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.
Protocol

Protocol for targeted modification of the rice genome using base editing

Meixia Wang,1,3,* Fang Yan,1 and Huanbin Zhou1,2,4,*

1State Key Laboratory for Biology of Plant Diseases and Insect Pests, Institute of Plant Protection, Chinese Academy of Agricultural Sciences, Beijing 100193, China
2Scientific Observing and Experimental Station of Crop Pests in Guilin, Ministry of Agriculture and Rural Affairs, Guilin 541399, China
3Technical contact
4Lead contact
*Correspondence: wangmx0921@gmail.com (M.W.), zhouhuanbin@caas.cn (H.Z.)
https://doi.org/10.1016/j.xpro.2022.101865

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 ddH2O, 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 ddH2O.
   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: Apal | 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)
## 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 ddH₂O, 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 ddH₂O, 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 ddH₂O. 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 ddH₂O. 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 ddH₂O. 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 4°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   |
| ddH₂O                  | 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   |
| ddH₂O                  | 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   |
| ddH₂O               | 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   |
| ddH₂O               | 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   |
| ddH₂O               | 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/Hygroycin 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   |
| Hygroycin B         | 0.05 g/L            | 1 mL   |
| ddH₂O               | 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 Hygroycin 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   |
| ddH₂O            | 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     |
| Agar gellan      | 6 g/L               | 6 g      |
| ddH₂O            | 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.

50x TAE buffer:

| Reagent          | Final concentration | Amount   |
|------------------|---------------------|----------|
| Tris             | 242 g/L             | 242 g    |
| Na₂EDTA-2H₂O     | 37.2 g/L            | 37.2 g   |
| CH₃COOH          | 57.1 mL/L           | 57.1 mL  |
| ddH₂O            | 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 1x working buffer before use.

2x CTAB DNA extraction solution:

| Reagent          | Final concentration | Amount   |
|------------------|---------------------|----------|
| Tris             | 12.11 g/L           | 12.11 g  |
| Na₂EDTA-2H₂O     | 7.44 g/L            | 7.44 g   |
| CTAB             | 20 g/L              | 20 g     |
| NaCl             | 81.8 g/L            | 81.8 g   |
| ddH₂O            | 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:**

```
GTGCTGAGCAGCCTCACGCCCGGCAGCTCCGCGGCGCGCGCGGCGGCGGCGGCGGCGGAG
TCGCGGGTCCCTTCGGGCCGGACCAGGGCGATGTCACGTCCTCCCTGCACTGGAGCTACGACG
CCGTCGCGCCCGCATGGAGTTCTTCAAGAACGACGAGGTTGTCTTCGATCTGGACGACATTATGGGT
TTGAGCTTT.
```

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

**Forward oligonucleotide:** 5'-GGAGCTACGAGCATGGAGTTCTTCAAGAACGACGAGGTTGTCTTCGATCTGGACGACATTATGGGT-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.

**WARNING:** 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'-tggttGGAGCTACGAGCATGGAGTTCTTCAAGAACGACGAGGTTGTCTTCGATCTGGACGACATTATGGGT-3'.

**CRITICAL:**
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   |
   | ddH₂O                       | 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   |
   | ddH₂O                       | 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)
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.
   b. Check for insertion of the dsDNA oligonucleotide by agarose gel (1%, w/v) electrophoresis.
   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 expected size of the PCR product will be 345 bp if the spacer is inserted.

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

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 Apal.
      Digestion reaction with FastDigest Apal 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 Apal | 0.5 U/μL         | 1 μL            |
      | ddH₂O                           | 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           |
| ddH₂O                           | 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 ddH2O, 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 ddH2O3–5 times.
c. Place the 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 Agrobacterium 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 Agrobacterium 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 ddH2O.

20. PCR amplification.
   a. Run standard PCR amplification (30–35 cycles) on a thermal cycler.

**PCR reaction:**

| Reagent             | Final concentration | Amount  |
|---------------------|---------------------|---------|
| 2X 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    |
| ddH2O               | 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.

Reference: GGAGCTACGACGCGGTCCGG

Line #1: GGAGCTACGACGCGGTCCGG A7>G.

GGAGCTACGACGCGGTCCGG A7>G.

GGAGCTACGACGCGGTCCGG reference.

