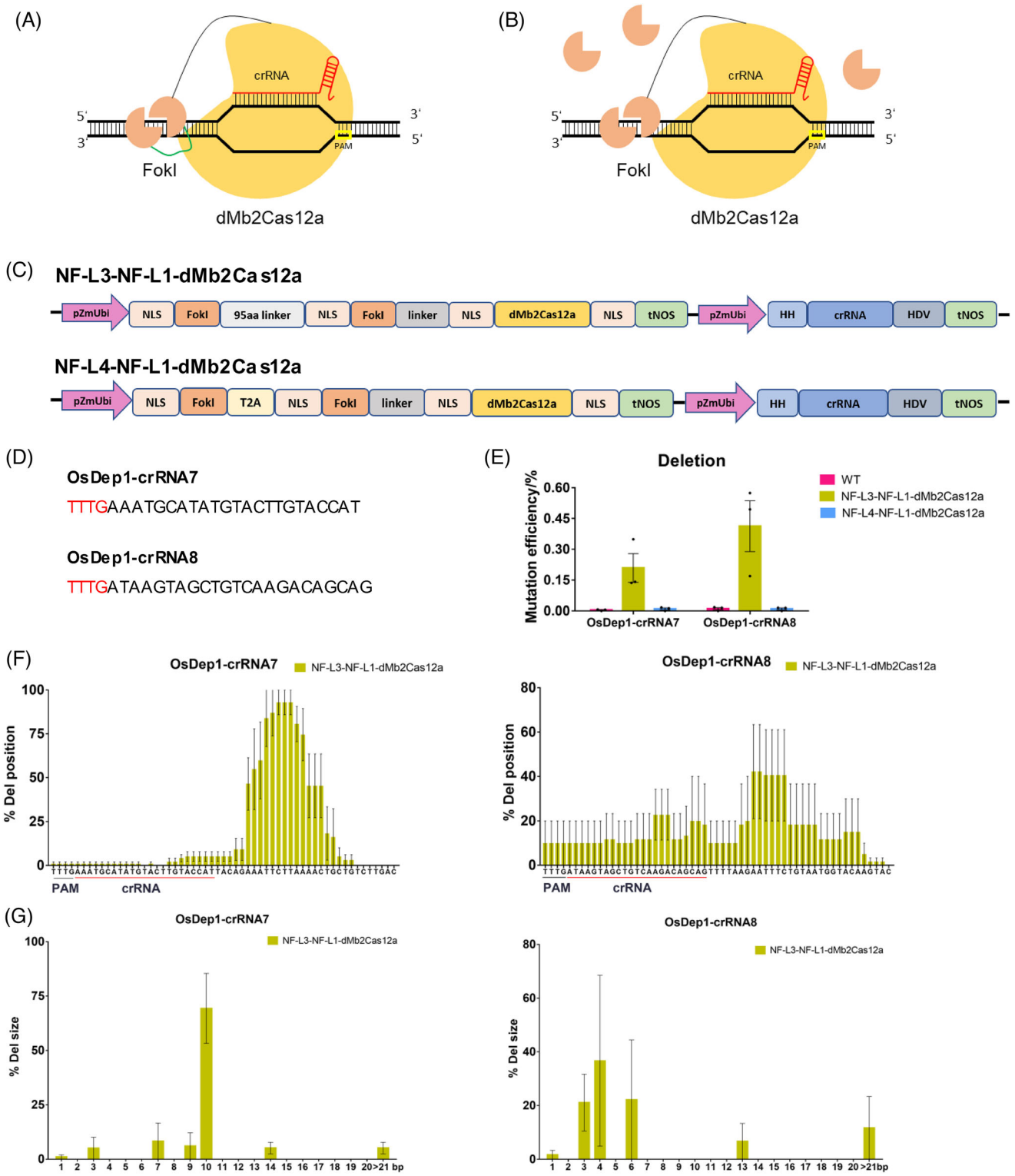


FIGURE 5 Development of single-chain Fok1-dMb2Cas12a systems for genome editing. (A, B) Architectures of single-chain FokI-dMb2Cas12a fusion systems based on a 95aa linker (A) or a T2A linker (B) between two FokI nucleases. The linker between FokI nuclease and dMb2Cas12a is the 5xGGGGS of 25aa. (C) Illustration of the single-chain FokI-dMb2Cas12a fusion constructs. (D) The paired target sites in the rice genome. The PAM is highlighted in red. (E) Comparison of deletion efficiency by NF-L3-NF-L1-dMb2Cas12a and NF-L4-NF-L1-dMb2Cas12a at the OsDep1-crRNA7 site and the OsDep1- crRNA8 site by amplicon deep sequencing. (F) Deletion positions by NF-L3-NF-L1-dMb2Cas12a at the two target sites. (G) Deletion sizes by NF-L3-NF-L1-dMb2Cas12a at the two target sites. L1 for linker GGGGSx5, L3 for linker 95aa, L4 for linker T2A. Data are presented as mean values ± SD of three biologically independent replicates

