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Letter to the Editor

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# Multiplex Gene Editing in Rice Using the CRISPR-Cpf1 System

Dear Editor,

The class 2/type II clustered regularly interspaced short palindromic repeat (CRISPR)/Cas9 system has been used successfully for simultaneous modification of multiple loci in plants. Two general strategies have been applied to coexpress multiple single guide RNAs (sgRNAs) to achieve multiplex gene editing in plant cells. One is to construct the multiple guide RNA expression cassettes into separate plasmids when direct gene delivery methods are adopted, such as biolistic bombardment and PEG-mediated protoplast transfection (Shan et al., 2013). The other is to assemble the multiple sgRNAs into a single vector when the *Agrobacteria*-mediated gene transformation method is used. These multiple sgRNAs can be driven by separate promoters (Ma et al., 2015; Zhang et al., 2016) or expressed as a single transcript for further processing by plant endogenous ribonucleases (Xie et al., 2015). Although these two strategies were reported to be efficient for introducing targeted gene modifications in plant genomes, the construction of CRISPR/Cas9 vectors was complicated and laborious.

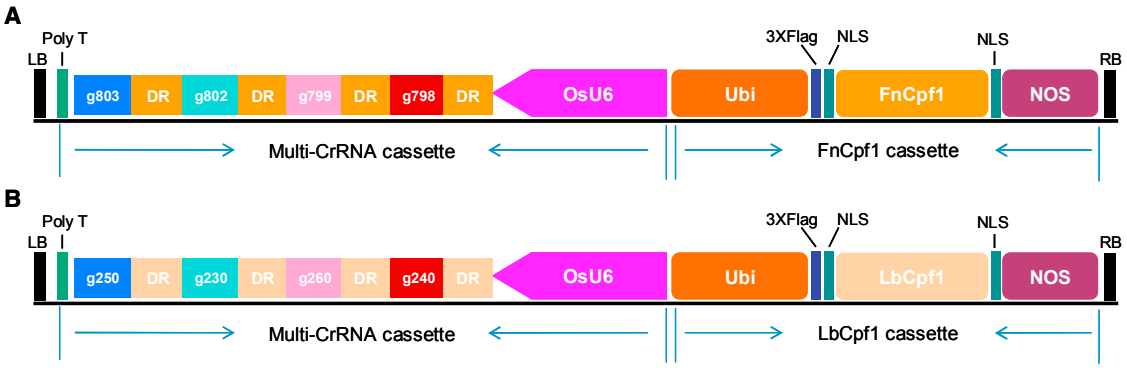
Recently, Cpf1 (CRISPR from *Prevotella* and *Francisella* 1) was characterized as a novel class 2 system component that has distinct features compared with Cas9. It is a single RNA-guided endonuclease, recognizes the thymidine-rich protospacer-adjacent motif (PAM), and produces staggered cuts distal to the PAM site (Zetsche et al., 2015). This type V CRISPR/Cpf1 system has been demonstrated to have robust genome editing activity in mammalian cells (Zetsche et al., 2015; Kim et al., 2016), and also generate target mutations in rice, *Arabidopsis*, soybean, and tobacco (Endo et al., 2016; Xu et al., 2016; Hu et al., 2017; Kim et al., 2017; Tang et al., 2017). Interestingly, Cpf1 is a dual nuclease that not only cleaves target DNA but also processes its own CRISPR RNA (crRNA) (Fonfara et al., 2016; Zetsche et al., 2016). In addition, the maturation of crRNA by Cpf1 does not require the assistance of *trans*-activating crRNA (tracrRNA). With these advantages, the natural structure of Cpf1 system was recently adopted for multiplex gene editing in mammalian cells, where up to four genes were simultaneously edited by Cpf1 using a single crRNA array spaced by mature direct repeats (DRs) (Zetsche et al., 2016).

To compare the gene editing efficiency of *Francisella novicida* Cpf1 (FnCpf1) and *Lachnospiraceae bacterium* ND2006 Cpf1 (LbCpf1) in rice, two T-DNA constructs were designed based on the pCambia binary vector to express FnCpf1 and LbCpf1 systems (Supplemental Figure 1). We chose 22–24 nt target sequences to induce mutations at six sites of three endogenous genes: *5-Enolpyruvylshikimate 3-Phosphate Synthase* (*OsEPSPS*, LOC_Os06g04280), *Bentazon Sensitive Lethal* (*OsBEL*, LOC_Os03g55240), and *Phytoene Desaturase* (*OsPDS*, LOC_Os03g08570). Our results showed that both FnCpf1 and LbCpf1 with their own mature DRs can introduce targeted gene mutations in transgenic plants (Supplemental Table 1). Mutations in *OsPDS* caused an albino phenotype, as expected (Supplemental Figure 2). Moreover, we found that the LbCpf1 system exhibited a higher editing efficiency than FnCpf1 in all of the six tested target sites, and both of them showed big differences in the frequency of induced mutations between two target sites within the same gene (Supplemental Table 1), which is consistent with a previous finding that the efficiency of Cpf1 may be influenced by factors such as the base content of the target sequence (Fonfara et al., 2016).

