Generation of targeted mutant rice using a CRISPR-Cpf1 system

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Summary

CRISPR-Cpf1 is a newly identified CRISPR-Cas system, and Cpf1 was recently engineered as a molecular tool for targeted genome editing in mammalian cells. To test whether the engineered CRISPR-Cpf1 system could induce the production of rice mutants, we selected two genome targets in the OsPDS and OsBEL genes. Our results show that both targets could be efficiently mutated in transgenic rice plants using CRISPR-Cpf1. We found that pre-crRNAs with a full-length direct repeat sequence exhibited considerably increased efficiencies compared with mature crRNAs. In addition, the specificity and transmission of the mutation were investigated, and the behaviours of crRNA-Cpf1-induced plant targeted genome mutagenesis were assessed. Taken together, our results indicate that CRISPR-Cpf1 expression via stable transformation can efficiently generate specific and heritable targeted mutations in rice and thereby constitutes a novel and important approach to specific and precise plant genome editing.

Introduction

The CRISPR-Cas9 system has emerged as a precise and versatile technique for genome editing, and mounting research demonstrates that efficient editing could be induced by this system in various plant organisms, such as Arabidopsis, rice, wheat, soya bean, maize, tobacco and tomato (Belhaj et al., 2015; Bortesi and Fischer, 2015; Liu et al., 2016). Furthermore, the development and customisation of plant CRISPR/Cas9 toolboxes or platforms result in more powerful molecular tools for fundamental research and crop breeding (Lowder et al., 2015; Ma et al., 2015; Mao et al., 2016; Tang et al., 2016; Wang et al., 2015; Xie et al., 2015; Xing et al., 2014). Rice is the most frequently used crop species for testing and applying CRISPR-Cas9 tools. Several agronomic traits of rice, including yield, fertility, architecture, biotic and abiotic stress response, and herbicide tolerance, have been successfully modified by targeted mutations in one or multiple major genes (Ikeda et al., 2016; Li et al., 2016a,b,c; Osakabe et al., 2016; Sun et al., 2016; Wang et al., 2016; Xu et al., 2014, 2016; Zhou et al., 2015).

The recently identified type V CRISPR-Cas system CRISPR-Cpf1 was found to mediate targeted genome modification in mammalian cells (Hur et al., 2016; Kim et al., 2016; Zetsche et al., 2015). Cpf1 is an RNA-guided endonuclease. Both crRNA and tracrRNA are required for Cas9-mediated targeting. In contrast, a single ~44-nucleotide (nt) crRNA with a 5′-located direct repeat sequence and a spacer sequence that complements the target sequence are sufficient to guide the DNA cleavage of Cpf1 (Zetsche et al., 2015). Moreover, Cpf1 recognizes the thymidine-rich protospacer-adjacent motif (PAM) and makes staggered cuts distal to the PAM site (Fontfara et al., 2016). Here, we demonstrated that efficient targeted mutagenesis can be achieved through the stable expression of certain types of crRNA and Cpf1 in rice (Oryza sativa), a major crop.

