Chapter 12

Targeted Plant Genome Editing via the CRISPR/Cas9 Technology

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Abstract

Targeted modification of plant genome is key for elucidating and manipulating gene functions in basic and applied plant research. The CRISPR (clustered regularly interspaced short palindromic repeats)/CRISPR-associated protein (Cas) technology is emerging as a powerful genome editing tool in diverse organisms. This technology utilizes an easily reprogrammable guide RNA (gRNA) to guide *Streptococcus pyogenes* Cas9 endonuclease to generate a DNA double-strand break (DSB) within an intended genomic sequence and subsequently stimulate chromosomal mutagenesis or homologous recombination near the DSB site through cellular DNA repair machineries. In this chapter, we describe the detailed procedure to design, construct, and evaluate dual gRNAs for plant codon-optimized Cas9 (pcoCas9)-mediated genome editing using *Arabidopsis thaliana* and *Nicotiana benthamiana* protoplasts as model cellular systems. We also discuss strategies to apply the CRISPR/Cas9 system to generating targeted genome modifications in whole plants.

Key words Plant genome editing, CRISPR/Cas9, Protoplast transient expression assay, Chromosomal mutagenesis, Homologous recombination

1 Introduction

Plant genome sequences are accumulating at a staggering rate due to the advent and rapid advance of whole-genome sequencing technologies, which in turn call for revolution of targeted genome editing tools. Using various designer endonucleases as "genomic scissors," researchers have the potential to generate loss-of-function mutants or desirable modifications in virtually any plant genes to elucidate their functions and regulatory mechanisms. The genome editing technologies also offer powerful genetic engineering tools to inactivate or modify desired plant genes to improve beneficial agricultural traits in crop plants.

Zinc finger nuclease (ZFN) and transcription activator-like effector nuclease (TALEN) represent earlier generations of targeted genome editing tools (reviewed in ref. [1]). Both engineered nucleases are translational fusions between a reprogrammable

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DNA-binding domain and the catalytic domain of *Fok*I restriction endonuclease. When a pair of ZFNs or TALENs is programmed to bind two closely positioned DNA sequences, each on one DNA strand, in a tail-to-tail orientation, two approaching *Fok*I nuclease domains are able to dimerize and cleave the chromosomal DNA between the two target sites. The resultant DNA double-strand break (DSB) will stimulate DNA repair through error-prone nonhomologous end joining (NHEJ) in the absence of a repair template, leading to nucleotide deletions, insertions, and substitutions around the DSB region and disrupting gene function. In the presence of a repair template, homologous recombination can occur to facilitate precise gene editing. Although these two genome-editing tools have been successfully implemented in diverse plant species [1, 2], two major challenges have hindered their broad application. One challenge is that engineering a pair of DNA-binding domains is needed each time for new genome targeting specificity, and the other is that both approaches are limited regarding the multiplexibility.

The CRISPR/Cas technology is emerging as a new promising genome editing tool originally inspired by the bacterial type-II CRISPR/Cas adaptive immune system [3]. In the CRISPR/Cas system, a single chimeric guide RNA (gRNA) containing a 20-nt guide sequence can guide the targeting of co-expressed *Streptococcus pyogenes* Cas9 endonuclease to an intended genomic N_{20}NGG sequence through base pairing (Fig. 1a). A DSB will then be generated through two separate nuclease domains of Cas9, each cleaving one DNA strand. During the DSB repair, gene mutagenesis or replacement can be obtained via the NHEJ pathway or homologous recombination pathway, depending on the availability of a DNA repair template. When compared to ZFNs and TALENs, the CRISPR/Cas9 system offers unparalleled simplicity and multiplexibility in genome editing because gRNAs can be easily designed and synthesized to achieve new DNA binding specificities and multiple gRNAs can work simultaneously with the same Cas9 nuclease on many different target sites.

Effective delivery of genome editing reagents including Cas9 nucleases, gRNAs, and homologous recombination DNA donors is key to the high efficiency of targeted genome modification, which remains challenging for most plant cells that are enclosed in cell walls. In this chapter, we introduce a detailed procedure for designing, assembling, and evaluating constructs for CRISPR/Cas9-mediated genome editing in *Arabidopsis thaliana* and tobacco (*Nicotiana benthamiana*) protoplasts (Fig. 1b), which support highly efficient DNA transfection for RNA and protein expression. The procedure is potentially adaptable to diverse plant species that are amenable to protoplast isolation and transfection [4]. Plant protoplasts offer a valuable system for rapidly evaluating the

