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An Efficient Targeted Mutagenesis System Using CRISPR/Cas in Monocotyledons

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Precise genome modification using artificial nucleases is a powerful tool for in-depth understanding of gene functions and for creating new varieties. The CRISPR/Cas system, derived from an adaptive immunity system in bacteria and archaea, can introduce DNA double-strand breaks (DSBs) into pre-selected genomic loci and lead to loss of gene function due to error-prone non-homologous end joining (NHEJ). RNA-guided nucleases have been widely used in several eukaryotic organisms. In this article, we provide a detailed protocol for designing and constructing gRNA targets, detecting nuclease activity in transient protoplast assays, and identifying mutations in transgenic plants (including rice, wheat and maize). Targeted mutations in T0 plants can be generated in 14 to 18 weeks.

Keywords: CRISPR/Cas • gene knock-out • maize • rice • wheat

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INTRODUCTION

Rice, wheat, and maize are major food crops worldwide; rice and maize are often used as model plants for genetic, cytological, and agricultural research. However, basic research and crop variety improvement are restricted by the limited availability of mutants and natural strains (Gao, 2015). The development of CRISPR/Cas technology has greatly reduced these problems and accelerated the process of crop breeding. Already CRISPR/Cas-mediated targeted genome modification has been successfully used in a number of model plants and crops, including *Arabidopsis* (Li et al., 2013), tobacco (Nekrasov et al., 2013), rice (Shan et al., 2013), wheat (Shan et al., 2013; Wang et al., 2014), maize (Liang et al., 2014), sorghum (Jiang et al., 2013), and soybean (Li et al., 2015). In the CRISPR/Cas system, a guide RNA (gRNA), a fusion of the trans-activating RNA (tracrRNA) and the CRISPR RNA (crRNA), recruits and activates the Cas9 nuclease. Cas9 cuts the target site and generates double-strand breaks (DSBs) 3 bp upstream of the PAM sequence (5′-NGG-3′) (Fig. 1A; Cong et al., 2013; Mali et al., 2013). The DSBs at the pre-selected site can be repaired by non-homologous end joining (NHEJ) or homologous recombination (HR). The HR mechanism can repair the DSBs precisely using the homologous template (donor) and generate a targeted gene insertion or replacement (Fig. 1B). However, HR in higher plants is very inefficient, and NHEJ is the major repair pathway. DSBs repaired by NHEJ can cause frame-shift mutations in the target site and thus generate loss-of function mutants. Here, we describe a protocol for CRISPR/Cas-mediated targeted mutagenesis in rice, wheat, and maize. This provides plant biologists with a routine method for making mutants.

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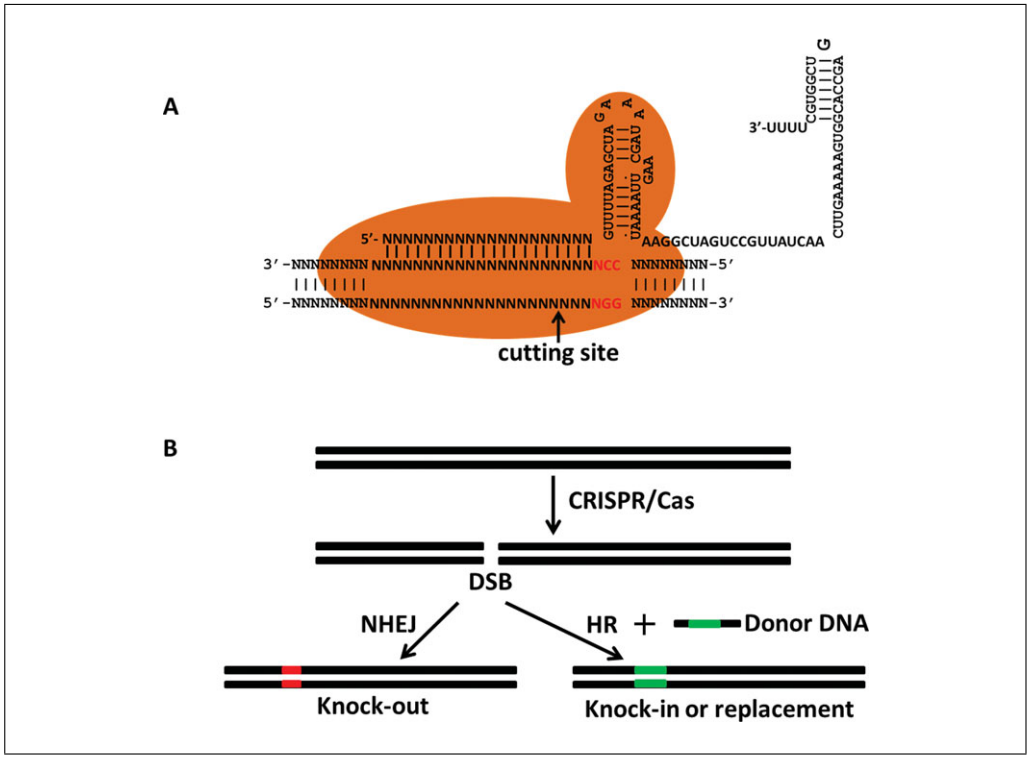

Figure 1 Genome editing mediated by CRISPR/Cas system. (**A**) Schematic illustration of the CRISPR/Cas9 system, containing Cas9 nuclease (brown) and gRNA. The Cas9 cuts 3 bp upstream of the PAM motif (red). (**B**) CRISPR/Cas-induced double-strand breaks (DSBs) can be repaired by either non-homologous end joining (NHEJ, left) or homologous recombination (HR, right), and result in random indels (deletion or insertion) and precise nucleotide substitution or gene insertion.

BASIC PROTOCOL

CRISPR/Cas-MEDIATED GENOME MODIFICATIONS IN MONOCOTYLEDONS

For targeted mutagenesis in crops using CRISPR/Cas technology, there are four steps: (1) selecting target sites for the gRNA; (2) constructing the gRNA vectors; (3) confirming nuclease activity in protoplast transient assays; and (4) screening mutations in T0 plants.

