Chapter 10

High-Efficiency Cas12a-Mediated A-to-G Base Editing in Rice

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Abstract

The development of CRISPR-Cas genome editing technology, notably the Cas9 and Cas12a systems, has significantly advanced biomedical studies and plant research. The Cas9-derived base editors have been effectively utilized for precise C-to-T, A-to-G, and C-to-G base editing in mammalian cells and plants, thanks to the availability of Cas9 nickase. Base editing scope can be further expanded with engineered variants of SpCas9 or other Cas9 orthologs with altered protospacer adjacent motif (PAM) requirements. CRISPR-Cas12a recognizes T-rich PAM, which can further expand the base editing range. However, the absence of an efficient Cas12a nickase presents a challenge in the development of efficient Cas12a-based base editors, necessitating the use of a deactivated Cas12a (dCas12a). Here, we present a detailed protocol for constructing T-DNA vectors using a deactivated *Lachnospiraceae bacterium* Cas12a (dLbCas12a) with the D156R mutation, coupled with an adenine base editor (ABE), which has demonstrated high efficiency in multiplexed A-to-G base editing in rice.

Key words CRISPR-Cas12a, dLbCas12a-D156R, Adenine base editor, Multiplexed editing, Rice

1 Introduction

The innovative CRISPR-Cas genome editing technologies, particularly Cas9 and Cas12a systems, have significantly aided plant research and crop improvement. Typical Cas9 and Cas12a tools function by inducing DNA double-strand breaks (DSBs) to edit targeted genes. Base editors (BE) are incredibly useful genome editing tools that generate precise nucleotide substitutions at specific DNA loci. Numerous base editors derived from Cas9 have been developed and demonstrated for highly precise C-to-T, A-to-G, and even C-to-G base editing in plants [1]. Adenine-based editors (ABEs) are composed of an adenine deaminase enzyme fused to a catalytically inactive SpCas9 protein (dSpCas9), a SpCas9 nickase (nSpCas9) or its engineered variants with altered protospacer adjacent motif (PAM) requirements. The adenine deaminase is derived from an enzyme that naturally occurs in

M. Tofazzal Islam et al. (eds.), *CRISPR-Cas Methods: Volume 3*, Springer Protocols Handbooks,
https://doi.org/10.1007/978-1-0716-4358-7_10,
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bacteria and can deaminate adenine (A) into inosine (I), which the cell's DNA repair systems interpret as guanine (G), thus leading to an A-to-G substitution after DNA repair and replication. This process also induces a complementary T-to-C edit on the opposite DNA strand [2]. The *Escherichia coli* transfer RNA adenosine deaminase (ecTadA) has been engineered for A-to-G editing [2], and its improved version, TadA-8e, has been further re-engineered to confer C-to-T editing [3–5]. Highly efficient ABEs have been demonstrated in a variety of plants, including rice [6–12], wheat [7], maize [13], poplar [14], *Arabidopsis* [15, 16], tomato [16, 17], and cotton [18], demonstrating their widespread use in plant genomic research and breeding.

CRISPR-Cas12a, a class II type V CRISPR nuclease that recognizes T-rich PAMs, offers a complementary targeting scope to SpCas9's G-rich PAMs, expanding the spectrum of genomic targets [19]. Cas12a system has been particularly effective in enabling multiplexed genome editing in plants, allowing for the simultaneous modification of multiple genetic sites [19, 20]. Despite the lack of an efficient Cas12a nickase, notable progress has been made in developing Cas12a base editors in mammalian cells, which cause low DNA damage due to the employment of deactivated Cas12a (dCas12a) [21–23]. However, efficient Cas12a-derived base editors have not been developed in plants until recently [24, 25]. We have successfully developed an efficient ABE by combining the high-efficiency LbCas12a-D156R variant, the potent adenine deaminase ecTadA8e, and an optimal flexible linker (8xGGGGS) [24]. This Cas12a-based ABE has proven to be as efficient as the Cas9 ABEs and achieved simultaneously editing at six endogenous sites in the rice genome [24].