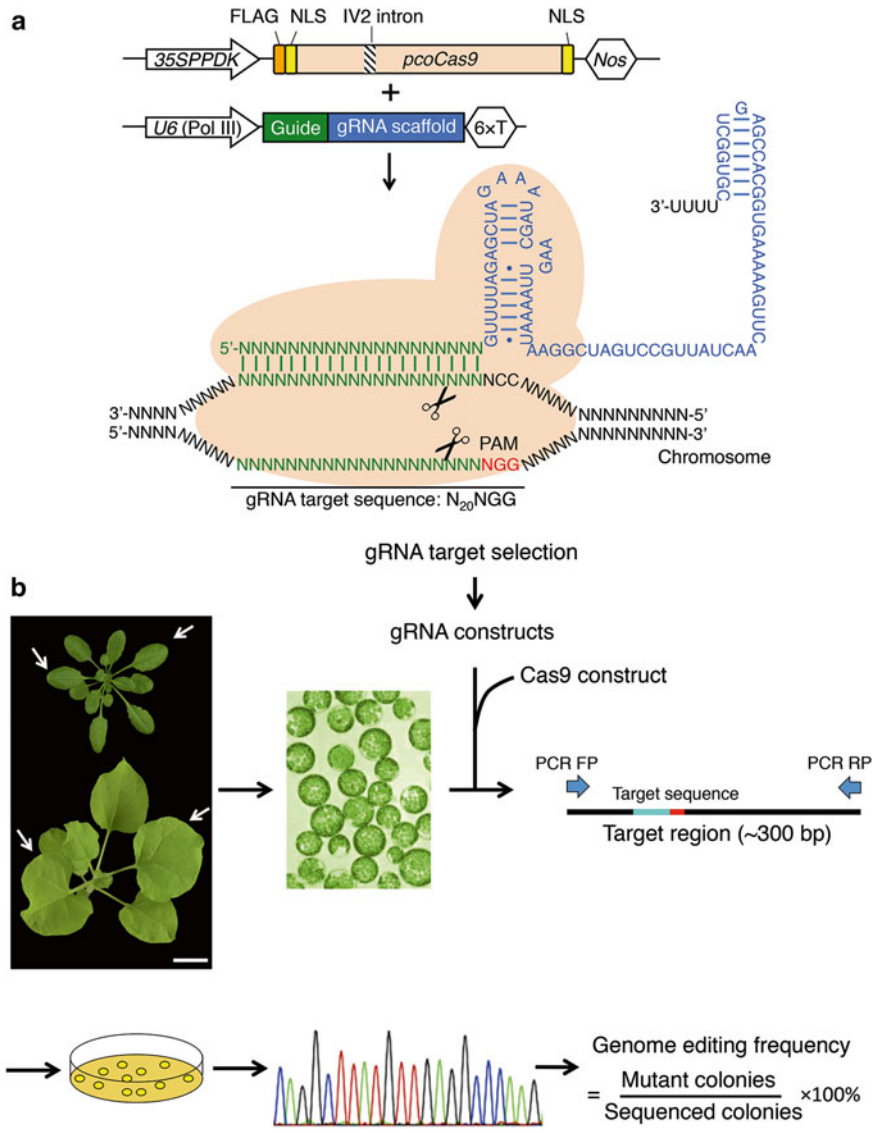


Fig. 1 Unbiased gRNA/Cas9-mediated genome editing in plant protoplasts. (**a**) Diagram of expression cassettes of *Cas9* and gRNA and their complex with the genomic N_{20}NGG target. Plant codon-optimized *Cas9* (pcoCas9) is fused to dual nuclear localization sequences (NLSs) and FLAG tags. The potato IV2 intron in the *pcoCas9* gene may minimize side effects of leaky *pcoCas9* expression on *E. coli* growth during cloning. The constitutive *35SPPDK* promoter and the *Arabidopsis U6-1* promoter were used to express *pcoCas9* and gRNA, respectively, in protoplasts. NGG, the protospacer adjacent motif (PAM), in the target sequence is highlighted in *red*. (**b**) Key procedure to generate and evaluate Cas9/gRNA-mediated genome editing in *Arabidopsis* and *tobacco* protoplasts. *White arrowheads* indicate the leaves at optimal developmental stage for protoplast isolation in 4-week-old plants. Scale bar = 2 cm. In the target region, the target sequence of N_{20} and NGG (the PAM) are represented in *cyan* and *red*, respectively. *E. coli* colonies containing PCR amplicons of the genomic target region were picked randomly for Sanger sequencing

performance of a given combination of gRNA/Cas9 at the genomic target. We discuss potential strategies to apply the CRISPR/Cas system for generating targeted genome modifications in whole plants.

2 Materials

2.1 Plant Tissue and Soil

1. Seeds of *Arabidopsis thaliana* Columbia-0.
2. Seeds of *Nicotiana benthamiana*.
3. Jiffy-7 peat soil pellets (Jiffy group).
4. Fafard soil (Fafard).

2.2 Bacterial Strain and Growth Medium

1. TOP10 chemically competent *Escherichia coli* and *Agrobacterium tumefaciens* strain GV3101 were used.
2. LB solid medium: 10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, and 15 g/L Bacto Agar.
3. Terrific broth (American Bioanalytical).

2.3 Plasmids

1. *pUC119-MCS*: a *pUC119*-derived cloning vector containing multiple cloning sites (MCSs) (Fig. 2a) for inserting single or multiple gRNA expression cassettes. This plasmid is available at Addgene (www.addgene.org, Plasmid #58807).
2. *pHBT-pcoCas9*: a transient expression plasmid for expressing the plant codon-optimized *Streptococcus pyogenes Cas9* (*pcoCas9*) gene [5] under the constitutive hybrid *35SPPDK* promoter (Fig. 2b). This plasmid is available at Addgene (Plasmid #52254).
3. *pUC119-gRNA*: the plasmid serves as the PCR template to assemble the expression cassette of a new gRNA with desired DNA targeting specificity, as it harbors the *Arabidopsis U6-1* promoter [6], which is an RNA polymerase III promoter required for gRNA expression, a gRNA guide sequence targeting to the *Arabidopsis PDS3* gene (target site: 5′ GGACTTTTG CCAGCCATGGT**CGG** 3′), a gRNA scaffold required for gRNA functionality, and a "TTTTTT" transcription terminator. This plasmid is available at Addgene (Plasmid #52255).
4. *pFGC-pcoCas9*: a binary plasmid expressing *pcoCas9* under the *35SPPDK* promoter and containing multiple cloning sites for inserting single or multiple gRNA expression cassettes (Fig. 2c). This plasmid is designed for *Agrobacterium*-mediated DNA delivery to plant nuclei, and is available at Addgene (Plasmid #52256). Sequencing primer (sequencing from *Eco*RI toward *Sma*I): 5′ AATAAAAACTGACTCGGA 3′.

