Brief Communication

Expanding the base editing scope to GA and relaxed NG PAM sites by improved xCas9 system

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Dear Editor,

Base editors have been developed to be powerful tools to generate precise point mutations. However, their applications are hindered by the strict canonical NGG PAM requirement of Streptococcus pyogenes Cas9 (SpCas9). Cas effectors recognizing different PAMs or relaxed PAMs have been employed to address this limitation. Recently, xCas9 and Cas9-N9 were both used to further broaden the editing scope to NG PAM sites (Hu et al., 2018; Nishimasu et al., 2018). xCas9 showed broader PAM recognition including GAA and GAT than Cas9-N9 and has been employed in base editors in human cells (Hu et al., 2018). However, no detected sites with these PAMs were edited by reported xCas9 involved cytosine base editors (xCas9-CBEs) in rice (Hua et al., 2019; Li et al., 2019; Ren et al., 2019; Zhong et al., 2019). In this study, we generated an efficient xCas9-CBE to achieve C-to-T mutation at GAA, GAT and even GAC, GAG PAM sites in rice, expanding the targeting scope and providing a reference for other plants and animals.

We first analyzed all reports on xCas9-CBEs and found that tRNA-sgRNA system was never used. Since xCas9 is a high-fidelity SpCas9 variant (Zhong et al., 2019), it may require perfectly matched 20-nucleotide (nt) target like other high-fidelity Cas9 variants (Zhang et al., 2017). Studies showed that tRNA–sgRNA system can produce precise 20-nt sequence completely complementary to the targets by the cellular enzymes RNase P and RNase Z (Figure 1a). Considering tRNA–sgRNA system can also improve sgRNA expression level (Xie et al., 2015; Zhang et al., 2017), and the scaffold modified sgRNA (known as esgRNA) was reported to enhance editing efficiency (Chen et al., 2013), we chose tRNA–sgRNA system including two sgRNA forms (Figure 1a) to develop xCas9-CBEs in rice.

A262Tr/R324L/S5409/E480K/E543D/M694/E1219V mutations were introduced into rice codon-optimized SpCas9 (Wu et al., 2019) to generate xCas9 3.7. We then fused the D10A nickase of xCas9 3.7 (xCas9n 3.7) with the commonly used rat cytidine deaminase rAPOBEC1 or Petromyzon marinus cytidine deaminase 1 (PmCDA1) and with uracil DNA glycosylase inhibitor (UGI) and subsequently placed them under control of the O. sativa ubiquitin (OsUbq) promoter in tRNA-sgRNA or tRNA-esgRNA system, generating four xCas9-CBEs designated as xCas9n-erBE, xCas9n-rBE, xCas9n-epBE and xCas9n-pBE, respectively (Figure 1a). We first chose two GAA PAM sites from two mitogen-activated protein kinases (OsMPK2 and OsMPK5) genes to test the systems. Rice resistant calli and stable transgenic T0 plants were generated by callus selection under hygromycin B after Agrobacterium-mediated transformation of rice as previously described (Wu et al., 2019). The data from rice resistant calli showed only xCas9n-epBE displayed base editing activity (Figure 1b). In T0 plants, xCas9n-epBE also showed robust base editing activity, with mutation rates of 44.4% and 28.6%, respectively, at these two sites (Figure 1c,d). These results suggest that xCas9n-epBE might be a promising cytosine base editor for GAA and GAT PAM sites in rice.

To further confirm the efficacy of xCas9n-epBE on targets with GAA and GAT PAMs, we tested the C-to-T substitution capability at another three GAA PAM target sites (T1-3, T1-4 and T1-5) in the OsMPK5, OsALS and OsNRT1.1B genes, respectively and three GAT PAM sites (T2-1, T2-2 and T2-3) in the OsMPK5 or OsWaxy genes. In T0 plants, at three GAA PAM sites, both T1-3 and T1-5 were edited with frequencies of 14.3% and 5%, respectively, while T1-4 was not edited (Figure 1c). Among all three target sites with GAT PAM, xCas9n-epBE also showed detectable base mutations, with frequencies ranging from 5.3% to 14.3% (Figure 1c). Collectively, these findings demonstrate that xCas9n-epBE can act as an effective cytosine base editor for genomic sites with GAA and GAT PAMs in rice.

Since the target sites harbouring GAA and GAT PAMs can be edited by xCas9n-epBE, we hypothesized that xCas9n-epBE could work at GAN PAM sites, including the other two PAMs, GAC and GAG. To test this, we used xCas9n-epBE to edit two GAC PAM target sites (T3-1 and T3-2) and two GAG PAM target sites (T4-1 and T4-2) in the OsALS, OsNRT1.1B or OsWaxy genes (Figure 1c). Each had one site being edited in T0 plants (Figure 1c). xCas9n-epBE showed robust editing activity at the GAG T4-2 site (Figure 1e), with frequencies over 70%, and showed low mutation frequency of 4.5% at the GAC T3-2 site (Figure 1c). Therefore, xCas9n-epBE could further broaden the targeting scope of cytosine base editing from GAA and GAT PAM sites to GAN PAM sites in rice.

