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Letter to the editor

Targeted mutagenesis in rice using CRISPR-Cpf1 system

Cpf1 is a class 2/type V CRISPR effector that has been recently harnessed for genome editing (Zetsche et al., 2015; Hur et al., 2016; Kim et al., 2016). Cpf1 recognizes thymidine-rich sequence as the protospacer-adjacent motif (PAM) at the 5′ end of target sequences. In addition, Cpf1 requires only a single shorter crRNA and cleaves DNA in a staggered fashion with 5′ overhangs (Zetsche et al., 2015). These unique features distinguish Cpf1 from type II CRISPR-Cas9 and provide for an expansion of the toolbox of genome editing. However, the function of Cpf1 in plants remains unexplored. We report here that CRISPR-Cpf1 is applicable to plants for genome editing by co-expression of Cpf1 protein and crRNA *in vivo*.

To test the potential of the CRISPR-Cpf1 system in genome editing in plants, we first codon-optimized *Lachnospiraceae bacterium ND2006 Cpf1* (*LbCpf1*) and *Acidaminococcus* sp. *BV3L6 Cpf1* (*AsCpf1*), and attached nuclear localization signals (NLSs) at both ends (Fig. 1A and Fig. S1). In contrast to Cas9 system, the spacer sequence, which is essential for the recognition of target sequence, is located at the 3′ end of the crRNAs in CRISPR-Cpf1 system (Fig. 1B). Currently, the widely used crRNA-expressing devices produce mature crRNAs with a 1–7-nt poly(U) tail in eukaryotic cells, which might result in mismatches between the crRNA and target sequence (Xie et al., 2015). To reduce the likelihood of mismatch, we used the endogenous tRNA-processing system to simultaneously produce multiple crRNAs for respective targets, which only contained the 1–4-nt "AACA" tails (Figs. 1C and S2) (Xie et al., 2015). This system contains tandem repeats of tRNA-crRNA under the control of a single rice *U3* promoter. The endogenous tRNA-processing RNases (RNase P and RNase Z) would recognize the structure and generate crRNAs (Fig. 1C). To completely get rid of potential disturbance of the mismatch of the tail, we screened the rice genome and selected one target DNA sequence (C1) harboring both the 3′-AACA tail and the indispensable 5′-TTTN (N = A, T, C or G) PAM (Fig. 1B and Table S1). Theoretically, the mature crRNA would completely match the target site no matter whether the 1–4-nt "AACA" tail is added or not. After assembling crRNA (C1) and *Cpf1* into one binary vector and conducting genetic transformation, 58 and 34 of independent *LbCpf1* and *AsCpf1* transgenic plants were obtained, respectively. By direct DNA sequencing of those plants, we identified mutations in 7 (12.1%) *LbCpf1* transgenic plants (Fig. 1D and E), while no mutations were detected in *AsCpf1* transgenic plants (Table S2). The result suggests that LbCpf1 can be used for genome editing in plants.

To further assess the capability of LbCpf1 in genome editing in plants, we screened the rice genome *in silico* using the same criterion described above and chose four target sites (C2–C5) (Table S1). The corresponding four tRNA-crRNA units were tandemly arrayed under control of a single *U3* promoter (Fig. S2), and were further assembled into one binary vector with *LbCpf1*. After conducting genetic transformation, a total of 20 independent transgenic plants were obtained. DNA sequencing of those plants revealed one mutation in the C2 and C5 targets, respectively (Figs. 1E and S3). The different mutation frequency between four targets implies that the efficiency of LbCpf1 might be correlated with the target sequence. Similar experiments targeting the same targets were also performed using AsCpf1 (Fig. S2), but no successful editing events were detected (Table S2), further indicating AsCpf1 might be less efficient than LbCpf1 in plants.