In this chapter, we describe a detailed protocol for constructing T-DNA vectors of this highly efficient dLbCas12a-D156R-ABE system and delivering the T-DNA using *Agrobacterium*-mediated transformation for multiplexed A-to-G editing in rice.

2 Materials

2.1 Plants and Bacterial

1. *Oryza sativa* L. japonica cv. Kitaake.
2. Chemically competent cells of *Escherichia coli* DH5α and *Agrobacterium tumefaciens* stain EHA105.

2.2 Solutions and Mediums

Stock solutions (sterilize using a 0.22 μm syringe filter or autoclave)

1. 20 mg/mL Tetracycline (in 70% ethyl alcohol, filter sterilization).
2. 50 mg/mL Spectinomycin (in H_2O, filter sterilization).
3. 50 mg/mL Kanamycin (in H_2O, filter sterilization).

4. 50 mg/mL Rifampicin (in DMSO).
5. 0.1 M Isopropyl β-D-1-thiogalactopyranoside (IPTG) (in H_2O, filter sterilization).
6. 20 mg/mL 5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal) (in DMSO).
7. 20 mg/mL Hygromycin (in H_2O, filter sterilization).
8. 200 mg/mL Timentin (in H_2O, filter sterilization).
9. 100 mM Acetosyringone (in DMSO, filter sterilization).
10. 2 mg/mL 2,4-Dichlorophenoxyacetic acid (add 2–3 mL of distilled ethanol or 1 M NaOH to dissolve the powder and then dilute the solution with distilled water to a final volume, filter sterilization).
11. 1 mg/mL α-Naphthaleneacetic acid (NAA) (add 2–3 mL of distilled ethanol or 1 M NaOH to dissolve the powder and then dilute the solution with distilled water to final volume, filter sterilization).
12. 2 mg/mL Kentin (in H_2O, filter sterilization).
13. 360 g/L Glucose (in H_2O, autoclave).
14. 100 g/L Glucose (in H_2O, autoclave).
15. 0.5 M KOH (in H_2O).
16. 1 M NaOH (in H_2O).
17. 1 M Tris–HCl pH 8.0 (in H_2O).
18. 0.5 M Ethylenediaminetetraacetic acid pH 8.0 (EDTA) (in H_2O).
19. 10 mM DL-Dithiothreitol (DTT) (in H_2O).

LB Medium for *E. coli* and *Agrobacterium*

1. 2.5% (w/v) LB Broth (Thermo Fisher Scientific).
2. 1.5% (w/v) Agar (Thermo Scientific) for preparing solid LB plates.

   Ensure to supplement medium with the correct antibiotics at suitable concentrations, as determined by the resistance markers present in plasmids.

2.3 Regents

1. BamHI (BamHI-HF®, New England Biolabs).
2. EcoRI-HF and rCut smart buffer (New England Biolabs).
3. Esp3I (BsmBI) (Thermo Fisher Scientific).
4. T4 DNA ligase and 10× T4 ligase buffer (New England Biolabs).
5. PCR polymerase and corresponding buffer (New England Biolabs).
6. 10 mM deoxy-ribonucleoside triphosphate (New England Biolabs).

7. Instant Sticky-end Ligase Master Mix (New England Biolabs).
8. Phire Plant Direct PCR Master Mix (Thermo Scientific).
9. Plasmid Miniprep kit (QIAGEN).
10. QIAquick Gel extraction kit (QIAGEN).
11. 1 kb DNA ladder and 6X gel loading dye (New England Biolabs).
12. Gateway™ LR Clonase™ II Enzyme Mix (Thermo Fisher Scientific).
13. Cetyl Trimethyl Ammonium Bromide (Fisher Scientific).
14. Agarose (PR1MA™).
15. Chu's N6 Basal Medium with Vitamins (PhytoTech Labs).
16. Casamino acids (Fisher bioreagents).
17. L-proline (Alfa Aesar).
18. Sucrose (Fisher Scientific).
19. Murashige & Skoog salt with Vitamins (Fisher Scientific).
20. D-Sorbitol (Thermo Scientific).
21. Myo-inositol (PhytoTech Labs).
22. Gelrite (Research Product International).

