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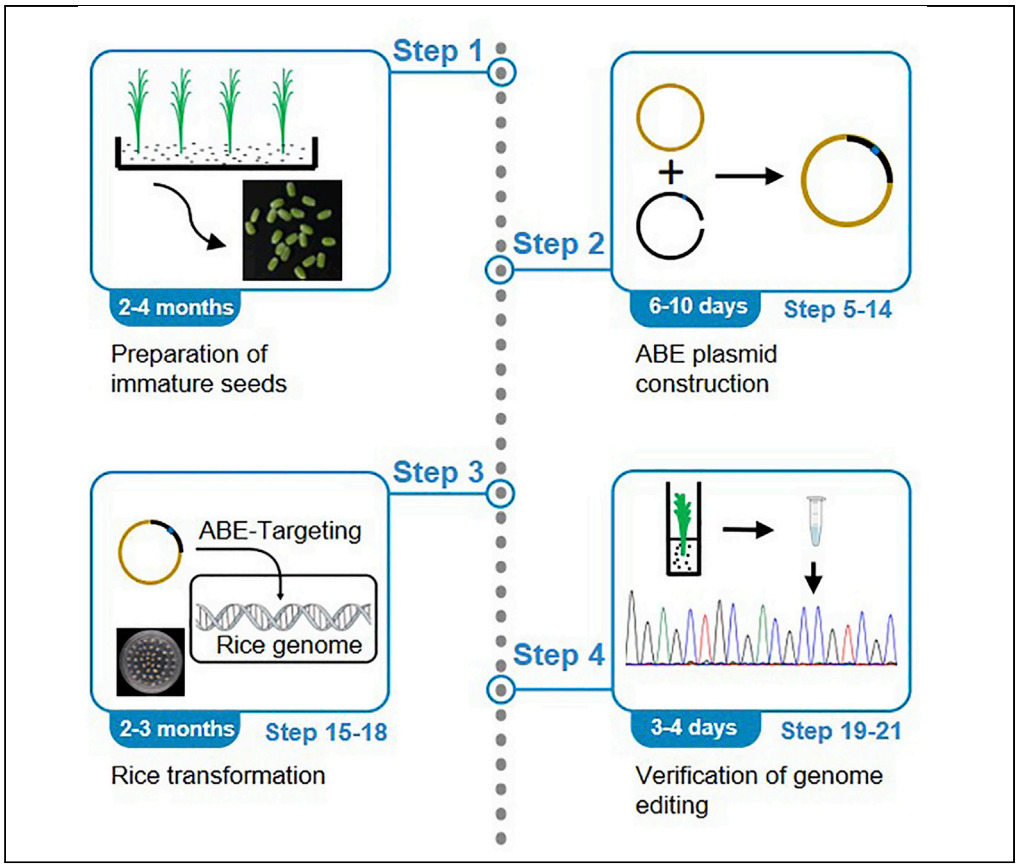
Protocol for targeted modification of the rice genome using base editing



Base editing is a precision genome-editing approach that is widely utilized to generate single-nucleotide variants (SNVs) in genomes. Here, we present a protocol to perform targeted adenine (A)-to-guanine (G) substitution in rice using adenine base editor (ABE). We detail the design of sgRNA, CRISPR plasmid construction, rapid genetic transformation of rice, and genotyping of editing events. This protocol can be applied to cytosine base editing in rice as well.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Description of high-speed tissue culture of rice

Steps to prepare CRISPR plasmids and perform *A. tumefaciens*-mediated rice transformation

Generation of precise nucleotide mutations by an adenine base editor

Genotyping of genome-edited plants to identify potential mutations in the target region

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Protocol for targeted modification of the rice genome using base editing

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SUMMARY

Base editing is a precision genome-editing approach that is widely utilized to generate single-nucleotide variants (SNVs) in genomes. Here, we present a protocol to perform targeted adenine (A)-to-guanine (G) substitution in rice using adenine base editor (ABE). We detail the design of sgRNA, CRISPR plasmid construction, rapid genetic transformation of rice, and genotyping of editing events. This protocol can be applied to cytosine base editing in rice as well.

For complete details on the use and execution of this protocol, please refer to Yan et al. (2021).[1]

BEFORE YOU BEGIN

Preparation of immature seeds

Timing: 12–16 weeks (for step 1)

This section describes how to grow rice plants and harvest immature seeds, which ensures fast and high- efficiency genetic transformation of rice.

1. Grow rice seedlings.
   a. Put 150 mature rice seeds into a 15-mL centrifuge tube, add 10 mL of 50% (v/v) sodium hypochlorite solution (10 mL Sodium hypochlorite, 10 mL ddH_2O, and one drop of Tween-20), and spin the tube on a rotating platform at 20 rpm for 45 min at room temperature (20°C–25°C).
   b. Wash the seeds thoroughly 3–5 times with ddH_2O.
   c. Germinate the seeds and grow rice seedlings on half-strength Murashige and Skoog (MS) medium in a growth chamber (16-h-light/8-h-dark photoperiod, 28°C or constant light, 28°C).
2. Transfer healthy seedlings into 50 × 80-cm plastic pots containing a 3:1 (w/w) mixture of field soil and vermiculite. Place all pots in a glasshouse (day/night temperatures of 28°C/20°C, 10-h-light/14-h-dark photoperiod).
3. Harvest rice spikelets with immature seeds on weeks 10–12, and dry spikelets in a 37°C incubator for 3–4 weeks. Seeds should be dehusked (Figure 1A) and stored at −80°C.

Note: Immature rice seeds have a better ability to generate callus than mature seeds and are critical for rice genetic transformation.