Materials

pEasy-Blunt cloning vector (TransGen Biotech)
pEasy-TI cloning vector (TransGen Biotech)
gRNA expression plasmids:
  pOsU3-gRNA (Addgene, Plasmid #53063)
  pTaU6-gRNA (Addgene, Plasmid #53062)
  pZmU3-gRNA (Addgene, Plasmid #53061)
10× annealing buffer (OriGene)
*Aar*I (Fermentas/Thermo Scientific) and corresponding 10× buffer
*Bbs*I (Fermentas/Thermo Scientific) and corresponding 10× buffer
10× Fast Green buffer(Fermentas/Thermo Scientific, supplied with FastDigest restriction enzyme)
DNA gel purification kit (Axygen)
T4 DNA ligase (Fermentas/Thermo Scientific) and 10× T4 DNA ligase buffer
Chemically competent *E coli* DH5α (TransGen Biotech)
LB medium and plates (see recipes) containing 100 μg/ml ampicillin
50% (v/v) glycerol
Primers (Table 1; prepared by DNA synthesis facility, e.g., BGI; also see Ellington and Pollard, 1998):

Table 1 Primers Used for gRNA Cloning and Validation

Name	Sequence	Purpose
OsU3F	5′-AAGGAATCTTTAAACATACGA-3′	Colony PCR and sequencing to validate the gRNA
TaU6F	5′-CATCTAAGTATCTTGGTAAAG-3′	Colony PCR and sequencing to validate the gRNA
ZmU3F	5′-CCCAAGCTTGACCAAGCCCG-3′	Colony PCR and sequencing to validate the gRNA
OsgRNA-F	5′-GGCANNNNNNNNNNNNNNNNN-3′	gRNA cloning
TagRNA-F	5′-CTTGNNNNNNNNNNNNNNNNN-3′	gRNA cloning
ZmgRNA-F	5′-AGCANNNNNNNNNNNNNNNNN-3′	gRNA cloning
gRNA-R	5′-AAACNNNNNNNNNNNNNNNNN-3′	gRNA cloning and colony PCR and sequencing to validate the gRNA

OsU3-F
TaU6-F
ZmU3-F
gRNA-R 5′-AAACN$_{(19)}$-3′
Plasmid miniprep kit (Axygen)
Wizard Plus midiprep kit (Promega)
pJIT163-Ubi-rCas9 (this plasmid can be obtained from the authors on request)
DNAquick plant system (Tiangen Biotech)
10× FastDigest buffer (Thermo Fisher Scientific)
T7 Endonuclease I (T7EI, ViewSolid Biotech) and 10× T7EI buffer

PCR thermal cycler (BioRad; also see Kramer and Coen, 2000)
NanoDrop spectrophotometer (Thermo Scientific)
Digital gel imaging system (BioDoc-It; UVP, cat. no. 97-0256-02)
Gel quantification software (e.g., ImageJ from the NIH)

Additional reagents and equipment for PCR (Kramer and Coen, 2000), general cloning techniques including sequencing (Ausubel et al., 2016), oligonucleotide synthesis (Ellington and Pollard, 1998), agarose gel electrophoresis (Voytas, 2000), and colony PCR (Woodman, 2008)

NOTE: The plasmid map and sequence for each of the plasmids listed above can be found in the supplementary materials file for this unit at *http://www.currentprotocols.com/protocol/PB20021*.

Selection of target sites for the gRNA

A 20-nt sequence within the gRNA determines the specificity of the Cas9 nuclease. In selecting a target site, several factors need to be taken into consideration: (1) the 20-nt sequence must precede the 5′-NGG PAM (protospacer-adjacent motif) sequence; (2) the danger of off-target activity must be considered; (3) the target site should be located in the first half of the target gene or within a key domain of that gene; (4) a restriction enzyme site should be present at the site cut by Cas9 (three bp upstream of the PAM sequence) for ease of detecting cutting (this is optional).

For wheat and maize, due to the lack of complete genome databases and the existence of SNPs between different strains, it is necessary to clone and sequence the target gene before selecting the target site. Hexaploid wheat is used as an example for the design. Proceed as follows.