Results and discussion

First, the coding sequence of a Cpf1 ortholog from Lachnocloaceae bacterium ND2006 (LbCpf1; NCBI accession number: NZ_JNKS01000011, gene locus_tag: T521_RS08385) was codon-optimized for expression in rice, and a nuclear-localization signal was attached to the 3′ ends (Data S1). To test whether crRNA-Cpf1 could induce genome mutagenesis in plants, we selected a target sequence in an exon of the rice phytoene desaturase (OsPDS) gene as a spacer to design crRNAs through the fusion of direct repeat sequences and constructed it into an engineered binary vector (Figures 1a and b and S1). After the transgenic plants were generated, the target region was examined using the T7 endonuclease (T7E1) assay (Figure S2a) and Sanger sequencing (Figure S3), and the results revealed that targeted mutagenesis could be achieved (Figure 1c and Table 1). Two putative null mutants were identified (Figure 1c) and, as expected, both exhibited an albino phenotype (Figure 1d). Another target located in the rice Bentazon-sensitive-lethal (OsBEL) gene was used to further confirm the genome editing of Cpf1 (Figures S2b and 4). Consistent with the OsPDS target, the mutants were efficiently induced through the transformation of LbCpf1 and its cognate crRNAs (Table 1). Cpf1 processes precursor crRNAs (pre-crRNAs) into mature crRNAs to guide the endonuclease (Fontfara et al., 2016). Most CRISPR-Cpf1 studies in mammalian cells have taken advantage of mature (processed) crRNAs (Hur et al., 2016; Kim et al., 2016; Zetsche et al., 2015). However, no mutant was identified from the transgenic plants generated by targeting either OsPDS or OsBEL (Table 1) directly using the mature crRNA sequence, suggesting that the mutagenesis efficiencies of mature crRNA-Cpf1 complexes were low in the plants. By contrast, mutants were more frequently identified in the lines transformed with pre-crRNAs (Table 1). We found that 13.6% and 20% of the transgenic plants targeting OsPDS and OsBEL, respectively, were...
mutated using pre-crRNAs with the full-length repeat-spacer sequence (pre-crRNA type I). Moreover, mutation frequencies of 21.4% and 41.2% were generated at the OsPDS and OsBEL targets, respectively, using longer pre-crRNAs with the full-length repeat-spacer-repeat sequence (pre-crRNA type II). The higher mutation frequency of pre-crRNAs in rice is consistent with the in vitro observation that the pre-crRNA-Cpf1 complexes exhibit stronger binding activity and increased nuclease activity than do mature crRNA-Cpf1 (Fonfara et al., 2016).

We identified several differences between the behaviour of Cpf1-mediated mutagenesis and that of the previously reported Cas9 system. Compared with the common short indels (1–2 base pairs, bp) generated by Cas9 in rice (Zhang et al., 2014), most of the mutations generated by Cpf1 were relatively long (Figures 1c and S4), which might be caused by nonhomologous end-joining repair on the 4–5 nt 5′ overhangs resulting from the staggered cutting of Cpf1. In addition, several reports have indicated that homozygous mutations can be easily generated by CRISPR-Cas9 in T0 plants (Shan et al., 2013; Zhang et al., 2014), which likely resulted from the Cas9-induced DNA cleavage in the first cell. However, homozygous mutated lines generated by Cpf1 were not observed; instead, the mutated lines were either chimeric or heterozygous (Table 2). Because a heterozygous genotype could be generated by mutation in only a portion of cells, the results suggest that the Cpf1-induced mutants might be largely somatic.

Similar mosaicism has been frequently observed in Cpf1-mutated mice (Kim et al., 2016), suggesting that Cpf1 should induce DNA cleavage after the first division of the embryonic cell and might require a longer acting period than Cas9 in vivo.

The off-target effect of CRISPR-Cpf1 might be minimal compared with that of CRISPR-Cas9 in mammalian cells (Hur et al., 2016; Kim et al., 2016; Kleinstiver et al., 2016). To determine the off-target effect of Cpf1 in plants, we selected genome sites with a high sequence similarity to the OsPDS or OsBEL targets (Table S1). For each target, five transgenic plants

![Figure 1](https://example.com/figure1.png)

**Figure 1** CRISPR-Cpf1 induces targeted genome mutagenesis in rice through genetic transformation. (a) Schematic illustration of the T-DNA region of the engineered CRISPR-Cpf1 binary vector. Three types of crRNAs were used to form different crRNA-Cpf1 combinations. The spacer and repeat regions of the crRNAs are marked in green and black, respectively. LB and RB, left board and right board of T-DNA, respectively. (b) Schematic of the OsPDS target. The PAM is labelled in red, and the spacer sequence is highlighted in green. Blue arrows mark putative sites of DNA cleavage. (c) Phenotype of the OsPDS target mutated lines. From left to right, a nonmutated transgenic plant (OsPDS:LbCpf1-pre-crRNA type I line #2), a plant with a heterozygous mutation (OsPDS:LbCpf1-pre-crRNA type I line #9), a bi-allelic mutant (OsPDS:LbCpf1-pre-crRNA type II line #8) and a putative null mutant with a chimeric mutation (OsPDS:LbCpf1-pre-crRNA type II line #19). Scale bar = 5 mm.