Given our results showing that both FnCpf1 and LbCpf1 with their own mature DRs induced mutations in transgenic plants, we tested whether these Cpf1 could be used for multiplex gene editing in rice. We used FnCpf1 to edit four members related to receptor-like kinases (*OsRLKs*): *OsRLK*-798 (LOC_Os02g04430), *OsRLK*-799 (LOC_Os02g07960), *OsRLK*-802 (LOC_Os01g39600), and *OsRLK*-803 (LOC_Os06g04370), and used LbCpf1 to edit four *OsBEL* genes of the CYP81A family: *OsBEL*-230 (LOC_Os03g55230), *OsBEL*-240 (LOC_Os03g55240), *OsBEL*-250 (LOC_Os03g55250), and *OsBEL*-260 (LOC_Os03g55260). The guide sequence targeting *OsBEL*-240 was the same as the *OsBEL*-1 site in the above single gene editing. In our design, four units of crRNAs in their mature form, 20 bp (FnCpf1) or 21 bp (LbCpf1) DR with 23–24 bp guide sequence, were ligated in tandem and driven by one OsU6 promoter in the same construct with FnCpf1 or LbCpf1 (Figure 1A and 1B, Supplemental Sequences). Our analysis of the resulting T0 rice transgenic plants showed robust multiplex gene editing activities; the mutation frequencies of the four *OsRLK* genes targeted by FnCpf1 were 43.8%–75%, and were 40%–60% for the four *OsBEL* genes targeted by LbCpf1 (Figure 1C). Approximately 44% and 67% of the mutants edited by FnCpf1 and LbCpf1, respectively, had all four target sites mutated simultaneously (Figure 1D and Supplemental Figures 3–8). The different target genes made it difficult to compare the efficiencies of the two Cpf1 systems. It is possible that the variations in editing efficiencies were due to the differences in target sequences.

The crRNA arrays with guides in different orders resulted in similar mutation frequencies in multiplex gene editing by Cpf1 in mammalian cells, suggesting that the positioning within an array is not crucial for activity (Zetsche et al., 2016). In our study, when the same locus in *OsBEL* gene (*OsBEL*-1 and *OsBEL*-240) was targeted by LbCpf1, the mutagenesis efficiencies of our multiplex gene editing system (53.3%) and

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Summary of the multiplex gene editing in rice by FnCpf1 and LbCpf1

| Target loci | PAM-guide sequence (5'-3') | Plantlets identify | Mutant | Mutation rate % | Genotype |
|---|---|---|---|---|---|
| **FnCpf1** | | | | | |
| *OsRLK*-798 | **TTT**CCATTCCCCATCCCGTTCTTTGGC | 32 | 24 | 75 | 3Ho+6Bi+6He+9Chi+8WT |
| *OsRLK*-799 | **TTT**GGAAGGACAGTTAGGCAGCCTGGG | | 20 | 62.5 | 6Bi+4He+10Chi+12WT |
| *OsRLK*-802 | **TTT**CGCAAGAGCGCCTCATCCACGCTT | | 21 | 65.6 | 8Bi+5He+8Chi+11WT |
| *OsRLK*-803 | **TTT**ACCTCGACGTCAACCACCTGACCG | | 14 | 43.8 | 2Bi+6He+6Chi+18WT |
| **LbCpf1** | | | | | |
| *OsBEL*-230 | **TTTG**CTCGGTGAGTACAAGTCTAAAAG | 30 | 18 | 60 | 5Bi+2He+11Chi+12WT |
| *OsBEL*-240 | **TTTC**ATCTCCTTCTAGAAGCACAAGCG | | 16 | 53.3 | 1Ho+4Bi+3He+8Chi+14WT |
| *OsBEL*-250 | **TTTG**TGCGGTGAGTGAGTACGTACTTTC | | 12 | 40 | 3Bi+4He+5Chi+18WT |
| *OsBEL*-260 | **TTTG**GTGCCGGAACAGAGACTACATCA | | 17 | 56.7 | 6Bi+3He+8Chi+13WT |

D

Distribution of the mutations in T0 plants derived from Cpf1-multiplex gene editing

| Mutation sites | 4 | 3 | 2 | 1 | 0 |
|---|---|---|---|---|---|
| Plantlets (FnCpf1) | 11(34.4%) | 7(21.9%) | 5(15.6%) | 2(6.3%) | 7(21.9%) |
| Plantlets (LbCpf1) | 12(40%) | 4(13.3%) | 1(3.3%) | 1(3.3%) | 12(40%) |

