Chapter 10

Seamless Genome Editing in Rice via Gene Targeting and Precise Marker Elimination

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Abstract

Positive–negative selection using hygromycin phosphotransferase (*hpt*) and diphtheria toxin A-fragment (*DT-A*) as positive and negative selection markers, respectively, allows enrichment of cells harboring target genes modified via gene targeting (GT). We have developed a successful GT system employing positive–negative selection and subsequent precise marker excision via the *piggyBac* transposon derived from the cabbage looper moth to introduce desired modifications into target genes in the rice genome. This approach could be applied to the precision genome editing of almost all endogenous genes throughout the genome, at least in rice.

Key word Gene targeting, Positive–negative selection, Marker excision, *piggyBac* transposon, Homologous recombination

1 Introduction

Homologous recombination (HR)-mediated gene targeting (GT) is currently the only universal technology that can introduce desired modifications precisely into a target gene. However, in most multicellular organisms, the repair of DNA double strand breaks (DSBs) by the HR pathway occurs at very low frequency, resulting in low GT efficiency. Thus, positive–negative selection has been applied in several organisms, including rice, to eliminate cells carrying the transgene integrated randomly into other gene loci and to enrich true GT cells harboring the HR-modified target locus. Terada et al. [1] succeeded in applying this system to establish an efficient GT system in rice by using the hygromycin phosphotransferase (*hpt*) gene as a positive selection marker and the diphtheria toxin A-fragment (*DT-A*) gene as a negative selection marker. Using positive–negative selection with *hpt* and *DT-A*, the designed modification of a target gene via GT has become a reproducible and general approach, at least in rice [2]. In addition, improved cytosine deaminase (codA D314A), which has increased

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deaminase activity for 5-fluorocytosin, or antisense RNA of an antibiotic resistance gene can also be used as negative selection markers for the enrichment of GT cells [3, 4].

To obtain transgenic plants harboring only the desired mutation in the target locus, the positive selection marker inserted into the targeted locus must be removed completely from the GT locus after the selection of GT cells. The *piggyBac* transposon—a DNA transposon derived from the moth (*Trichoplusia ni*)—inserts into the host genome at TTAA elements and excises without leaving a footprint at the excised site [5]. The *piggyBac* transposon has been used for transgenesis, insertional mutagenesis, and marker excision not only in insects but also in mammalian cells [6, 7]. We found that *piggyBac* can transpose accurately and efficiently also in rice cells [8]. More recently, *piggyBac* has been applied successfully to the excision of the positive selection marker from a GT locus [9]. Thus, seamless genome editing via GT and subsequent precise *piggyBac*-mediated marker elimination could be applied universally in rice (*see* **Note 1**).

Here, we provide protocols for the targeted modification of endogenous genes via GT with positive–negative selection using *hpt* and *DT-A* and subsequent marker excision via *piggyBac* transposon in rice (Figs. 1 and 2).

2 Materials

2.1 Agrobacterium-Mediated Transformation

1. Mature rice. seeds (*Oryza sativa L.* cv. Nipponbare).
2. *Agrobacterium tumefaciens* strain EHA105 [10].

2.1.1 Plant and Agrobacterium Materials

2.1.2 GT Vector

The GT vector (Fig. 1) carries the *DT-A* gene (under the control of the maize poly-ubiquitin 1 gene promoter or rice elongation factor promoter) at both ends of the T-DNA flanking the left and right border sequences, respectively, as well as a region of ca. 6.0-kb homologous to the target gene locus harboring the desired mutation. The *piggyBac* transposon carrying a rice actin terminator and *hpt* expression cassette is inserted into a central TTAA site in the homology arm of the target gene locus. If there are no TTAA sites adjacent to the target region of a desired mutation, the *piggyBac* transposon can be inserted into an artificially created TTAA site in the intron of the target gene. This artificially synthesized TTAA site remains after *piggyBac* excision in the target gene.