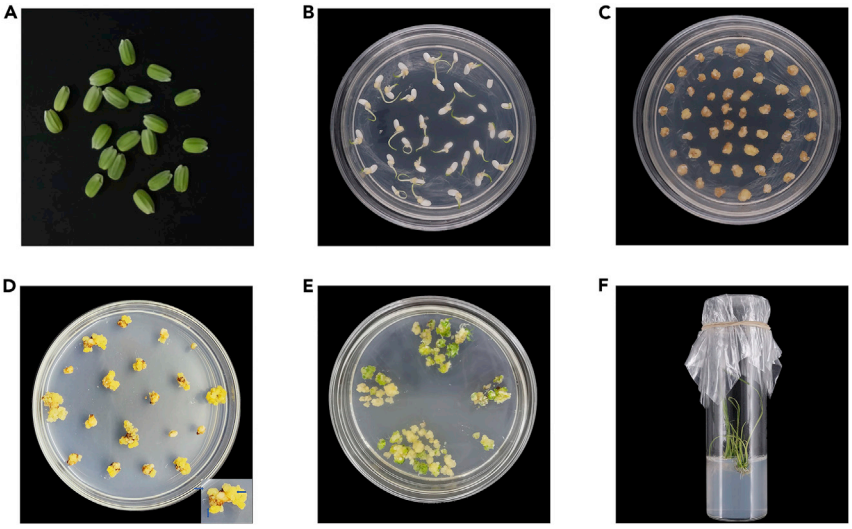

Figure 1. *Agrobacterium*-mediated transformation of *Japonica* rice variety Kitaake with immature embryos

(A) Immature rice seeds.

(B) Callus induction.

(C) Rice calli infected by *Agrobacterium*.

(D) Proliferation of Hygromycin-resistant calli.

(E) Shoot regeneration of Hygromycin-resistant calli.

(F) Root regeneration.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
TSINGKE TSC-C03 TSach1-T1 Chemically Competent Cells	TSINGKE	Cat#TSC-C03
TSINGKE TSC-C07 JM109 Chemically Competent Cells	TSINGKE	Cat#TSC-C07
EHA105 Chemically Competent Cells	TSINGKE	Cat#TSC-A03
Chemicals, peptides, and recombinant proteins		
BtgZI	New England Biolabs	Cat#R0703S
10×CutSmart Buffer	New England Biolabs	Cat#R0703S
FastDigest ApaI	Thermo Scientific	Cat#ER1411
10×FastDigest Green Buffer	Thermo Scientific	Cat#ER1411
T4 DNA ligase	Thermo Scientific	Cat#15224025
10×T4 buffer	Thermo Scientific	Cat#15224025
T4 polynucleotide kinase	Thermo Scientific	Cat#EK0031
10×T4 PNK buffer A	Thermo Scientific	Cat#EK0031
Gateway™ LR Clonase™ II Enzyme Mix	Thermo Scientific	Cat#11791020
Kanamycin (Kan)	Sigma-Aldrich	Cat#E004000
Rifampin (Rif)	Sigma-Aldrich	Cat#R3501
Hygromycin B	Roche	Cat#10843555001
Agargellan	PhytoTechnology	A133
Timentin	Solarbio	Cat#T8660
Sorbitol	Aladdin	Cat#S104840
MS (Murashige & Skoog modified basal medium with Gamborg vitamins)	PhytoTechnology	M404
Sucrose	Sigma-Aldrich	Cat#V900116
Tris (hydroxymethyl) aminomethane	Sigma-Aldrich	Cat#252859

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
$Na_2EDTA{\cdot}2H_20$	Sigma-Aldrich	Cat#E9884
CH_3COOH	Sigma-Aldrich	Cat#A6283
Hexadecyltrimethylammonium bromide (CTAB)	Sigma-Aldrich	Cat#H6269
Sodium hypochlorite	Sigma-Aldrich	Cat#239305
Agar Plant TC	PhytoTechnology	A111
Acetosyringone (AS)	Sigma-Aldrich	Cat#D134406
Tween 20	Coolaber	Cat#11551
Yeast extract	OXOID	Cat#LP0021
Tryptone	OXOID	Cat#LP0042
Agar powder	Sigma-Aldrich	Cat#A1296
Naphthalene acetic acid (NAA)	Sigma-Aldrich	Cat#N0640
2,4-Dichlorophenoxyacetic acid (2,4-D)	Sigma-Aldrich	Cat#40330
6-Benzyladenine (6-BA)	Sigma-Aldrich	Cat#B3408
Agarose	Sigma-Aldrich	Cat#A9539
Critical commercial assays		
Plasmid DNA Mini Kit	Axygen	AP-MN-P-250
DNA Gel Recovery Kit	Axygen	AP-GX-250
2× Rapid Taq Master Mix	Vazyme	Cat# P222
Experimental models: Organisms/strains		
Rice cultivar Kitaake	Ikegaya et al.[2]	Kept in our laboratory
Oligonucleotides		
Target-oligonucleotide 1:	Yan et al.[1]	5′-tgttGGAGCTACGACGCCGTCGC-3′
Target-oligonucleotide 2:	Yan et al.[1]	5′-aaacGCGACGGCGTCGTAGCTCC-3′
U6p-F1:	Zhou et al.[3]	5′-AAGAACGAACTAAGCCGGAC-3′
Recombinant DNA		
pENTR:sgRNA4	Zhou et al.[3]	Obtained from Dr Bing Yang's laboratory
rBE49d	Yan et al.[1]	Kept in our laboratory
Software and algorithms		
Rice genome information website	http://rice.uga.edu/	N/A
Cas-OFFinder	CRISPR RGEN Tools (rgenome.net)	N/A
Other		
0.2 μm Acrodisc Syringe Filters	PALL	Cat#4612
Centrifuge 5425	Eppendorf	Cat#5405000204
T100 Thermal Cycle	Bio-Rad	Cat#1861096
Constant Temperature Cycler ZX-10B	Shanghai Zhixin	N/A
TissueLyser II	QIAGEN	Cat#85300
Biomedical Upright Plasma Freezer MDF-U5412	Panasonic	N/A
Medical Refrigerator HYC-940	Haier	N/A
Electric Heating Constant Temperature Incubator DRP-9162	Shanghai Senxin	F9E0BA48
Microvolume Spectrophotometer NanoPhotometer-N60	IMPLEN	ASO000919
Ultra-low Temperature Freezer Forma 900	Thermo Scientific	Cat#902GP-ULTS
Gel Doc-XR Imaging Systems	Bio-Rad	Cat#1708195
PowerPac Basic Power Supply	Bio-Rad	Cat#1645050
Ultra clean bench	Thermo Scientific	Cat#51900900
Constant Temperature Oscillator IS-RDS4	Crystal	N/A

