Expanding the base editing scope in rice by using Cas9 variants

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Summary

Base editing is a novel genome editing strategy that enables irreversible base conversion at target loci without the need for double stranded break induction or homology-directed repair. Here, we developed new adenine and cytosine base editors with engineered SpCas9 and SaCas9 variants that substantially expand the targetable sites in the rice genome. These new base editors can edit endogenous genes in the rice genome with various efficiencies. Moreover, we show that adenine and cytosine base editing can be simultaneously executed in rice. The new base editors described here will be useful in rice functional genomics research and will advance precision molecular breeding in crops.

Introduction

Base editing is a newly developed genome engineering tool that enables gene editing through irreversible base conversion, without the need for double stranded break (DSB) induction or homology-directed repair (HDR; Komor et al., 2016). The first member in the base editing toolbox is the cytosine base editors (CBEs) that consist of a cytidine deaminase domain fused with a Streptococcus pyogenes Cas9 nickase (nSpCas9 (D10A)) or catalytically deficient Cas9 (dSpCas9; Komor et al., 2017; Lu and Zhu, 2017). One major limitation of cytidine deaminase mediated base editing is its inability to induce other forms of base conversion beyond the C-G to T-A mutation. Recently, David R. Liu’s group addressed this limitation by developing the adenine base editors (ABEs) that can convert A-T to G-C in a programmable manner in mammalian cells (Gaudelli et al., 2017). We and others showed that the ABEs can be adapted for applications in plants (Hua et al., 2018; Yan et al., 2018). The combination of adenine and cytosine base editors now can generate all four base transition mutations.

However, efficient adenine and cytosine base editing requires the presence of a specific protospacer adjacent motif (PAM) sequence (NGG PAM for SpCas9) that places the target base(s) within a narrow base editing window (Gaudelli et al., 2017; Komor et al., 2016). This PAM requirement significantly limits the genomic sites that can be targeted by the ABEs and CBEs. Here, we circumvent this limitation by developing new ABEs and CBEs with SpCas9 and Staphylococcus aureus Cas9 (SaCas9) variants, thus substantially increasing the target scopes for base editing in the rice genome.

Results and discussion

Previous studies have shown that the wild type SpCas9 protein can recognize not only the canonical NGG PAM but also the non-canonical NAG PAM sequence in the rice genome and exhibits robust editing efficiencies at some sites with NAG PAMs (Meng et al., 2018). We wondered whether our ABE-P1 (adenine base editor plant version 1) vector with a wild type SpCas9 (D10A) nickase (Hua et al., 2018) can also edit genome sequences with NAG PAMs. First, we designed sgRNA1 that targeted the OsmiRNA156 binding sequence in OsSPL14 followed by an NAG PAM (Figure S1a). From 46 independent transgenic lines generated from Agrobacterium-mediated transformation, only two lines showed A-G substitutions at the expected base editing window (Tables 1 and 2). Line SG1-5 harboured an A-G substitution at position 5 of the protospacer (scoring the NAG PAM as positions 21–23), whereas line SG1-24 had an A-G substitution at position 7 of the protospacer (T-C conversions in opposite strand were shown in Figure S1b). To confirm the Sanger sequencing results, line SG1-5 was selected for TA cloning. Three out of 20 randomly selected clones showed an A-G substitution at position 5 of the protospacer, suggesting that line SG1-5 was chimeric (T-C conversions in opposite strand are shown in Figure S1c). To further test the base editing capability of ABE-P1 at target sites with NAG PAMs, we designed two other sgRNAs. The second sgRNA (sgRNA2) was selected to simultaneously target OsmiRNA145 binding sites in OsSPL16 and OsSPL18 (Figure S1d). We genotyped 34 transgenic lines and found that only lines SG2-15 and SG2-18 had an A-G base editing event at position 9 of the protospacer in the OsSPL16 target site, whereas the target site in OsSPL18 was not edited (Tables 1 and 2 and T-C conversions in opposite strand are shown in Figure S1e). The third sgRNA (sgRNA3) was chosen to target the OsmiR396 binding site in GRF4. From the 40 transgenic lines that we obtained, we did not find any base editing event at this target site (Table 1). Taken together, the above results indicated that in contrast to the highly efficient cutting activity of the SpCas9 at NAG PAMs, the base editing efficiency of ABE-P1 was low at target sites with NAG PAMs in rice.