2.1.3 piggyBac Transposase Expression Vector

piggyBac transposes accurately and efficiently in rice upon expression of a mammalian codon-optimized hyper-active *piggyBac* transposase (hyPBase) carrying seven amino acid substitutions [11]. The hyPBase expression vector carries an expression cassette consisting of

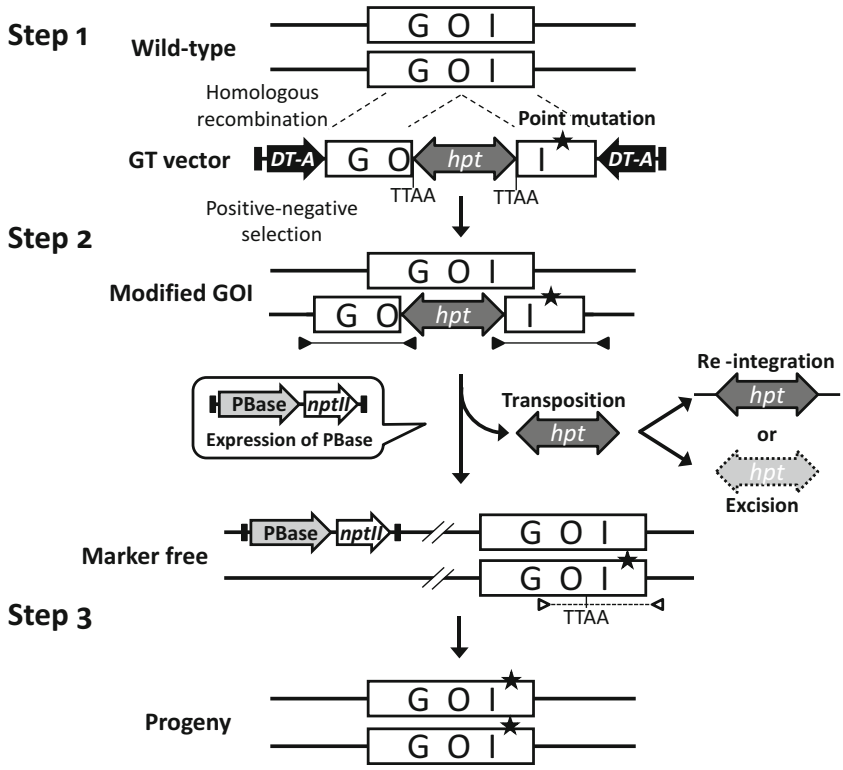

Fig. 1 Strategy to introduce a desired mutation into a target gene via GT and subsequent marker excision from the GT locus using the *piggyBac* transposon. (Step 1) Introduction of point mutations into the target gene via GT. *Wild-type* and *GT vector* schematics show the structure of the wild-type gene of interest (GOI) locus and the GT vector, respectively. The GT vector comprises target gene sequences with a point mutation (*star*), the *piggyBac* transposon carrying the *hpt* gene as a positive selectable marker, and expression cassettes of diphtheria toxin fragment A (*DT-A*) as a negative selectable marker. GT cells isolated using positive/negative selection are subjected to PCR analysis with the primer set indicated by *black arrowheads*. (Step 2) Marker excision from the GT locus using *piggyBac* transposon. *Modified GOI* and *Marker free* schematics reveal the structure of the target locus modified via GT and the modified target locus containing only the desired mutations following hyPBase-mediated marker excision, respectively. *White arrowheads* indicate the primer sets used for PCR to verify the presence of the TTAA element at the *piggyBac*-excised site and the desired mutation in the target gene. Since the hyPBase expression cassette might have integrated into the host genome, marker-free and homozygous plants with the modified GOI can be obtained via self-pollinating or out-crossing (Step 3)

hyPBase driven by the maize polyubiquitin1 gene promoter and a neomycin phosphotransferase II (*nptII*) expression cassette.