**Figure 1. T-DNA Constructs of FnCpf1 and LbCpf1 for Multiplex Gene Editing and the Resulting Mutagenesis Efficiency in Rice.**
**(A)** The construct of the FnCpf1 multiplex gene editing system contains an FnCpf1 expression cassette and a multi-crRNA expression cassette. FnCpf1 was inserted downstream of the *Zm*Ubi promoter. A NOS terminator was placed at the end of FnCpf1 ORF. The SV40-derived nuclear localization signal (NLS) was fused translationally to both the N and C termini of FnCpf1. A 3XFlag was in-frame fused to the N terminus of NLS. The multi-crRNA expression cassette contains four DR guide units, and each unit includes one mature DR and 23–24 bp of guide sequence (g). This array is controlled by the *Os*U6 promoter and terminated by a 7 bp polyT sequence.
**(B)** The construct of the LbCpf1 multiplex gene editing system. The elements included are similar to those of FnCpf1.
**(C)** Summary of the target sequences and mutations in rice T0 plants transformed with the FnCpf1 and LbCpf1 multiplex gene editing systems. Ho, possible homozygote; Bi, possible bi-allele; He, possible heterozygote; Chi, Chimera; WT, wild-type. The PAM motif (TTN for FnCpf1 and TTTN for LbCpf1) is marked in bold.
**(D)** Distribution of the mutations in T0 plants derived from Cpf1 multiplex gene editing.

the single gene editing system (62.5%) were comparable. The result indicated that crRNA array structure does not significantly affect the cleavage efficiency of Cpf1 systems in plants. Nevertheless, we found that the last DR-guide unit at both the FnCpf1 and LbCpf1 crRNA arrays caused the lowest mutagenesis efficiency (targets for *OsRLK*-803 and *OsBEL*-250, Figure 1A–1C). The last DR-guide unit may need to be extended, or an additional DR element may be added at the end of crRNA for better processing of the tail in the array by Cpf1.

Different from the common 1–2 bp short indels generated by Cas9 in rice (Zhang et al., 2014), most of the mutations derived from multiplex gene editing by both FnCpf1 and LbCpf1 in rice were 3–30 bp deletions at 3′ of the target sequence (Supplemental Figures 3–8). The mutation spectrum was similar with recently reported single gene editing using FnCpf1 or LbCpf1 in rice (Endo et al., 2016; Xu et al., 2016).

Cpf1 was shown to be highly specific in gene editing in human cells (Kim et al., 2016). No off-target mutations were found at potential off-target sites, when LbCpf1 was used to edit several single genes in rice (Xu et al., 2016). However, slight off-target effects were found at highly homologous sequences that have only one mismatch with their on-target sites, when some single loci were edited using FnCpf1 in rice (Endo et al., 2016). To test the off-target effect of Cpf1 in multiplex gene editing, we assayed three most likely off-target sites for each target by PCR and sequencing. The test for each potential off-target site was carried out in at least five transgenic lines that had all four target sites mutated. We did not find any off-target mutations from any of

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the selected potential off-target sites for either FnCpf1 or LbCpf1. These results illustrated the faithfulness of CRISPR-Cpf1 in multiplex gene editing in rice.

Cpf1 recognizes the crRNA through a combination of sequence-specific and structural features of the stem loop, and that the DR sequences of Cpf1 are highly conserved and functionally interchangeable (Zetsche et al., 2015, 2016; Fonfara et al., 2016). The FnCpf1 and LbCpf1 systems have the same repeat recognition sequence (RRS) and stem regions in their mature DRs, but the size of the loop in LbDR is one nucleotide larger than FnDR (Zetsche et al., 2015). It has been illustrated that RRS and stem structure are crucial for the cleavage activity of Cpf1; however, changes in the loop region resulted in reduced cleavage activity for a shorter loop, whereas an increased loop length did not influence cleavage (Fonfara et al., 2016). To investigate the specificity of DR sequences in rice, we interchanged the crRNA cassette between the FnCpf1 and LbCpf1 constructs (Supplemental Figure 9), and then used the new constructs to target the *OsPDS*-1 and *OsBEL*-2 sites. We found that both interchanged Cpf1 systems caused targeted mutations with efficiencies comparable with their original ones, although the FnCpf1-LbDR system showed an increased mutation efficiency at the *OsBEL*-2 site (21.6% versus 12.5%) and the LbCpf1-FnDR system showed a decreased mutation efficiency at the *OsPDS*-1 site (36.7% versus 73.5%) (Supplemental Table 2).

