CHAPTER TWENTY-ONE

Cas9-Based Genome Editing in *Arabidopsis* and Tobacco

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Abstract

Targeted modification of plant genome is key to elucidating and manipulating gene functions in plant research and biotechnology. The clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein (Cas) technology is emerging as a powerful genome-editing method in diverse plants that traditionally lacked facile and versatile tools for targeted genetic engineering. This technology utilizes easily reprogrammable guide RNAs (sgRNAs) to direct *Streptococcus pyogenes* Cas9 endonuclease to generate DNA double-stranded breaks in targeted genome sequences, which facilitates efficient mutagenesis by error-prone nonhomologous end-joining (NHEJ) or sequence replacement by homology-directed repair (HDR). In this chapter, we describe the procedure to design and evaluate dual sgRNAs for plant codon-optimized Cas9-mediated genome editing using mesophyll protoplasts as model cell systems in *Arabidopsis thaliana* and *Nicotiana benthamiana*. We also discuss future directions in sgRNA/Cas9 applications for generating targeted genome modifications and gene regulations in plants.

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1. INTRODUCTION

The CRISPR/Cas9 technology is derived from the bacterial type-II CRISPR/Cas adaptive immune system (Jinek et al., 2012). The technology uses a single chimeric guide RNA (sgRNA) containing a 20-nt guide sequence to direct coexpressed *Streptococcus pyogenes* Cas9 endonuclease to an intended genomic N_{20}NGG sequence through base pairing. Two separate nuclease domains of Cas9 each cleave one DNA strand to generate a DSB in the targeted sequence. During the DSB repair, site-specific gene mutagenesis or replacement can be obtained via the NHEJ pathway or homologous recombination pathway, the later depending on the availability of a DNA repair template (Cong et al., 2013; Li et al., 2013; Mali et al., 2013). Among the designer nucleases for genome editing, the CRISPR/Cas9 system exhibits unparalleled simplicity and multiplexibility in genome editing because sgRNAs can be easily modified to achieve new DNA binding specificities and multiple sgRNAs can work simultaneously with the same Cas9 nuclease on many different target sites (Gaj, Gersbach, & Barbas, 2013; Li et al., 2013; Sander & Joung, 2014).

Effective delivery of genome-editing reagents, including Cas9 nucleases, sgRNAs, 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 describe the detailed procedure for designing and evaluating constructs using the CRISPR/Cas9 system for genome editing in *Arabidopsis thaliana* and tobacco (*Nicotiana benthamiana*) mesophyll protoplasts (Fig. 21.1), which support highly efficient DNA transfection and RNA and protein expression (Li, Zhang, & Sheen, 2014; Yoo, Cho, & Sheen, 2007). The procedure is potentially adaptable to diverse plant species that are amenable to protoplast isolation and transfection (Li et al., 2014). Plant protoplasts offer a valuable system for rapidly evaluating the performance of a given combination of sgRNA and Cas9 at the genomic target site. To enhance the rate of generating null mutations, dual sgRNAs are designed and evaluated. We discuss promising strategies to apply the CRISPR/Cas system for generating targeted and inheritable genome modifications in plants. The CRISPR/Cas system has the potential to generate loss-of-function mutations or desirable modifications and regulations in virtually any plant genes and sequences to elucidate their functions and regulatory mechanisms. The new technologies also offer powerful genetic engineering tools to inactivate or modify desired plant genes and traits for agricultural improvement.