2.1.4 Media for Agrobacterium-Mediated Transformation

1. AB medium: 5 g/L glucose, 3 g/L K_2HPO_4, 1.3 g/L $NaH_2PO_4{\cdot}2H_2O$, 1 g/L NH_4Cl, 150 mg/L KCl, 10 mg/L $CaCl_2{\cdot}2H_2O$, 2.5 mg/L $FeSO_4{\cdot}7H_2O$ and 15 g/L Bacto-agar, pH 7.2. After autoclaving, add 1.2 mL of 1 M $MgSO_4{\cdot}7H_2O$ and the appropriate selective agent depending on the vector and *Agrobacterium* strain.

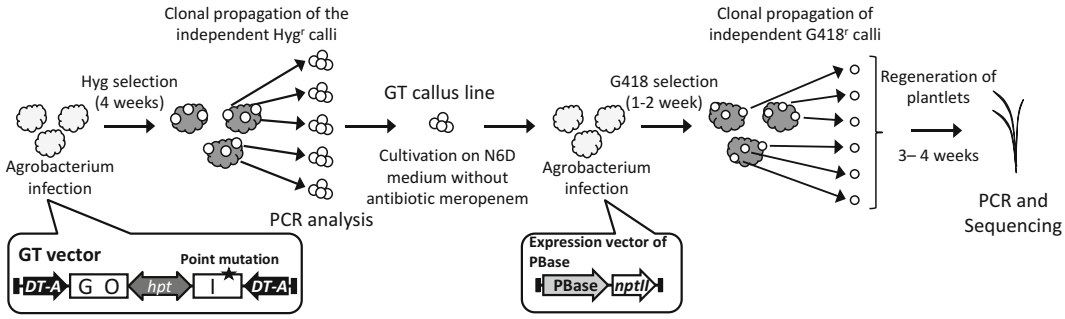

Fig. 2 Experimental strategy for precise genome modification using the *piggyBac* transposon. Four-week-old rice calli were inoculated with *Agrobacterium* harboring the GT vector and were selected on N6D medium containing 50 mg/L hygromycin sulfate and 25 mg/L meropenem for 4 weeks. Genomic DNA extracted from hygromycin-resistant calli was subjected to PCR analysis with primer sets shown in Fig. 1 to identify transgenic calli in which a GT event had occurred at the target locus. GT callus lines were transferred to N6D medium without meropenem and cultured for 4 weeks. GT calli were infected with *Agrobacterium* harboring a PBase expression vector. PBase transgenic calli were selected and regenerated on medium with 35 mg/L G418 and 25 mg/L meropenem. Regenerated plants were subjected to marker excision analysis by PCR and sequencing