To expand the targeting scope of our ABE-P1 base editor in rice, we sought to replace the wild type SpCas9 in ABE-P1 with...
Table 1 Summary of base editing efficiencies of different adenine base editors

| Base editor ABE/P2 | Target gene (base editor sgRNA) | PAM sequence | Number of total transgenic lines | Number of edited lines | Editing efficiency |
|-------------------|--------------------------------|--------------|---------------------------------|------------------------|-------------------|
| ABE-P1            | OsSPL14 (sgRNA1)              | GAG          | 46                              | 2                      | 4.3%              |
|                   | OsSPL16 (sgRNA2)              | GAG          | 34                              | 2                      | 5.9%              |
|                   | OsSPL18 (sgRNA3)              | GAG          | 0                               | 0                      |                  |
| ABE-P3            | Grf4 (sgRNA4)                 | CAG          | 40                              | 0                      |                  |
|                   | OsSPL14 (sgRNA5)              | GGA          | 39                              | 26                     | 66.7%             |
|                   | OsSPL17 (sgRNA6)              | GGA          | 9                               | 9                      | 30%               |
|                   | OsSPL18 (sgRNA7)              | GGA          | 20                              | 20                     | 66.7%             |
| ABE-P4            | OsTOE1 (sgRNA8)               | AGCG         | 39                              | 1                      | 2.6%              |
|                   | OsIDS1 (sgRNA9)               | AGCG         | 1                               | 1                      | 2.6%              |
|                   | OMTN1 (sgRNA10)               | GGCG         | 8                               | 0                      |                  |
| ABE-P5            | SNB (sgRNA11)                 | TGCAGT       | 46                              | 3                      | 6.5%              |
|                   | OMTN1 (sgRNA12)               | TTAGGT       | 21                              | 0                      |                  |

*Two genomic sites are simultaneously targeted by sgRNA2, sgRNA4, sgRNA5 and sgRNA6.

Table 2 Base editing activity window for different ABEs and CBEs at different target sites

| Base editor ABE/P4 | Target gene (base editor sgRNA) | Editing sites in the protospacer | Editing form |
|-------------------|--------------------------------|---------------------------------|--------------|
| ABE-P1            | OsSPL14 (sgRNA1)              | 5, 7                            | A-G conversion |
|                   | OsSPL16 (sgRNA2)              | 9                               | A-G conversion |
| ABE-P2            | OsSPL14 (sgRNA3)              | 8, 10, 14                       | A-G conversion |
|                   | OsSPL17 (sgRNA4)              | 6, 8, 10, 14                    | A-G conversion |
| ABE-P3            | OsSPL14 (sgRNA5)              | 3, 4, 6, 8, 10                  | A-G conversion |
|                   | OsSPL17 (sgRNA6)              | 4, 6, 8, 10                     | A-G conversion |
|                   | OsSPL18 (sgRNA7)              | 3, 4, 6, 8, 10                  | A-G conversion |
| ABE-P4            | OsTOE1 (sgRNA8)               | 5                               | A-G conversion |
|                   | OsIDS1 (sgRNA9)               | 5                               | A-G conversion |
| ABE-P5            | SNB (sgRNA10)                 | 4, 8                            | A-G conversion |
| CBE-P1            | SNB (sgRNA11)                 | 4, 5                            | C-T conversion |
| CBE-P3            | PMS3 (sgRNA12)                | 8, 11, 15                       | C-T conversion |

*Two genomic sites are simultaneously targeted by sgRNA2, sgRNA4, sgRNA5, sgRNA6, sgRNA10, sgRNA11, and sgRNA12.

Base editing sites in the protospacer were counted from the PAM-distal end, scoring the PAM as positions 21–23 for base editors with SaCas9 variants and scoring the PAM as positions 22–27 for base editors with SaCas9 variants.

Adenines in positions 3, 4, 6, 8, 10 of the protospacer could be substituted to guanines in the OsSPL14 and/or OsSPL18 target sites. sgRNA was designed to target the OsmiRNA156 binding sites in OsSPL16 and OsSPL18 (Figure S2a). The base editing efficiencies at these two target sites were also high, up to 66.7% (20/30) at the OsSPL18 target site and 30% (9/30) at the OsSPL16 target site (Table 1). Furthermore, nine transgenic lines were edited simultaneously at both sites and sequencing chromatograms of two representative lines are shown in Figure S2b. Interestingly, even adenine in position 10 of the protospacer could be efficiently edited at the OsSPL18 target site (Table 2).