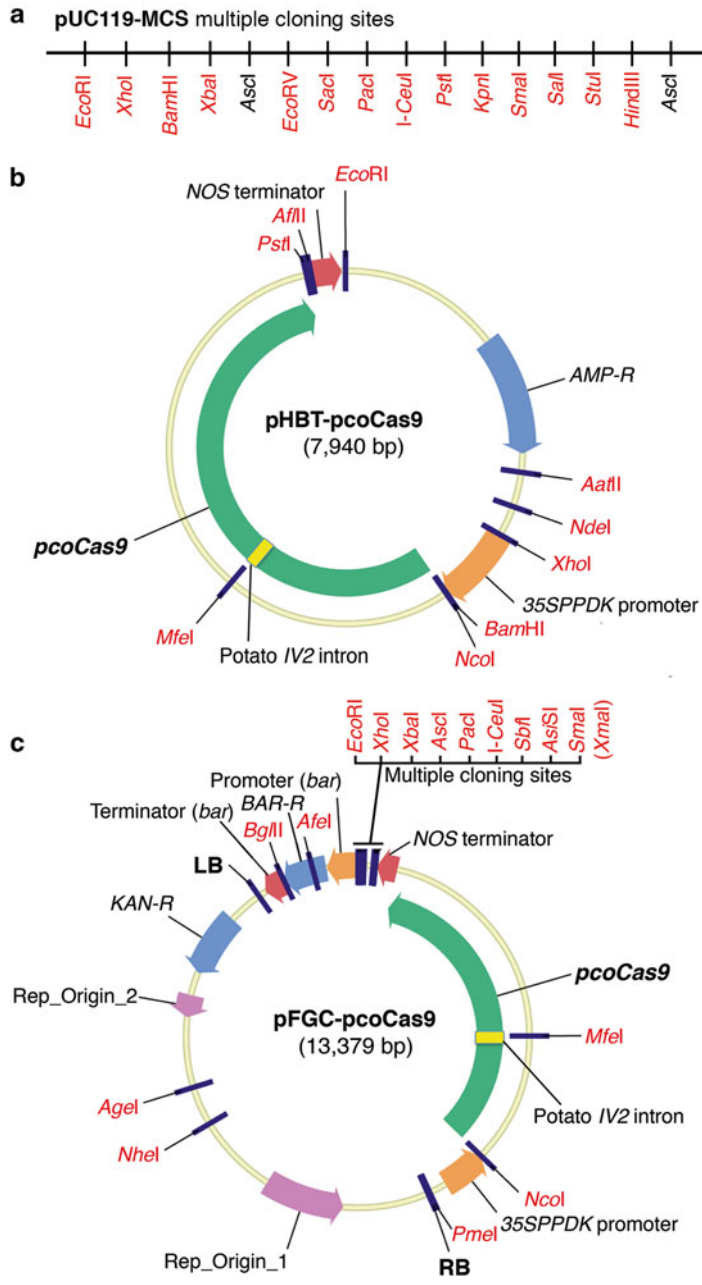


Fig. 2 Cloning maps for gRNA and *pcoCas9* expression vectors. (**a**) The multiple cloning sites in the *pUC119-MCS* vector for dual gRNA cloning. This plasmid confers ampicillin resistance to *E. coli*. (**b**) The *pHBT-pcoCas9* plasmid for protoplast-based transient expression of *pcoCas9*. This plasmid confers ampicillin resistance (AMP-R) to *E. coli*. (**c**) Binary plasmid *pFGC-pcoCas9* for *Agrobacterium*-mediated stable or transient expression of gRNAs and *pcoCas9*. This plasmid confers kanamycin resistance (KAN-R) to bacteria and Basta resistance (BAR-R) to plants. In (**a**)–(**c**), restriction sites in *red* are single-cut in the indicated plasmids. LB, T-DNA left border. RB, T-DNA right border

5. *pHBT-HA* vector: the plasmid is used for cloning and sequencing PCR products of the genomic target region, and is available from the authors upon request. Sequencing primer: 5′ GTCACGTAGTAAGCAGCTCTCGG 3′.

2.4 Solutions for Protoplast Isolation and Transfection

1. Cell wall digestion solution: 1 % (wt/vol) cellulase R10 (Yakult Pharmaceutical Ind. Co., Ltd., Japan), 0.2 % (wt/vol) macerozyme R10 (Yakult Pharmaceutical Ind. Co., Ltd., Japan), 0.4 M mannitol, 20 mM KCl, 20 mM MES, pH 5.7, 10 mM $CaCl_2$, and 0.1 % (wt/vol) BSA. Heat the enzyme solution including everything except $CaCl_2$ and BSA at 55 °C for 10 min to dissolve enzymes and to inactivate proteases from cellulase and macerozyme products. Cool down the solution to room temperature before adding $CaCl_2$ and BSA and filter the solution through a 0.45 μm filter. This solution should be freshly made before use.
2. W5 solution: 154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl, 2 mM MES, and pH 5.7.
3. MMg solution: 0.4 M mannitol, 15 mM $MgCl_2$, 4 mM MES, and pH 5.7.
4. WI solution: 0.5 M mannitol, 20 mM KCl, 4 mM MES, and pH 5.7.
5. PEG solution: 40 % (wt/vol) PEG4000 (Sigma 81240 or 95904), 0.2 M mannitol, and 100 mM $CaCl_2$. This solution should be freshly made before use.
6. Bovine calf serum (Sigma A7906).