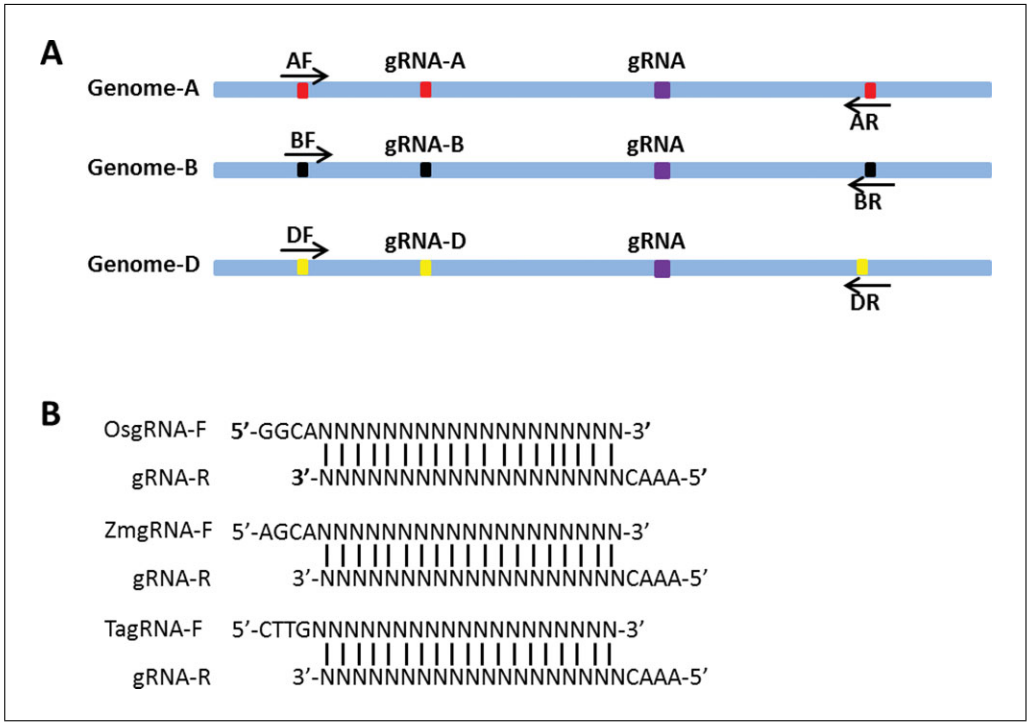

Figure 2 Strategy for designing gRNA in wheat (hexaploid as an example) and gRNA cloning. (**A**) The gRNA located in the conserved region can knock out the three homologous copies in genomes A, B, and D simultaneously. The gRNA-A, gRNA-B, and gRNA-D can disrupt the corresponding allele specifically. (**B**) Sequence of the annealed target oligos for gRNA cloning.

1. As shown in Figure 2A, design primers using the genomic database or the sequences of genes in model plants that are homologous to the gene of interest and amplify the target gene by PCR (Kramer and Coen, 2000) using a high-fidelity polymerase.
2. Clone the PCR product into pEasy-Blunt vector (see Ausubel et al., 2016, for cloning techniques) and sequence several independent clones (ten clones for wheat, but five clones are sufficient for maize because it is diploid; see Ausubel et al., 2016, Chapter 7, for sequencing techniques).

   Bread wheat is an allohexaploid, and most genes exist in 3 similar copies in the A, B, and D genome. After sequence alignment, conserved regions can be selected in order to design a gRNA to knock out all three copies simultaneously. In addition, target sites can be selected in non-conserved regions to knock out the gene of interest in individual genomes as desired (Fig. 2A).
3. Design the gRNA manually or with a Web-based tool such as "sgRNA Designer" (*http://www.broadinstitute.org/rnai/public/analysis-tools/sgrna-design*; Doench et al., 2014), which can generate gRNA scores based on sequence features that may improve on-target fidelity.

   We recommend constructing two to three gRNAs as candidates to disrupt the target gene.
4. In order to minimize potential off-target activity, run a BLAST analysis of the pre-selected target sequences (*http://blast.ncbi.nlm.nih.gov/*) in the reference genome.
5. Synthesize the target oligos as shown in Figure 2B (see Ellington and Pollard, 1998, for synthesis techniques).

   The reverse oligo for the target site is 5′-AAACN($_{19}$)-3′. The forward oligo primers for target sites differ according to the species: 5′-GGCAN($_{19}$)-3′ for rice; 5′-AGCAN($_{19}$)-3′ for maize; and 5′-CTTGN($_{19}$)-3′ for wheat (Table 1).

Cloning the target sequence into the gRNA expression vector

To construct a gRNA vector, we only need to insert a 20-nt nucleotide target into the gRNA expression vector, which can be done in a single cloning step. We use pOsU3-gRNA (Addgene, Plasmid #53063) for rice, pTaU6-gRNA (Addgene, Plasmid #53062) for wheat, and pZmU3-gRNA (Addgene, Plasmid #53061) for maize. The gRNA expression plasmid needs to be co-transformed with the pJIT163-Ubi-rCas9 vector (available from the authors on request). The Cas9 sequence is codon-optimized for monocots to increase its expression and is driven by the ubiquitin promoter. See Ausubel et al., 2016, for cloning techniques.

6. To prepare the gRNA oligo insert, dilute the gRNA oligo to a final concentration of 10 μM and set up the following annealing reaction:

   5 μl 10× annealing buffer
   20 μl 10 μM Ta/Zm/Os-Target-F (synthesized at step 5)
   20 μl 10 μM gRNA-R (Table 1; synthesized at step 5)
   5 μl distilled, deionized H_2O.

   Incubate 5 min at 95°C in a thermal cycler, then ramp temperature down to75°C at −1°C/cycle over 20 min, then keep at 75°C for 30 min, then ramp down to 45°C at −1°C/cycle over 30 min, then hold indefinitely at 4°C.

7. Prepare the corresponding vectors for cloning. Digest the gRNA expression vector with *Aar*I (pOsU3-gRNA) or *Bbs*I (pZmU3-gRNA and pTaU6-gRNA) according to substep a or b below.

   a. For pOsU3-gRNA, prepare the following reaction mix:

      2 μg of pOsU3-gRNA
      5 μl of 10× *Aar*I buffer (supplied with *Aar*I)
      1 μl of 10× oligonucleotides (supplied with *Aar*I)
      2 μl of 2 U/μl *Aar*I
      Distilled, deionized H_2O to 50 μl.

      Incubate the digest reaction at 37°C for 3 to 4 hr.

      Longer times are better for total digestion.

   b. For pZmU3-gRNA and pTaU6-gRNA, prepare the following reaction mix:

      2 μg of pZmU3-gRNA or pTaU6-gRNA
      5 μl 10× Fast Green buffer
      2 μl of 10 U/μl *Bbs*I
      Distilled, deionized H_2O to 50 μl.

      Incubate the digest reaction at 37°C for 1 to 2 hr.

8. After digestion, run all the digest reactions on a 1.2% agarose gel in TAE buffer at 140 V for 20 min (Voytas, 2000). Only one band will show in the gel (2.9 kb for pTaU6-gRNA, 3.2 kb for pOsU3-gRNA, or 4.8 kb for pZmU3-gRNA). Cut out the observed band and purify it using a gel purification kit.

   The final concentration of the purified product should be >10 ng/μl. The purified product should be eluted with distilled deionized water instead of the elution buffer provided with the kit.

9. Ligate the oligo insert into the purified vector using the following ligation reaction:

7 μl oligo inserts (step 6)
Variable volume (30 ng) purified vector (step 8)
2 μl 10× T4 DNA ligase buffer
1 μl 5 U/μl T4 DNA ligase.