### Table 1

| Genomic target | crRNA type* | No. of events examined† | No. of events with mutation† | Mutation rate (%) |
|----------------|-------------|-------------------------|-----------------------------|------------------|
| OsPDS          | Mature crRNA| 85                      | 0                           | 0                |
|                | Pre-crRNA type I | 22                   | 3                           | 13.6             |
|                | Pre-crRNA type II| 28                   | 6                           | 21.4             |
| OsBEL          | Mature crRNA| 48                      | 0                           | 0                |
|                | Pre-crRNA type I | 20                   | 4                           | 20               |
|                | Pre-crRNA type II| 34                   | 14                          | 41.2             |

*The details of the crRNA types are given in Figures 1a and S1.
†Independent T0 events were used to examine the target mutation.

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Table 2 Zygosity in T₀ rice mutants

| Genome target | crRNA type | No. of mutants | Zygosity (%)* |
|---------------|------------|----------------|---------------|
| OsPDS:Pre-crRNA type I 3 | 0 | 0 | 2(66.7) 1(33.3) |
| OsPDS:Pre-crRNA type II 6 | 0 | 1(16.7) 2(33.3) 3(50) |
| OsBEL:Pre-crRNA type I 4 | 0 | 2(50) 2(50) |
| OsBEL:Pre-crRNA type II 14 | 0 | 2(14.3) 4(28.6) 8(57.1) |

*Plant zygosity is considered homozygous (Ho) or bi-allelic (Bi-al) if the two copies of the target are mutated to the same or different types, respectively. If more than two genotypes are found in the target, the zygosity is believed to be chimeric (Ch). The zygosity of the T₀ plant is putative. The percentage in brackets indicates the frequency of the corresponding genotype.

with on-target mutations were separately analysed by direct Sanger sequencing of the potential off-target sites. After examining the homologous sites with fewer than 7-nt mismatches, we did not detect off-target mutations. These results further support the finding that the CRISPR-Cpf1 system, with careful target selection, is highly specific in vivo.

To determine whether the Cpf1-generated mutation could be transmitted through the germ-line, we analysed the T₁ generation populations of several mutated T₀ plants and found that the majority of the progeny were still mutants (Table 3). Furthermore, most of the T₀ mutation types were identified in T₁ plants, suggesting that CRISPR-Cpf1-mediated genome mutagenesis is heritable. Fragment-specific PCR showed that the T-DNA insertion could be segregated out in certain T₁ mutants (Fig. S5), suggesting that similar to the CRISPR-Cas9 system, CRISPR-Cpf1 could rapidly generate transgene-free, genome-modified crops (Zhang et al., 2014).

The mouse was the first organism to be mutated with the CRISPR-Cpf1 system. The mutants were obtained by transiently introducing a crRNA-Cpf1 protein complex or a crRNA-Cpf1 mRNA mixture (Hur et al., 2016; Kim et al., 2016). The data obtained in this study show that the expression of CRISPR-Cpf1 through stable transformation of the engineered vector can generate heritable targeted modifications in the rice genome. We believe that in plant species with reliable genetic transformation systems, the stable expression of CRISPR-Cpf1 should efficiently produce mutants more rapidly and with a lower cost compared with the transient strategy and that this technique constitutes a robust and ready-to-use approach for plant genome mutagenesis. Due to the substantial differences between Cpf1 and Cas9 in terms of target recognition and DNA cleavage, CRISPR-Cpf1 not only provides a new and alternative method for plant targeted mutagenesis but also greatly enhances the scope and precision of crop genome editing.

Experimental procedures

Vector construction

The coding sequence of LbCpf1 was codon-optimized for rice and synthesized by GeneWiz (Suzhou). The CRISPR-Cpf1 binary vector was developed from the pHSN400 plasmid (Xing et al., 2014). The Ubi promoter, rice codon-optimized Cpf1 and 35S terminator (35-T) were used instead of the 35S promoter, Cas9 and Nos terminator in pHSN400 through HindIII/NorI, NtII/Sacl and SacII/EcoRI double digestions, respectively. A cassette for OsU3-driven crRNA expression was synthesized (Data S1) and inserted into the plasmid by HindIII digestion to construct the CRISPR-Cpf1 binary vector.