MATERIALS AND EQUIPMENT

Preparation of stock solutions

Timing: 1–2 days

This section describes the preparation of concentrated stock solutions used in this protocol.

Kan (50 mg/mL): Dissolve 2.5 g of kanamycin sulfate in 45 mL of ddH_2O, make the final volume to 50 mL. Filter-sterilize with a 0.2-μm filter and store in 1-mL aliquots at −20°C for no more than 6 months.

Rif (50 mg/mL): Dissolve 2.5 g of rifampin in 45 mL of dimethyl sulfoxide (DMSO), make the final volume to 50 mL and store in 1-mL aliquots at −20°C for no more than 12 months.

Timentin (200 mg/mL): Dissolve 3.2 g of Timentin in 12 mL of ddH_2O, make the final volume to 16 mL. Filter-sterilize with a 0.2-μm filter and store in 1-mL aliquots at −20°C for no more than 12 months.

6-BA (1 mg/mL): Add 0.5 M KOH dropwise to 10 mg of 6-BA powder until completely dissolved and make up to 10 mL with ddH_2O. Filter-sterilize with a 0.2-μm filter and store in 1-mL aliquots at −20°C for no more than 12 months.

NAA (1 mg/mL): Add 0.5 M KOH dropwise to 10 mg of NAA powder until completely dissolved and make up to 10 mL with ddH_2O. Filter-sterilize with a 0.2-μm filter and store in 1-mL aliquots at −20°C for no more than 6 months.

2,4-D (1 mg/mL): Dilute 5 mg/mL 2,4-D solution to 1 mg/mL with ddH_2O. Filter-sterilize with a 0.2-μm filter and store in 1-mL aliquots at 4°C for no more than 6 months.

AS (200 mM): Dissolve 392.4 mg of AS in 10 mL of dimethyl sulfoxide (DMSO) and store in 1-mL aliquots at −20°C for no more than 12 months.

Prepare growth media and buffers

Timing: 1–2 days

This section describes the formulation of growth media and buffers.

LB broth:

Reagent	Final concentration	Amount
Bacto tryptone	10 g/L	10 g
Bacto yeast extract	5 g/L	5 g
NaCl	10 g/L	10 g
ddH_2O	N/A	To 1 L
Total volume	N/A	1 L

Autoclave at 121°C for 20 min and store at room temperature (20°C–25°C) for no more than 4 weeks.

LB agar medium:

Reagent	Final concentration	Amount
Bacto tryptone	10 g/L	10 g
Bacto yeast extract	5 g/L	5 g
NaCl	10 g/L	10 g
Agar	15 g/L	15 g
ddH_2O	N/A	To 1 L
Total volume	N/A	1 L

Autoclave at 121°C for 20 min. Pour ~25 mL of medium into each Petri dish (100 × 15 mm). Store at 4°C for no more than 4 weeks.

TY broth:

Reagent	Final concentration	Amount
Bacto Tryptone	5 g/L	5 g
Bacto yeast extract	3 g/L	3 g
Kan	0.05 g/L	1 mL
Rif	0.05 g/L	1 mL
AS	200 μM	1 mL
ddH_2O	N/A	To 1 L
Total volume	N/A	1 L

Autoclave at 121°C for 20 min. Add filter-sterilized Kan, Rif, and AS solutions before use. Store at room temperature (20°C–25°C) for no more than 4 weeks.

MSD plates:

Reagent	Final concentration	Amount
MS	4.43 g/L	4.43 g
Sucrose	30 g/L	30 g
Agar Plant TC	8 g/L	8 g
2,4-D	0.002 g/L	2 mL
ddH_2O	N/A	To 1 L
Total volume	N/A	1 L

Adjust pH to 5.8 with 1 M KOH, and then autoclave at 121°C for 20 min. Pour ~25 mL of medium into each Petri dish (100 × 15 mm). Store at 4°C for no more than 4 weeks.

MSD+AS plates:

Reagent	Final concentration	Amount
MS	4.43 g/L	4.43 g
Sucrose	30 g/L	30 g
Agar plant TC	8 g/L	8 g
2,4-D	0.002 g/L	2 mL
AS	200 μM	1 mL
ddH_2O	N/A	To 1 L
Total volume	N/A	1 L

Adjust pH to 5.2 with 1 M KOH, then autoclave at 121°C for 20 min. Add filter-sterilized AS solution before pouring the medium into Petri dishes. Store at 4°C for no more than 4 weeks.