Incubate the ligation reaction at 22°C for 1 to 2 hr.

If you choose the T4 DNA ligase by NEB, the incubation temperature should be 16°C.

10. Incubate competent *E. coli* with 10 μl of the ligation product under standard conditions on LB plates containing 100 μg/ml ampicillin at 37°C overnight.

    Generally, we use strain DH5α.

11. Identify the desired colonies on the plates by colony PCR (Woodman, 2008) using primers OsU3-F/TaU6-F/ZmU3-F and gRNA-R 5′-AAACN$_{(19)}$-3′. Three to four colonies are enough for identifying at least one correct one. Pick one of these into 5 ml of LB medium containing 100 mg/ml ampicillin and shake at 37°C and 200 rpm overnight.

    Detailed information on the primer sequences is given in Table 1.

12. Pipet 500 μl of the culture into a 1.5-ml sterile microcentrifuge tube. Add an equal volume of 50% (v/v) glycerol, and store at −80°C for further use. Isolate the plasmid from the rest of the culture using a miniprep kit.

13. Sequence the isolated plasmid (see Ausubel et al., 2016, Chapter 7) with the corresponding primer: OsU3-F, TaU6-F, or ZmU3-F (Table 1). Align the sequence obtained along with the gRNA expression cassette using BLAST to check if the 20-nt target sequence is inserted into the plasmid correctly.

14. Inoculate 0.1 ml of the stored culture (step 12) into 100 ml LB medium containing 100 μg/ml ampicillin and shake at 37°C overnight.

15. Isolated the plasmid from the 100 ml culture using a midiprep kit.

    The plasmid can be used for protoplast assays and explant transformation.

    The final concentration of the plasmid should be at least 1 μg/μl. If the concentration is less, it should be concentrated.

Confirm gRNA activity in the protoplast assay

Due to the time-consuming nature of plant tissue culture and transformation, you should first confirm the activity of the gRNA in the protoplast transient assay—which can be done in 3 days. To detect the mutations in the target sites, we usually use PCR/RE (recommended) or T7EI assays. For PCR/RE, there needs to be a unique restriction enzyme site in the Cas9 cutting site. The T7EI assay can detect the mutations when there is no restriction enzyme site in the target site.

16. Co-transfect the gRNA vector from step 15 and pJIT163-Ubi-rCas9 into protoplasts and incubate for 40 to 48 hr (see Support Protocol).

    The detailed protoplast transformation procedure is shown in the Support Protocol.

17. Collect the transformed protoplasts and extract the genomic DNA using the DNAquick Plant System from Tiangen Biotech. Dilute the genomic DNA with distilled, deionized water; the concentration of the DNA is usually 20 to 60 ng/μl.

18. Perform PCR (Kramer and Coen, 2000) to amplify the target region containing the target site.

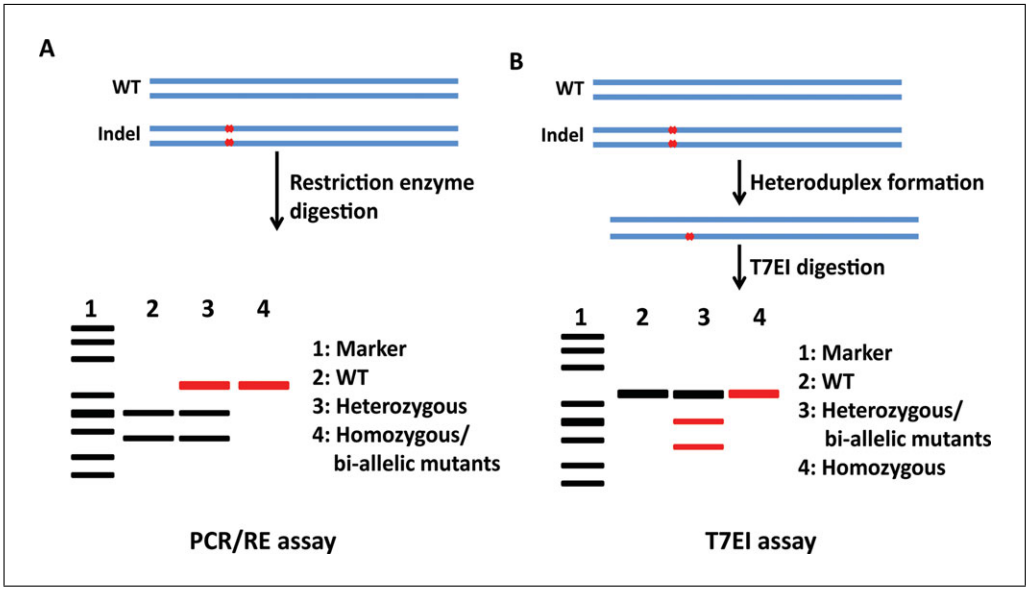

Figure 3 Schematic of the strategy to detect mutations. (**A**) Schematic illustration of the PCR/RE assay. The heterozygous mutations can be partially digested and the homozygous mutations and bi-allelic mutations can be completely undigested. (**B**) Schematic illustration of the T7EI assay. The heterozygous and bi-allelic mutations can be cleaved by T7EI, while homozygous mutants remain intact and cannot be distinguished from the wild type. The red bands represent the mutations.

We recommend primers that can amplify a 350- to 800-bp fragment starting >100 bp from the target site; fragments of this size can be easily identified on 2% gels after digestion.

19. Run 5 μl of the PCR product on a 1.2% agarose gel (Voytas, 2000) and check the yields of the amplified fragment.

20. For PCR/RE assays, use the following reaction system:

    5 μl PCR product from step 19
    2 μl 10× FastDigest buffer
    1 μl of 10 U/μl of the appropriate restriction enzyme (according to the sgRNA sequence)
    12 μl distilled, deionized H_2O.

    Incubate the mixture at 37°C for 2 hr.

