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Letter

Systemic evaluation of the inhibitory effects of 4 anti-CRISPR systems on different Cas12a nucleases in rice

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Dear Editor,

The emergence of clustered regularly interspaced short palindromic repeats-CRISPR associated protein (CRISPR-Cas) system has brought a revolutionary leap forward in genome editing. The type II and V CRISPR-Cas systems are the most extensively studied and widely applied technologies in the field of genome editing (Liu et al. 2022; Tang and Zhang 2023; Zheng et al. 2023; Zhou et al. 2023; Zheng et al. 2024). Faced with the CRISPR-Cas defense mechanism of bacteria, bacteriophages have evolved a series of small anti-CRISPR proteins to effectively inhibit the function of the CRISPR-Cas systems (Bondy-Denomy et al. 2013). The anti-CRISPR proteins targeting Cas12a are classified as Type V-A, known as AcrVAs. To date, 5 AcrVA proteins have been reported, but only AcrVA1, AcrVA4, and AcrVA5 have shown the ability to inhibit the enzymatic activity of the CRISPR-Cas12a system through distinct mechanisms (Choudhary et al. 2023). In plants, the AcrVA1 protein has been demonstrated to effectively suppress the gene editing and transcriptional activation functions of LbCas12a in *Nicotiana benthamiana* (Calvache et al. 2022), as well as those of LbCas12a and Mb2Cas12a in rice (He et al. 2024). Similarly, the AcrVA5 protein has shown the capability to inhibit gene editing based on LbCas12a in rice (He et al. 2024). In addition, our recent study identified AcrC03 as a putative anti-CRISPR protein for LbCas12a (He et al. 2024). However, the inhibitory effects of different AcrVA proteins on other Cas12a orthologs in plants require further in-depth research and exploration. In this study, we evaluated the inhibitory effects of various AcrVA proteins on 3 Cas12a orthologs, including AsCas12a, which recognizes canonical VTTT PAM (protospacer adjacent motif) and is very temperature sensitive (Zetsche et al. 2015; Tang et al. 2017; Malzahn et al. 2019; Tang et al. 2019), FnCas12a, which recognizes simpler VTT PAM (Zetsche et al. 2015; Zhong et al. 2018; Tang and Zhang 2023), and Mb3Cas12a (Zetsche et al. 2020), a Cas12a that we recently found to confer PAM-relaxed and temperature-tolerant genome editing in plants (Liu et al. 2024).

Firstly, to investigate the inhibitory activity of 4 V-A type Acr (AcrVA) proteins (AcrVA1, AcrVA4, AcrVA5, and AcrC03) in prokaryotic cells, we employed a compatible plasmid interference system that we recently developed (He et al. 2024) (Supplementary Methods and Materials). With this system, we were able to quantitatively assess the inhibitory impact of Acr proteins on the editing activity of the CRISPR-Cas12a system (Fig. 1A). AcrVA1 exhibited a strong inhibitory effect on AsCas12a, while AcrVA4, AcrVA5, and AcrC03 did not show significant inhibitory activity against AsCas12a in *Escherichia coli* (Fig. 1B). For FnCas12a, we found that both AcrVA1 and AcrVA4 exhibited strongly inhibitory effects, while AcrVA5 and AcrC03 showed almost no inhibitory activity in *E. coli* (Fig. 1C). Overall, our plasmid interference assays in *E. coli* identified AcrVA1 as a potent Acr protein for both AsCas12a and FnCas12a, and AcrVA4 as a potent Acr protein for FnCas12a.

Next, we assessed the inhibitory effects of these 4 Acr proteins on Cas12a-mediated gene editing in rice protoplasts (Supplementary Methods and Materials). For this purpose, we designed 5 all-in-one vectors: 1 containing expression units of Cas12a and crRNA, and the others containing additional expression units of different Acr proteins (Fig. 1D). We selected 6 gene loci in rice for multiplexed editing by different Cas12a nucleases. The experimental results showed that the AsCas12a protein had editing efficiency of 11.7%, 9.9%, 26.9%, 56.7%, 47.3%, and 35.7% at these sites when not inhibited by any Acr (Fig. 1E). However, when AcrVA1 was co-expressed, the editing efficiency of AsCas12a significantly decreased to 0.8%, 2.4%, 1.9%, 9.6%, 1.7%, and 6.6% (Fig. 1E). AcrC03 also showed inhibitory effects on AsCas12a at 4 loci, while AcrVA4 and AcrVA5 proteins did not (Fig. 1E). For FnCas12a, the results showed that in the absence of Acr protein, the editing efficiency at 6 specific genomic loci reached 23.8%, 10.2%, 31.9%, 39.2%, 36.6%, and 31.6%, respectively (Fig. 1F). The genome editing activity of FnCas12a was significantly inhibited by AcrVA1, with the efficiency decreasing to