The second FokI-based CRISPR system that we developed is a dimeric FokI-dMb2Cas12a system. Like the FokI-dCas9 systems,[18–20] FokI-dMb2Cas12a systems worked in a PAM-out orientation, not in a PAM-in orientation (Figure 2C). When the Mb2Cas12a nuclease was assessed in a PAM-in orientation, we found the editing was largely concentrated at the cleavage sites of both gRNAs (Supplementary Figure S1E), suggesting steric hindrance which might have prevented simultaneous editing at the two paired target sites. When the Mb2Cas12a was assessed in a PAM-out orientation, large deletions predominated the editing outcomes, indicating simultaneous cleavage at the paired target sites (Supplementary Figure S1C). These observations based on nuclease-active Mb2Cas12a provided a mechanistic explanation for why only the PAM-out orientation worked for the development of functional FokI-dMb2Cas12a systems (Figure 2C). Impressively, the editing efficiencies of FokI-dMb2Cas12a based on the GGGGSx5 linker in a PAM-out orientation (Figure 2C) were comparable to that of Mb2Cas12a (Supplementary Figure S1B and D), making this FokI-dMb2Cas12a system an appealing system for genome editing in plants and beyond. Interestingly, we found the GGGGSx5 linker is better than the XTEN linker that was previously used for developing highly efficient FokI-dCas9 systems.[18,19] Also, five copies of GGGGS as a linker between FokI and dMb2Cas12a used in our study could provide more flexibility than one copy of GGGGS used in the earlier study on developing FokI-dCas9 systems.[20] In our FokI-dMb2Cas12a system based on a GGGGSx5 linker, we found spacer lengths between 17 to 20 bp showed good activity. In the previous study, FokI-dCas9 system based on a short GGGGS linker showed an optimal spacer length between 14 and 17 bp.[20] These data suggest that the length of the linker between FokI and dMb2Cas12a may define the optimal length of spacers between two gRNA target sites as well as the editing activity of the system. Further engineering of dimeric FokI-dMb2Cas12a systems could explore the relationship between linkers and spacer length, which should provide a useful guidance on using such tools for high-efficiency genome editing.

The third system that we developed is a single-chain FokI-FokI-dMb2Cas12a system, which has multiple implications for future engineering and use of such systems. A most striking feature that we observed for the single-chain FokI-FokI-dMb2Cas12a system is that it largely induced mutations outside its target site or protospacer (Figure 5F). Thus, the mutations generated in the PAM-distal region would not prevent the nuclease system from further cleavage. The capability of exerting repeated cleavage at the same target would allow the FokI-FokI-dMb2Cas12a system to be useful for generating larger deletions, promoting homology-directed repair for precise genome editing, and creating a series of different mutations across cell cycles for lineage tracing. Furthermore, the single-chain FokI-FokI-dMb2Cas12a system represents a new class of Type II restriction enzymes, which can be programmed by gRNAs for targeting different DNA sequence. This provides endless opportunity for molecular cloning, making such systems very suitable for Golden Gate Cloning.[37] While the demonstrated editing activity for the single-chain FokI-FokI-dMb2Cas12a so far is one magnitude lower than the dimeric FokI-dMb2Cas12a system, the activity however could be improved by testing different linkers and using FokI variants of high nuclease activity. By contract, the FokI-T2A-FokI-dMb2Cas12a system failed to edit the target sites (Figure 5E), which indicates that autonomous dimerization did not happen without bringing the two FokI nucleases into proximity through DNA targeting. This data further supports that a dimeric FokI-dCas system is highly specific.

The use of FokI nuclease, not the nuclease domain of Cas9 or Cas12a, for generating DNA DSBs would render the systems more suitable for genome editing in nonmammalian organisms such as plants. For example, both Cas9 and Cas12a are temperature sensitive, and their nuclease activity is suboptimal at room temperature.[38–41] To improved editing efficiency of Cas9 and Cas12a in animals and plants, high-temperature treatment regimens were used.[38–40] High-temperature treatment however may not be universally applicable in all organisms. Interestingly, unlike Cas9, editing activities of ZFNs and TALENs were plateaued at 30°C in human cells,[41] suggesting that FokI based SSNs could be more suitable for genome editing at lower temperatures. Hence, dimeric FokI-dSpRY and FokI-dMb2Cas12a systems as well as single-chain FokI-FokI-dMb2Cas12a systems represent a suite of promising genome editing tools suitable for genome editing in nonmammalian organisms that occur at lower temperatures.

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CONLICT OF INTEREST

Y.Z. and Y.Q. are inventors for a U.S. patent application concerning the CRISPR-Cas12a genome editing systems. Y.Q. is a consult for Inari Agriculture and CTC Genomics. Other authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The 5 Gateway compatible Cas vectors used in this study have been deposited to Addgene: pYPQ-NF1-L1-dSpRY (#177681), pYPQ-NF1-L2-dSpRY (#177682), pYPQ-NF-L1-dMb2Cas12a (#177683), pYPQ-NF-L2-dMb2Cas12a (#177684), pYPQ-NF-L3-NF-L1-dMb2Cas12a (#177686). The high-throughput sequencing data sets have been submitted to the National Center for Biotechnology information (NCBI) database under Sequence Read Archive (SRA) BioProject ID: PRJNA769620.

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