Multiplex gene editing provides a powerful tool for targeting members of multigene families. Although previous studies have shown that multiplex gene editing in plants is possible with CRISPR-Cas9 (Ma et al., 2015; Xie et al., 2015; Zhang et al., 2016), the Cas9 system requires large constructs to express multiple sgRNA cassettes, which are more laborious to construct and may cause instability and reduce transformation efficiency. The current study has demonstrated the feasibility of high-efficiency multiplex gene editing in plants using engineered CRISPR-Cpf1 with a simple short DR-guide array. The flexibility of the DR sequence of Cpf1 will help to further optimize the DR-guide array to achieve higher editing efficiency. Although future studies are needed to examine the germline transmission of the Cpf1 multiplex gene-editing-induced mutations and to further evaluate the potential off-target effect genome-wide, our results show that the CRISPR/Cpf1 system can significantly simplify multiplex gene editing in plants.

## SUPPLEMENTAL INFORMATION

Supplemental Information is available at *Molecular Plant Online*.


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## REFERENCES


**Endo, A., Masafumi, M., Kaya, H., and Toki, S.** (2016). Efficient targeted mutagenesis of rice and tobacco genomes using Cpf1 from *Francisella novicida*. Sci. Rep. **6**:38169.

**Fonfara, I., Richter, H., Bratovi, M., Le, R.A., and Charpentier, E.** (2016). The CRISPR-associated DNA-cleaving enzyme Cpf1 also processes precursor CRISPR RNA. Nature **532**:517–521.

**Hu, X., Wang, C., Liu, Q., Fu, Y., and Wang, K.** (2017). Targeted mutagenesis in rice using CRISPR-Cpf1 system. J. Genet. Genomics **44**:71–73.

**Kim, D., Kim, J., Hur, J.K., Been, K.W., and Yoon, S.H.** (2016). Genome-wide analysis reveals specificities of Cpf1 endonucleases in human cells. Nat. Biotechnol. **34**:863–868.

**Kim, H., Kim, S.T., Ryu, J., Kang, B.C., Kim, J.S., and Kim, S.G.** (2017). CRISPR/Cpf1-mediated DNA-free plant genome editing. Nat. Commun. **8**:14406.

**Ma, X., Zhang, Q., Zhu, Q., Liu, W., Chen, Y., Qiu, R., Wang, B., Yang, Z., Li, H., Lin, Y., et al.** (2015). A robust CRISPR/Cas9 system for convenient, high-efficiency multiplex genome editing in monocot and dicot plants. Mol. Plant **8**:1274–1284.

**Shan, Q., Wang, Y., Li, J., Zhang, Y., Chen, K., Liang, Z., Zhang, K., Liu, J., Xi, J.J., Qiu, J., et al.** (2013). Targeted genome modification of crop plants using a CRISPR-Cas system. Nat. Biotechnol. **31**:686–688.

**Tang, X., Lowder, L.G., Zhang, T., Malzahn, A.A., Zheng, X., Voytas, D.F., Zhong, Z., Chen, Y., Ren, Q., Li, Q., et al.** (2017). A CRISPR-Cpf1 system for efficient genome editing and transcriptional repression in plants. Nat. Plants **3**:17018.

**Xie, K., Minkenberg, B., and Yang, Y.** (2015). Boosting CRISPR/Cas9 multiplex editing capability with the endogenous tRNA-processing system. Proc. Natl. Acad. Sci. USA **112**:3570–3575.

**Xu, R., Qin, R., Li, H., Li, D., Li, L., Wei, P., and Yang, J.** (2016). Generation of targeted mutant rice using a CRISPR-Cpf1 system. Plant Biotechnol. J. http://dx.doi.org/10.1111/pbi.12669.

**Zetsche, B., Gootenberg, J.S., Abudayyeh, O.O., Slaymaker, I.M., Makarova, K.S., Essletzbichler, P., Volz, S.E., Joung, J., van der Oost, J., Regev, A., et al.** (2015). Cpf1 is a single RNA-guided endonuclease of a class 2 CRISPR-Cas system. Cell **163**:759–771.

**Zetsche, B., Heidenreich, M., Mohanraju, P., Fedorova, I., and Kneppers, J.** (2016). Multiplex gene editing by CRISPR-Cpf1 using a single crRNA array. Nat. Biotechnol. **35**:31–34.

**Zhang, H., Zhang, J., Wei, P., Zhang, B., Gou, F., Feng, Z., Mao, Y., Yang, L., Zhang, H., Xu, N., et al.** (2014). The CRISPR/Cas9 system produces specific and homozygous targeted gene editing in rice in one generation. Plant Biotechnol. J. 1–11.

**Zhang, Z., Mao, Y., Si, H., Liu, W., and Botella, J.R.** (2016). A multiplex CRISPR/Cas9 platform for fast and efficient editing of multiple genes in *Arabidopsis*. Plant Cell Rep. **35**:1519–1533.