2.5 Other Reagents

1. Phusion high-fidelity PCR master mix (New England BioLabs).
2. Restriction enzymes (New England BioLabs).
3. T4 DNA ligase master mix (New England BioLabs).
4. Ampicillin (Gold Biotechnology) 1,000× stock solution: 100 mg/mL in Milli-Q water.
5. Kanamycin (Gold Biotechnology) 1,000× stock solution: 50 mg/mL in Milli-Q water.
6. Alkaline phosphatase (Roche).
7. Plasmid DNA miniprep kit (Zymo Research).
8. PureLink HiPure plasmid maxiprep kit (Life Technologies).
9. Gel/PCR DNA purification kit (Qiagen).

2.6 Equipment

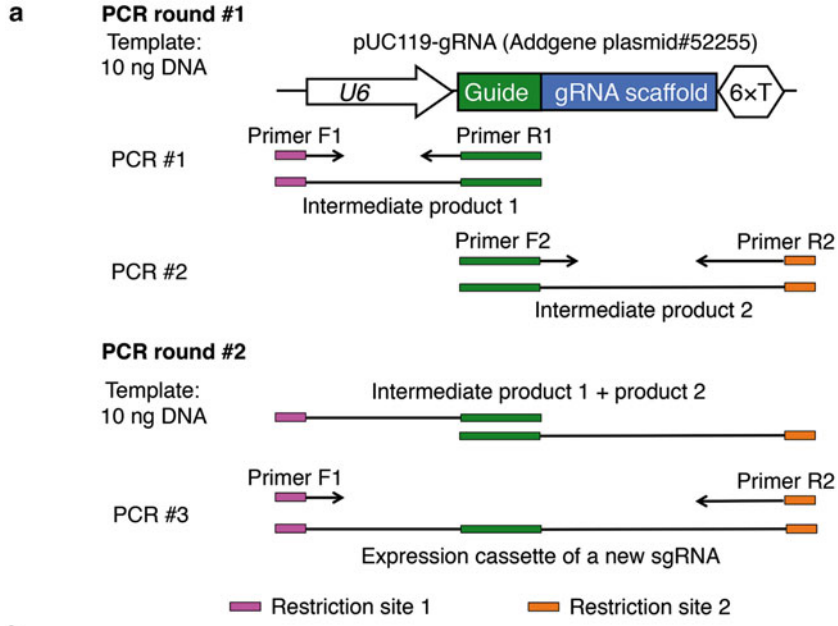
1. CL2 clinical centrifuge (Thermo Scientific).
2. Heating block (Fisher Scientific).
3. Microcentrifuge tubes, 1.5 mL (USA Scientific).
4. Round-bottom microcentrifuge tubes, 2 mL (USA Scientific).

5. Culture plates, six-well (Falcon).
6. Disposable inoculating loop (BD Biosciences).
7. Petri dish, 100 × 25 mm^2 (VWR Scientific).
8. Round-bottom tube, 30 mL (Sarstedt).
9. Hemocytometer (Hausser Scientific).
10. Razor blade (VWR Scientific).
11. Aluminum foil (Novelis).
12. Nylon filter, 70 μm pore size (Sefar Filtration Inc.).
13. Flasks, 2 L (Corning).

3 Methods

3.1 Designing and Constructing gRNAs

1. Select a pair of closely located gRNA targets in an *Arabidopsis* gene of interest (*see* **Note 1**) by referring to a preexisting database of *Arabidopsis* gene-specific gRNA targets [5] or a gRNA target list generated upon request by the CRISPR-Plant Web server ([7], www.genome.arizona.edu/crispr/CRISPRsearch.html) (*see* **Note 2**).
2. Design PCR primers according to Fig. 3 for PCR-based seamless assembly of new gRNA expression cassettes (*see* **Notes 3** and **4**).
3. Generate expression cassettes of gRNAs by an overlapping PCR strategy (Fig. 3a) (*see* **Note 5**) using Phusion high-fidelity DNA polymerase in a 50 μL reaction mixture according to the PCR conditions described in Fig. 3c. Two different products of PCR round #1 were gel-purified and 5 ng of each product were combined as templates for PCR round #2.
4. Insert one gRNA expression cassette into the multiple cloning sites of *pUC119-MCS* vector to obtain the *pUC119-one-gRNA* plasmid by restriction digestion of both the vector and gel-purified product of PCR round #2 (Fig. 3a) with the same restriction enzyme(s) and subsequent ligation.
5. Transform *E. coli* and the next day inoculate a few single colonies from ampicillin-containing LB solid medium for plasmid miniprep using a commercial plasmid miniprep kit or home-made silica resins as previously described [8].
6. Verify sequence accuracy of the cloned gRNA expression cassette by Sanger sequencing. Sequencing primer F (sequencing from *Eco*RI toward *Hin*dIII in *pUC119-MCS*) is 5′ ATTAAGTTGG GTAACGCC 3′ and primer R (sequencing from *Hin*dIII toward *Eco*RI) is 5′ TGGAATTGTGAGCGGATA 3′.
7. Insert a second gRNA expression cassette into the multiple cloning sites of the *pUC119-one-gRNA* plasmid to obtain the



b

Primer name	Primer sequence (5' to 3') for a gRNA targeting to N_{20}NGG
F1	XXX + restriction site 1 + AGAAATCTCAAAATTCCG
R1	Reverse complement of N_{20} + AATCACTACTTCGTCTCT 3' (if N_1 = G)
	Reverse complement of N_{20} + CAATCACTACTTCGTCTCT 3' (if N_1 = C, A, T)
F2	N_{20} + GTTTTAGAGCTAGAAATAGC
R2	XXX + restriction site 2 + AAAAAAGCACCGACTCGGTGC