2. AAM medium (liquid medium): 68.5 g/L sucrose, 36 g/L glucose, 250 mg/L $MgSO_4 \cdot 7H_2O$, 150 mg/L $CaCl_2 \cdot 2H_2O$, 150 mg/L $NaH_2PO_4 \cdot 2H_2O$, 3 g/L KCl, 40 mg/L Fe-EDTA, 10 mg/L $MnSO_4 \cdot 4\text{-}6H_2O$, 2 mg/L $ZnSO_4 \cdot 7H_2O$, 0.025 mg/L $CuSO_4 \cdot 5H_2O$, 0.025 mg/L $CoCl_2 \cdot 6H_2O$, 0.75 mg/L KI, 3 mg/L H_3BO_3, 0.25 mg/L $Na_2MoO_4 \cdot 2H_2O$, 100 mg/L myo-inositol, 1 mg/L nicotinic acid, 1 mg/L pyridoxine HCl, 10 mg/L thiamine HCl, 0.5 g/L casamino acids, 7.5 mg/L L-glycine, 176.7 mg/L L-arginine, 0.9 g/L L-glutamine and 0.3 g/L L-aspartic acid, pH 5.2. Autoclave.
3. 2N6AS medium: 30 g/L sucrose, 10 g/L glucose, 0.3 g/L casamino acids, 2 mg/L L-glycine, N6-vitamin, N6 salts, 2 mg/L 2,4-D and 4 g/L gelrite, pH 5.2. After autoclaving, add 0.2 mL of 100 mg/mL acetosyringone.
4. N6D medium: 30 g/L sucrose, 0.3 g/L casamino acids, 2.878 g/L L-proline, 2 mg/L L-glycine, N6-vitamin (100 mg/L myo-inositol, 0.5 mg/L nicotinic acid, 0.5 mg/L pyridoxine HCl, 1 mg/L Thiamine HCl), N6 salts [Chu(N6)Medium Salt Mixture, Wako Pure Chemical Industries, Osaka, Japan], 2 mg/L 2,4-D and 4 g/L gelrite (for hygromycin selection) or 8 g/L Bacto Agar (for G418 selection), pH 5.8. Autoclave. After autoclaving, add 2 mL of 12.5 mg/mL meropenem and 1 ml of 50 mg/L Hygromycin B or 35 mg/L Geneticin (G418).
5. ReIII medium: 30 g/L sucrose, 30 g/L sorbitol, 2 g/L casamino acids, MS Medium salt Mixture (Wako Pure Chemical Industries, Japan), 2 mg/L glycine, N6-vitamins, 20 μg/L 1-naphthalene acetic acid, 2 mg/L kinetin and 4 g/L gelrite

(for hygromycin selection) or 8 g/L Bacto Agar (for G418 selection), pH 5.8. After autoclaving, add 2 mL of 12.5 mg/mL meropenem and 1 ml of 50 mg/L Hygromycin B or 35 mg/L Geneticin (G418).

6. HF medium: 30 g/L sucrose, MS Medium salt Mixture, 2 mg/L glycine, N6-vitamins and 4 g/L gelrite (for hygromycin selection) or 8 g/L Bacto Agar (for G418 selection), pH 5.8.

2.1.5 Stock Solutions

1. 2,4-Dichlorophenoxy acetic acid (2,4-D): 5 mg/mL. Dissolve the powder in dimethyl sulfoxide (DMSO). After filter-sterilization, store at 4 °C.
2. Kinetin: 0.2 mg/mL. Dissolve in a small amount of 1 N KOH or 1 N NaOH, make up volume with water, and store at 4 °C.
3. Naphtalene acetic acid (NAA): 0.2 mg/mL. Dissolve in a small amount of 1 N KOH or 1 N NaOH, make up volume with water, and store at 4 °C.
4. Acetosyringone (3′,5′-dimethoxy-4′hydroxy-acetophenone): 100 mg/mL. Dissolve the powder in DMSO. After filter-sterilization, store at 4 °C.
5. Meropenem: 12.5 mg/mL. Dissolve the powder in hot ddH_2O. After filter-sterilization, store at 4 °C.
6. Hygromycin B: 50 mg/mL. Dissolve the powder in ddH_2O. After filter-sterilization, store at 4 °C.
7. G418: 35 mg/mL. Dissolve the powder in ddH_2O. After filter-sterilization, store at 4 °C.

2.2 Identification of GT Calli

2.2.1 DNA Extraction

Agencourt chloropure (Beckman Coulter, USA).

2.2.2 Polymerase Chain Reaction

KOD FX neo (TOYOBO, Japan).

2.2.3 Purification of PCR Products for Sequencing Analysis

Agencourt AMPure XP (Beckman Coulter, USA).

2.3 Selection of Marker-Free Plants

2.3.1 Southern Blot Analysis

Nucleon PhytoPure (GE Healthcare, USA).

2.3.2 Polymerase Chain Reaction

PrimeSTAR GXL DNA polymerase (Takara, Japan).