For the base editor ABE-P4, we tested two sgRNAs (sgRNA6 and sgRNA7) that targeted three different genomic sites. We found that the base editing efficiencies of ABE-P4 at the selected target sites were much lower than that of ABE-P3 (Table 1). The mR172 binding sites in OsIDS1 and OsTOE1 were selected as target sites of sgRNA6 (Figure S3a). From 39 transgenic lines that we genotyped, only one line (SG7-9) was found to harbour an expected A-G substitution at position 5 of the protospacer in OsIDS1, while another line, SG7-17, had an A-G substitution at position 5 of the protospacer in OsTOE1 (C-T conversions in opposite strand are shown in Figure S3b and Table 2). We did not detect any mutations in the OsR164 binding site in the OMTN1 gene, which was targeted by sgRNA7 (Table 1).

Recently, studies have also shown that an engineered SaCas9 variant (SaKKH-Cas9) can recognize a more relaxed PAM sequence, NNNRRT (Kleinstiver et al., 2015a). To broaden the target range of our ABE-P2 base editor that uses SaCas9 (Hua et al., 2018), we constructed a base editor, ABE-P5, which has a SaKKH-Cas9 (D10A) nickase to replace the SaCas9 (D10A) nickase in ABE-P2 (Figure 1a). We designed two sgRNAs (sgRNA8 and sgRNA9) to test the activity of ABE-P5 against endogenous genes in the rice genome. For sgRNA8 that targets the OsmiR172 binding site in the SNB gene, the base editing efficiency was 6.5% (3/46; Figure S4a,b and Table 1). We did not find any mutation for sgRNA9 that targets the OsmiR164 binding site in OsSPL13 (Table 1).

Cytosine base editors with SaCas9 and SaCas9 variants have been shown to work efficiently in mammalian cells (Kim et al., 2017). So we sought to expand the target range of the cytosine base editors in plants. Previous studies in rice have identified two genetic loci, PMS1 and PMS3, that are responsible for the
environment sensitive genic male sterity (EGMS) phenotype of the NK58S mutant and both loci encode long noncoding RNAs (Fan and Zhang, 2018). The causal mutations in NK58S were G-T and C-G substitutions in \( PMS1 \) and \( PMS3 \), respectively (Fan and Zhang, 2018). We surmised that a G-A mutation in \( PMS1 \) and a C-T substitution in \( PMS3 \) may also lead to the same EGMS phenotype in the rice variety Nipponbare. We designed sgRNA10 for CBE-P5 that targets \( PMS1 \) and sgRNA11 for CBE-P3 that targets \( PMS3 \) (Figure 1a,d and Figure S5b). These two vectors were then separately transformed into rice by \textit{Agrobacterium}-mediated transformation or co-transformed by particle bombardment. From the single transformation experiment, five lines were edited at the \( PMS3 \) target site from just seven transgenic lines generated (Figure 1e and Table 3). However, we did not find any mutation at the \( PMS1 \) target site from 52 transgenic lines (Table 3). From the co-transformation experiment, 18 co-transformants were obtained from 28 transgenic lines. Again, we found that the \( PMS3 \) target site was edited with high efficiency in the co-transformants, up to 61.1% (11/18; Table 3). All edited lines had expected C-T substitutions, except that one line had an additional 1 bp insertion. We found that cytosines even at position 15 in the protospacer could be edited (Figure 1e and Table 2). Therefore, the base editing windows for ABEs and CBEs at some target sites in our study (Table 2) are larger than previously defined in mammalian systems (Gaudelli et al., 2017; Komor et al., 2016). Two recent \textit{in vivo} base editing studies in...
Table 3  Base editing or mutation efficiencies of sgRNA10-sgRNA13