XXX: Additional nucleotides to ensure efficient restriction digestion of PCR products

c

PCR conditions for Phusion high-fidelity DNA polymerase

98°C	30 sec	
98°C	10 sec	10-30 cycles
55°C	30 sec	
72°C	30 sec	
72°C	5 min	

Fig. 3 Seamless assembly of gRNA expression cassette through overlapping PCR. (**a**) Diagram of the overlapping PCR strategy. (**b**) Primer design instruction. Primer names and colors are consistent with those in (**a**). Note that, when N_1 of the target sequence is C, A, or T, an additional "C" (*red*) was introduced into the primer R1 to generate a "G" upstream of N_1 during PCR #1 to optimize transcription initiation by the *Arabidopsis U6-1* promoter. (**c**) PCR conditions used for Phusion high-fidelity DNA polymerase. Cycle numbers depend on the quantity of template DNA used in PCR

pUC119-dual-gRNA plasmid by restriction digestion of both the *pUC119-one-gRNA* plasmid and gel-purified final PCR products (Fig. 3a) with the same restriction enzyme(s) and subsequent ligation (*see* **Note 6**).

8. Transform *E. coli* and the next day inoculate a few single colonies on ampicillin-containing LB solid medium for plasmid miniprep.

9. Verify sequence accuracy of the latter gRNA expression cassette in the *pUC119-dual-gRNA* plasmid by Sanger sequencing using primers described in **step 6**.
10. Keep the *E. coli* strain with the *pUC119-dual-gRNA* plasmid obtained in **step 8** on LB agar plates supplemented with 100 μg/mL of ampicillin or in frozen 20 % glycerol stock for plasmid DNA preparation.
11. Inoculate *E. coli* cells with the *pUC119-dual-gRNA* plasmid or *pHBT-pcoCas9* into one 2-L flask containing 200 mL of Terrific broth with ampicillin using a sterile disposable inoculating loop and shake the flask vigorously at 37 °C for 8–12 h.
12. Harvest bacterial cells and purify individual plasmid DNA (*see* **Note 7**).

3.2 Growing Arabidopsis and Tobacco Plants

1. Wet Jiffy-7 peat soil pellets (*see* **Note 8**) or Fafard soil thoroughly with water and drain excess water.
2. For even germination, conduct stratification of *Arabidopsis* seeds at 4 °C for 2 days.
3. For *Arabidopsis*, sow 3–4 seeds onto the top of each swollen Jiffy-7 pellet. For tobacco, sow the seeds onto the Fafard soil surface.
4. Grow *Arabidopsis* and tobacco plants under the conditions of 60 % humidity, 75 μmol/m^2/s light intensity, and photoperiods of 12 h light at 23 °C and 12 h dark at 20 °C.
5. Within 1 week, keep only one healthy *Arabidopsis* seedling on each Jiffy-7 pellet or one healthy tobacco seedling in each Fafard pot by removing other seedlings.
6. After 4 weeks, select well-expanded leaves (Fig. 1b) in *Arabidopsis* and tobacco plants for protoplast isolation.

3.3 Isolating Protoplasts from Arabidopsis or Tobacco Plants

1. Detach 4–6 well-expanded leaves from 4-week-old *Arabidopsis* or tobacco plants with a razor blade and pile the leaves on several layers of white paper on the lab bench.
2. Cut the middle section of leaves horizontally into 1 mm strips with a clean razor blade.
3. Use a sterile disposable inoculating loop to help submerge leaf strips into 10 mL of cell wall digestion solution in a 100×25 mm^2 petri dish and incubate the digestion mixture in the dark at room temperature (23–25 °C) for 3 h without shaking.
4. Shake the digestion mixture on a horizontal shaker at 60 rpm for 3 min to facilitate protoplast release (*see* **Note 9**).
5. Add 10 mL of W5 solution to the digestion mixture and mix well.

6. Filter the slurry through a nylon filter (pore size: 70 μm) and collect the flow-through into a 30-mL round-bottom tube or 50-mL Falcon tube.
7. Centrifuge the flow-through at 100 × *g* for 2 min using a CL2 swing-bucket clinical centrifuge and remove the supernatant by pipetting.
8. Add 10 mL of W5 solution to gently resuspend the protoplast pellet by swirling the tube and rest the slurry on ice for at least 30 min.
9. Centrifuge the slurry at 100 × *g* for 30 s using a CL2 swing-bucket clinical centrifuge and remove the supernatant by pipetting.
10. Add 2 mL of MMg solution at room temperature to gently resuspend the protoplast pellet and take 9 μL of protoplast resuspension for cell counting under a microscope using a hemocytometer.
11. Dilute protoplasts with additional MMg solution to a final density of 2×10^5 cells/mL and keep the protoplasts at room temperature.