3 Methods

3.1 Agrobacterium-Mediated Transformation of GT Vector into Rice Calli

1. Dehulled mature seeds of rice are sterilized and inoculated on callus induction N6D medium [12]. Seeds are incubated at 33 °C for 2–3 weeks.
2. Actively proliferating rice calli (approximately 5 mm in diameter) are subcultured on fresh N6D medium for 4 days before *Agrobacterium* infection.
3. *Agrobacterium* (EHA105) harboring the GT vector is inoculated onto AB medium containing appropriate antibiotics in Petri dishes and incubated at 28 °C in the dark for 3 days before *Agrobacterium* infection.
4. Approximately 5 g of calli (in a volume of approximately 5 mL) are collected in a 50 mL tube (BD falcon, USA). *Agrobacterium* harboring the GT vector is suspended in 40 mL of AAM medium ($OD_{600} = 0.03–0.1$) supplemented with 20 mg/L acetosyringone in a fresh 50 mL tube, and is then added to the 50 mL tube containing calli. The bacterial suspension and calli are mixed gently by inverting for 1.5 min. We usually use a strainer to discard the bacterial suspension solution. After discarding the bacterial suspension, calli are placed on three or four pieces of sterilized filter paper (70 mm) in Petri dishes to remove excess bacterial suspension and are transferred onto a sterilized filter paper that is then placed on 2N6-AS medium and moistened with 0.5 mL of AAM medium supplemented with 20 mg/L acetosyringone. Calli on 2N6-AS medium are co-cultivated at 25 °C in the dark for 3 days.
5. After 3 days of co-cultivation, calli are collected in a 50 mL tube and washed ten times with sterilized water with 25 mg/L meropenem solution. Washed calli are placed onto three or four pieces of sterilized filter paper in Petri dishes to remove excess solution. Approximately 100 pieces of calli are placed onto N6D medium containing 50 mg/L hygromycin and 25 mg/L meropenem and grown at 33 °C.
6. After a 2-week selection period, all calli are transferred onto fresh N6D medium containing 50 mg/L hygromycin and 25 mg/L meropenem and grown at 33 °C for another 2 weeks. Hygromycin-resistant calli do not appear at this time.
7. Hygromycin-resistant calli are transferred onto fresh N6D medium containing 50 mg/L hygromycin and 25 mg/L meropenem and are clonally propagated at 33 °C for 2 weeks.

3.2 Identification of GT Calli Using PCR Analysis

1. Genomic DNA is extracted from small pieces of hygromycin-resistant calli using Agencourt chloropure (Beckman Coulter, USA) according to the manufacturer's protocol.

2. PCR amplifications targeting both upstream and downstream of the insertion site of the positive selection marker are performed with KOD FX neo (TOYOBO, Japan) using primer sets that anneal to the positive selection marker and the endogenous target locus, which is not present on the GT vector (Fig. 1). Transgenic calli yielding positive PCR signals derived from the target locus carrying *piggyBac* with the positive selection marker are regarded as true GT lines. The proportion of PCR-positive calli per total hygromycin-resistant calli is approximately 1 % [1].
3. To confirm the introduction of a desired mutation into the target gene and TTAA elements located in the insertion site of the *piggyBac* transposon, PCR fragments targeting both upstream and downstream of the insertion site of the positive selection marker are purified by Agencourt AMPure XP (Beckman Coulter, USA) according to the manufacturer's protocol, and sequenced using a primer annealing approximately 300-bp distant from the mutation position or TTAA elements (*see* **Notes 2** and **3**).
4. True GT calli are transferred onto fresh N6D medium containing 50 mg/L hygromycin without meropenem and grown at 33 °C for 2–4 weeks to remove the meropenem (*see* **Note 4**).