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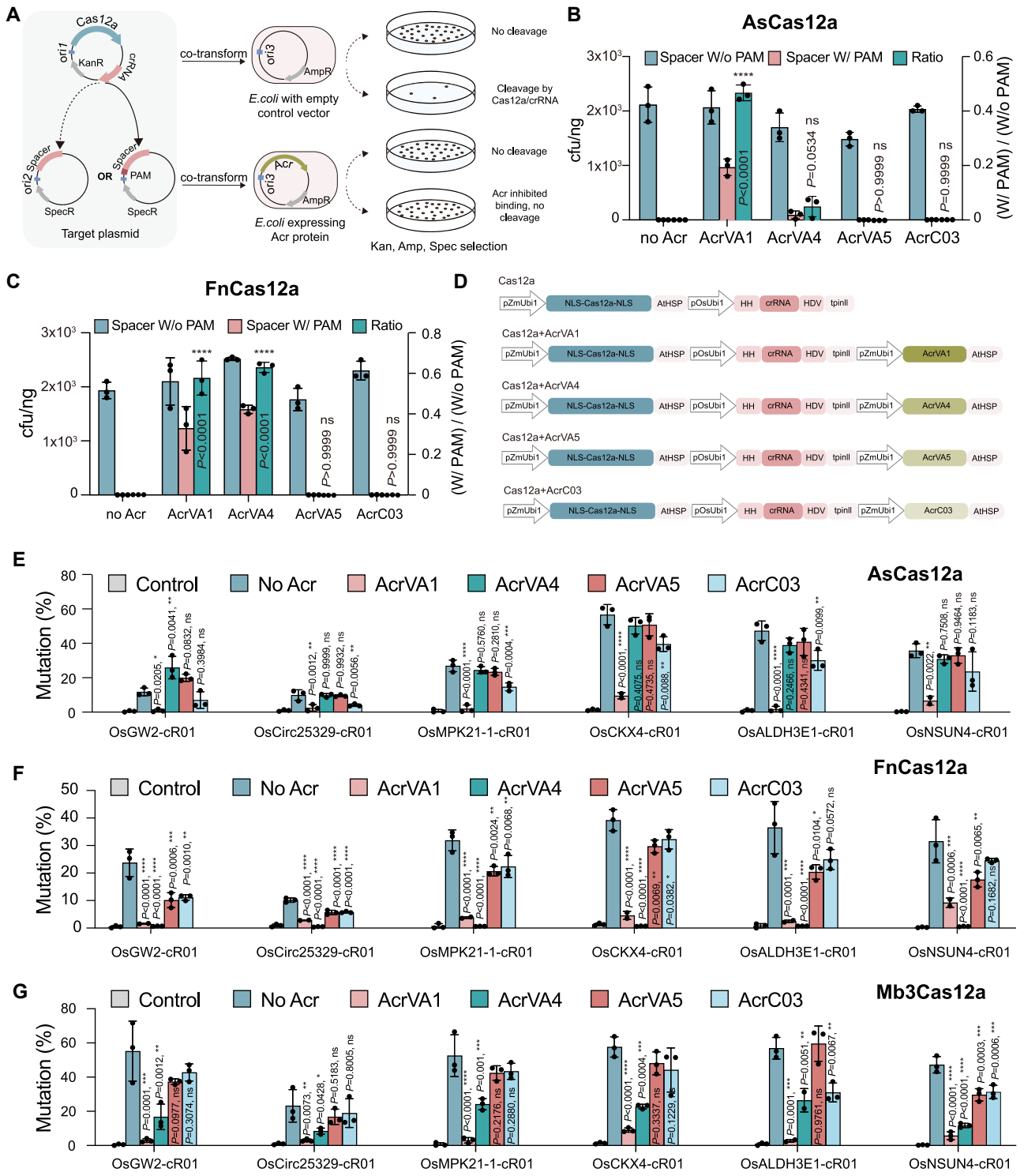
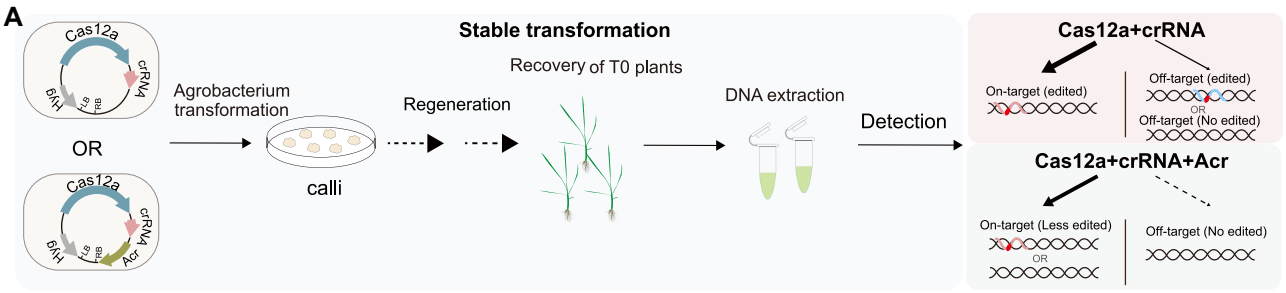