    A wild-type sample is needed as a negative control.

    a. Run 20 μl of the digest products on a 2% agarose gel in TAE buffer at 140 V for 15 to 20 min (Voytas, 2000).

        As shown in Figure 3A, the sample should yield an undigested band, whereas the negative control will be totally digested. The mutagenesis frequency can be measured by determining the percentage of the sample that remains undigested band using ImageJ or some other gel-quantification software.

    b. Purify the undigested band and clone it into pEasy-T1 according to the manufacturer's instructions. On the next day, pick 5 to 10 positive clones at random for sequencing.

    c. Run a BLAST analysis of the sequencing results along with the wild-type sequence (*http://blast.ncbi.nlm.nih.gov/*), as shown in Figure 4B.

    There should be small deletions, insertions, or substitutions at the target site.

21. For the T7EI assay.

    In the T7EI assay, the T7EI nuclease cuts mismatched sites in DNA heteroduplexes.

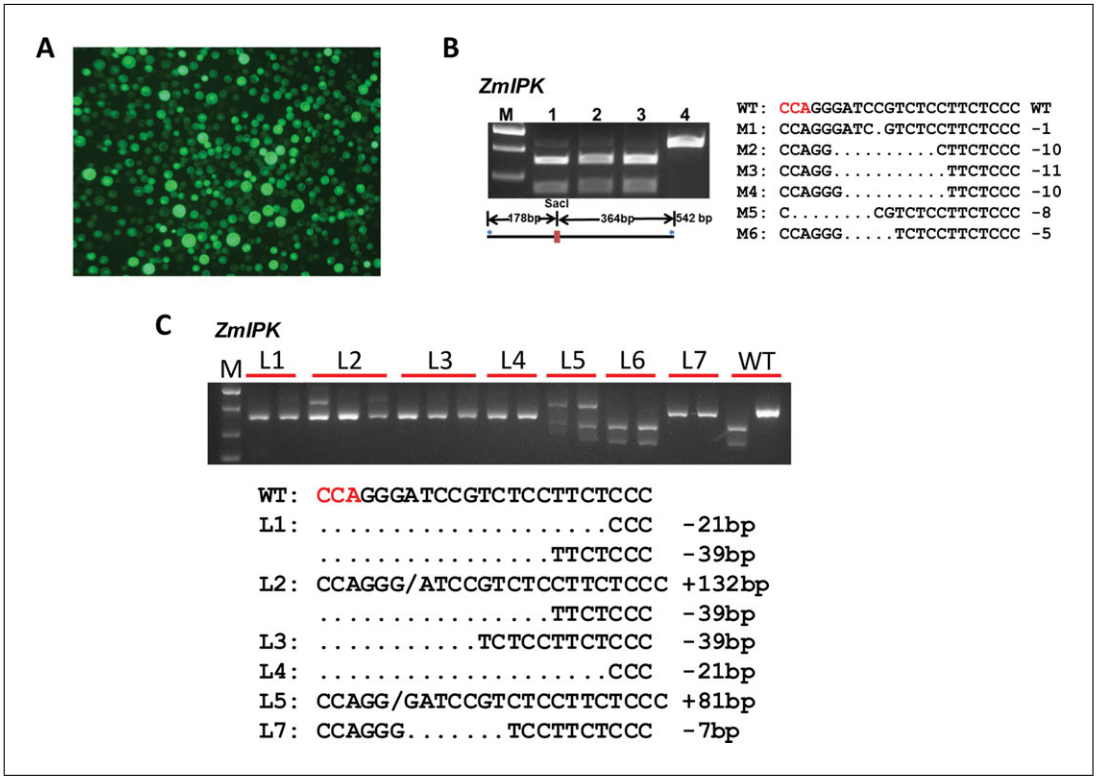

Figure 4 Anticipated results for CRISPR/Cas induced mutations. (**A**) The protoplast transformation efficiency should be up to 60%. (**B**) Agarose gels illustrating mutations at the *ZmIPK* loci in protoplast. This is detected by PCR/RE assay and the mutation efficiency is about 10%. (**C**) Agarose gel showing mutations at *ZmIPK* loci in T0 transgenic plants detected by the PCR/RE assay. L1, L3, L4, and L7 represent the homozygous mutants; L2 represents bi-allelic mutants; L5 represents heterozygous mutants; and L6 represents wild-type. The homozygous mutation efficiency can be more than 70%.

a. Use the following annealing reaction to form heteroduplexes in the PCR product from step 19.

5 μl PCR product from step 19
1.1 μl of 10× T7EI buffer
4.4 μl distilled, deionized H_2O.

Incubate the mixture at 95°C for 5 min, and cool it to room temperature.

b. Add 0.5 μl T7EI nuclease to the annealed products and digest at 37°C for 30 min.
c. Immediately run the digested products on a 2% agarose gel (Voytas, 2000).

A negative control is needed. The sample should be partially digested, demonstrating the presence of mutations, while the wild-type control should be totally undigested (Fig. 3B).

d. To confirm the mutations, purify the PCR product and clone into pEasy-T1 for sequencing.

We recommend testing 30 to 50 positive clones.

The PCR/RE assay requires testing fewer clones because the unaltered sequence is digested by the RE, and the mutant sequence is enriched. For the T7EI assay, the PCR products contains both wild-type and mutant sequence, and so additional clones must be picked and tested.

Generate transgenic plants and screen for mutations in the T0 plants

To generate gene-knockout mutants, Agrobacterium- or particle-bombardment-mediated transformation of rice (Nipponbare), wheat (Kenong199), or maize (HiII) is performed.

Generally, 20 to 30 transgenic lines are enough to obtain mutants. Also see Gao et al. (2016).

22. Extract genomic DNA from the individual transgenic lines using the DNAquick Plant System.

23. Perform PCR to amplify the fragment encompassing the target site as described in steps 19 to 20.

24. Detect mutations in the transgenic lines by PCR/RE (step 21) or T7EI assays (step 22; also see Fig. 3).

    In the PCR/RE assay, the resulting gel can yield any one of three patterns: a totally undigested sample indicates a bi-allelic or homozygous mutation; a partially digested sample indicates a heterozygous mutation; a totally digested sample implies the absence of any mutation.