Given that 3′ tail will be added to the mature crRNA when it is expressed in eukaryotic cells, we therefore wanted to know whether the 3′ tail affects the function of CRISPR-Cpf1 system. Four target sites with the 5′-TTTN PAMs for LbCpf1 but without the 3′-AACA were selected, two (C6 and C7) in *Narrow Leaf1* (*NAL1*) and two (C8 and C9) in *LIGULELESS1* (*LG1*) (Table S1). The expression cassette containing tandemly arrayed four tRNA-crRNAs units for C6 to C9, was also driven by a single *U3* promoter, but lacked the tRNA behind the tRNA-crRNA (C9). Thus, this cassette would generate three mature crRNAs (C6–C8) carrying 1–4-nt "AACA" tails and one mature crRNA (C9) carrying a 1–7-nt poly(U) tail (Figs. S2 and S4). By sequencing of the 35 transgenic lines obtained, we detected no mutation in all those sites (Table S3). The results suggest that the 3′ tail generated by endogenous crRNA expression system may affect the function of CRISPR-Cpf1 system and must be taken into account in Cpf1-mediated genome editing.

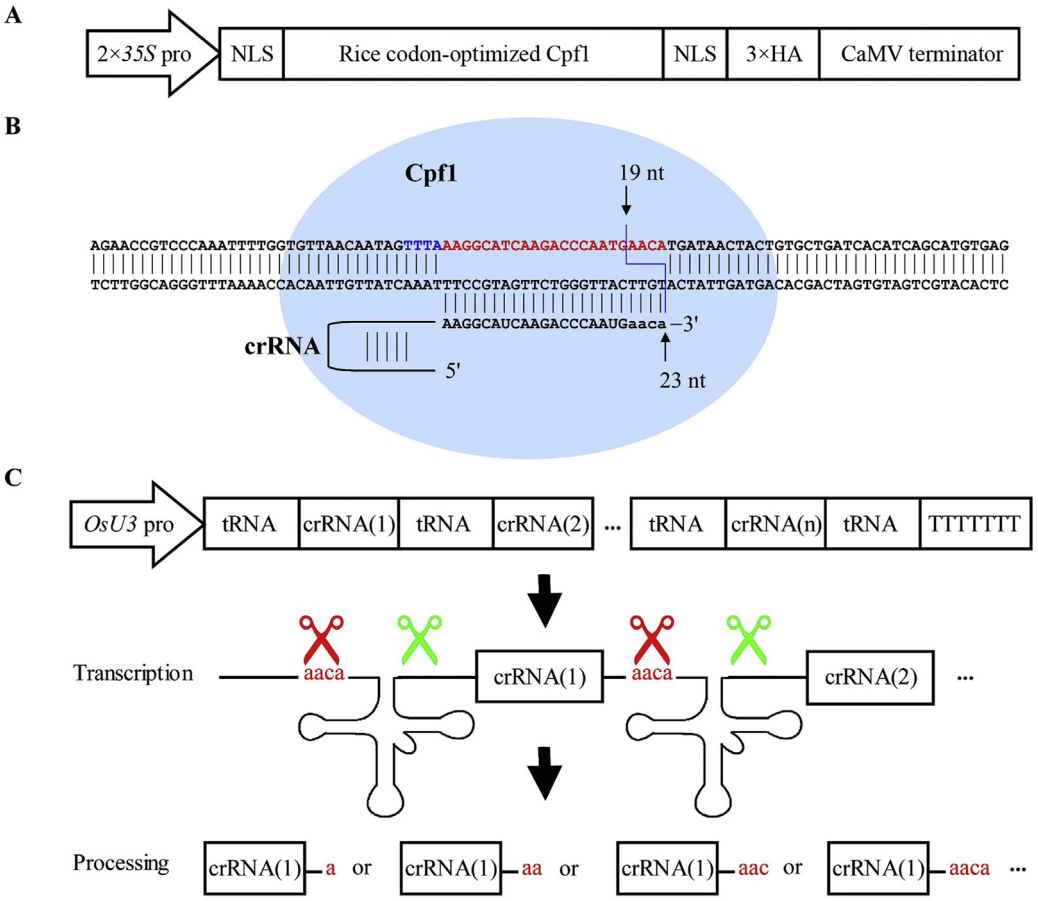
Unlike Cas9 that creates blunt ends within the target DNA, Cpf1 generates sticky overhangs. To elucidate whether staggered cuts introduced by Cpf1 would lead to mutation types different from those caused by Cas9, we analyzed the mutation types of all successfully edited plants. All mutations generated by Cpf1 were deletions at the target sites, ranging from 6 to 38 nt around sticky overhangs (Figs. 1D and S3). No small insertions or deletions (indels) ($\leq$5 nt) were identified. Therefore, in contrast to CRISPR-Cas9 system that frequently introduce small indels (Shan et al., 2013), the CRISPR-Cpf1 system is likely be more proficient at generating long nucleotide deletions in plants. To evaluate potential off-target effects of Cpf1, we examined all five possible off-target sites (Off-C1 to Off-C5) as we previously described (Hu et al., 2016). However, no off-target mutation was found at those sites (Table S4).