To target the specific genome sites of OsPDS and OsBEL, we used 25- to 31-nt sequences with the PAM sequence at their 5'-ends as spacers. The spacers were fused with different repeat sequences to construct crRNAs (Figure S1). In accordance with a previous protocol (Xing et al., 2014), the double-stranded crRNAs were assembled from two complementary oligos (Table S2) and inserted into the vector by Bsal digestion. The colonies were positively selected using kanamycin and negatively selected using spectinomycin and then further confirmed by sequencing.

Rice transformation and plant growth

The vectors were introduced into a pSOUPL-containing agrobacterium strain EH105 (Hellens et al., 2000). For each vector, 600 (for the mature crRNA vector) or 300 (for the pre-crRNA vector) embryonic calluses of rice (Oryza sativa L. ssp. japonica cv. Nipponbare) were infected with the agrobacterium according to a previously described protocol (Duan et al., 2012). The transgenic plants were selected and regenerated under selection with 50 mg/L hygromycin. After two to three weeks of rooting, the T₀ plants were transplanted into soil and grown in a glasshouse at 30 °C. The progeny were generated through strict self-pollination of each individual T₀ event.

Table 3 Transmission of Cpf1-induced mutations from T₀ transgenic rice to the T₁ generation

| Lines | Mutation types (bp) | Zygosity | Mutation types (bp) | No./Test No. | T-DNA segregation |
|-------|-------------------|----------|-------------------|-------------|------------------|
| OsPDS: LbCpf1-crRNA type I #3 | −7 | He | −7, −5 | 9/12 (75%) | 10=2− |
| OsPDS: LbCpf1-crRNA type I #9 | −28/+6 | He | −28/6, −7 | 10/12 (83.3%) | 8=4− |
| OsPDS: LbCpf1-crRNA type I #17 | −10, −32 | He | −10, −32 | 12/12 (100%) | 12+ |

*The genotype of each line is also indicated in Fig. 1c. The zygosity of the T₀ plant is putative.

A total of 12 T₁ plants of each T₀ mutant line were used to examine the targeted mutation. The frequency in the bracket was calculated using the number of T₁ mutants as the numerator.

‡, number of T₁ plants in which T-DNA regions could be detected; −, number of T₁ plants in which T-DNA regions could not be detected.
Genotyping of targeted mutations

The genomic DNA from the leaves of rice plants was isolated using the CTAB method. The genomic region containing the target site was amplified in the corresponding transgenic plants using site-specific primers (Table S2) and the high-fidelity HiFi PCR mixture (TransGen Biotech, Beijing). To detect the mutation, we mixed the PCR product of each transgenic plant with the product generated from the wild-type plant and then analysed it using the T7E1 assay following a standard protocol (New England Biolabs, MA, USA). Moreover, the PCR products were sequenced directly using the respective forward primer or after cloning into a pEASY-T vector (TransGen Biotech, Beijing). For each putative mutant event, at least 10 colonies were Sanger sequenced. Specific primers for the LbCpf1 crRNAs and hygromycin phosphotransferase (HP) cassette were also designed (Table S2) to detect the presence of the T-DNA fragment.

Off-target effect detection

The potential off-target sites were predicted using the online tool Cas-OFFinder (Bae et al., 2014). The sites of each target in five randomly selected mutant lines were examined by site-specific PCR and direct Sanger sequencing. The sequences of the sites and related PCR primers are listed in Tables S1 and S3.