2.4 Equipment

1. Heating block or water bath.
2. Hotplate stirrer.
3. Temperature-controlled shaker incubator.
4. PCR Thermocycler.
5. pH meter.
6. Electrophoresis apparatus.
7. Gel imager.
8. NanoDrop™ One UV-Visible spectrophotometer or other equipment for DNA quantification.
9. Laminar flow hood.
10. Autoclave.
11. Vortexer.
12. Centrifuge.
13. Growth chamber.

2.5 Targets and Plasmids

1. The genomic sequence information for the target genes is available through the Rice Genome Annotation Project database (http://rice.uga.edu/).
2. DNA oligonucleotides for CRISPR RNAs (crRNAs). crRNAs can be designed using various online tools including CRISPR-P v2.0 (http://crispr.hzau.edu.cn/CRISPR2/), CRISPR-DT

(http://bioinfolab.miamioh.edu/CRISPR-DT), Benchling (https://benchling.com), and so on. Potential crRNA-dependent off-target sites can be predicted by Cas-OFFinder (http://www.rgenome.net/cas-offinder/). The crRNAs used in this protocol are listed in Table 1.

3. All plasmids mentioned in this protocol are available from Addgene (https://www.addgene.org): pYPQ131-STU-Lb (Addgene #138096), pYPQ132-STU-Lb (Addgene #138099), pYPQ133-STU-Lb (Addgene #138102), and pYPQ134-STU-Lb (Addgene #138105), pYPQ144-ZmUBI-pT (Addgene #138108), dpYPQ230-D156R-ABE5 (ecTadA8e-8xGGGGS-dLbCas12a-D156R, #195371), and pYPQ203 (Addgene # 86207).

3 Methods

3.1 Vector Construction for dLbCas12a-ABE System

1. Design crRNAs for Cas12a-mediated multiplexed A-to-G editing. crRNAs can be designed using a CRISPR crRNA design tool. Generally, the target sequence should be approximately 23 nucleotides in length and should be directly adjacent to the LbCas12a PAM sequence as 5′-TTTV-3′, targeting either strand of double-stranded genomic DNA. It's important to consider the specific base editing window of the base editor being used; for the dpYPQ230-D156R-ABE5 base editor used here, the active editing window ranges from A8 to A12, downstream of PAM sequence.
2. Synthesize crRNAs as single-strand oligonucleotides. Create forward and reverse oligos as follows: the forward oligo (5′-TAGATNNNNNNNNNNNNNNNNNNNN -3′) and the reverse oligo (5′-GGCCNNNNNNNNNNNNNNNNNNNNNA-3′). Rehydrate the lyophilized DNA oligos with DNase-free water (*see* **Note 1**) to a final concentration of 100 μM.
3. Phosphorylate and anneal the oligos. Perform this process by incubating the mixture at 37 °C for 30 min; 95 °C for 5 min; ramp down to 25 °C at a rate of 5 °C per min using a thermocycler (alternatively incubate in boiled water and then cool down to room temperature) (Table 2).
4. Prepare crRNA cloning plasmids. Digest the plasmids of pYPQ131/2/3/4-STU-Lb with *Esp3I* (*BsmBI*). Mix each regent (Table 3) and incubate at 37 °C for at least 1 h. Run digested plasmids on a 1% agarose gel, and then excise the correct band size and purify digested plasmids using a gel extraction kit. Quantify the concentration of linear plasmids using Nanodrop (*see* **Notes 2** and **3**).