In summary, we successfully generated genome-edited plants

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      ATAGTTTAAAGGCATCAAGACCCAATGAACATGATAACTACTGTGCTGATCACATCAGCATGTGAG WT
#15   ATAGTTTAAAGGCATCAAGACCCAATGAACATGATAACTACTGTGCTGATCACATCAGCATGTGAG WT
      ATAGTTTAAAGGCATCAAGACCCA-------TGATAACTACTGTGCTGATCACATCAGCATGTGAG -7
#17   ATAGTTTAAAGGCATCAAGACCCAATGAACATGATAACTACTGTGCTGATCACATCAGCATGTGAG WT
      ATAGTTTAAAGGCATCAAGACC------------------TGTGCTGATCACATCAGCATGTGAG -19
#22   ATAGTTTAAAGGCATCAAGACCCAATGAACATGATAACTACTGTGCTGATCACATCAGCATGTGAG WT
      ATAGTTTAAAGGCATCAAGACCCA-------TGATAACTACTGTGCTGATCACATCAGCATGTGAG -7
#28   ATAGTTTAAAGGCATCAAGACCCAATGAACATGATAACTACTGTGCTGATCACATCAGCATGTGAG WT
      ATAGTTTAAAGGCATCAAGAC------AACATGATAACTACTGTGCTGATCACATCAGCATGTGAG -6
#39   ATAGTTTAAAGGCATCAAGACCCAATGAACATGATAACTACTGTGCTGATCACATCAGCATGTGAG WT
      ATAGTTTAAAGGCATCAAGACCCA-------TGATAACTACTGTGCTGATCACATCAGCATGTGAG -7
#56   ATAGTTTAAAGGCATCAAGACCCAATGAACATGATAACTACTGTGCTGATCACATCAGCATGTGAG WT
      ATAGTTTAAAGGCATCAAGAC-----------------------------------ATGTGAG -38
#57   ATAGTTTAAAGGCATCAAGACCCAATGAACATGATAACTACTGTGCTGATCACATCAGCATGTGAG WT
      ATAGTTTAAAGGCATCAAGA----------------ACTACTGTGCTGATCACATCAGCATGTGAG -16
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E

	crRNA				
	C1	C2	C3	C4	C5
Number of plants detected	58	20	20	20	20
Number of modified plants	7	1	0	0	1
Mutation rate (%)	12.1	5	0	0	5

Fig. 1. Cpf1-mediated genome editing *in planta* by co-expression of Cpf1 protein and crRNA *in vivo*. **A**: Schematic diagram of the vector expressing rice codon-optimized Cpf1 protein. NLS, nuclear localization signal. **B**: Schematic illustrating of CRISPR-Cpf1 system. The PAM (5′-TTTA) and target sequence (C1) are denoted in blue and red, respectively. The blue line shows a potential staggered cut caused by Cpf1. The lowercase indicates the 1–4-nt tail, which are randomly produced by endogenous tRNA-processing system. **C**: The endogenous tRNA-processing system produces mature crRNAs with 1–4-nt "AACA" tails. The system contains tandem repeats of tRNA-crRNA under the control of a single rice *U3* promoter. The transcript is processed by endogenous tRNA-processing RNases to release multiple crRNAs with 1–4-nt "AACA" tails. The red and green scissors represent RNase P and RNase Z, respectively. **D**: Sequencing assay of mutations around C1 site. The PAM and target sequence are denoted in blue and red, respectively. – indicates deleted nucleotides. **E**: Mutation rates of C1 to C5 with LbCpf1.

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using Cpf1. During the revision of our manuscript, one other group published similar studies in rice (Xu et al., 2016). Although the editing efficiency is still low and needs further improvement, both findings demonstrate that Cpf1 can be utilized as an alternative system for genome editing in plants.

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Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jgg.2016.12.001.

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