3.4 Transfecting and Expressing Cas9/gRNAs in Protoplasts

1. Mix 10 μL of the *pHBT-pcoCas9* plasmid (2 μg/μL) and 10 μL of the *pUC119-dual-gRNA* plasmid (2 μg/μL) in a 2 mL round-bottom microcentrifuge tube (*see* **Note 10**).
2. Add 200 μL of protoplasts (40,000 cells) to the microcentrifuge tube containing the DNA cocktail.
3. Add 220 μL of PEG solution and gently tap the bottom of the tube a few times to completely mix DNA, protoplasts, and PEG solution.
4. Incubate the transfection mixture at room temperature for 5 min.
5. Stop transfection by gently adding 800 μL of W5 solution to the tube and inverting the tube twice.
6. Centrifuge the tube at 100 × *g* for 2 min using a CL2 swing-bucket clinical centrifuge and pipet most of the supernatant away without disturbing the protoplast pellet (*see* **Note 11**).
7. Add 100 μL of W5 solution to resuspend the protoplasts.
8. Coat a six-well culture plate by adding and subsequently removing 1 mL of 5 % bovine calf serum and then add 1 mL of WI solution to each well (*see* **Note 12**).
9. Transfer transfected protoplasts into one well of the 6-well plate and mix well with the WI solution.
10. Incubate transfected protoplasts at room temperature for 36 h in the dark by covering the plate with aluminum foil.

3.5 Evaluating the Frequency of Targeted Genome Modifications

1. Design and synthesize a pair of genomic PCR primers (PCR FP and PCR RP, Fig. 1b) for amplifying a ~300 bp genomic region covering the two gRNA target sites in the target gene and introduce a *Bam*HI site and a *Stu*I site into the forward primer and the reverse primer, respectively (*see* **Note 13**).
2. Transfer protoplasts from the six-well plate to a 1.5 mL microcentrifuge tube and harvest protoplasts by centrifugation at 100 × *g* for 2 min using a CL2 swing-bucket clinical centrifuge and subsequent removal of the supernatant by careful pipetting.
3. Freeze protoplasts immediately in liquid nitrogen.
4. Add 50 μL of sterile water to resuspend protoplasts by vortexing.
5. Heat the resuspended protoplasts using a heating block at 95 °C for 10 min.
6. Take 2 μL of heated protoplast suspension as the PCR template to amplify the genomic target region in a 50 μL reaction using Phusion high-fidelity DNA polymerase and the PCR conditions (30 cycles) described in Fig. 3c.
7. Purify PCR products corresponding to the expected genomic amplicons using a commercial gel purification kit or homemade silica resins [8] and digest the PCR products with 2 units of *Bam*HI/*Stu*I restriction enzymes in a 50 μL reaction mixture with the NEB buffer 2 at 37 °C for 1 h.
8. Digest 300 ng of the *pHBT-HA* vector with 2 units of *Bam*HI/*Stu*I restriction enzymes in a 50 μL reaction mixture with the NEB buffer 2 at 37 °C for 1 h and add 10 units of alkaline phosphatase to the digestion mixture and incubate at 37 °C for another 1 h (*see* **Note 14**).
9. Purify *Bam*HI/*Stu*I digested genomic PCR products and *pHBT-HA* vector using a commercial DNA purification kit or homemade silica resins [8] and ligate two DNA fragments in an approximate molar ratio of insert–vector as 3:1 in a 10 μL reaction mixture using T4 ligase master mix according to the manufacturer's instructions (*see* **Note 15**).
10. Transform TOP10 chemically competent *E. coli* with 5 μL of ligation mixture and the next day randomly select 20–30 single colonies from ampicillin-containing LB solid medium for plasmid miniprep.
11. Conduct Sanger sequencing for plasmids extracted from individual colonies using an appropriate sequencing primer (e.g., for *pHBT-HA* vector, sequencing primer is 5′ GTCACGTAGTAAGCAGCTCTCGG 3′).