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References

Bae, S., Park, J. and Kim, J.S. (2014) Cas-OFFinder: a fast and versatile algorithm that searches for potential off-target sites of Cas9 RNA-guided endonucleases. Bioinformatics, 30, 1473–1475.
Belha, K., Chaparro-Garcia, A., Kamoun, S., Patron, N.J. and Nekrassov, V. (2015) Editing plant genomes with CRISPR/Cas9. Curr. Opin. Biotechnol. 32, 76–84.
Bortesi, L. and Fischer, R. (2015) The CRISPR/Cas9 system for plant genome editing and beyond. Biotechnol. Adv. 33, 41–52.
Duan, Y., Zhai, C., Li, H., Li, J., Mei, W., Gui, H., Ni, D. et al. (2012) An efficient and high-throughput protocol for Agrobacterium-mediated transformation based on phosphomannose isomerase positive selection in Japonica rice (Oryza sativa L.). Plant Cell Rep. 31, 1611–1624.
Fonfrara, I., Richter, H., Bratovic, M., Le Rhun, A. and Charpentier, E. (2016) The CRISPR-associated DNA-cleaving enzyme Cpf1 also processes precursor CRISPR RNA. Nature, 532, 517–521.
Hellens, R.P., Edwards, E.A., Leyland, N.R., Bean, S. and Mullineaux, P.M. (2000) pgGreen: a versatile and flexible binary Ti vector for Agrobacterium-mediated plant transformation. Plant Mol. Biol. 42, 819–832.
Hur, J.K., Kim, K., Been, K.W., Baek, G., Ye, S., Hur, I.W., Ryu, S.M. et al. (2016) Targeted mutagenesis in mice by electroperforation of Cpf1 ribonucleoproteins. Nat. Biotechnol. 34, 807–808.
Ikeda, T., Tanaka, W., Mikami, M., Endo, M. and Hirano, H.Y. (2016) Generation of artificial drooping leaf mutants by CRISPR-Cas9 technology in rice. Genes Genet. Syst. 90, 231–235.
Kim, Y., Cheong, S.A., Lee, J.G., Lee, S.W., Lee, M.S., Baek, I.J. and Sung, Y.H. (2016) Generation of knockout mice by Cpf1-mediated gene targeting. Nat. Biotechnol. 34, 808–810.
Kleinstever, B.P., Tsai, S.Q., Prev, M.S., Nguyen, N.T., Welch, M.M., Lopez, J.M., McCaw, Z.R. et al. (2016) Genome-wide specificities of CRISPR-Cas Cpf1 nucleases in human cells. Nat. Biotechnol. 34, 869–874.
Li, J., Meng, X., Zong, Y., Chen, K., Zhang, H., Liu, J., Li, J. et al. (2016a) Gene replacements and insertions in rice by intron targeting using CRISPR-Cas9. Nature Plants, 2, 16139.
Li, M., Li, X., Zhou, Z., Wu, P., Fang, M., Pan, X., Lin, Q. et al. (2016b) Reassessment of the four yield-related genes Gna1a, DEP1, G53, and IPAI in rice using a CRISPR/Cas9 system. Front. Plant Sci. 7, 377.
Li, Q., Zhang, D., Chen, M., Liang, W., Wei, J., Qi, Y. and Yuan, Z. (2016c) Development of japonica photo-sensitive genic male sterile rice lines by editing carbon starved anther using CRISPR/Cas9. J. Genet. Genomics, 43, 415–419.
Liu, D., Hu, R., Palla, K.J., Tsukun, G.A. and Yang, X. (2016) Advances and perspectives on the use of CRISPR/Cas9 systems in plant genomics research. Curr. Opin. Plant Biol. 30, 79–77.
Lowder, L.G., Zhang, D., Baltes, N.J., Paul, J.W. 3rd, Tang, X., Zheng, X., Voytas, D.F. et al. (2015) A CRISPR/Cas9 toolbox for multiplexed plant genome editing and transcriptional regulation. Plant Physiol. 169, 971–985.
Ma, X., Zhang, Q., Zhu, Q., Liu, W., Chen, Y., Qiu, R., Wang, B. 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.
Mao, Y., Zhang, Z., Feng, Z., Wei, P., Zhang, H., Botella, J.R. and Zhu, J.K. (2016) Development of germ-line-specific CRISPR-Cas9 systems to improve the production of heritage gene modifications in Arabidopsis. Plant Biotechnol. J. 14, 519–532.