    In the T7EI assay, there are two possible outcomes: a totally undigested sample indicates the presence of the wild type sequence or a homozygous mutation; partial digestion implies the presence of a heterozygous or bi-allelic mutation.

25. Sequence the candidate mutants (Ausubel et al., Chapter 7) to confirm them and identify the type of allele involved. Select the suitable mutation types for further study.

    Homozygous mutations can be detected easily by sequencing the PCR products; bi-allelic and heterozygous mutations should be sequenced by cloning.

PROTOPLAST TRANSFORMATION ASSAY

SUPPORT PROTOCOL

The protoplast transformation procedure is derived from the *Arabidopsis thaliana* transformation assay with some modifications (Yoo et al., 2007). All the procedures are performed at room temperature. The protoplast assay comprises three parts: (1) plant preparation; (2) protoplast isolation; and (3) PEG-mediated protoplast transformation.

Materials

Rice cultivar: Nipponbare (IGDB, CAS)
Wheat cultivar: Kenong 199 (IGDB, CAS)
Maize cultivar: Hi-II (IGDB, CAS)
75% ethanol
2.55 (w/v) sodium hypochlorite
1/2 MS solid medium (see recipe)
Nutrient-rich soil (Pindstrup sphagnum)
0.6 M mannitol, sterilized with a 0.45-μm filter (store at room temperature)
Enzyme solution (for rice and wheat or for maize; see recipes)
W5 solution (for rice and wheat or for maize; see recipes)
MMG solution (see recipe)
gRNA expression plasmids:
  pOsU3-gRNA (Addgene, Plasmid #53063)
  pTaU6-gRNA (Addgene, Plasmid #53062)
  pZmU3-gRNA (Addgene, Plasmid #53061)
pJIT163-Ubi-rCas9 (this plasmid can be obtained from the authors on request)
PEG solution (see recipe)

Environmentally controlled incubators (Eppendorf)
Shaker
Round glass cup with diameter 66 mm and height 180 mm
9-cm Petri dishes

Blotting paper
Single-edge razor blade (Feiying)
150-ml conical flask
Vacuum desiccator and vacuum pump
40-μm nylon mesh (BD Falcon)
50-ml round-bottom centrifuge tubes (Haimeng)
2-ml round-bottom microcentrifuge tubes
6-well tissue culture plates
Fluorescence microscope (Olympus)

Plant preparation

1a. To prepare rice plants for protoplast assay:

i. Prewash the seeds by immersing in 75% ethanol for 1 min.
ii. Discard the supernatant and sterilize the seeds by immersing in 2.5% (w/v) sodium hypochlorite for 20 min, shaking gently during this procedure.
iii. Drain the seeds and wash them with sterile water five to seven times.
iv. Plate 12 to 15 seeds in a round glass cup (diameter 66 mm and height 180 mm) containing 50 ml 1/2 MS solid medium. Grow the plants at 28°C for 2 weeks in an environmentally controlled incubator with a long day photoperiod of LD 16:8.

In our laboratory, we generally use the rice cultivar Nipponbare for protoplast isolation. Other cultivars may also work with this protocol.

1b. To prepare wheat plants for protoplast assays, grow the wheat seeds in nutrient-rich soil at 24°C for 2 weeks with a long day photoperiod of LD 16:8.

We usually use the wheat cultivar Kenong 199 for protoplast isolation, and this protocol also works with other cultivars.

1c. To prepare maize plants for protoplast assays

i. Soak the seeds in water at 28°C overnight.
ii. Transfer 10 to 15 seeds into a 9-cm Petri dish containing blotting paper and incubate at room temperature for 2 days to germinate.
iii. Grow the germinated seeds in nutrient-rich soil at 24°C for 2 weeks with a long day photoperiod of LD 16:8.

We generally use the maize cultivar Hi-II for protoplast isolation, as this cultivar is easy to transform. This protocol also works with other cultivars.

Protoplast isolation

2. Bundle together the stems and sheaths of 30 to 40 rice seedlings and cut them into 0.5-mm strips using a sharp razor blade. For maize and wheat, use healthy fresh leaves to isolate the protoplasts.

Generally 150 rice seedlings (or 20 to 30 maize or wheat seedlings) can generate 1 × 10^7 protoplasts and can be used to introduce 20 different plasmids.

3. Immerse the strips (rice or wheat) in a Petri dish containing 0.6 M mannitol and incubate the dish in the dark for 10 to 15 min (optional for maize).

4. Transfer the immersed strips into a 150-ml conical flask containing 50 ml filter-sterilized enzyme solution.

5. Wrap the conical flask in aluminum foil and put it in a vacuum desiccator. Apply vacuum for 30 min at −15 to −20 mmHg. Following this treatment, incubate in the dark for 4 to 6 hr with gentle shaking (90 rpm).

6. After enzymatic digestion, add an equal volume of W5 solution and shake gently by hand for 10 sec.

7. Filter the mixture through a 40-μm nylon mesh into three 50-ml round bottom centrifuge tubes to collect the protoplasts.

8. Centrifuge 3 min at 250 × *g* for rice (or 100 × *g* for maize and wheat), room temperature, to pellet the protoplasts. Remove the supernatant and re-suspend the pellets in 10 ml W5 solution in one 50-ml round bottom centrifuge tube.

9a. *For rice:* Centrifuge 3 min at 250 × *g*, room temperature, and remove as much as possible of the supernatant. Resuspend the protoplasts in 4 ml MMG solution.

The final concentration should be about 2.5 × 10^6 protoplasts per ml.

9b. *For wheat and maize:* Keep the re-suspended protoplasts on ice for 30 min to allow them to settle to the bottom of the tube by gravity. Remove as much as possible of the supernatant by pipetting. Resuspend the pellet to a final concentration of 2.5 × 10^6 cells per ml in the appropriate volume of MMG solution.