Table 1
Examples of crRNA sequences and DNA oligonucleotides

Target gene	Target site	crRNA target sequence with PAM (5′ - 3′)	DNA oligonucleotides (5′ - 3′)
LOC_Os09g26999	TTTTC04	**TTTC**CAGAAAGAGAAGGAGGCACAGAT	Forward: tagatCAGAAAGAGAAGGAGGCACAGAT Reverse: ggccATCTGTGCCTCCTTCTCTTTCTGa
LOC_Os07g40404	CTTTG02	**TTTG**GCACCATATGCTTGCTGATCAAA	Forward: tagatGCACCATATGCTTGCTGATCAAA Reverse: ggccTTTGATCAGCAAGCATATGGTGCa
LOC_Os02g43194	ATTTA02	**TTTA**CCGGTGAAAAGGACCTTGTCCCA	Forward: tagatCCGGTGAAAAGGACCTTGTCCCA Reverse: ggccTGGGACAAGGTCCTTTTCACCGGa
LOC_Os07g10860	CTTTA01	**TTTA**TACGTGGAAACAATGACAGTTCA	Forward: tagatTACGTGGAAACAATGACAGTTCA Reverse: ggccTGAACTGTCATTGTTTCCACGTAa

Table 2
Oligos phosphorylation

Component	Volume
gRNA oligo forward (100 μM)	1 μL
gRNA oligo reverse (100 μM)	1 μL
T4 polynucleotide kinase (10 U/μL)	0.2 μL
10X T4 polynucleotide kinase buffer	1 μL
ddH2O	6.8 μL
Total	**10 μL**

Table 3
Empty crRNA cloning plasmid digestion

Component	Volume
pYPQ131/2/3/4-STU-Lb	2 μg
Buffer Tango (10X)	5 μL
DTT (10 mM)	5 μL
Esp3I (BsmBI) (10 U/μL)	2 μL (20 U)
Water	To 50 μL
Total	**50 μL**

5. Ligate the crRNA oligos. Insert the annealed crRNA oligos into *Esp3I* (*BsmBI*)-linearized crRNA cloning plasmids with Instant Sticky-end Ligase Master Mix and incubate at room temperature for 15–30 min (*see* **Note 4**).
6. Transform the ligation mixture. Use the heat shock method to introduce the ligated plasmids into *E. coli* DH5α competent cells. Then culture on LB plate supplemented with 10 mg/L tetracycline and incubate overnight at 37 °C.
7. Select and verify colonies. Pick 2 to 3 colonies per construct for overnight culture and plasmid preparation. Verify correct clones (pYPQ131-STU-Lb-TTTTC04; pYPQ132-STU-Lb-CTTTG02; pYPQ133-STU-Lb-ATTTA02; and pYPQ134-STU-Lb-CTTTA01) by Sanger sequencing using the sequencing primer pTC14-F2 (5′-CAAGCCTGATTGGGAGAAAA-3′).
8. Assemble four crRNA cassettes. Use the Golden Gate assembly to insert four crRNAs into the pYPQ144-ZmUBI-pT (Table 4) and incubate reactions in a thermocycler (Table 5).
9. Transform *E. coli* and screen colonies. Transform the assembly mixture into *E. coli* DH5α using the heat shock method, and

Table 4
Golden Gate assembly of four crRNAs

Component	Volume
pYPQ131-STU-Lb-crRNA1	100 ng
pYPQ132-STU-Lb-crRNA2	100 ng
pYPQ133-STU-Lb-crRNA3	100 ng
pYPQ134-STU-Lb-crRNA4	100 ng
pYPQ144-ZmUbi-pT	100 ng
T4 DNA Ligase Buffer (10X)	2 μL
BsaI-HF®v2	2 μL (20 U)
T4 DNA Ligase (400 U μL-1)	2 μL (800 U)
Nuclease-free water	To 20 μL
Total	**20 μL**

Table 5
Golden Gate reaction program

Temperature (°C)	Time (min)	Cycles
37 16	5 10	10 ~ 12
50	5	1
80	5	1
10	∞	/

culture the transformed cells on LB medium with 50 mg/L spectinomycin. To enable blue-white screening, add 50 μL of 20 mg mL-1 X-gal and 50 μL of 0.1 M IPTG on LB plate surface in advance. Pick 2 to 3 white colonies and culture for miniprep. Confirm correct clones by restriction enzyme digestion of the plasmids and sanger sequencing using primers M13-F1 (5′-TTCCCAGTCACGACGTTGTAAAAC-3′) and M13-R1 (5′-TTTGAGACACGGGCCAGAGCTGC-3′).