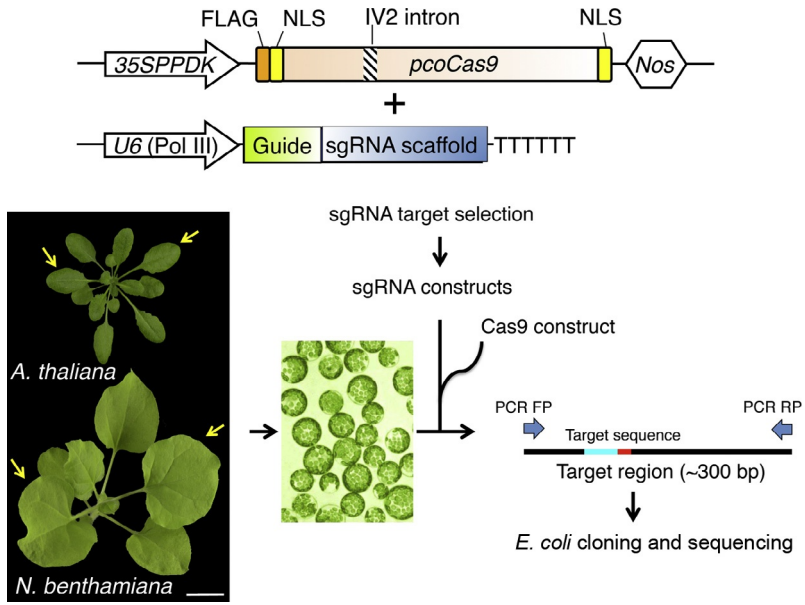

Figure 21.1 Unbiased sgRNA/Cas9-mediated genome editing in plant protoplasts. The expression cassettes of Cas9 and sgRNA are shown. Plant codon-optimized Cas9 (pcoCas9) is fused to dual nuclear localization sequences (NLSs) and FLAG tags. The constitutive *35SPPDK* promoter and the *Arabidopsis U6-1* promoter were used to express pcoCas9 and sgRNA, respectively, in protoplasts. NGG, the protospacer adjacent motif (PAM), in the target sequence is highlighted in red. The diagram illustrates the key procedure to generate and evaluate Cas9/sgRNA-mediated genome editing in *Arabidopsis* and tobacco protoplasts. Yellow arrows indicate the leaves at optimal developmental stage for protoplast isolation from 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. Genomic DNA from protoplasts was PCR amplified and cloned into a sequencing vector. *E. coli* colonies were picked randomly for PCR amplification and sequencing.

2. Cas9 AND sgRNA EXPRESSION

1. *p35SPPDK-pcoCas9*: a plant transient expression plasmid for expressing the plant codon-optimized *Streptococcus pyogenes Cas9* (*pcoCas9*) gene (Li et al., 2013) under the constitutive and strong hybrid *35SPPDK* promoter (Fig. 21.2A). This hybrid plant promoter (Sheen, 1993) and potentially the potato IV2 intron alleviated problems associated with cloning of the *pcoCas9* coding sequence in *Escherichia coli*. This plasmid is available at Addgene (www.addgene.org; Plasmid #52254).