MSD/Hygromycin B/Timentin plates:

Reagent	Final concentration	Amount
MS	4.43 g/L	4.43 g
Sucrose	30 g/L	30 g
Agar plant TC	8 g/L	8 g
2,4-D	0.002 g/L	2 mL
Timentin	0.2 g/L	1 mL
Hygromycin B	0.05 g/L	1 mL
ddH_2O	N/A	To 1 L
Total volume	N/A	1 L

Adjust pH to 5.8 with 1 M KOH, then autoclave at 121°C for 20 min. Add filter-sterilized Hygromycin B and Timentin solutions before pouring the medium into Petri dishes. Store at 4°C for no more than 4 weeks.

RM plates:

Reagent	Final concentration	Amount
MS	4.43 g/L	4.43 g
Sucrose	30 g/L	30 g
Agar plant TC	12 g/L	12 g
Sorbitol	25 g/L	25 g
Timentin	0.2 g/L	1 mL
Hygromycin B	0.05 g/L	1 mL
6-BA	0.03 g/L	3 mL
NAA	0.0005 g/L	0.5 mL
ddH_2O	N/A	To 1 L
Total volume	N/A	1 L

Adjust pH to 5.8 with 1 M KOH, then autoclave at 121°C for 20 min. Add filter-sterilized Hygromycin B, 6-BA, NAA, and Timentin solutions before pouring the medium into Petri dishes. Store at 4°C for no more than 4 weeks.

Half-strength MS medium:

Reagent	Final concentration	Amount
MS	2.2 g/L	2.2 g
Sucrose	30 g/L	30 g
Agargellan	6 g/L	6 g
ddH_2O	N/A	To 1 L
Total volume	N/A	1 L

Adjust pH to 5.8 with 1 M KOH, then autoclave at 121°C for 20 min. Store at 4°C for no more than 4 weeks.

50× TAE buffer:

Reagent	Final concentration	Amount
Tris	242 g/L	242 g
$Na_2EDTA{\cdot}2H_20$	37.2 g/L	37.2 g
CH_3COOH	57.1 mL/L	57.1 mL
ddH_2O	N/A	To 1 L
Total volume	N/A	1 L

Store at room temperature (20°C–25°C) for no more than 12 months and dilute to 1× working buffer before use.

2× CTAB DNA extraction solution:

Reagent	Final concentration	Amount
Tris	12.11 g/L	12.11 g
$Na_2EDTA{\cdot}2H_20$	7.44 g/L	7.44 g
CTAB	20 g/L	20 g
NaCl	81.8 g/L	81.8 g
ddH_2O	N/A	To 1 L
Total volume	N/A	1 L

Store at room temperature (20°C–25°C) for no more than 12 months.

⚠ CRITICAL: All culture media must be sterilized and stored for no more than 4 weeks, otherwise it would decrease the transformation efficiency of rice.

STEP-BY-STEP METHOD DETAILS

sgRNA design

Timing: 1 day (for step 1)

This section describes how to design sgRNA and choose appropriate base editors for gene targeting in the rice genome.

1. Retrieve the sequence of the target gene from the rice genome information website (http://rice.uga.edu/).
2. Identify putative PAM sequences by scanning the target gene sequence on both strands near the target base, which refines the choice of an appropriate base editor.

Note: Select appropriate base editors based on the target base and PAM sequence. Indeed, different *Cas* proteins recognize distinct PAM sequences. For example, SpCas9 from *Streptococcus pyogenes* requires a 20-bp spacer followed by the consensus PAM sequence NGG. When the target base is A or T and the PAM sequence is NGG, SpCas9 and Cas9-NG can be selected as the ABE base editor, such as rBE49d that fused TadA variant with SpCas9.

Note: The target base should be located within the activity window of the base editor.

3. Select the appropriate base editor as a function of the target base: ABE for As or CBE for Cs.
4. Design and synthesize oligonucleotide pairs.

Example: Sequences marked in red represent the target site; bold nucleotides indicate the PAM.

LOC_Os05g25770 gene sequence:

GTGCTGAGCAGCCTCACGCCCGGCAGCTCCGCGGCGCGCGGCGGCGGCGGCGGCGGAG
TCGCGGGTCCCTTCGGGCCGGACCAGGGCGATGTCACGTCCTCCCTGCACTGGAGCTACGACG
CCGTCGC**CGG**CATGGAGTTCTTCAAGAACGACGAGGTTGTCTTCGATCTGGACGACATTATGGGT
TTGAGCTTT.

Based on the gene sequence, design a pair of oligonucleotides are:

Forward oligonucleotide: 5′-GGAGCTACGACGCCGTCGC-3′.

Reverse oligonucleotide: 5′-GCGACGGCGTCGTAGCTCC-3′.

Note: Predict potential off-targets using websites such as CRISPR RGEN Tools (rgenome.net). Spacers without potential off-target are desirable.

Note: Design primer pairs at NCBI (National Center for Biotechnological Information, ncbi.nlm.nih.gov) to amplify PCR fragments that include the target site.

⚠ CRITICAL: The sgRNA cassette is driven by the U6 promoter. The first nucleotide of the spacer must be G; if it is not G, it should be replaced by a G. Add a 4-bp adapter sequence complementary to the sticky end of the BtgZI restriction site to the 5′ end of the oligonucleotide (indicated as lowercase letters).

Target oligonucleotide 1: 5′-tgttGGAGCTACGACGCCGTCGC-3′.

