Brief Communication

Targeted base editing in rice with CRISPR/ScCas9 system

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The CRISPR/Cas system has rapidly become the preferred tool for genome engineering in various organisms due to high efficiency, specificity, simplicity and versatility. Currently, CRISPR/Cas-mediated base editing, a novel genome editing strategy that enables irreversible nucleotide changes at target loci without double-stranded DNA cleavage or any donor template, has been widely adopted for generating gain-of-function germplasms in functional genomics research and crop genetic improvement (Hu et al., 2019; Ren et al., 2018; Yan et al., 2018). However, the recognition of a specific protospacer adjacent motif (PAM) for Cas protein restricts the targeting range of these tools, especially base editors, given that it requires a functional PAM for Cas protein interaction to localize the target base in the editing window within the protospacer for nucleotide deamination (Ren et al., 2017; Ren et al., 2018; Yan et al., 2018). The PAM specificity is therefore the key limitation of the application of the CRISPR system in genome editing. Since the commonly used Streptococcus pyogenes Cas9 (SpCas9) recognizes canonical NGG PAM (Hu et al., 2018), many efforts have been directed towards the identification of new Cas proteins for different PAM specificity in years (Li et al., 2019; Qin et al., 2019). Intriguingly, a promising candidate, an orthologous Cas9 protein from Streptococcus canis (ScCas9), which shares 89.2% sequence similarity with SpCas9, has been identified and characterized. ScCas9 recognizes minimal NNG PAM sequences, and it is capable of efficient genome editing in human cells (Chatterjee et al., 2018). However, its PAM specificity has not been verified in other systems and its application in plants has not been previously reported. Here we show that ScCas9 achieves efficient target gene mutagenesis at NAG sites in comparison with NGG, NTG and NCG sites. Moreover, we also show that ScCas9 can be used in multiplex genome editing and base editing in rice plants.

To test PAM preference of ScCas9 in rice, we first assessed its nuclease activity, side-by-side with SpCas9, towards twelve endogenous genomic loci with NGG, NAG, NCG and NTG PAMs (Figure 1a). ScCas9 gene was codon-optimized, fused with a nuclear localization signal at both termini and expressed in stable transgenic rice plants. Subsequently, individual lines were genotyped by Sanger sequencing as previously reported (Ren et al., 2019). For NGG PAM sites, the editing efficiency of ScCas9 was comparable to that of SpCas9 at the OsCPK6 target site, averaging 97.92% indels dominated with mono-allelic mutations compared to 94.12% indels dominated with bi-allelic mutations for SpCas9. On the other two NGG sites in OsMPK9 and OsMPK17, ScCas9 showed no activity, whereas SpCas9 did well. For NAG PAM sites, ScCas9 resulted in 91.18% efficiency on OsMPK16, 94.74% on OsCPK7, while ScCas9 yielded 7.69% and 92.31%, respectively. On three NTG and NCG PAM sites tested, indel frequencies were more variable, ranging from 0% to 46.67% (all plants were mono-allelic mutants). Interestingly, the editing efficiency of ScCas9 was genomic locus-dependent, since 7 out of 12 target sites tested were resistant to ScCas9. Taken together, these results indicate that ScCas9 nuclease recognizes NNG PAM on a locus-dependent manner in targeted plant genome editing, and it is more suitable for editing NAG target sites in rice.

To further validate the capacity and efficacy of ScCas9 towards the NAG PAM, we tested ScCas9 in multiplex genome editing in transgenic rice plants. One sgRNA targeting both OsMPK14 and OsMPK15 at the conserved genomic region was transferred into rice. Genotyping data showed that ScCas9 achieved comparable activity to SpCas9 at both target sites (Figure 1b). Alternatively, two different sgRNAs targeting OsCPK9 and OsCPK10 simultaneously were used as well. ScCas9 showed notably improved genome editing, averaging 94.12% editing of OsCPK9 and 89.36% editing of OsCPK10. By contrast, SpCas9 achieved 73.33% and 12.12% editing under the same condition tested, respectively (Figure 1b). In view of all data, we conclude that ScCas9 outperforms SpCas9 on target sites with NAG PAMs in rice.