10. Construct the T-DNA expression vector. Assemble the T-DNA expression vector by a three-way Gateway LR reaction (Table 6). Incubate the reaction at room temperature for at least 3 h.
11. Transform *E. coli* and confirm clones. Transform all reaction mixtures into *E.coli* DH5α and culture on LB medium with 50 mg/L kanamycin. Pick 2–3 colonies for miniprep. Confirm

Table 6
Three-Way Gateway LR reaction

Component	Amount
dpYPQ230-D156R-ABE5	150 ng
pYPQ144-ZmUBI-crRNA1-4	150 ng
pYPQ203	200 ng
LR Clonase II	1 μL

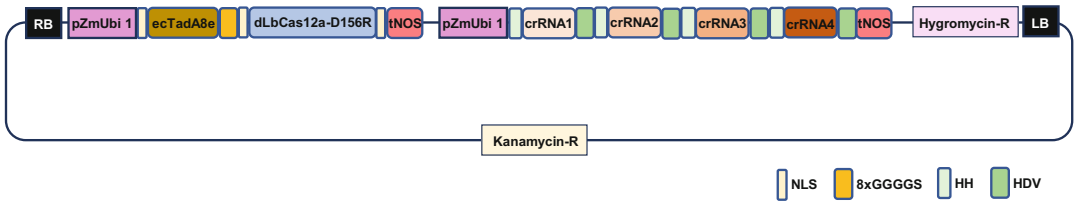

Fig. 1 Diagram illustrating the T-DNA construct of engineered dCas12a-based Adenine Base Editor (ABE) with a dual Pol II promoter system. The system utilizes an 8xGGGGS linker between ecTadA8e and dLbCas12a-D156R. Abbreviations: pZmUbi1 maize ubiquitin promoter, NLS nuclear localization signal, tNOS nopaline synthase (NOS) terminator, HH hammerhead ribozyme, HDV hepatitis delta virus ribozyme

correct T-DNA clones by restriction enzyme digestion of the plasmids and/or whole plasmid sequencing (Fig. 1). Freeze the confirmed *E.coli* cells harboring correct T-DNA plasmids in 25% glycerol at −80 °C for long-term storage.

12. Transform *Agrobacterium*. Transfer the correct final T-DNA plasmids into *Agrobacterium EHA105* (*see* **Note 5**) by a freeze-thaw transformation method or electroporation. Confirm correct Argo clones by colony PCR (*see* **Note 6**).
13. Transform rice callus cells by *Agrobacterium*-mediated method. Perform *Agrobacterium*-mediated transformation as detailed in a previous publication [20]. The steps can be simplified as follows: Sterilize healthy Kitaake rice seeds and place them on rice callus induction medium to induce calli. After 14 days, cut and collect healthy calli from germinated seeds. Mix and co-cultivate calli and *Agrobacterium* cells for 3–5 days at 26 °C in the dark. Next, wash calli thoroughly to remove any remaining *Agrobacterium* cells. Culture washed calli on hygromycin-containing medium for several weeks at 32 °C to select transformed calli. Once new calli emerge, transfer them to regeneration medium I and incubate at 29 °C with a 16 h/8 h light/dark cycle. Lastly, transfer emerging seedlings to regeneration medium II for further growth (*see* **Note 7**).