Target oligonucleotide 2: 5′-aaacGCGACGGCGTCGTAGCTCC-3′.

sgRNA plasmid construction

Timing: 3–5 days (for step 5)

Detailed description of inserting spacer into pENTR:sgRNA4, the sgRNA-expressing cassette in this entry construct can be gatewayed to any destination vector carrying *Cas* or engineered *Cas* through recombination reaction.

5. The synthesized oligonucleotides are phosphorylated and annealed to form a double-stranded DNA molecule, which is then inserted into the pENTR:sgRNA4 plasmid digested with BtgZI.
   a. The phosphorylation reaction is mixed and incubated at a constant temperature of 37°C for 30 min to phosphorylate the oligonucleotides. Place the mixture in boiling water for 5 min to denature the DNA, before allowing the mixture to cool down naturally to 20°C–25°C.

   Each phosphorylation reaction consists of:

Reagent	Final concentration	Amount
Target oligonucleotide 1 (10 μM)	3 μM	3 μL
Target oligonucleotide 2 (10 μM)	3 μM	3 μL
10×T4 PNK buffer A	1×	1 μL
1 mM ATP	0.1 mM	1 μL
T4 polynucleotide kinase	1 U/μL	1 μL
ddH_2O	N/A	1 μL
Total	N/A	10 μL

   b. The annealed oligonucleotide pair forms double-stranded DNA (dsDNA), which is then kept at −20°C for ligation into the linearized vector.
6. Linearized pENTR:sgRNA4 plasmid.
   a. The circular pENTR:sgRNA4 vector is digested with the restriction enzyme BtgZI at 60°C for 1 h.
   b. Run the restriction digest on an agarose gel (1%); excise the linearized band and purify with a DNA Gel Recovery Kit; store at −20°C.

   BtgZI restriction enzyme setup:

Reagent	Final concentration	Amount
10×CutSmart Buffer (NEB)	1×	2 μL
Plasmid	50 ng/μL	10 μL
Restriction Enzyme BtgZI	0.05 U/μL	1 μL
ddH_2O	N/A	7 μL
Total	N/A	20 μL

   Note: The expected size of linearized pENTR:sgRNA4 vector is 3,156 bp.

7. Ligate the linearized pENTR:sgRNA4 vector with the phosphorylated dsDNA oligonucleotide with T4 DNA ligase, resulting in pENTR:gRNA-Target.

Ligation reaction between spacer and linearized pENTR:gRNA:

Reagent	Final concentration	Amount
Annealed oligo duplex	N/A	4 μL
Linearized pENTR:sgRNA4	3 ng/μL	30 ng

(*Continued on next page*)

Continued

Reagent	Final concentration	Amount
10×T4 buffer	1×	1 μL
T4 DNA ligase	0.1 U/μL	1 μL
ddH_2O	N/A	To 10 μL

Note: The reaction time is preferably no more than 2 h, as longer incubation periods may cause multiple dsDNA oligonucleotide copies to be inserted into the linearized vector.

8. Transform competent cells with the ligation reaction:
   a. Thaw *E. coli* T1 competent cells on ice after removing from −80°C.
   b. Add 10 μL of the ligation reaction to 100 μL *E. coli* T1 competent cells and mix gently. Place on ice for 30 min.
   c. Heat shock for 45 s at 42°C. Then immediately chill on ice for 2–3 min.
   d. Add 700 μL of LB medium to the samples and shake at 180 rpm for 1 h at 37°C.
   e. Centrifuge the tube at 4,000 g for 1 min to pellet the bacteria. Discard 600 μL of the supernatant, resuspend the cells in the remaining medium, and spread on LB agar plates containing 50 mg/L Kan.
   f. Incubate plates at 37°C for 8–10 h. Multiple visible single colonies will appear on the plate.
9. PCR detection and sequencing.
   a. Pick individual colonies for PCR with the following conditions. Run standard PCR amplification (25–30 cycles) on a thermal cycler.

   PCR conditions:

Reagent	Final concentration	Amount
2×Rapid Taq Master Mix	1×	5 μL
U6p-F1 (10 μM)	1 μM	1 μL
Target oligonucleotide 2 (10 μM)	1 μM	1 μL
DNA	N/A	1 μL
ddH_2O	N/A	2 μL
Total	N/A	10 μL

PCR cycling conditions

Steps	Temperature	Duration	Number of cycles
Initial denaturation	95°C	30 s	1
Denaturation	95°C	10 s	25–30 cycles
Annealing	55°C	20 s	
Extension	72°C	10 s	
Final extension	72°C	1 min	1
Hold	16°C	forever	

   b. Check for insertion of the dsDNA oligonucleotide by agarose gel (1%, w/v) electrophoresis.

Note: The expected size of the PCR product will be 345 bp if the spacer is inserted.

   c. According to the electrophoresis results above, select colonies and inoculate 2 mL sterile LB containing 50 mg/L Kan and grow at 37°C with 200 rpm shaking for 8–12 h.
   d. Extract the recombinant plasmid using a Plasmid DNA mini kit for sequencing.

Note: The orientation and copy number of inserted oligos should be verified by Sanger sequencing.

ABE plasmid construction

Timing: 3–5 days (for step 10)

This section describes generation of destination construct containing both sgRNA and ABE by Gateway cloning.

10. Obtain the sgRNA cassette.
    a. The plasmid mentioned in step d above is digested with ApaI.
    Digestion reaction with FastDigest ApaI restriction enzyme:

Reagent	Final concentration	Amount
10×FastDigest Green Buffer	1×	2 μL
Plasmid or PCR product	50 ng/μL	10 μL
FastDigest Restriction Enzyme ApaI	0.5 U/μL	1 μL
ddH_2O	N/A	To 20 μL

    b. Run 1% agarose gel with the digestion products; excise the band of interest and purify with DNA Gel Recovery Kit; store at −20°C.