Osakabe, Y., Watanabe, T., Sugano, S.S., Ueta, R., Ishihara, R., Shinozaki, K. and Osakabe, K. (2016) Optimization of CRISPR/Cas9 genome editing to modify abiotic stress responses in plants. Sci. Rep. 6, 26685.
Shan, Q., Wang, Y., Li, J., Zhang, Y., Chen, K., Liang, Z., Zhang, K. et al. (2013) Targeted genome modification of crop plants using a CRISPR-Cas system. Nat. Biotechnol. 31, 686–688.
Sun, Y., Zhang, X., Wu, C., He, Y., Ma, Y., Hou, H., Guo, X. et al. (2016) Engineering herbicide-resistant rice plants through CRISPR/Cas9-mediated homologous recombination of acetolactate synthase. Mol. Plant, 9, 628–631.
Tang, Z., Zheng, X., Qi, Y., Zhang, D., Cheng, Y., Tang, A., Voytas, D.F. et al. (2016) A single transcript CRISPR/Cas9 system for efficient genome editing in plants. Mol. Plant, 9, 1088–1091.
Wang, Z.P., Xing, H.L., Dong, L., Zhang, H.Y., Han, C.Y., Wang, X.C. and Chen, Q.J. (2015) Egg cell-specific promoter-controlled CRISPR/Cas9 efficiently generates homozygous mutants for multiple target genes in Arabidopsis in a single generation. Genome Biol. 16, 144.
Wang, F., Wang, C., Liu, P., Lei, C., Hao, W., Gao, Y., Liu, Y.G. et al. (2016) Enhanced rice blast resistance by CRISPR/Cas9-targeted mutagenesis of the ERF transcription factor gene OsERF922. PLoS ONE, 11, e0154027.
Xie, K., Minkenberg, B. and Yang, Y. (2015) Boosting CRISPR/Cas9 multiplex editing capability with the endogenous RNA-processing system. Proc. Natl Acad. Sci. USA, 112, 3570–3575.
Xing, H.L., Dong, L., Wang, Z.P., Zhang, H.Y., Han, C.Y., Liu, B., Wang, X.C. et al. (2014) A CRISPR/Cas9 toolkit for multiplex genome editing in plants. BMC Plant Biol. 14, 327.
Xu, R., Li, H., Qin, R., Wang, L., Li, L., Wei, P. and Yang, J. (2014) Gene targeting using the Agrobacterium tumefaciens-mediated CRISPR-Cas system in rice. J. Biol. 7, 1.
Xu, R., Yang, Y., Qin, R., Li, H., Qiu, C., Li, L., Wei, P. et al. (2016) Rapid improvement of grain weight via highly efficient CRISPR/Cas9-mediated multiplex genome editing in rice. J. Genet. Genomics, 43, 529–532.
Zetsche, B., Gootenberg, J.S., Abudayeh, O.O., Slaymaker, I.M., Makarova, K.S., Essletzbichler, P., Volz, S.E. et al. (2015) Cpf1 is a single RNA-guided endonuclease of a Class 2 CRISPR-Cas system. Cell, 163, 759–771.
Zhang, H., Zhang, J., Wei, P., Zhang, B., Gou, F., Feng, Z., Mao, Y. et al. (2014) The CRISPR/Cas9 system produces specific and homoyzgous targeted gene editing in rice in one generation. Plant Biotechnol. J. 12, 797–807.
Zhou, J., Peng, Z., Long, J., Sosso, D., Liu, B., Eom, J.S., Huang, S. et al. (2015) Gene targeting by the TAI effector PnK2 reveals cryptic resistance gene for bacterial blight of rice. Plant J. 82, 632–643.

Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:
Figure S1 Design of different crRNA variants for Cpf1-induced plant genome targeting.

Figure S2 T7E1 assay to detect transgenic plants carrying target mutations.

Figure S3 Sequencing chromatograms of Cpf1-induced mutations in the OsPDS target.

Figure S4 Cpf1-induced mutations in the OsBEL target in the T0 generation transgenic rice plants.

Figure S5 Segregation of the T-DNA fragment in representative T1 mutants.

Table S1 Potential off-target sites of the Cpf1-targeting OsPDS and OsBEL.

Table S2 Primers used for vector construction and genotyping.

Table S3 Primers used to detect off-target effects in rice plants.

Data S1 Sequence of the rice codon-optimized LbCpf1 and crRNA expression cassette.