3.2 NGS Sequencing and Analysis of Base Editing Efficiency

1. Collect leaf tissue from the regenerated rice seedlings for genomic DNA extraction using the CTAB method as described by Stewart and Via (1993) [26].
2. Prepare the extracted DNA for Next-Generation Sequencing (NGS) through a two-round barcoded PCR amplification as previously described [20, 27].
   (a) First Round: Amplify the target region using target-specific primers that include bridge sequences for later barcoding.
   (b) Second Round: Use 2 μL of the first-round PCR products as a template and amplify it using HiTom barcoding primers [27].
   (c) Pool less than 96 different PCR samples together and purify them using a column-based method.
   (d) Prepare purified PCR products (<500 bp) with partial Illumina® adapter sequences. Normalize the concentration to 20 ng/μL, with a minimum total of 500 ng of double-stranded DNA to ensure successful sequencing.
   (e) Perform a 2 × 250 bp sequencing run without fragmenting the amplicons at GeneWiz, USA.
3. Post-sequencing, analyze the NGS data using the CRISPR Rgen BE analyzer tool available online (http://www.rgenome.net/be-analyzer/#!).
4. For the presentation and statistical analysis of the data, use software GraphPad Prism v.9.4.1 (Fig. 2). Display the genotypes of the T0 generation plants with their corresponding zygosity patterns as shown in Fig. 3.

4 Notes

1. All water used in the protocol is sterilized and of molecular grade to prevent contamination.
2. If the plasmid digestion results in fragments too small to be effectively recovered using a gel extraction kit, skipping agarose gel electrophoresis may be considered. However, performing electrophoresis can help remove undigested plasmids, potentially enhancing the efficiency of crRNA cloning.
3. This protocol outlines the construction of the dLbCas12 a-D156R-ABE5 system, which is designed to multiplex four crRNAs. For assembling a higher number of crRNAs, please refer to our previous work for detailed procedures [20].

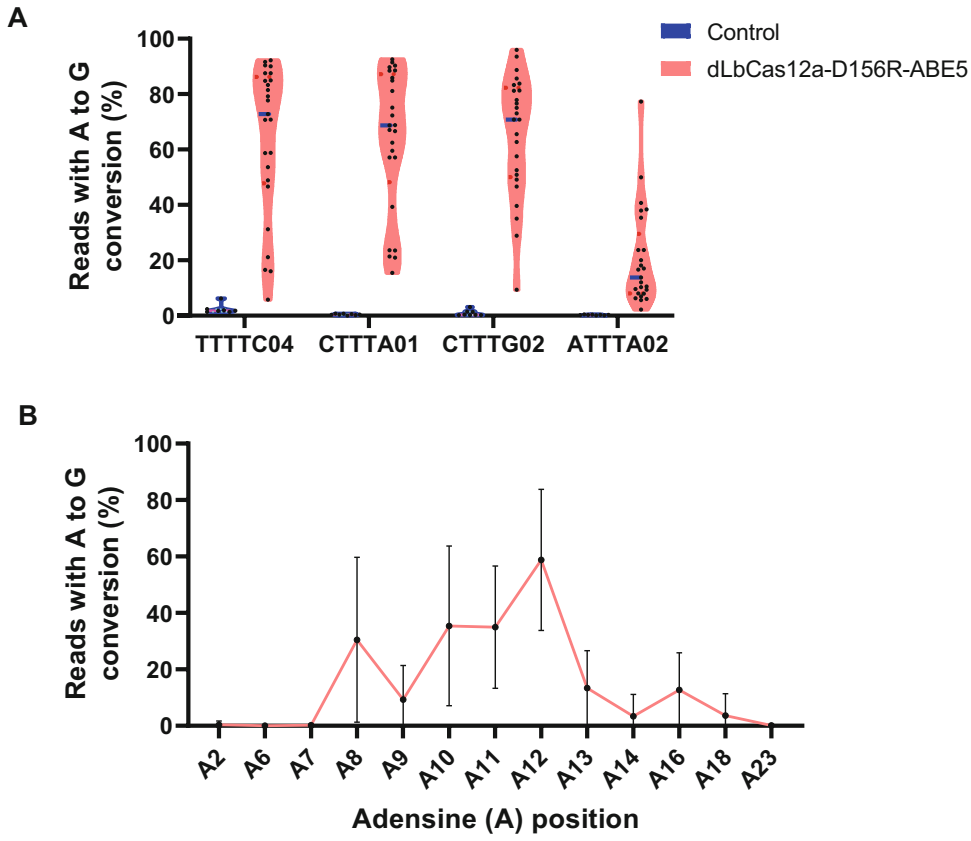

Fig. 2 Evaluation of A-to-G base editing frequency by dLbCas12a-D156R-ABE5 editor at four sites in T0 rice plants. (**a**) The distribution of editing frequencies. The first quartile, median, and third quartile are shown as doted lines. Each black dot represents a distinct line. The GFP-expressing transgenic plants served as a control. (**b**) The editing window of dLbCas12a-D156R-ABE5 across the four target sites. NGS of PCR amplicons was used to detect the mutations at the target sites in regenerated T0 rice lines