PEG-mediated protoplast transformation

10. Add 10 μg of gRNA expression vector (pOsU3-gRNA, pTaU6-gRNA, or pZmU3-gRNA) and 10 μg of Cas9 expression vector (pJIT163-Ubi-rCas9) to a 2-ml round bottom microcentrifuge tube and mix well.

We generally use a GFP expression vector (pJIT163-Ubi-GFP) as a positive control to measure the protoplast transformation efficiency.

11. Add 200 μl protoplasts (5 × 10^5 cells) and mix well by gentle tapping of the tube.

12. Add 220 μl PEG solution and mix thoroughly by inverting several times or tapping gently. Incubate the mixture at room temperature for 15 min in the dark.

The PEG solution should be fresh—no more than 2 days old.

13. Add 880 μl W5 solution and mix well by inverting the tube several times to stop the transfection reaction.

14. Centrifuge 3 min at 250 × *g* (100 × *g* for wheat and maize), room temperature. Remove the supernatant and resuspend the pellet in 2 ml WI solution (W5 for wheat and maize).

15. Transfer the transfected protoplasts to 6-well plates to incubate.

16. On the next day, check the transformation efficiency by counting GFP-positive cells in the positive control with a fluorescence microscope.

REAGENTS AND SOLUTIONS

Use MilliQ-purified water in all procedures.

$CaCl_2$, 1 M

Prepare 1 M and sterilize with a 0.45-μm filter. Store up to 3 months at room temperature (22° to 25°C).

Enzyme solution (for maize)

1.5% (w/v) cellulase R10 (Yakult Pharmaceutical)
0.3% (w/v) macerozyme R10 (Yakult Pharmaceutical)
0.6 M mannitol (Ameresco)

continued

10 mM N-morpholino)ethanesulfonic acid (MES), pH 5.7 (add from 0.2 M stock; see recipe)
Warm solution 10 min at 55°C to enhance enzyme solubility
Cool to room temperature (22° to 25°C), then add:
1 mM $CaCl_2$ (from 1 M stock; see recipe)
0.1% (w/v) BSA
Prepare fresh

Enzyme solution (for rice and wheat)

1.5% (w/v) cellulase R10
0.75% macerozyme R10
0.6 M mannitol (Ameresco)
10 mM N-(morpholino)ethanesulfonic acid (MES), pH 5.7 (add from 0.2 M stock; see recipe)
Warm the solution at 55°C for 10 min to enhance enzyme solubility
Cool to room temperature (22° to 25°C), then add:
10 mM $CaCl_2$ (from 1 M stock; see recipe)
0.1% (w/v) BSA
Prepare fresh

LB medium

10 g tryptone
5 g yeast extract
5 g NaCl
1 ml 1 N NaOH
Appropriate antibiotics
Store indefinitely at 4°C

LB plates

10 g tryptone
5 g yeast extract
5 g NaCl
1 ml 1 N NaOH
15 g agar or agarose
Appropriate antibiotics
Store indefinitely at 4°C wrapped in plastic to prevent desiccation

MES [N-(morpholino)ethanesulfonic], 0.2 M

Prepare 0.2 M MES, pH 5.7 and sterilize with a 0.45-μm filter. Store up to 3 months at room temperature (22° to 25°C).

$MgCl_2$, 1 M

Prepare 1 M $MgCl_2$ and sterilize with a 0.45-μm filter. Store up to 3 months at room temperature (22° to 25°C).

MMG solution

0.4 M mannitol
15 mM $MgCl_2$ (add from 1 M stock; see recipe)
4 mM N-(morpholino)ethanesulfonic acid (MES), pH 5.7 (add from 0.2 M stock; see recipe)
Store up to 3 days at room temperature

1/2 MS solid medium

Add 2.215 g MS salts, 30 g sucrose, and 0.1 g inositol to 700 ml distilled water, and make up the volume to 1000 ml. Adjust the pH to 5.8 and add 3 g of phytagel (Sigma, cat. no. P8169). Autoclave at 121°C for 20 min. Pour ~55 ml of medium into a round glass cup (diameter 66 mm and height 180 mm). Store up to several weeks at 4°C.

PEG solution

40% (w/v) PEG
0.2 M mannitol
0.1 M $CaCl_2$ (add from 1 M stock; see recipe)
Store up to 3 days at room temperature

W5 solution (for rice and wheat)

154 mM NaCl
125 mM $CaCl_2$ (add from 1 M stock; see recipe)
5 mM KCl
2 mM N-(morpholino)ethanesulfonic acid (MES), pH 5.7 (add from 0.2 M stock; see recipe)
Store up to 3 months at room temperature

W5 solution (for maize)

154 mM NaCl
125 mM $CaCl_2$
5 mM KCl
4 mM N-(morpholino)ethanesulfonic acid (MES), pH 5.7 (add from 0.2 M stock; see recipe)
Store up to 3 months at room temperature

WI solution

0.5 M mannitol
20 mM KCl
4 mM
N-(morpholino)ethanesulfonic acid (MES), pH 5.7 (add from 0.2 M stock; see recipe)
Store up to 3 months at room temperature

COMMENTARY

Background Information

In recent years, the development of sequence-specific nucleases (SSNs), including ZFNs, TALENs, and CRISPR/Cas, has led to a revolution in basic research and applications in plant science. SSNs have been used to introduce frame-shift mutations, single nucleotide changes, deletions, substitutions, and insertions at pre-selected target sites in a wide range of plant species (Voytas, 2013). In addition, SSN derivatives can be used to regulate gene expression and alter the epigenetic status of chromatin in a sequence-specific way.