3.3 Marker Excision via piggyBac Transposition

1. *Agrobacterium* (EHA105) harboring the hyPBase expression vector is inoculated onto AB medium containing appropriate antibiotics in Petri dishes and incubated at 28 °C in the dark for 3 days before *Agrobacterium* infection.
2. Cultured true GT calli on N6D medium without meropenem are transformed with *Agrobacterium* harboring the hyPBase vector and are co-cultivated according to Subheading 3.1 **step 4**. Co-cultivated calli with *Agrobacterium* are washed according to Subheading 3.1 **step 4**, then transferred onto N6D medium containing 35 mg/L G418 and 25 mg/L meropenem and grown at 33 °C for 2 weeks.
3. G418-resistant calli are transferred onto fresh N6D medium containing 35 mg/L G418 and 25 mg/L meropenem and are propagated clonally at 33 °C for 2 weeks.
4. To analyze the copy number of the hyPBase expression vector using Southern blot analysis, genomic DNA is extracted from approximately 20 lines of hyPBase transgenic calli using Nucleon PhytoPure (GE Healthcare, USA) in accordance with the manufacturer's protocol. Southern blot analysis is performed with specific DNA probes for the hyPBase gene according to a standard protocol (*see* **Note 5**).
5. Transgenic calli carrying a single copy of the hyPBase expression cassette are selected and transferred onto regeneration

medium (ReIII) containing 35 mg/L G418 and 25 mg/L meropenem and grown at 28 °C under constant light for 10–14 days (*see* **Note 6**).

6. Some pieces of green callus are transferred to fresh ReIII medium and grown at 28 °C under constant light for 2 weeks.
7. Shoots arising from callus on ReIII medium are transferred to HF medium containing 35 mg/L G418 and 25 mg/L meropenem and grown at 28 °C under constant light for 2 weeks.

3.4 Selection of Marker-Free Plants

1. Shoots derived from hyPBase transgenic calli are divided into individual plants and each is assigned a number.
2. Genomic DNA is extracted from leaves of regenerated plants using Agencourt chloropure (Beckman Coulter) according to the manufacturer's protocol.
3. To confirm whether the selection marker has been eliminated from GT-modified target locus via transposition of *piggyBac*, PCR amplifications are performed with KOD FX neo (TOYOBO) using the primer set shown in Subheading 3.2 **step 2** (Fig. 1). In addition, to detect the existence of re-integrated *piggyBac* transposon, positive selection marker-specific fragments are amplified with KOD FX neo. If the selection marker is excised from the target locus without concomitant re-integration of *piggyBac* transposon, the DNA fragment cannot be amplified with the PCR conditions used here.
4. DNA fragments carrying the *piggyBac*-excised site and the desired mutation inserted into the target gene via GT are amplified with PrimeSTAR GXL DNA polymerase (Takara) using a primer set specific for the endogenous target gene, and are purified using Agencourt AMPure XP. Subsequently, these fragments are cloned into the vector pCR-Blunt II-TOPO using TOPO cloning methods (Life Technologies, USA) and sequenced to check the TTAA element at the *piggyBac*-excised site and the presence of the desired mutation in the target gene.
5. To analyze segregation of the target gene modified via GT and the hyPBase expression vector, T_1 progeny plants are obtained from self-pollinating regenerating plants.

4 Notes

1. We find that a combination of introduction of DSBs by sequence-specific nucleases and their repair via single-strand annealing (SSA) also allows for marker excision without leaving unnecessary sequences from the GT locus (Unpublished data).
2. The desired mutations are not always inserted into the target locus via GT.

3. If mutations introduced into the target gene via GT generate recognition sites for a restriction enzyme, the presence of the mutation in the target gene can be detected by cleaved amplified polymorphic sequences (CAPS) analysis combined with PCR amplification of the target gene and digestion with that restriction enzyme.
4. The expected band size derived from modified target gene locus via GT in PCR-positive calli should also be checked by Southern blot analysis.
5. To obtain progenies in which a hyPBase expression cassette segregates out, transgenic calli lines carrying a single copy of the hyPBase expression cassette should be selected by Southern blot analysis.
6. If G418-resistant regenerated plants are not obtained, use ReIII and MSFH medium solidified with 4 g/L gelrite containing 25 mg/L meropenem without G418.

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