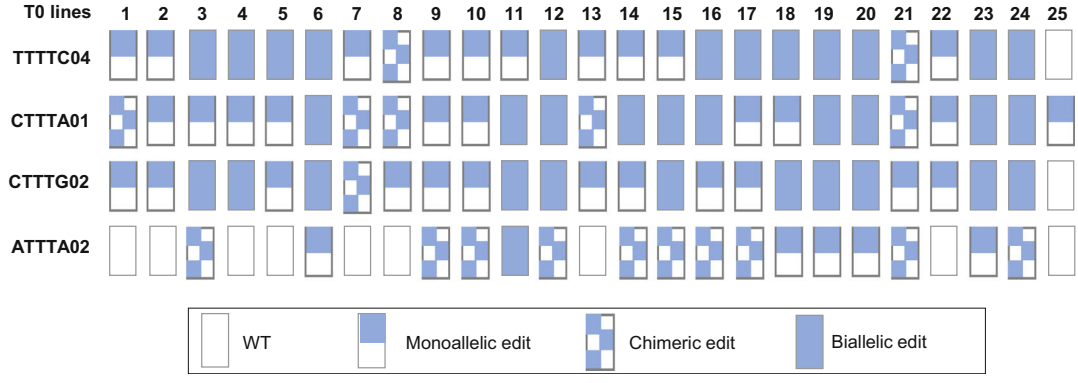

Fig. 3 Representation of the editing outcomes in T0 rice plants edited at four target sites using the dLbCas12a-D156R-ABE5 editor. Genotypes are categorized based on mutation frequencies, with wild type (WT) shown as empty rectangles, chimeric edits as dotted rectangles, monoallelic edits as half-filled rectangles, and biallelic edits as fully filled rectangles. Plants with less than 10% A-to-G mutation frequency are classified as WT, those with 10–30% as chimeric edits, 30–75% as monoallelic edits, and above 75% as biallelic edits. NGS of PCR amplicons was performed to detect the mutations at the target sites in regenerated T0 rice lines

4. When using standard T4 DNA ligase, prepare the ligation mixture and incubate at the room temperature for at least 1 h, or at 16 °C overnight. If the transformation is not conducted immediately after ligation step, deactivate the ligation reaction by heating it to 65 °C for 10 min to terminate the process.
5. Although the T-DNA plasmids can also be used for polyethylene glycol (PEG)-mediated rice protoplast transformation, this method is generally less efficient for base editing due to lack of cell division in protoplasts. Therefore, stable transformation is recommended for optimal base editing results.
6. It is not uncommon that T-DNA vectors might be recombined in *Agrobacterium* cells during transformation. Often, PCR cannot distinguish the correct vectors from recombined ones, as the recombination rate depends on the *Agrobacterium* strains and the transformation methods. To confirm the T-DNA vector is intact in transformed *Agrobacterium* cells, one can mini-prep the T-DNA plasmid from transformed *Agrobacteria* and retransform the resulting T-DNA plasmid into *E. coli*, followed by another miniprep and whole plasmid sequence.
7. To maintain healthy growth, transfer the calli to fresh selection or regeneration medium every two weeks, ensuring that the calli remain viable and contamination-free.

Acknowledgments

This work was supported by NSF Plant Genome Research Program grants (award nos. IOS-2029889 and IOS-2132693) and the USDA-AFRI Agricultural Innovations Through Gene Editing Program (award no. 2021-67013-34554).

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