In our previous studies, we developed a series of cytidine and adenosine base editors for targeted base editing using the nickase version of SpCas9 and its variants (Ren et al., 2019; Ren et al., 2017). Thus, we speculated that ScCas9 could broaden the targeting scope of base editors considering the preference of NAG PAM. Therefore, we constructed the hAID·Δ·ScCas9n-UGI-NLS chimeric gene, cytidine base editor named rBE25 (Figure 1c), and tested its activity towards an NAG PAM at the OsBZR1 site in transgenic rice plants (Figure 1d). Of 46 independent lines confirmed by Sanger sequencing, 17 heterozygous lines (36.96% efficiency) were identified with nucleotide changes in the target region and 2 lines carried indel mutations (Figure 1e and f). Meanwhile, the chimeric gene Tada-Tada7.10·ScCas9n-NLS, adenosine base editor named rBE26 (Figure 1g), was constructed and used to target the endogenous OsG51 gene for generating potential herbicide-resistant rice germplasm (Figure 1h). As a result, 19 of 40 independent lines (47.5% efficiency) were identified with a single A to G conversion at the desired site. All the mutated lines were heterozygous, and no indels were
(a) | PAM Seq | Gene | Target site | Nuclease | Mutation efficiency | Mono-allelic mutation | Bi-allelic mutation |
|---|---|---|---|---|---|---|
| OsMPK9 | GGTTATAGCCCTTGATCACA | SpCas9 | 12/30 (40.00%) | 12 | 0 |
| | OsMPK17 | CCACCAGCAGTCTACAGGAGAT | ScCas9 | 0/48 (0.00%) | 0 | 0 |
| OsCPK6 | ATGGCAGCTACTACACTGTCGCC | ScCas9 | 2/26 (7.69%) | 2 | 0 |
| OsMPK15 | TGAGAAGTGTGATCAAGAAG | ScCas9 | 1/33 (3.05%) | 1 | 0 |
| OsMPK16 | TGTGACTTCGCGCTTGCTAG | ScCas9 | 22/30 (73.33%) | 22 | 0 |
| OsCPK7 | GCCAGAACGAGCTTCTGGGGAG | ScCas9 | 24/26 (92.31%) | 24 | 0 |

(b) | PAM Seq | Gene | Target site | Nuclease | Mutation efficiency | Mono-allelic mutation | Bi-allelic mutation |
|---|---|---|---|---|---|---|
| OsMPK14 | GATACAGACTTCTACACGACGAG | ScCas9 | 23/30 (76.67%) | 23 | 1 |
| OsMPK15 | GCACCCGGACACGATACCGGAG | ScCas9 | 18/30 (60.00%) | 18 | 2 |
| OsCPK9 | TGACTACCTGATTACACTACCTGAC | ScCas9 | 8/10 (80.00%) | 8 | 5 |
| OsCPK10 | CAGCAAGACCGCTTCTCCAG | ScCas9 | 4/33 (12.12%) | 4 | 0 |

(c) NOS

(d) Ubi

(e) CACGCGTATTT (H205Y/P206L) in OsBZR1

(f) rBE25: OsBZR1

(g) TadA7.10

(h) NAG

(i) rBE26: OsGS1

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identified (Figure 1i and j). Taken together, our data indicate that ScCas9 is compatible with nucleotide deaminases and might serve as a useful RNA-guided DNA-targeting platform for other modification enzymes for genome engineering.

In this study, we have extensively investigated the nuclease activity of ScCas9 on different NNG PAM sequences in rice plants. We found that the cleavage activity of ScCas9, different to the report from human cells, is lower at NGG sites and more robust at NAG sites as compared to SpCas9. Furthermore, ScCas9 is less active at NTG and NCG sites. Interestingly, the performance of ScCas9 nuclease is locus-dependent. It has previously been reported that SpCas9 is sensitive to chromatin state, DNA and/or histone modifications at the target region (Kallimasioti-Pazi et al., 2018). Therefore, we presume that ScCas9 might be more sensitive than SpCas9 to these factors. Further experiments with more target sites are required to address this question. Nevertheless, our data show that ScCas9 is a new genome editing player regarding NAG PAM, achieving considerable editing efficiency in multiplex genome editing, and in cytidine as well as adenosine base editing. In conclusion, ScCas9 nuclease and its derived editing tools expand the CRISPR toolbox for targeted genome editing in plants.

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**Competing interests**

The authors declare no conflict of interests.

**Author contributions**

H.Z., X.Z., C.Z., F.Y. and C.S. designed the experiments. M.W., G.G., Z.X., B.R., Y.C and Y.K. conducted the experiments and performed the data analysis. H.Z., C.S and G.G wrote the paper with input of all other authors. All authors participated in discussion and revision of the manuscript.

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