Figure 1. Efficient in bacteria and planta inhibition of Cas12a genome editing by anti-CRISPR systems. **A)** Schematic overview of designed plasmids and procedure of plasmid interference assays for anti-CRISPR activity analysis in *E. coli*. AmpR, ampicillin resistance; KanR, kanamycin resistance; SpecR, spectinomycin resistance. **B)** The bar plot of colony forming units (cfu) of each anti-CRISPR against AsCas12a in *E.coli*. **C)** The bar plot of colony forming units (cfu) of each anti-CRISPR against FnCas12a in *E.coli*. **D)** Schematic of the dual RNA polymerase II promoter system for Cas12a and crRNA expression and the Acr expression cassette. **E)** Editing efficiency of protoplasts transformed with AsCas12a nuclease without anti-CRISPR or with 4 different anti-CRISPR proteins in 6 sites. **F)** Editing efficiency of protoplasts transformed with FnCas12a nuclease without anti-CRISPR or with 4 different anti-CRISPR proteins in 6 sites. **G)** Editing efficiency of protoplasts transformed with Mb3Cas12a nuclease without anti-CRISPR or with 4 different anti-CRISPR proteins in 6 sites. Each dot represents a biological replicate. Each target contains 3 biological replicates. Error bars represent the mean values ± SD (ns, $P > 0.05$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$, Ordinary one-way ANOVA, Dunnett's test).

1.6%, 2.9%, 3.8%, 4.6%, 2.5%, and 9.3%, respectively (Fig. 1F). It is noteworthy that the presence of AcrVA4 protein significantly inhibited the editing activity of FnCas12a, and its effect even exceeds that of AcrVA1 protein. Specifically, the AcrVA4 protein reduced the editing efficiency of FnCas12a at 6 sites to 0.7%, 0.5%, 0.6%, 0.7%, 0.7%, and 0.6%, respectively (Fig. 1F).



B

Targeted sites	FnCas12a		FnCas12a+AcrVA1		FnCas12a+AcrVA4		FnCas12a+AcrVA5		FnCas12a+AcrC03	
	Tested T0 lines	Mutation*	Tested T0 lines	Mutation	Tested T0 lines	Mutation	Tested T0 lines	Mutation	Tested T0 lines	Mutation
OsGW2-cR01	19	9 ; 47.4%	20	0 ; 0.0%	18	0 ; 0.0%	17	4 ; 23.5%	15	5 ; 33.3%
OsCirc25329-cR01	19	4 ; 21.1%	20	0 ; 0.0%	18	0 ; 0.0%	17	1 ; 5.9%	15	3 ; 20.0%
OsMPK21-1-cR01	19	8 ; 42.1%	20	0 ; 0.0%	18	1 ; 5.6%	17	2 ; 11.8%	15	8 ; 53.3%
OsCKX4-cR01	19	12 ; 63.2%	20	2 ; 10.0%	18	0 ; 0.0%	17	9 ; 52.9%	15	7 ; 46.7%
OsALDH3E1-cR01	19	11 ; 57.9%	20	1 ; 5.0%	18	0 ; 0.0%	17	8 ; 47.1%	15	7 ; 46.7%
OsNSUN4-cR01	19	8 ; 42.1%	20	3 ; 15.0%	18	1 ; 5.6%	17	7 ; 41.2%	13	7 ; 46.7%

