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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 (Hua *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 SpCas9 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 adenine 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*, adenine base editor named *rBE26* (Figure 1g), was constructed and used to target the endogenous *OsGS1* 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

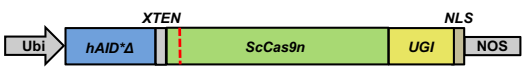
(a)

PAM Seq	Gene	Target site	Nuclease	Mutation efficiency	Mono-allelic mutation	Bi-allelic mutation
NGG	*OsMPK9*	GGTTATTAGCCTTTGATCCAAAGG	SpCas9	12/30 (40.00%)	12	0
			ScCas9	0/48 (0.00%)	0	0
	OsMPK17	CCACCCGCATCCTTAGGGAGAT	SpCas9	23/24 (95.83%)	1	22
			ScCas9	0/48 (0.00%)	0	0
	OsCPK6	ATGGGCAACTACTACTCGTGCGG	SpCas9	16/17 (94.12%)	1	15
			ScCas9	47/48 (97.92%)	32	15
NAG	*OsMPK15*	TGAGAAAGTTGCTATCAAGAAG	SpCas9	1/39 (2.56%)	1	0
			ScCas9	0/23 (0.00%)	0	0
	OsMPK16	TGTGACTTCGGCCTTGCTCGAG	SpCas9	2/26 (7.69%)	2	0
			ScCas9	31/34 (91.18%)	1	30
	OsCPK7	GCCAGAACGGGACTCTTGGGAG	SpCas9	24/26 (92.31%)	2	22
			ScCas9	18/19 (94.74%)	1	17
NCG	*OsMPK17*	GAGTTATGGTGTTGTGGCTGCCG	ScCas9	0/48 (0.00%)	0	0
	OsCPK5	CGTCACGCTTAGATCCAAGTACT	ScCas9	0/16 (0.00%)	0	0
	OsCPK6	GGGCAACTACTACTCGTGCGGCG	ScCas9	14/30 (46.67%)	14	0
NTG	*OsMPK9*	TGATCCAAAGGACCGTCCAACTG	ScCas9	4/56 (7.14%)	4	0
	OsMPK15	GGATACTTCGTGAAATCAAGTTG	ScCas9	0/48 (0.00%)	0	0
	OsCPK8	CAGGTACGCGTGCAAGTCGATA	ScCas9	0/48 (0.00%)	0	0

(b)

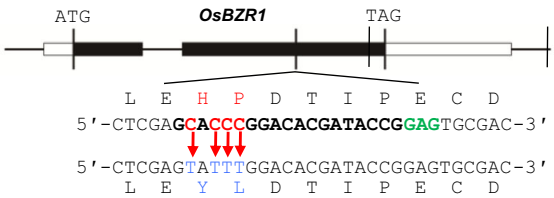
PAM Seq	Gene	Target site	Nuclease	Mutation efficiency	Mono-allelic mutation	Bi-allelic mutation
NAG	*OsMPK14*	GATCAACAGCAGCTCCACCTGAG	SpCas9	24/30 (80.00%)	23	1
			ScCas9	14/17 (82.35%)	11	3
	OsMPK15		SpCas9	18/30 (60.00%)	16	2
			ScCas9	10/20 (50.00%)	5	5
	OsCPK9	TCGCTCCGGCTACCACCGATGAG	SpCas9	22/30 (73.33%)	8	14
			ScCas9	32/34 (94.12%)	21	11
	OsCPK10	CAGCAAGAACGGCTTCTTCCAG	SpCas9	4/33 (12.12%)	4	0
			ScCas9	42/47 (89.36%)	23	19

(c)

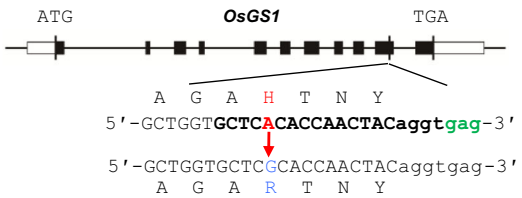


(g)