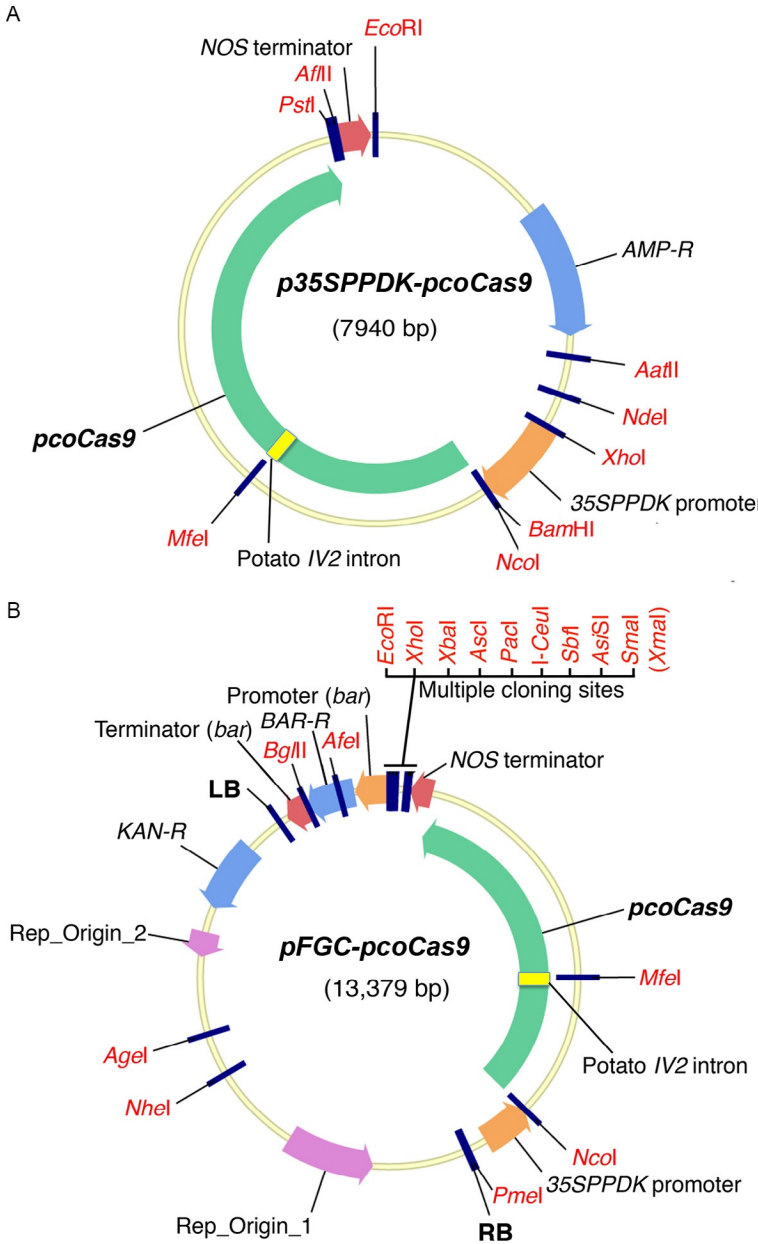

Figure 21.2 Expression plasmid maps. (A) *p35SPPDK-pcoCas9* plasmid for protoplast transient expression. (B) Binary plasmid *pFGC-pcoCas9* for *Agrobacterium*-mediated stable or transient expression analyses.

2. *pUC119-sgRNA*: the plasmid serves as the PCR template to assemble the expression cassette of a new sgRNA with desired DNA targeting specificity. It harbors the *Arabidopsis U6-1* promoter (Li et al., 2007; Waibel & Filipowicz, 1990), an RNA polymerase III promoter required for sgRNA expression, a sgRNA targeting to the *Arabidopsis PDS3* gene (target site: 5′ GGACTTTTGCCAGCCATGGT**CGG** 3′), and a "TTTTTT" transcription terminator (Li et al., 2013). This plasmid is available at Addgene (Plasmid #52255).
3. *pFGC-pcoCas9*: a binary plasmid expressing *pcoCas9* under the *35SPPDK* promoter and containing multiple cloning sites (MCSs) for inserting single or multiple sgRNA expression cassettes (Fig. 21.2B). This plasmid is designed for *Agrobacterium*-mediated DNA delivery to the plant nuclei and available at Addgene (Plasmid #52256). Sequencing primer (sequencing from *Eco*RI toward *Sma*I): 5′ AATAAAAACTG ACTCGGA 3′.

3. DUAL sgRNA-GUIDED GENOME EDITING

3.1. Designing and constructing dual sgRNAs

1. Select a pair of closely located sgRNA targets in an *Arabidopsis* gene of interest (see *Note 1*) by referring to a preexisting database of *Arabidopsis* gene-specific sgRNA targets (Li et al., 2013) or a sgRNA target list generated upon request via the CRISPR-Plant web server (Xie, Zhang, & Yang, 2014, www.genome.arizona.edu/crispr/CRISPRsearch.htmL; see *Note 2*).
2. Design PCR primers for PCR-based seamLess assembly of new sgRNA expression cassettes (Li et al., 2013; see *Notes 3 and 4*).
3. Generate expression cassettes of sgRNAs, including the *U6-1* promoter, sgRNA, and the terminator, by an overlapping PCR strategy (see *Note 5*) using Phusion high-fidelity DNA polymerase (Li et al., 2013).
4. Insert one sgRNA expression cassette into any MCSs of a vector (e.g., *pUC119-MCS*, Addgene Plasmid #58807) to obtain the *pUC119-one-sgRNA* plasmid by restriction digestion of both the vector and the final PCR products with the same restriction enzyme(s) and subsequent ligation. The MCS are EcoRI, XhoI, BamHI, XbaI, AscI, EcoRV, SacI, PacI, I-CeuI, PstI, KpnI, SmaI, SalI, StuI, HindIII, and AscI.
5. Transform *E. coli* and inoculate a few single colonies from ampicillin-containing LB solid medium for plasmid miniprep.

