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Hypercompact CRISPR–Cas12j2 (CasΦ) enables genome editing, gene activation, and epigenome editing in plants

CRISPR-Cas9, -Cas12a, -Cas12b, and -Cas13 have been harnessed for genome engineering in human and plant cells (Liu et al., 2022). However, the large size of these Cas proteins (e.g. ~190 kDa for SpCas9) makes them difficult to deliver into cells via a viral vector. The development of smaller Cas proteins will lead to reduced viral vector sizes that can be more widely adopted in versatile genome engineering systems. Recently, a CRISPR-Cas12j2 (CasΦ) system was discovered in huge phages and developed into a hypercompact genome editor due to the small size of Cas12j2 (~80 kDa) (Pausch et al., 2020). Unfortunately, the gene editing efficiency of Cas12j2 in *Arabidopsis* protoplasts using ribonucleoprotein delivery was less than one percent (Pausch et al., 2020). Further optimization of this system is clearly required if CRISPR-Cas12j2-mediated editing in plant genomes is to be adopted by the plant sciences community.

To develop an efficient CRISPR-Cas12j2 genome editing system in plants, we used an efficient dual Pol II promoter DNA-based expression system previously applied for CRISPR-Cas12a (Tang et al., 2017) and CRISPR-Cas12b (Ming et al., 2020). In this system, the crRNA is processed by HH and HDV ribozymes (Figure 1A). Cas12j2 prefers T-rich protospacer adjacent motifs (PAMs) according to PAM-depletion assays in bacteria (Pausch et al., 2020). To assess the PAM requirements of Cas12j2 in plants, we selected 17 protospacer sequences that were each present twice in the rice genome but contained slightly different PAMs: VTTV or VTTTV. Rice protoplast assays demonstrated that about half of the 34 target sites exhibited genome edits as determined by next-generation sequencing (NGS) with an efficiency of up to 40% (Figure 1B). Strikingly, on most occasions, the target sites with the VTTV/VTTTV PAM pairs of the same protospacer sequence showed similar editing tendency (Figure 1B), suggesting important roles of protospacer sequences in Cas12j2-mediated genome editing. The VTTV PAM was clearly preferred by Cas12j2 over the VTTTV PAM (Figure 1B). We next investigated the importance of the flanking "V" nucleotides in the VTTV PAM by testing 35 target sites in rice cells. The majority of target sites containing NTTA, NTTC, and NTTG PAMs showed detectable genome editing (up to 25%) whereas most NTTT PAM sites showed no editing (Figure 1C). Targeting protospacer sequences with four non-canonical TSN (S = G or C) PAM sites (Pausch et al., 2020) exhibited minimal editing activity (Supplemental Figure 1). These data collectively support that NTTV is the preferred PAM of Cas12j2 in plants.

We next turned our attention to developing a multiplexed Cas12j2 genome editing system using the RNA polymerase II promoter and HH-HDV ribozyme systems (Ming et al., 2020; Zhang et al., 2021) (Figure 1D). Multiplexed editing at four select sites showed high genome editing efficiency (~15% to ~50%) in rice protoplasts (Figure 1E). A total of 16 VTTV PAM sites in the tomato genome were targeted by four multiplexed Cas12j2 constructs. Genome editing was detected at 12 out of 16 sites with variable efficiencies (Figure 1F and Supplemental Figure 2). In both rice and tomato cells, the editing outcomes were deletions nearly every time (Figure 1E-F and Supplemental Figure 2). In both rice and tomato, these deletions were frequently found at target bases distal to the PAM (Figure 1G-H and Supplemental Figure 3). Deletion sizes were typically 7–13 bp (Supplemental Figure 4). These results demonstrate that staggered cleavage is created by Cas12j2 as previously observed with Cas12a and Cas12b (Zetsche et al., 2015; Tang et al., 2017; Ming et al., 2020).

To assess the specificity of Cas12j2, we used a single high-activity protospacer that has two target sites in the rice genome differing by a VTTV or VTTTV PAM site. Protospacers with lengths of 24 (only with the VTTV PAM), 22, 20 (the default protospacer length), 18, 16, and 14 nucleotides were used. The results showed that protospacers of 18 bp or longer resulted in optimal editing efficiencies, whereas 16 bp and 14 bp protospacers resulted in reduced and undetectable editing, respectively (Figure 1I). Permutations of every two nucleotides across the 20 bp protospacer demonstrated that mutations at PAM-distal sites could be well tolerated (Figure 1J). However, simultaneous introduction of two adjacent mutations at positions 1–14 of the protospacer completely abolished the nuclease activity of Cas12j2 (Figure 1J). These data demonstrate that Cas12j2 is a small but highly specific nuclease.