Due to the ease of cloning, the wide range of target sites in the genome, and its high cleavage activity, the CRISPR/Cas system is the most popular and fastest-growing of the three major genome editing tools. Although CRISPR/Cas has been widely used in many organisms, off-target effects cannot be ignored (Fu et al., 2013; Kuscu et al., 2014). Several methods have been described for reducing this problem. Pairs of gRNA with Cas9 nickase (Ran et al., 2013) and dimeric gRNA-guided Cas9-FokI (Tsai et al., 2014) reduce the off-target efficiency by increasing the length of the target site. Both these methods need two highly active gRNAs. Truncated gRNA has also been reported to enhance the specificity of genome editing (Fu et al., 2014). Generally speaking, we tend to search target sites in the reference genome and avoid those that contain less than the full complement of five nucleotides, so as to enhance on-target efficiency.

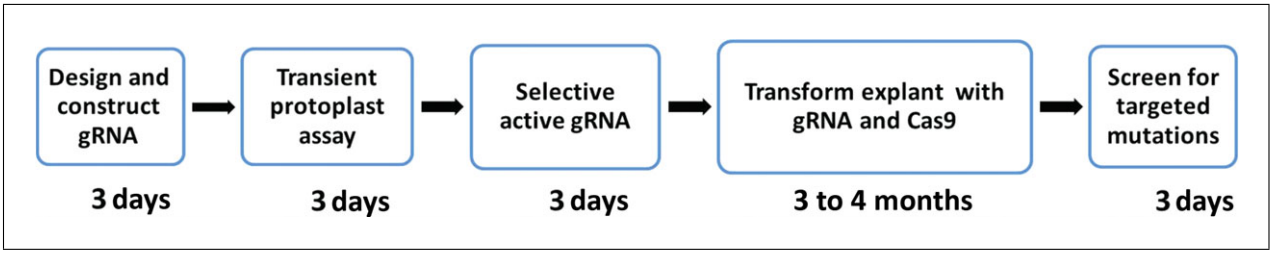

Figure 5 Procedure for recovering mutants by CRISPR/Cas.

Homologous recombination is one of the most powerful tools to alter the genome precisely. For HR, also referred to as gene targeting, it is necessary to introduce a DNA fragment (donor DNA) combined with the SSN into the cells. The donor DNA comprises homology sequence flanking the pre-selected target, the so-called homology arms, and determines the types of sequences that can be introduced into the genome (Fig. 1B). When a DSB occurs, it can be repaired by the repair template through the homologous recombination mechanism. This approach can be used for single nucleotide changes, targeted insertions, and trait stacking. Several groups have successfully applied this approach in plants (Shukla et al., 2009; Townsend et al., 2009). However, the frequency of HR in plants is still very low. There is still a long way to go to solve this problem.

In this protocol, the gRNA and Cas9 expression vector are co-transformed into the explant by particle bombardment (Alfonso-rubí, 1999; Wang and Frame, 2009; Zhang et al., 2015). The gRNA and Cas9 can also be cloned into a binary vector and introduced via *Agrobacterium*.

Critical Parameters

1. It is better to have more than five base mismatches between the target site and putative off-target loci since CRISPR/Cas can tolerate up to five mismatches, and even a single-base mismatch in the seed region (12-bases upstream of the PAM sequence) can be tolerated (unpub. observ.).

2. The final concentration of the gRNA and Cas9 expression vector should be at least 1 μg/μl. The quality of the plasmids is very important for both transient and stable transformation by particle bombardment. Low plasmid concentrations yield few transformants.

3. The protoplast transformation efficiency should be at least 50%. The PCR/RE and T7EI assays may fail to detect mutations if the protoplast transformation efficiency is low.

4. In the transient protoplast assay, the mutation efficiency of CRISPR/Cas should exceed 10%. Otherwise, no mutants are produced in the T0 generation, or large populations are needed to generate mutants.

5. For the PCR/RE assay, the restriction enzyme sites must be located precisely at the site of cutting by Cas9 (3 bp upstream of PAM). Otherwise, mutants that do not alter the restriction enzyme site will be missed.

6. For the T7EI assay, we use different primers to amplify the relevant allele in the A, B, and D genomes, respectively, of wheat, because the alleles often contain SNPs that affect the assay.

Troubleshooting

1. If no gRNA-positive colonies appear on the selective plates, the reason may be that the plasmid has been incompletely digested or the ligation time is too short. Try to digest and ligate for longer.

2. If the T7EI digested PCR products form smeary bands or there is a high background in the negative control, the reason may be that SNPs exist in the amplified regions, or there has been inadequate annealing or over-digestion by T7EI. Optimize the primers for PCR and re-anneal the templates. Note that T7EI digestion should not proceed for longer than 30 min.

3. If the protoplast yield is low or the protoplasts are cracked, the most probable reason is that the conditions for the seedlings are poor. Try to optimize the conditions in the greenhouse, namely photoperiod, humidity, and temperature.

4. If there is a low protoplast transformation efficiency, the reasons may include impure DNA, low concentrations of DNA, and a sub-optimal PEG solution. We use a Promega midiprep plasmid kit to extract the plasmid DNA. Make sure that the PEG solution is freshly prepared and completely dissolved.

5. If you cannot detect mutations in the T0 plants, the most likely reason is the use of an inefficient gRNA. Re-design more gRNAs and select the gRNA that has the highest activity.

Anticipated Results

Based on this protocol, we can successfully obtain gene knock-out mutations in crops within 4 to 5 months. Generally, we select three to four gRNAs to target the gene of interest and choose the gRNA with highest activity. The protoplast transformation efficiency can reach 80% to 90% (Fig. 4A). The mutation yield in protoplast assays ranges from 10% to 30% (Fig. 4B; Shan et al., 2014). For rice and maize, it is easy to obtain bi-allelic or homozygous mutations in the T0 generation (with an efficiency of 80% to 90% (Fig. 4C). For wheat, the yield of simultaneous mutations in the A, B, and D genome can be lower, maybe due to the complex genome of wheat. One can obtain homozygous wheat mutants by crossing and selfing.

Time Considerations

Figure 5 shows the timeline of the procedure for the recovery of mutants from CRISPR/Cas as described in this article.

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