Note: Expected size of bands are 2,125 and 1,054 bp; excise the 2,125-bp band.

11. The *sgRNA*-expressing cassette (purified band above) is recombined into the binary vector rBE49d via LR clonase reaction to construct the rBE49d-gRNA vector.

Gateway reaction:

Reagent	Final concentration	Amount
Gateway LR Clonase II Enzyme	N/A	2 μL
sgRNA-expressing cassette	5–10 ng/μL	50–100 ng
rBE49d	7.5 ng/μL	75 ng
ddH_2O	N/A	To 10 μL

12. As above, add 2.5 μL of the Gateway reaction to 100 μL of JM109 competent cells for transformation and isolation of the resulting plasmid.

Note: The rBE49d vector contains the ccdB gene, which is lethal to all *E. coli* strains except DB3.1. After Gateway cloning, the ccdB gene is replaced by the sgRNA expression cassette, and the recombined vector can be transformed into JM109 cells.

13. Pick single colonies for colony PCR on a thermal cycler. Refer to step 8 for PCR conditions.
14. Extract the recombinant plasmid using a Plasmid DNA Mini Kit.

A. tumefaciens-mediated genetic transformation of rice

Timing: 10–12 weeks (for step 15)

This section describes detailed procedures of rice tissue culture used for *A. tumefaciens*-mediated transformation.

15. Transform the rBE49d-gRNA plasmid into *Agrobacterium tumefaciens*.

Note: There are two ways to introduce the vector into rice cells: *Agrobacterium*-mediated genetic transformation or particle bombardment of cells. In this protocol, *Agrobacterium*-mediated genetic transformation is used.

a. Incubate 100 μL of *Agrobacterium* competent cells (strain EHA105) at 37°C for 5 min.
b. Add 1 μg plasmid to the competent cells, mix gently, and place on ice for 30 min.
c. Freeze the tube in liquid nitrogen for 1 min, take it out, and incubate at 37°C for 5 min.
d. Add 700 μL of LB medium to the tubes and shake them at 180 rpm for 3 h at 30°C.
e. Centrifuge the tube at 5,000 g for 1 min to collect the bacteria. Discard 600 μL of the supernatant and resuspend the cells in the remaining liquid.
f. Spread the resuspended cells on LB agar plate containing 50 mg/L Kan and 50 mg/L Rif. Incubate plates at 30°C for 2–3 d. Multiple visible single colonies should appear.
g. Pick single colonies for colony-PCR (25–30 cycles) on a thermal cycler.

Pause point: *A. tumefaciens* strains can be stored at −80°C for a long time.

16. Callus induction.
    a. Select a 15-mL centrifuge tube and surface sterilize 30 dehusked immature rice seeds in 50% (v/v) sodium hypochlorite solution (10 mL Sodium hypochlorite, 10 mL ddH_2O, and one drop of Tween-20) on a shaker at 20 rpm for 45 min at 20°C–25°C.
    b. Wash the seeds with ddH_2O 3–5 times.
    c. Place seeds onto MSD plate (∼30 seeds/petri dish) and incubate for 10 days (Figure 1B) in a growth chamber (16-h light/8-h dark photoperiod at 28°C, or constant light at 28°C) for callus tissue induction.
    d. The embryos and seed coats are removed with forceps, and the calli are transferred to new MSD plates and incubated for 3–5 days before use for *Agrobacterium*-mediated transformation.
17. *Agrobacterium* infection.
    a. Inoculate fresh bacterial colonies into a 125-mL conical flask containing 20 mL TY with 50 mg/L Rif, 50 mg/L Kan, and 200 μM AS.
    b. Grow overnight (8–12 h) at 120 rpm at 30°C until OD600 reaches 1.0–2.0.
    c. Transfer *Agrobacterium* cultures to 1.5-mL Eppendorf tubes and centrifuge at 5,000 g for 1 min to pellet the bacteria; resuspend the pellet in ∼20 mL of liquid MSD medium containing 200 μM AS for a final OD600 of ∼0.15.

    Note: The final OD600 needs to be in the range of 0.1–0.2 for effective transformation.

    d. Soak the calli in *Agrobacterium* suspension for 30–60 min in a Petri dish at room temperature (20°C–25°C) and shake it gently every 10 min.
    e. Remove *Agrobacteria* suspension with a pipette and blot the calli dry on sterile filter paper.
    f. Place the calli onto a new MSD plate containing 200 μM AS and co-culture with *Agrobacteria* for 3–5 days at 20°C–25°C in the dark (Figure 1C).
18. Selection and regeneration of transgenic rice calli.
    a. Transfer the calli onto new MSD/Hygromycin B/Timentin plates (∼30 calli/plate) and incubate for 2 weeks in a growth chamber (16-h light/8-h dark photoperiod at 28°C, or constant light at 28°C).

    Note: Calli will turn brown at this step. Check plates every day for overgrowth of *Agrobacteria*.

    b. The calli are sub-cultured on new MSD/Hygromycin B/Timentin plates (∼12–15 calli/plate) from 2 weeks to 2 months until tiny nodular embryos grow on the surface of resistant calli (Figure 1D).

    Note: True transgenic calli grow fast and as a loose mass; false positives grow as a very dense mass that is sticky.

c. Transfer the Hygromycin-resistant calli (light yellow, dry, and growing loosely) to regeneration RM plates and grow for 2 weeks.