* : (number ; ratio)

C

Targeted sites	Mb3Cas12a		Mb3Cas12a+AcrVA1		Mb3Cas12a+AcrVA4		Mb3Cas12a+AcrVA5		Mb3Cas12a+AcrC03	
	Tested T0 lines	Mutation*	Tested T0 lines	Mutation	Tested T0 lines	Mutation	Tested T0 lines	Mutation	Tested T0 lines	Mutation
OsGW2-cR01	20	12; 60.0%	17	0 ; 0.0%	19	2 ; 10.2%	17	4 ; 23.5%	17	7 ; 41.2%
OsCirc25329-cR01	20	11; 55.0%	17	1 ; 5.9%	19	3 ; 15.8%	17	3 ; 17.6%	17	4 ; 23.5%
OsMPK21-1-cR01	20	13; 65.0%	17	0 ; 0.0%	19	6 ; 31.6%	17	5 ; 29.4%	17	6 ; 35.3%
OsCKX4-cR01	20	16; 80.0%	17	0 ; 0.0%	19	11 ; 57.9%	17	7 ; 41.2%	17	9 ; 52.9%
OsALDH3E1-cR01	20	12; 60.0%	17	0 ; 0.0%	19	11 ; 57.9%	17	8 ; 47.1%	17	10; 58.8%
OsNSUN4-cR01	20	8 ; 40.0%	17	0 ; 0.0%	19	8 ; 42.1%	17	8 ; 47.1%	17	7 ; 41.2%

* : (number ; ratio)