| sgRNA  | Base editor/vector | Target gene | Single or double transformation* | Number of transgenic lines | Number of co-transformants | Number of edited lines | Editing efficiency |
|--------|--------------------|-------------|----------------------------------|---------------------------|---------------------------|------------------------|-------------------|
| sgRNA10| CBE-P5             | PMS1        | single                           | 52                        | –                         | 0                      | 0                 |
| sgRNA11| CBE-P3             | PMS3        | single                           | 7                         | –                         | 5                      | 71.4%             |
| sgRNA10| CBE-P5             | PMS1        | double                           | 28                        | 18                        | 0                      | 0                 |
| sgRNA11| CBE-P3             | PMS3        | double                           | 28                        | 18                        | 11                     | 61.1%             |
| sgRNA10| pSaKKH-Cas9        | PMS1        | double                           | 19                        | 14                        | 5                      | 35.7%             |
| sgRNA11| pVQR-Cas9          | PMS3        | double                           | 19                        | 14                        | 0                      | 0                 |
| sgRNA12| ABE-P2             | OsSPL14     | double                           | 51                        | 20                        | 5                      | 25%               |
| sgRNA12| ABE-P2             | OsSPL17     | double                           | 51                        | 20                        | 9                      | 45%               |
| sgRNA13| CBE-P1             | SNB         | double                           | 51                        | 20                        | 16                     | 80%               |

*Double transformation means that two vectors are co-transformed into rice by particle bombardment.

mouse and rabbit also showed that ABEs and CBEs with SpCas9 can edit target adenines and cytosines outside of the canonical base editing activity windows at some target sites (Liu et al., 2018a, b). As the base editing windows characterized in previous studies were derived from limited target sites and were mainly from cell-based assays which had relatively a short time for base editors to function (Gaudelli et al., 2017; Komor et al., 2016), more target sites need to be tested, and in vivo base editing studies are required to accurately define the base editing windows for both adenine and cytosine base editors. Furthermore, it has been reported that base editors with SaCas9 have larger base editing activity windows than those of base editors with SpCas9, perhaps due to greater strand exposure to deaminases after formation of the SaCas9-sgRNA-DNA R-loop complex (Hua et al., 2018; Kim et al., 2017).

It was interesting that the PMS1 target site was still resistant to editing in the co-transformants, indicating that sgRNA10 had poor activity. The sgRNA activity is affected by many factors, such as base composition, GC content and chromatin states of the target site. To test the activities of sgRNA10 and sgRNA11, we also co-transformed rice with the corresponding DSB-inducing CRISPR/Cas9 vectors (pSaKKH-Cas9 and pVQR-Cas9) as a control (Figure S5a). To our surprise, this time we found that five lines were mutated at the sgRNA10 target site from 14 co-transformants, whereas no mutations were identified at the sgRNA11 target site (Figure S5c and Table 3). Therefore, the sgRNA activities for inducing indel mutations and for base editing are not correlated, at least at the PMS1 and PMS3 target sites. We speculate that a mechanistic difference between CRISPR-Cas9 mediated mutagenesis and base editing may account for their different mutation rates at the PMS1 and PMS3 target sites. The key mechanistic difference between CRISPR-Cas9 mediated mutagenesis and base editing is whether or not they rely on DSB formation. The DSB induced by Cas9 is mainly repaired by the non-homologous end-joining pathway in plant cells, which usually results in indel mutations (Michael and Holger, 2017). However, the base editors do not induce DSB formation and are designed to manipulate the cellular DNA repair pathways by inhibiting base-excision repair and stimulating mismatch repair to improve base conversion efficiency (Gaudelli et al., 2017; Komor et al., 2016). Thus, different DNA repair pathways are involved in indel formation and base conversion. It is possible that the sequence context and/or other features of a target site may determine whether one repair pathway is more efficient than the other at the target site. The different mutation rates between CRISPR-Cas9 mediated mutagenesis and base editing at the same target site may be a reflection of this difference.

Thus far, multiple Cas9 orthologues have been identified and repurposed for genome engineering. We hypothesized that the adenine and cytosine base editing may be simultaneously executed in plants by fusing the cytidine and adenine deaminase to orthogonal Cas9 enzymes. To test our hypothesis, we designed sgRNA12 for ABE-P2 and sgRNA13 for CBE-P1. sgRNA12 targets the Osmir156 binding sites in OsSPL14 and OsSPL17 (Figure 1b), whereas sgRNA13 targets the Osmir172 binding site in SNB (Figure S4a). The two vectors were co-transformed into rice by particle bombardment. After genotyping, we obtained 20 co-transformants from 51 transgenic lines (Table 3). Then the three target sites in the 20 co-transformants were individually PCR amplified and subjected to Sanger sequencing. We found that 5 and 9 lines harboured the expected A-G substitutions at the target sites in OsSPL14 and OsSPL17, respectively (Table 3). Eighty percent of the co-transformants (16/20) had C-T substitutions at the target site in SNB without inducing any indels or other base transition or transversion mutations (Table 3). Importantly, we obtained 5 lines that were edited simultaneously at the three target loci. Sequencing chromatograms at the three target sites of a representative line, line 14, are shown in Figure 1f. These results suggested that the ABE and CBE can efficiently work together in plants.