Note: Number each callus on the side wall of the new plate to help distinguish them. The calli should be gently embedded in the agar surface by pressing the agar with forceps; this step will prevent later confusion caused by calli sliding along the surface; separate new resistant calli as much as possible. Transgenic calli will gradually turn green.

d. Sub-culture green callus tissue on new regeneration RM plates (Figure 1E) every 2 weeks until plantlets grow.

Note: Four transfers are sufficient; if no green shoots appear after this step, start over.

e. Transfer the regenerated plantlets to culture tubes containing half-strength MS medium (Figure 1F) for root regeneration. Cover each tube with breathable sealing film for 1–2 weeks.
f. Remove sealing film and harden off tender seedlings for 4–6 days.
g. Transfer healthy seedlings into 50 × 80-cm plastic pots containing a 3:1 (w/w) mixture of field soil and vermiculite. Place all pots in a glasshouse (day/night temperatures of 28°C/20°C, 10-h-light/14-h-dark photoperiod).

Genotyping of genome-edited plants

Timing: 3–4 days (for step 19)

This section describes genome DNA extraction and PCR amplification to identify potential mutations in the target region in transgenic lines.

19. Rice genomic DNA extraction.
    a. Collect leaf blades in 1.5-mL Eppendorf tubes, add a 5-mm-diameter stainless steel bead to each tube, and immediately freeze in liquid nitrogen.
    b. Remove Eppendorf tubes from liquid nitrogen and quickly place them on a Tissuelyser (50 Hz, 1 min).
    c. Add 600 μL 2× CTAB DNA extraction buffer.
    d. Incubate the mixture at 65°C for 45 min and mix every 10–15 min.
    e. Add 500 μL of chloroform and mix for 1 min.
    f. Centrifuge at 12,000 g for 10 min at room temperature (20°C–25°C).
    g. Transfer the upper aqueous phase into new tubes, add 400 μL of isopropanol, and mix well.
    h. Centrifuge at 12,000 g for 10 min at room temperature (20°C–25°C).
    i. Wash the pellet with 75% (v/v) ethanol for 5 min.
    j. Air dry the pellets and resuspend genomic DNA in 50 μL ddH_2O.
20. PCR amplification.
    a. Run standard PCR amplification (30–35 cycles) on a thermal cycler.

    PCR reaction:

Reagent	Final concentration	Amount
2× Rapid Taq Master Mix	1×	12.5 μL
Forward primer (10 μM)	0.4 μM	1 μL
Reverse primer (10 μM)	0.4 μM	1 μL
DNA	N/A	1 μL
ddH_2O	N/A	9.5 μL
Total	N/A	25 μL

PCR cycling conditions:

Steps	Temperature	Duration	Number of cycles
Initial Denaturation	95°C	30 s	1
Denaturation	95°C	10 s	30–35 cycles
Annealing	55°C	20 s	
Extension	72°C	10 s	
Final extension	72°C	1 min	1
Hold	16°C	Forever	

b. Separate PCR products on a 1% (w/v) agarose gel.

21. Sequencing. The PCR products were sent for Sanger sequencing. Align the sequencing results to the genome sequence to confirm changes.

EXPECTED OUTCOMES

Using the base editor rBE49d and the target gene *LOC_Os05g25770* as a practical example of base editing, the possible mutation types of the target sites in independent transgenic lines are shown below. The target region and PAM sequence are underlined in blue and red, and detected nucleotide substitutions are marked by arrows in the sequencing chromatograms, respectively (Figures 2, 3, 4, and 5). The target base and detected nucleotide substitutions are in red and green in the following sequences, respectively.

GGAGCTACGACGCCGTCGC**CGG** reference.

Line #1 GGAGCTGCGACGCCGTCGC**CGG** A7>G.

GGAGCTGCGACGCCGTCGC**CGG** A7>G.

GGAGCTACGACGCCGTCGC**CGG** reference.

Line #2 GGAGCTACGACGCCGTCGC**CGG** WT.

GGAGCTGCGACGCCGTCGC**CGG** A7>G.

GGAGCTACGACGCCGTCGC**CGG** reference.

Line #3 GGAGCTACGACGCCGTCGC**CGG** WT.

GGGGCTGCGACGCCGTCGC**CGG** A3,7>G.

GGAGCTACGACGCCGTCGC**CGG** reference.

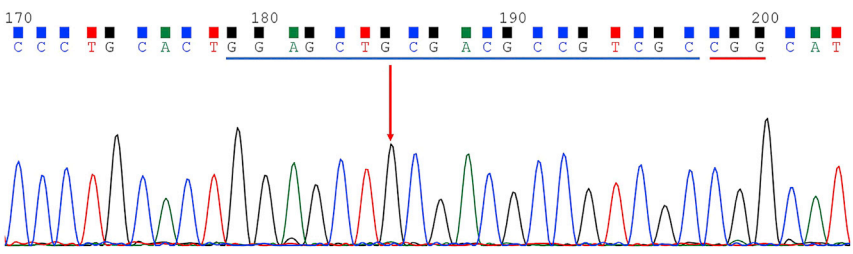

Figure 2. Representative Sanger sequencing chromatogram of the target region in T0 transgenic line #1

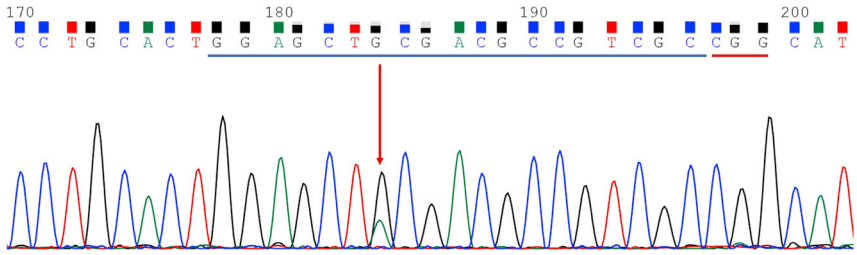

Figure 3. Representative Sanger sequencing chromatogram of the target region in T0 transgenic line #2

Line #4 GGAGCTGCGACGCCGTCGC**CGG** A7>G.