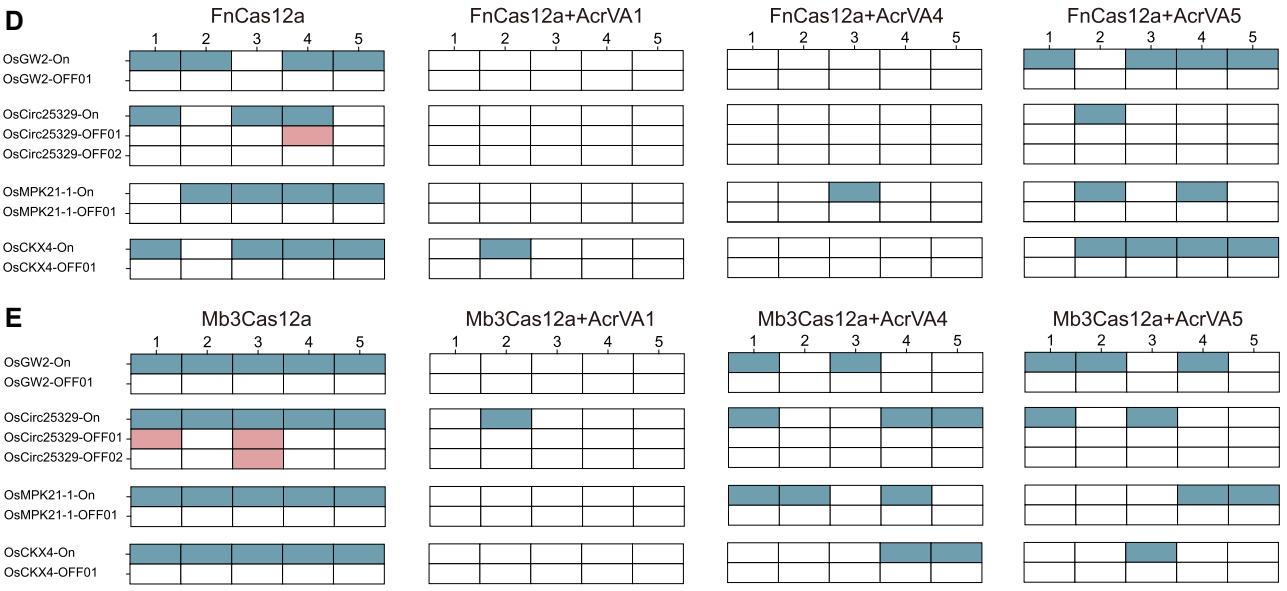


Figure 2. Quantitative regulation of Mb3Cas12a and FnCas12a genome editing in rice plants through anti-CRISPR systems. **A)** The workflow for evaluating the quantitative regulation of Cas12a by anti-CRISPR system in stable rice transformation. **B)** The mutation rates of FnCas12a nuclease without anti-CRISPR or with 4 different anti-CRISPR in 6 sites in rice T_0 lines. **C)** The mutation rates of Mb3Cas12a nuclease without anti-CRISPR or with 4 different anti-CRISPR in 6 sites in rice T_0 lines. **D)** Mutation analysis of gRNA dependent off-target mutations in 5 edited plants based on FnCas12a. **E)** Mutation analysis of gRNA dependent off-target mutations in 5 edited plants based on Mb3Cas12a. The white blocks indicate unedited sites, whereas the blue and red blocks represent edited sites. Specifically, the blue blocks denote the target sites, and the red blocks highlight the potential off-target sites.

Interestingly, AcrVA5 and AcrC03 displayed comparable but milder inhibitory effects on FnCas12a-mediated genome editing in rice protoplasts (Fig. 1F). For Mb3Cas12a, our data showed that its editing efficiency at these 6 sites was 55.2%, 23.2%, 52.5%, 57.6%, 56.9%, and 47.2%, respectively, which are overall higher than those of AsCas12a and FnCas12a (Fig. 1G). However, when

AcrVA1 was co-expressed, the editing efficiency of Mb3Cas12a significantly decreased to 2.9%, 3.0%, 3.0%, 9.0%, 2.7%, and 5.7% (Fig. 1G). Similarly, the co-expression of AcrVA4 also resulted in a decrease, to a lesser extent, in the editing efficiency of Mb3Cas12a to 16.7%, 8.4%, 24.2%, 22.8%, 26.5%, and 11.7% at these sites, respectively (Fig. 1G). In contrast, the co-expression of AcrVA5 and AcrC03 did not show significant inhibitory effects at most target sites (Fig. 1G). In addition, we found all tested Acr proteins did not alter the genome editing profiles of these 3 Cas12a gene editing systems as revealed by deletion positions (Supplementary Figs. S1–S3). Based on the above results, we further revealed differential inhibitory effects of different Acr proteins on different Cas12a proteins. For example, AcrVA1 is generally a strong inhibitor for all 3 Cas12a proteins, consistent with the observation with LbCas12a (He et al. 2024). However, AcrVA4 showed divergent inhibitory effects on different Cas12a proteins, as a strong inhibitor for FnCas12a, a mild inhibitor for Mb3Cas12a, and with no inhibitory effect on AsCas12a.

We wanted to test the inhibitory effects of different AcrVA proteins on Cas12a homologs in stably transformed rice plants, as the inhibitory effects could potentially help reduce off-target effects in genome editing (Fig. 2A). To this end, FnCas12a and Mb3Cas12a were tested for multiplexed editing of 6 target sites in stable rice lines (Supplementary Methods and Materials). FnCas12a generated genome editing efficiency of 47.4%, 21.1%, 42.1%, 63.2%, 57.9%, and 42.1% at the 6 target sites, respectively (Fig. 2B). When AcrVA1 was co-expressed, gene editing was not observed at 3 out of 6 target sites (Fig. 2B), and no single edit could be detected at 4 target sites when AcrVA4 was co-expressed (Fig. 2B). Effectively a reduction of genome editing efficiency was observed with AcrVA5 at 3 target sites, while co-expression of AcrC03 protein had no effect on FnCas12a (Fig. 2B). All these stable plant data is consistent with the results of protoplasts (Fig. 1F). Mb3Cas12a generated genome editing efficiency of 60.0%, 55.0%, 65.0%, 80.0%, 60.0%, and 40.0% at the 6 target sites, respectively (Fig. 2C). When AcrVA1 was co-expressed, gene editing was not observed at 5 out of 6 target sites (Fig. 2C). Mild reduction of genome editing efficiency was observed with AcrVA5, AcrVA4, and AcrC03 across 5 target sites other than *OsNSUN4*-cR01 (Fig. 2C). These data confirmed AcrVA1 as a strong inhibitor on both FnCas12a and Mb3Cas12a in plants, while AcrVA4, although having an effectively inhibitory effect on FnCas12a, has a relatively weak effect on Mb3Cas12a. In addition, AcrVA5 can to some extent, inhibit the activity of FnCas12a and Mb3Cas12a in transgenic plants. By contract, AcrC03 was only able to inhibit Mb3Cas12a to some extent, but could not inhibit FnCas12a in stable rice plants.