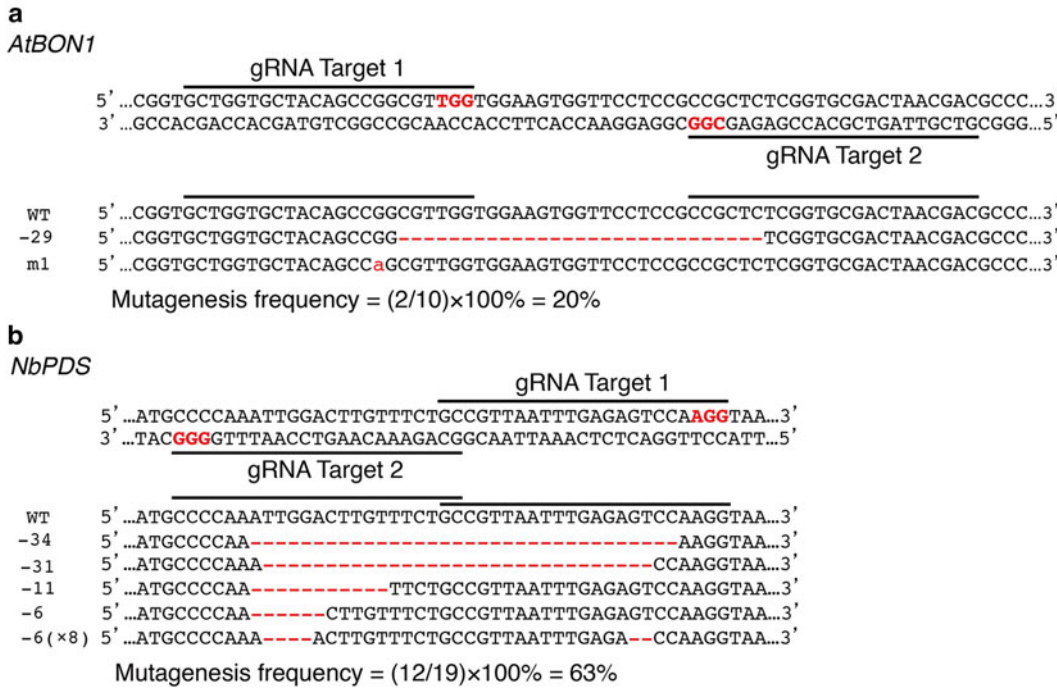


Fig. 4 Representative results of gRNA/Cas9-mediated genome editing in protoplasts. (**a**) Dual gRNA-induced mutagenesis in the *AtBON1* gene in *Arabidopsis* protoplasts. (**b**) Dual gRNA-induced mutagenesis in the *NbPDS* gene in tobacco protoplasts. The *black line* marks each target sequence, in which the protospacer adjacent motif "NGG" is highlighted in *red* and *bold*. Nucleotide deletions and substitutions are shown in *red* as *dashes* and *lower case letters*, respectively

12. Visualize genome modifications in the target sequence by aligning DNA sequencing results to the native genomic target sequence (Fig. 4).
13. Calculate genome modification frequency using the following formula: genome modification frequency = (number of mutant colonies/number of total sequenced colonies) × 100 %.

3.6 Generating Targeted Genome Modifications in Whole Plants

After evaluation of the editing efficacy mediated by several different pairs of gRNAs for the target gene of interest in *Arabidopsis* protoplasts, the most efficient gRNA pair can be further used for generating targeted modifications in the target gene in *Arabidopsis* plants to obtain inheritable mutations. A commonly used strategy is to clone the *Cas9* and gRNA expression cassettes into a single binary vector and then generate transgenic *Arabidopsis* plants stably expressing *Cas9* and two gRNAs using the *Agrobacterium*-mediated floral-dip transformation method. The T1 transgenic *Arabidopsis* will express Cas9 and two gRNAs to facilitate mutagenesis in the target gene predominantly in somatic cells and occasionally in shoot apical meristem cells and germ line cells, and the latter can eventually lead to heritable homozygous mutations in

the target gene in some of the T2 transgenic *Arabidopsis*, as reported very recently [9, 10]. A DNA repair donor with homology to the target region can also be co-delivered into transgenic *Arabidopsis* via the same binary plasmid [11] to facilitate homologous recombination-mediated genome modifications in transgenic *Arabidopsis*. Major limitations of such a strategy are: (1) the entire procedure to generate and screen targeted homozygous mutations is time- and labor-consuming; (2) permanent integration of *Cas9* and gRNA expression cassettes into the *Arabidopsis* genome and constant production of these genome editing reagents, even after the generation of intended target mutagenesis, is not desirable and may increase risk of off targets.

A potentially more straightforward but technically more demanding strategy is to convert *Arabidopsis* protoplasts harboring targeted genome modifications (Fig. 4a) into plants through protoplast regeneration [12]. Attractive advantages of this strategy are: (1) homozygous mutations in the target gene may be obtained within one generation; (2) *Cas9*, gRNA and DNA repair donor constructs can be easily and efficiently delivered and expressed; (3) there should be no integration of any foreign DNA into the *Arabidopsis* genome. However, protoplast regeneration for *Arabidopsis* ecotype Columbia-0 remains technically challenging [12].

For tobacco, targeted genome modification in whole plants can be achieved through transient expression of *Cas9* and gRNAs from a single binary plasmid via *Agrobacterium*-mediated leaf infiltration and a subsequent tissue regeneration procedure [13]. A DNA repair donor can also be co-delivered into tobacco leaf cells through the same binary plasmid [14] to facilitate homologous recombination-mediated genome modifications in tobacco plants.

To simplify the assembly of a single binary plasmid for co-expressing *Cas9* and gRNAs and co-delivering a DNA repair donor, we generated the binary plasmid *pFGC-pcoCas9* (Fig. 2b, Addgene plasmid #52256), which contains a *35SPPDK* promoter-driven *pcoCas9* expression cassette and multiple cloning sites to accommodate multiple gRNA expression cassettes and the DNA repair donor. Moreover, after the initial *Cas9*/gRNA evaluation in protoplasts, preassembled expression cassettes of the most efficient gRNA pair between the two *Asc*I sites in the *pUC119-MCS* vector can be directly transferred into the *Asc*I site of the *pFGC-pcoCas9* plasmid through one-step cloning (Fig. 2a, c).

4 Notes

1. Although targeting an *Arabidopsis* gene with a single gRNA may be sufficient in triggering loss-of-function mutagenesis in some cases, we generally recommend using two closely targeting gRNAs (i.e., target sites with a spacer less than 30 bp)

for a single gene to trigger efficient genomic deletion to ensure the disruption of target gene function. Despite the databases for genome-wide gRNA candidate prediction [5, 7], different gRNAs targeting to the same gene may work with variable efficiency due to many unknown factors. It is suggested to evaluate 3–4 pairs of gRNAs targeting the 5′ half of the coding region of each gene to ensure the disruption of functional protein products using the simple and rapid protoplast transient expression system. An optimal pair of gRNAs can be rapidly identified within a week for the target gene prior to the time- and labor-consuming endeavor of generating plants with CRISPR/Cas9 to obtain inheritable mutations. For targeted homologous recombination, we recommend the use of a single gRNA whose target sequence is overlapping with or closest to the intended genomic modification site to reduce mutagenesis via NHEJ DNA repair.