GGGGCTGCGACGCCGTCGC**CGG** A3,7>G.

The most widely cultivated rice is diploid and may harbors different mutations on each chromosome in base editing which would be characterized by mono-, di-allelic (homozygous or heterozygous) or chimeric mutations. According to the Sanger sequencing results, line #1 and # 4 are representative of typical homozygous and heterozygous plants with di-allelic mutation, respectively. Line #2 and #3 have mono-allelic mutations but carry different base editing events. It should be further confirmed by sequencing in the progeny, such as Sanger sequencing, Hi-Tom analysis,[4] *etc.* Generally, most of the edited plants obtained via ABE editing are heterozygous plants, the T-DNA-free, homozygous progenies with edited gene can be easily obtained in T1 population by self-pollination.

LIMITATIONS

The application of a base editor is mainly affected by the PAM sequence and the editing activity window. *Cas* enzymes modify nucleotides at specific positions by recognizing specific PAMs near the target site. However, different *Cas* proteins recognize different PAM sequences, such as NGG for SpCas9,[5] NG for Cas9-NG,[6] NNG for ScCas9[7] and NNN for SpRY.[8] The activity windows of ABEs are also quite variable. For example, the base editor based on the deaminase TadA7.10 showed high activities at sgRNA positions 4–7 (the first nucleotide of the spacer being position 1).[9] The window of TadA9 spans from positions 1–12, with variation at different genomic loci.[1] TadA8e showed high activities at sgRNA positions 4–12.[8] The appropriate base editor should therefore be selected based on the type of targeted base, the potential PAM sequences, and the editing activity window. CBEs containing UGI (uracil DNA glycosylase inhibitor) may produce more by-products compared to ABEs. However, CBEs are deployed in a similar way as ABEs. Theoretically, both ABE and CBE can be applied to any plant species, but codon optimization of base editors for enhanced protein expression is highly recommended in each case. Furthermore, potential sgRNA-dependent off-targets of base editing can be easily predicted by bioinformatic analysis, and backcross can be performed in case if needed.

TROUBLESHOOTING

Problem 1

No PCR products are obtained (step 9b).

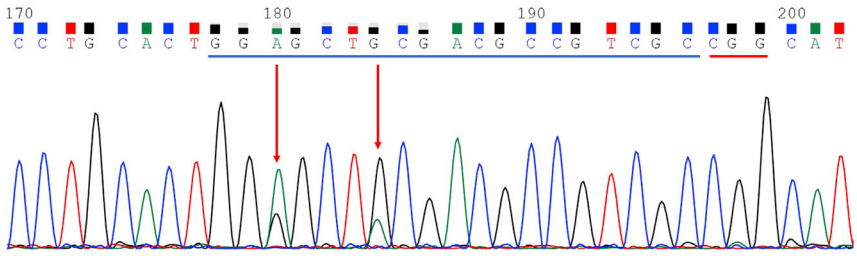

Figure 4. Representative Sanger sequencing chromatogram of the target region in T0 transgenic line #3

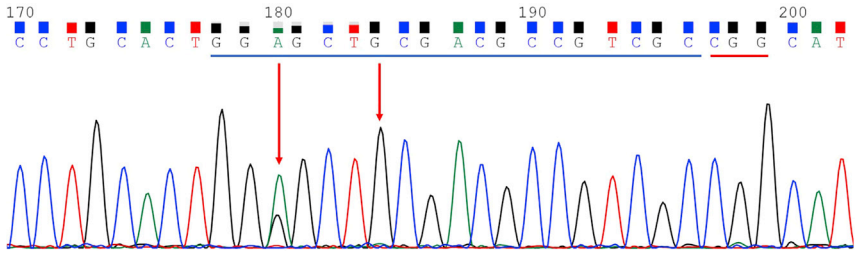

Figure 5. Representative Sanger sequencing chromatogram of the target region in T0 transgenic line #4

Potential solution

Partial digestion of pENTR:sgRNA4 in step 6. One solution is to transform the DNA obtained after step 6b into T1 *E. coli* competent cells and repeat step 6 if multiple single colonies appear. In addition, 2% agarose gel can be used in step 6b.

Problem 2

Tandem repeats of spacers are inserted into the pENTR:sgRNA4 (step 9d).

Potential solution

Reduce the amount of annealed oligo duplex or shorten the duration of the ligation reaction.

Problem 3

Agrobacterium overgrows in callus (step 18a).

Potential solution

Shorten the incubation time in step 17f to 2–3 d.

Problem 4

Multiple base editing events occur in single independent Hygromycin-resistant line (step 18c).

Potential solution

Pick and transfer Hygromycin-resistant callus clumps timely and separate them well on fresh media each time.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Huanbin Zhou (zhouhuanbin@caas.cn).

Materials availability

All wild-type plant materials in this study are available upon request.

Data and code availability

This study did not generate/analyze datasets.

ACKNOWLEDGMENTS

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AUTHOR CONTRIBUTIONS

The authors conceived and wrote the manuscript.

DECLARATION OF INTERESTS

Two Chinese patents have been filed for "An artificial base editing system for rice" (ZL 202110061201.2) and "Adenosine deaminase and its related biological materials and applications" (ZL202011308944.7).

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