Two Cas12j2 variants, nCas12j2 and vCas12j2, were previously shown to have enhanced *in vitro* nuclease kinetics over Cas12j2 (Pausch et al., 2021). We tested both variants on 15 target sites in rice protoplasts and found that nCas12j2 had significantly improved editing activity at 6 out of 15 target sites while vCas12j2 had similar activity to the wild-type Cas12j2 (Figure 1K and Supplemental Figure 5). These results suggest that *in vivo* genome editing by Cas12j2 can be improved through protein engineering and that nCas12j2 is better suited for genome editing in plants and likely other eukaryotic cells.

The improved nCas12j2 was next tested for its ability to perform genome edits in whole transgenic plants. Low genome editing efficiencies of 1.5%, 2.5%, 6%, and 20% were detected by NGS

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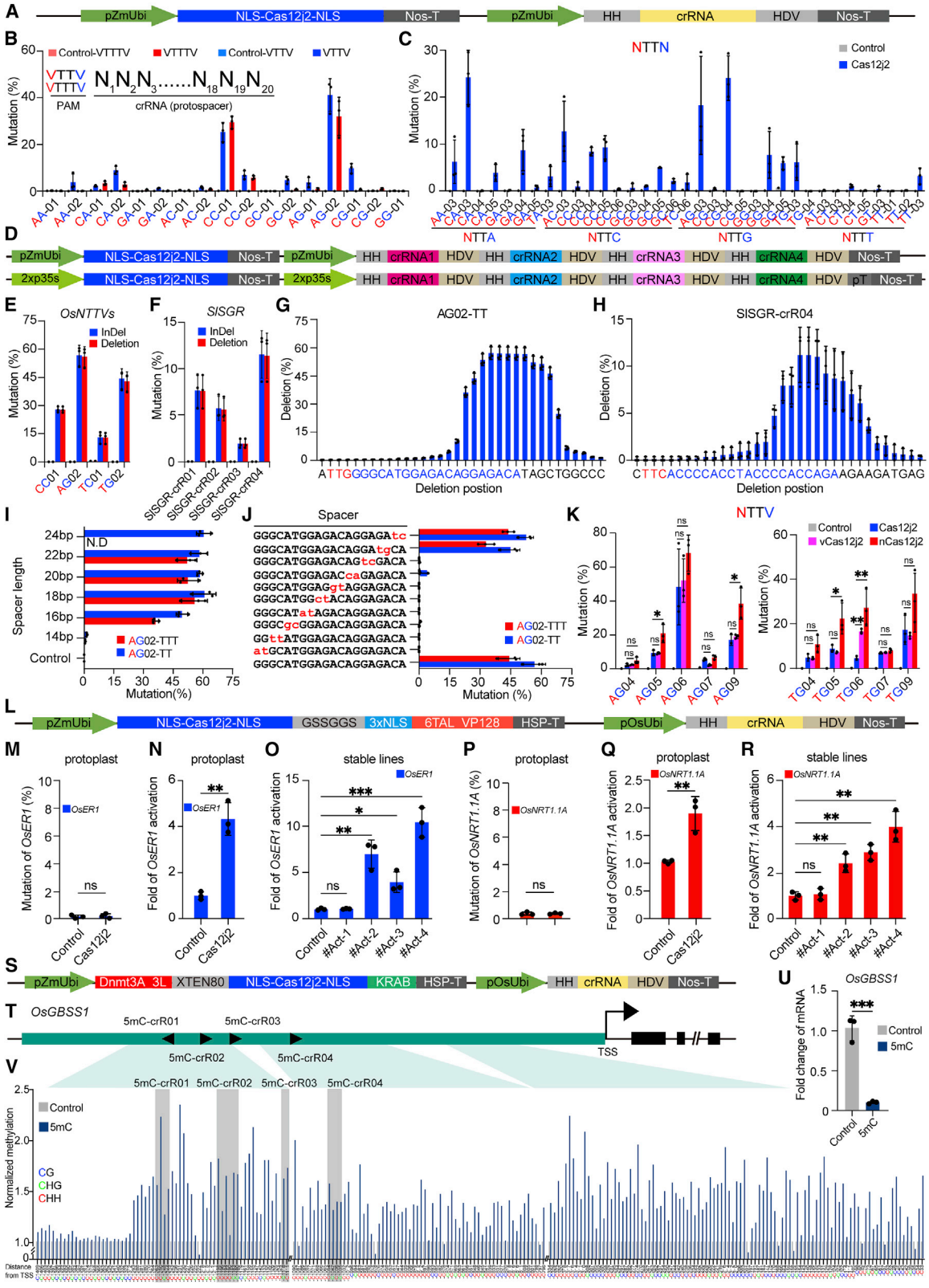