(d)

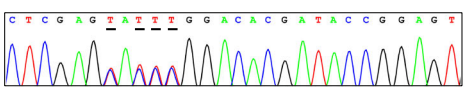


(h)



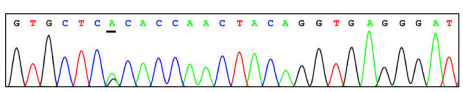
(e)

GCACCC>GTATTT (H205Y/P206L) in *OsBZR1*



(i)

CAC>CGC (H249R) in *OsGS1*



(f)

rBE25: OsBZR1	His>Tyr				Pro>Leu/Trp			Asp			Thr			Ile			Pro			Glu			Indels
%	G	C_{-18}	A	C_{-16}	C_{-15}	C_{-14}	G	G	A	C	A	C	G	A	T	A	C	C	G	G	A	G	4.35
A	2.2	0.0	100.0	0.0	0.0	0.0	0.0	0.0	100.0	0.0	100.0	0.0	0.0	100.0	0.0	100.0	0.0	0.0	0.0	0.0	100.0	0.0	
T	0.0	32.6	0.0	34.8	32.6	23.9	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	
C	0.0	67.4	0.0	65.2	65.2	76.1	0.0	0.0	0.0	100.0	0.0	100.0	0.0	0.0	0.0	0.0	100.0	100.0	0.0	0.0	0.0	0.0	
G	97.8	0.0	0.0	0.0	2.2	0.0	100.0	100.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0	0.0	0.0	0.0	100.0	100.0	0.0	100.0	

(j)

rBE26: OsGS1	Ala			His>Arg			Thr			Asn			Tyr									Indels
%	G	C	T	C	A_{-15}	C	A	C	C	A	A	C	T	A	C	a	g	g	t	g	a	g	0.0
A	0.0	0.0	0.0	0.0	52.5	0.0	100.0	0.0	0.0	100.0	100.0	0.0	0.0	100.0	0.0	100.0	0.0	0.0	0.0	0.0	100.0	0.0	
T	0.0	0.0	100.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	
C	0.0	100.0	0.0	100.0	0.0	100.0	0.0	100.0	100.0	0.0	0.0	100.0	0.0	0.0	100.0	0.0	0.0	0.0	100.0	0.0	0.0	0.0	
G	100.0	0.0	0.0	0.0	47.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0	100.0	0.0	100.0	0.0	100.0	

Figure 1 Targeted genome editing in rice using ScCas9, rBE25 and rBE26. (a) Summary of the frequencies of mutations induced by SpCas9 and ScCas9 towards different PAM sequences in T0 transgenic rice plants. (b) Summary of the simultaneous editing frequencies of the target genes induced by SpCas9 and ScCas9 towards NAG PAM in T0 transgenic rice plants. (c) Gene construct of cytidine base editor rBE25. (d) The target site in the *OsBZR1* gene in rice. (e) Representative Sanger sequencing chromatogram of the rBE25-edited *OsBZR*1 allele in a T0 transgenic line. (f) Summary of nucleotide changes in the editing window of the endogenous *OsBZR1* gene caused by rBE25 in independent T0 transgenic lines. (g) Gene constructs of adenine base editor rBE26. (h) The target site in the *OsGS1* gene in rice. (i) Representative Sanger sequencing chromatogram of the rBE26-edited *OsGS1* allele in a T0 transgenic line. (j) Summary of nucleotide changes in the editing window of the endogenous *OsGS1* gene caused by rBE26 in independent T0 transgenic lines. The intron is depicted as lower-case letters. The PAM sequences, target sequences, candidate bases in the putative editing window and detected nucleotide changes/corresponding amino acids are highlighted in green, bold, red and blue, respectively. In (e) and (i), the nucleotide changes are underlined in the sequencing chromatograms

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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