To see whether type V-A Acr proteins can reduce off-target effects of Cas12a genome editing in plants, we used the Cas-OFFinder (Bae et al. 2014) to predict potential off-target sites of the 6 target sites with mismatches ≤3 (Supplementary Fig. S4). Based on this analysis, *OsALDH3E1*-cR01 and *OsNSUN4*-cR01 sites have no off-target sites with ≤3 mismatches, and we hence focused on the other 4 target sites. For each editing system, we selected 5 plants and evaluated off-target editing at 4 off-target sites (Supplementary Fig. S4). In plants edited with FnCas12a, off-target mutations were detected at one off-target site (Fig. 2D). However, with co-expression of AcrVA1, AcrVA4, or AcrVA5, there were no mutations at off-target sites in the FnCas12a-edited lines (Fig. 2D). In Mb3Cas12a-edited plants, off-target mutations were detected at 2 off-target sites (Fig. 2E). However, when AcrVA1, AcrVA4, or AcrVA5 was co-expressed in the construct, no mutations occurred at off-target sites in these Mb3Cas12a-edited line (Fig. 2E). These results indicate that AcrVA1, AcrVA4, and AcrVA5 can alleviate off-target effects of FnCas12a and Mb3Cas12a in rice.

In summary, this study thoroughly investigated the quantitative inhibitory effects of 4 V-A type Acr proteins (AcrVA) on 3 different Cas12a nucleases in rice. It was found that AcrVA1 exhibited significant inhibitory effects on AsCas12a, FnCas12a, and Mb3Cas12a, while AcrVA4 had strong inhibitory effects on FnCas12a and relatively weak inhibitory effects on Mb3Cas12a. Also, AcrVA5 and AcrC03 have weak inhibitory effects on FnCas12a and Mb3Cas12a. Furthermore, AcrVA1, AcrVA4, and AcrVA5 can effectively alleviate off-target effects of FnCas12a and Mb3Cas12a in rice. From the experimental results, it can be seen that the inhibitory effect of the same Acr protein on different Cas12a proteins varies. This could be due to structural differences between different Cas12a proteins or their Cas12a/crRNA ribonucleoprotein complexes, which may affect their interactions with different Acr proteins. However, this hypothesis warrants future investigations; together, our results and discoveries will aid future use of these Acr proteins for fine-tuning the activities of different CRISPR-Cas12a systems for genome engineering in plants and beyond.

Author contributions

Y.Z. proposed the project and designed the experiments. Y.H. and S.L. designed all the constructs. Y.H. and S.L. generated all the constructs. Y.H. and S.L. did plasmid interference assays. Y.H. and S.L. conducted rice protoplast transformation. Y.H. analyzed the mutation frequencies in protoplasts. Y.H. and X.Z. conducted rice stable transformation and analyzed the mutation frequencies in rice stable T_0 lines. Y.Z., Y.Q., and Y.H. analyzed the data and wrote the paper with input from other authors. All authors read and approved the final version of the manuscript.

Supplementary data

The following materials are available in the online version of this article.

Supplementary Methods and Materials.

Supplementary Figure S1. Anti-CRISPR does not affect the positions of deleted bases by AsCas12a.

Supplementary Figure S2. Anti-CRISPR does not affect the positions of deleted bases by FnCas12a.

Supplementary Figure S3. Anti-CRISPR does not affect the positions of deleted bases by Mb3Cas12a.

Supplementary Figure S4. Potential off-target sites with mismatches ≤3 of 4 target sites dependent on crRNAs.

Supplementary Table S1. Oligos and sequence used in this study.

Supplementary Table S2. Constructs used in this study.

Supplementary Table S3. Target sites used in this study.

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Conflict of interest statement. None declared.

Data availability

Backbone vector pTrans_210d (Addgene Plasmid #91109) is available from Addgene. The deep sequencing data sets generated from this study are available at NCBI Sequence Read Archive under Bioproject PRJNA1162663.

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