2. The priority in gRNA target selection should be given to the 5′ exons of target gene because mutagenesis in 3′ exons or all the introns may not lead to null mutations. There is currently no database or Web server to aid the prediction for gene-specific gRNA target sites in *N. benthamiana*. Genomic N_{20}NGG sequences can be manually identified from a tobacco gene of interest as the gRNA target sites based on the draft genome sequence for *N. benthamiana* (http://solgenomics.net/organism/Nicotiana_benthamiana/genome).
3. The RNA polymerase III promoter (e.g., the *Arabidopsis U6-1* promoter [6] or *U6-26* promoter [9, 10]) is required to drive gRNA transcription to ensure the generation of precise gRNA sequence without the polyA tail to facilitate nuclear retention and genomic target recognition. Optimal transcription by the *Arabidopsis U6* promoter is initiated with "G". Therefore, if the selected gRNA target sequence (N_{20}NGG) is not initiated with "G" (N_1 as "C," "A," or "T"), an additional "G" can be introduced at the junction between the *Arabidopsis U6-1* promoter and the guide sequence through the primer R1 (Fig. 3b) to enhance transcription.
4. Restriction sites of *Sac*I, *Pac*I, *Pst*I, *Kpn*I, *Sma*I, or *Hin*dIII are highly recommended to be used in the primers F1 and R2 because: (1) they are present in the *pUC119-MCS* vector as cloning sites flanked by two *Asc*I sites, so stacked gRNA expression cassettes in the *pUC119-MCS* vector through these sites can be easily subcloned into the binary plasmid *pFGC-pcoCas9* through *Asc*I digestion and insertion (Fig. 2a, c); (2) they are readily digestible when located at the ends of PCR products. In any case, additional nucleotides (as "XXX" in Fig. 3b) should be added at the 5′ of restriction sites in primers F1 and R2 to facilitate restriction digestion of resultant PCR products

during cloning. Avoid using *Stu*I in primer F1 or R2 because the *Arabidopsis U6-1* promoter contains an internal *Stu*I site.

5. A gRNA expression cassette from the *Arabidopsis U6-1* promoter to the TTTTTT terminator flanked by desired restriction sites can also be synthesized as a gBlocks Gene Fragment at Integrated DNA Technologies (www.idtdna.com), but this option takes longer time and is more expensive. A simplified gRNA expression cassette based on the *U6-26* promoter and dual BbsI cloning strategy is now available from Fauser et al. [10].
6. One can also clone individual gRNA expression cassettes into the *pUC119-MCS* vector to obtain separate gRNA expression plasmids and then achieve gRNA co-expression by protoplast co-transfection with two different gRNA expression plasmids. However, cloning a pair of gRNA expression cassettes into the same *pUC119-MCS* vector ensures even co-expression of two gRNAs in transfected protoplasts.
7. High-quality and concentrated (2 μg/μL) plasmid DNA is key for high protoplast transfection efficiency. It is highly recommended to use CsCl gradient ultracentrifugation method to purify plasmid DNA by following the protocol on the Sheen laboratory website (http://molbio.mgh.harvard.edu/sheenweb/protocols_reg.html). Plasmid DNA purified by homemade silica resin [8] or by commercial DNA maxiprep kits is acceptable but may lead to slightly lower protoplast transfection efficiency.
8. Jiffy-7 peat soil pellet can swell to its full size after soaking up water.
9. Tobacco leaves release mesophyll protoplasts more readily than *Arabidopsis* leaves after the same treatment with the cell wall digestion solution.
10. In the case of obtaining targeted homologous recombination in protoplasts, 20 μL of DNA transfection cocktail is composed of 8 μL of the *pHBT-pcoCas9* plasmid (2 μg/μL), 8 μL of the *pUC119-one-gRNA* plasmid (2 μg/μL) and 4 μL of DNA repair template (~2 μg/μL), which can be double-stranded DNA (e.g., PCR products) containing a desired mutation flanked by two homology arms, each with at least 100 bp identical to the genomic target region. Longer homology arms are likely to enhance the efficiency of homologous recombination.
11. After centrifugation, transfected tobacco protoplasts are not pelleted as tightly as the *Arabidopsis* protoplasts, so removal of the supernatant should be conducted with caution and ~30 μL supernatant can be kept in the tube so that the pellet is not disturbed.

12. Coating the plates with 5 % calf serum can substantially prevent protoplasts from attaching to the bottom of the culture plate. This step can also be done before protoplast transfection.
13. Design of genomic PCR amplicons with sizes around 300 bp allows efficient PCR amplification using crudely prepared genomic DNA as template and makes PCR products clearly distinguishable from possible primer dimers. In addition, keeping the PCR amplicons short minimizes the possibility of PCR-introduced DNA mutagenesis.
14. Alkaline phosphatase is active in all NEB restriction digestion buffer systems and its treatment significantly reduces the background *E. coli* transformants resulting from vector self-ligation.
15. One can also clone the genomic PCR products into any other cloning vector available in his/her lab via appropriate restriction sites for Sanger sequencing.

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