Figure 1. Development of the CRISPR–Cas12j2 system for genome editing, gene activation, and DNA methylation-based gene silencing in plants.

(A) Schematic of the dual RNA polymerase II promoter system for *Cas12j2* and crRNA expression.

(B) Assessment of the effects of PAM and protospacer sequence on genome editing efficiency with CRISPR-Cas12j2 at 34 target sites in rice protoplasts.

(legend continued on next page)

among 39 rice lines generated with four T-DNA constructs (Supplemental Figure 6A–D). This contrasts with the relatively higher editing efficiencies in rice protoplasts. qRT–PCR analysis of select T0 lines showed that the expression level of *Cas12j2* was relatively high (Supplemental Figure 6E), which suggests that low genome editing efficiency in stable lines was not due to low *Cas12j2* expression. We next generated four multiplexed Cas12j2 constructs for editing 16 target sites in four genes from poplar (four sites per gene). An analysis of 16 T0 transgenic plants per construct revealed very low editing efficiencies (up to 1.2%) at all target sites (Supplemental Figure 7A–D) even though *Cas12j2* expression was normal (Supplemental Figure 7E). These data point to a disconnect between Cas12j2-mediated genome editing in stable lines versus protoplasts. To solve this puzzle, we selected three T0 rice lines that express *nCas12j2* and crRNAs for editing the AG17–TT site. Although we could not detect successful genome editing in the leaves of these seedlings by Sanger sequencing (Supplemental Figure 8A), we did detect genome editing efficiencies of roughly 50% in protoplasts derived from these seedlings after 48 h or 72 h of resting (Supplemental Figure 8B-C). These data suggest that it may be more efficient to obtain nCas12j2-edited plants via protoplast regeneration rather than stable plant transformation (Yue et al., 2021).

To engineer a CRISPR-Cas12j2 system that can activate the transcription of target genes, we fused a potent TV (6TAL–VP128) activation domain (Li et al., 2017) to the C-terminus of Cas12j2 (Figure 1L). The resulting Cas12j2 activator was tested for transcriptional activation of *OsER1* and *OsNRT1.1A* using 16 bp protospacers in rice protoplasts. While no edits were detected in either gene, four- and two-fold increases in gene expression were observed for *OsER1* and *OsNRT1.1A*, respectively (Figure 1M-P). Even higher gene expression was detected in stable transgenic plants: a ten-fold increase for *OsER1* and a four-fold increase for *OsNRT1.1A* (Figure 1O and Figure 1R). We further demonstrated that two additional target genes (*OsCHS* and *OsGBSS1*) could be transcriptionally activated by Cas12j2 without being edited (Supplemental Figure 9A-B).

Finally, a CRISPRoff configuration was adopted to develop a Cas12j2-derived epigenome editing system for targeted DNA methylation in plants (Nunez et al., 2021) (Figure 1S). Four crRNAs were designed for multiplexed targeting of the promoter region of *OsGBSS1* (Figure 1T). Transformation of the Cas12j2 epigenome editor (5mC) in rice cells resulted in a drastic reduction of the mRNA level for *OsGBSS1*, which coincided with methylation of the promoter as determined by bisulfite sequencing (Figure 1U-V).