Line #2: GGAGCTACGACGCGGTCCGG WT.

GGAGCTACGACGCGGTCCGG A7>G.

GGAGCTACGACGCGGTCCGG reference.

Line #3: GGAGCTACGACGCGGTCCGG WT.

GGGGCTACGACGCGGTCCGG A3,7>G.

GGAGCTACGACGCGGTCCGG reference.

**Figure 2.** Representative Sanger sequencing chromatogram of the target region in T0 transgenic line #1
The most widely cultivated rice is diploid and may harbor 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, 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, NG for Cas9-NG, NNG for ScCas9 and NNN for SpRY. The activity windows of ABEs are also quite variable. For example, the base editor based on the deaminase TadA showed high activities at sgRNA positions 4–7 (the first nucleotide of the spacer being position 1). The window of TadA spans from positions 1–12, with variation at different genomic loci. The TadABe showed high activities at sgRNA positions 4–12. 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 3. Representative Sanger sequencing chromatogram of the target region in T0 transgenic line #2

Figure 4. Representative Sanger sequencing chromatogram of the target region in T0 transgenic line #3
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
This study was supported by grants from the Hainan Yazhou Bay seed Lab (B21HJ0215) and the Central Public-interest Scientific Institution Basal Research Fund (No. Y2022PT24).

AUTHOR CONTRIBUTIONS
The authors conceived and wrote the manuscript.
DEVELOPMENT 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).

REFERENCES

1. Yan, D., Ren, B., Liu, L., Yan, F., Li, S., Wang, G., Sun, W., Zhou, X., and Zhou, H. (2021). High-efficiency and multiplex adenine base editing in plants using new TadA variants. Mol. Plant 14, 722–731. https://doi.org/10.1016/j.molp.2021.02.007.

2. Ikegaya, T., and Ashida, K. (2021). Genetic region responsible for the differences of starch properties in two glutinous rice cultivars in Hokkaido, Japan. Breed Sci. 71, 375–383. https://doi.org/10.1270/jsbbs.20163.

3. Zhou, H., Liu, B., Weeks, D.P., Spalding, M.H., and Yang, B. (2014). Large chromosomal deletions and heritable small genetic changes induced by CRISPR/Cas9 in rice. Nucleic Acids Res. 42, 10903–10914. https://doi.org/10.1093/nar/gku806.

4. Liu, Q., Wang, C., Jiao, X., Zhang, H., Song, L., Li, Y., Gao, C., and Wang, K. (2019). Hi-TOM: a platform for high-throughput tracking of mutations induced by CRISPR/Cas systems. Sci. China Life Sci. 62, 1–7. https://doi.org/10.1007/s11427-018-9402-9.

5. Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J.A., and Charpentier, E. (2012). A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. Science 337, 816–821. https://doi.org/10.1126/science.1225829.

6. Ren, B., Liu, L., Li, S., Kuang, Y., Wang, J., Zhang, D., Zhou, X., Lin, H., and Zhou, H. (2019). Cas9-NG greatly expands the targeting scope of the genome-editing toolkit by recognizing NG and other atypical PAMs in rice. Mol. Plant 12, 1015–1026. https://doi.org/10.1016/j.molp.2019.03.010.

7. Wang, M., Xu, Z., Gosavi, G., Ren, B., Cao, Y., Kuang, Y., Zhou, C., Spetz, C., Yan, F., Zhou, X., and Zhou, H. (2020). Targeted base editing in rice with CRISPR/ScCas9 system. Plant Biotechnol. J. 18, 1645–1647. https://doi.org/10.1111/pbi.13330.

8. Xu, Z., Kuang, Y., Ren, B., Yan, D., Yan, F., Spetz, C., Sun, W., Wang, G., Zhou, X., and Zhou, H. (2021). SpiRY greatly expands the genome editing scope in rice with highly flexible PAM recognition. Genome Biol. 22, 6. https://doi.org/10.1186/s13059-020-02231-9.

9. Jeong, Y.K., Lee, S., Hwang, G.H., Hong, S.A., Park, S.E., Kim, J.S., Woo, J.S., and Bae, S. (2021). Adenine base editor engineering reduces editing of bystander cytosines. Nat. Biotechnol. 39, 1426–1433. https://doi.org/10.1038/s41587-021-00943-2.