In summary, we have developed new adenine and cytosine base editors with engineered SpCas9 and SaCas9 variants that substantially expand the targetable sites in the rice genome. Moreover, we have demonstrated that adenine and cytosine base editing can be simultaneously executed in rice. We anticipate that our new base editors described here will be useful for molecular genetics research and precision molecular breeding in rice and other crops.

Experimental procedures

Vector construction

Adenine base editing vectors with SpCas9 and SaCas9 variants used in this study were modified from the pRABEsp-OsU6 (also named as ABE-P1) and pRABEsa-OsU6sa (also named as ABE-P2) vectors (Hua et al., 2018). Briefly, specific point mutations described by Kleinstiver et al. (2015a, b) were introduced into the wild type SpCas9 and SaCas9 by PCR to convert them to

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bombardments were performed on PDS-1000/He
rice calli were pre-treated on osmotic medium (induction
and 12-h darkness at 22
°C) in the dark. Then, the hygromycin resistant calli were
induce shoot regeneration. The shoots that grew up to 4
weeks) in the dark. Then, the hygromycin resistant calli were
Nipponbare (Oryza sativa L. japonica. cv. Nipponbare) were used
for transformation. The shoots that grew up to 4–5 cm
length were transferred to rooting medium for root induction.
Two weeks later, the plantlets were transplanted to greenhouse
and grew under the standard conditions for rice (12-h light 28°C
and 12-h darkness at 22°C).

For particle bombardment-mediated co-transformation, two
vectors were pre-mixed together in a 1:1 ratio. Then the
vectors were coated on 0.6 μm gold particles, following the
protocol described by Shan et al. (2014). Before bombardment,
rice calli were pre-treated on osmotic medium (induction medium
with 0.5 mol/L mannitol) for four hours. Particle
bombardments were performed on PDS-1000/He™ system
(Bio-Rad, California) according to the manufacturer’s instructions.
After bombardment, rice calli were kept on osmotic medium for another eighteen hours under the dark condition at 28°C. Thereafter, the rice calli were transferred to the selection medium for two rounds selection. Then the
hygromycin resistant calli were selected for regeneration. The shoot regeneration and root induction steps were the same as described above.

Genotyping base editing and mutation events
Rice genome DNA was extracted from the fresh leaves of all transgenic lines. The target regions were amplified by Taq DNA polymerase and the PCR products were send for Sanger sequencing by a next primer. For base edited lines that need to be confirmed by TA cloning, the target regions were re-amplified by KOD DNA polymerase (TOBOYO, OSAKA, Japan) and the PCR products were cloned into the p-EASY Blunt Zero vector (TransGen Biotech, Beijing, China). At least 20 clones were randomly selected for sequencing. For rice co-transformation experiments, all transgenic lines were first genotyped with the M13F and the corresponding sgRNA reverse primers. Then the co-
transformants were selected for sequencing at the target sites. Base editing ratio at each target site was calculated by dividing the number of plants with base editing events to the total number of genotyped transgenic lines. All the primers used for genotyping are listed in Table S2.

Acknowledgements
This research was supported by the Chinese Academy of Sciences.

Conflict of interest
The authors declare no conflict of interests.

Author contributions
K.H. performed most of the experiments, analysed the data and wrote the manuscript. X.T. performed rice transformation. J.-K. Z. supervised the project and wrote the manuscript.

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Supporting information
Additional supporting information may be found online in the Supporting Information section at the end of the article.

Data S1 Complete DNA sequences of all vectors used in this study.
Figure S1 Base editing by ABE-P1 at the genomic sequences with NAG PAMs.
Figure S2 Targeted base editing at OsSPL16 and OsSPL18 by ABE-P3.
Figure S3 Targeted base editing at OsTOE1 and OsIDS1 by ABE-P4.
Figure S4 Targeted base editing at the SNB gene by ABE-P5.
Figure S5 Targeted mutation at the PMS1 and PMS3 genes by pSaKKH-Cas9 and pVQR-Cas9.
Table S1 Primers for sgRNAs used in this study.
Table S2 Primers for target sites amplification and sequencing.