In summary, we demonstrate that the hypercompact Cas12j2 can be used for genome editing in a variety of plant species. Furthermore, we repurposed Cas12j2 for gene activation and epigenome editing to fine-tune target gene expression in plants. Our observation that Cas12j2 has a unique preference for editing in non-dividing cells warrants further investigation to determine the exact mechanism by which this occurs. Furthermore, we repurposed Cas12j2 for gene activation and epigenome editing to fine-tune target gene expression in plants. With further improvement of Cas12j2, we anticipate that this small Cas protein will become widely adopted for versatile applications in plant genome engineering (Lyu, 2020).

SUPPLEMENTAL INFORMATION

Supplemental information is available at *Plant Communications Online*.

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(C) Refined analysis of NTTN PAM requirements for CRISPR-Cas12j2 in rice protoplasts.
(D) Schematics of the dual RNA polymerase II promoter-based and multiplexed CRISPR-Cas12j2 systems for genome editing in rice and tomato, respectively.
(E) Multiplexed editing of four target sites in rice protoplasts.
(F) Multiplexed editing of four target sites in tomato protoplasts.
(G) Deletion position profile for a representative target site in rice.
(H) Deletion position profile for a representative target site in tomato.
(I) Assessment of protospacer length requirements at two PAM sites with the same protospacer in rice protoplasts (N.D., not detected).
(J) Assessment of targeting specificity using mismatched crRNAs at two PAM sites with the same protospacer in rice protoplasts.
(K) Improvement of genome editing efficiency using engineered Cas12j2 variants.
(L) Schematic of a CRISPR-Cas12j2 transcriptional activation system.
(M–R) Transcriptional activation of *OsER1* and *OsNRT1.1A* in rice.
(M and P) Testing of genome editing efficiency.
(N and Q) Testing of transcriptional activation in rice protoplasts.
(O and R) Testing of transcriptional activation in transgenic rice lines.
(S) Schematic of a CRISPR-Cas12j2-based targeted DNA methylation system.
(T) Schematic of targeted DNA methylation of the *OsGBSS1* promoter. Four target sites are indicated by black arrow heads. The three regions chosen for bisulfite sequencing are indicated by green arrows.
(U) Targeted gene silencing of *OsGBSS1* with directed DNA methylation in rice protoplasts.
(V) DNA methylation at target regions determined using bisulfate sequencing of PCR products. For experiments in rice and tomato protoplasts, three biological replicates were used. The error bars denote standard deviations. Asterisks are used to denote statistical significance by Student's *t*-test (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ns, not significant).

AUTHOR CONTRIBUTIONS

Y.Z. and Y.Q. designed the experiments. S.L. and S.S. designed and made the constructs. S.L., S.S., T.F., X.T., A.Q., Y.X., and Z.Z. generated all T-DNA vectors. S.L. and T.F. conducted rice protoplast isolation and transformation. S.L., Y.X., Y.H., Y.L., Q.H., and X.Z. did NGS analysis for genome editing in rice protoplasts and stable lines. W.G. and X.G. analyzed the rice methylation NGS data. Y.C. and S.S. did the tomato protoplast transformation and NGS analysis of editing. G.L. conducted stable transformation of poplar and NGS analysis of editing. Y.Z. and Y.Q. analyzed the data and wrote the manuscript. All authors participated in discussion and revision of the manuscript.

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Shishi Liu[1,5], Simon Sretenovic[2,5], Tingting Fan[1,5], Yanhao Cheng[2], Gen Li[2], Aileen Qi[2], Xu Tang[1], Yang Xu[1], Weijun Guo[3], Zhaohui Zhong[1], Yao He[1], Yanling Liang[1], Qinqin Han[1], Xuelian Zheng[1], Xiaofeng Gu[3], Yiping Qi[2,4,] and Yong Zhang[1,*]*

[1]Department of Biotechnology, School of Life Sciences and Technology, Center for Informational Biology, University of Electronic Science and Technology of China, Room 216, Main Building, No. 4, Section 2, North Jianshe Road, Chengdu 610054, China
[2]Department of Plant Science and Landscape Architecture, University of Maryland, College Park, MD 20742, USA
[3]Biotechnology Research Institute, Chinese Academy of Agricultural Sciences, Beijing 100081, China
[4]Institute for Bioscience and Biotechnology Research, University of Maryland, Rockville, MD 20850, USA
[5]These authors contributed equally to this article.
***Correspondence: Yiping Qi (Yiping@umd.edu), Yong Zhang (zhangyong916@uestc.edu.cn)**
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