The previously reported xCas9-CBEs were not efficient at NG PAM sites in rice regenerated plants, with the exception of canonical NGG PAM sites (Hua et al., 2019; Li et al., 2019; Ren et al., 2019; Zhong et al., 2019). Given the good performance of xCas9n-epBE at editing sites with GA PAM, we envisioned that xCas9n-epBE could also work on NG PAM sites in rice. Two target sites for each PAM (NGG, NGT, NGA and NGC) were tested (Figure 1c). xCas9n-epBE could edit all six targets with NGG, NGT and NGA PAMs, with frequencies ranging from 5% to 64.3% (Figure 1c), but for two NGC PAM sites, no mutation was
Improving xCas9 system for enlarged base editing scope

(a) Diagram showing the xCas9 system components and their interactions.

(b) Graph showing the C-to-T frequency distribution for different xCas9 variants.

(c) Table summarizing the editing results:

| Target site | Target gene | PAM sequence | Tested T0 plants | Edited T0 plants | C-to-T frequency (%) | Homozygous T0 Plants |
|-------------|-------------|--------------|------------------|------------------|----------------------|---------------------|
| T1-1        | OsMPK2      | GAA          | 9                | 4                | 44.4                 | 0                   |
| T1-2        | OsMPK5      | GAA          | 7                | 2                | 28.6                 | 0                   |
| T1-3        | OsMPK5      | GAA          | 7                | 1                | 14.3                 | 0                   |
| T1-4        | OsALS       | GAA          | 20               | 0                | 0                    | 0                   |
| T1-5        | OsNRT1.1B   | GAA          | 20               | 1                | 5                    | 0                   |
| T2-1        | OsMPK5      | GAT          | 10               | 1                | 14.3                 | 0                   |
| T2-2        | OsWaxy      | GAT          | 20               | 0                | 5.3                  | 0                   |
| T2-3        | OsWaxy      | GAT          | 19               | 1                | 5.3                  | 0                   |
| T3-1        | OsALS       | GAC          | 41               | 1                | 0                    | 0                   |
| T3-2        | OsNRT1.1B   | GAC          | 44               | 1                | 4.5                  | 0                   |
| T4-1        | OsWaxy      | GAG          | 44               | 1                | 4.5                  | 0                   |
| T4-2        | OsWaxy      | GAG          | 26               | 2                | 0                    | 0                   |
| T5-1        | OsMPK2      | TGG          | 9                | 3                | 33.3                 | 0                   |
| T5-2        | OsMPK5      | TGG          | 10               | 1                | 14.3                 | 0                   |
| T6-1        | OsMPK5      | TGT          | 8                | 1                | 12.5                 | 0                   |
| T6-2        | OsMPK5      | GGT          | 14               | 9                | 64.3                 | 0                   |
| T7-1        | OsNRT1.1B   | TGA          | 20               | 1                | 5                    | 0                   |
| T7-2        | OsWaxy      | TGG          | 19               | 2                | 10.5                 | 0                   |
| T8-1        | OsMPK5      | GGC          | 7                | 0                | 0                    | 0                   |
| T8-2        | OsMPK2      | TGC          | 58               | 0                | 0                    | 0                   |

(d) Diagram showing the reference sequences for different target sites.

(e) Another diagram showing additional reference sequences.

(f) Graph illustrating mutation profiles across different positions.

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Figure 1  Cytosine base editing by xCas9n-epBE in rice. (a) Schematic view of xCas9n-erBE, xCas9n-rBE, xCas9n-epBE and xCas9n-pBE base editors. erBE/ rBE: APOBEC1 based cytosine base editor (CBE) with or without esgRNA. epBE/pBE: PmCDA1 based CBE with or without esgRNA. n in (tRNA-esgRNA)n or (tRNA-sgRNA)n means the target number in the vector. The sequences of trNA-esgRNA/sgRNA units are given. tRNA is cleaved by RNase P and RNase Z at specific sites. Different nucleotides between the scaffold of esgRNA and sgRNA are marked in pink. (b) Efficiency comparison of four base editors at two genomic sites, T1-1 and T1-2, in rice resistant calli. Number of detected calli is 20. (c) Base editing efficiencies of xCas9n-epBE in rice T0 plants. Homozygous T0 plant was designated when all C-to-T mutations were homozygous. T1 to T8 represent the target sites with different PAMs. (d) Sequencing chromatograms at T1-1 and T1-2 sites of all mutated T0 lines produced by xCas9n-epBE. Arrows indicate the edited bases. The PAM sequences are reported in this paper.

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Conflict of interest

The authors submitted patent applications based on the results reported in this paper.

Author contributions

J.Y. and Y.L. designed the experiments. C.Z., W.X., F.W., G.K., S.Y., X.L. and L.L. performed all the experiments. C.Z. and W.X. analyzed the results. C.Z., W.X., Y.L. and J.Y. wrote the manuscript. J.Y. and Y.L. supervised the project.

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