6. Verify sequence accuracy of the cloned sgRNA expression cassette by Sanger sequencing.
7. Insert a second sgRNA expression cassette into the MCSs of the *pUC119-one-sgRNA* plasmid to obtain the *pUC119-dual-sgRNA* plasmid by restriction digestion and subsequent ligation (see *Note 6*).
8. Transform *E. coli* and inoculate a few single colonies on ampicillin-containing LB solid medium for plasmid miniprep.
9. Verify sequence accuracy of the second sgRNA expression cassettes in the *pUC119-dual-sgRNA* plasmid by Sanger sequencing.
10. To obtain high plasmid DNA yield, retransform *E. coli* with the *pUC119-dual-sgRNA* plasmid and the *p35SPPDK-pcoCas9* plasmid (Addgene plasmid #52254), respectively.
11. Scrape off overnight grown bacteria from the ampicillin-containing LB plate into 200 mL of Terrific broth with ampicillin using a sterile disposable inoculating loop and shake the culture vigorously at 37 °C for 8 h.
12. Maxiprep the plasmid DNA of both constructs (see *Note 7*).

3.2. Transfecting and expressing Cas9/sgRNAs in protoplasts

1. Mix 10 μL of the *p35PPDK-pcoCas9* plasmid (2 μg/μL) and 10 μL of the *pUC119-dual-sgRNA* plasmid (2 μg/μL) in a 2-mL round-bottom microcentrifuge tube (see *Note 8*).
2. Add 200 μL of protoplasts (40,000 cells) to the microcentrifuge tube containing the DNA cocktail. *Arabidopsis* and tobacco mesophyll protoplasts are isolated by the established protocol (Yoo et al., 2007).
3. Add 220 μL of PEG4000 solution (40% PEG4000, v/v, 0.2 *M* mannitol, 100 m*M* $CaCl_2$; Yoo et al., 2007) 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 (154 m*M* NaCl, 125 m*M* $CaCl_2$, 5 m*M* KCl, 2 m*M* MES, pH 5.7; Yoo et al., 2007) to the tube and inverting the tube twice.
6. Centrifuge the tubes at 100 × *g* for 2 min using a CL2 clinical centrifuge and remove the supernatant without disturbing the protoplast pellet (see *Note 9*).
7. Add 100 μL of W5 solution to resuspend the protoplasts.
8. Coat a 6-well culture plate with 5% bovine calf serum, remove the serum and add 1 mL of W5 or WI solution (0.5 *M* mannitol, 4 m*M* MES, pH 5.7, 20 m*M* KCl; Yoo et al., 2007) to each well.

9. Transfer transfected protoplasts to one well of the 6-well plate and mix well with the W5 or WI solution.
10. Incubate transfected protoplasts in the dark at 23–25 °C up to 36 h by covering the plate with aluminum foil.

3.3. Evaluating the frequency of targeted genome modifications

1. Design and synthesize a pair of genomic PCR primers (PCR FP and PCR RP, Fig. 21.1) for amplifying a ~300-bp genomic region covering the two sgRNA target sites in the target gene and introduce restriction sites into the forward primer and the reverse primer, respectively (see *Note 10*).
2. Transfer protoplasts from the 6-well plate to a 1.5 mL microcentrifuge tube and harvest protoplasts by centrifugation at 100 × *g* for 2 min using a CL2 clinical centrifuge and subsequent removal of the supernatant.
3. Freeze protoplasts immediately in liquid nitrogen.
4. Add 50 μL of sterile water to resuspend protoplasts by vortexing.
5. Heat resuspended protoplasts 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 volume using Phusion high-fidelity DNA polymerase.
7. Purify PCR products corresponding to the expected genomic amplicons and digest the PCR products with restriction enzymes at 37 °C for 1–3 h before cloning into any sequencing vector.
8. Transform *E. coli* and the next day randomly select 20–30 single colonies from ampicillin-containing LB solid medium for plasmid miniprep.
9. Conduct Sanger sequencing for plasmids extracted from individual colonies.
10. Visualize genome modifications in the target sequence by aligning DNA sequencing results to the native genomic target sequence (Fig. 21.3).
11. Calculate genome modification frequency using the following formula: genome modification frequency = (number of mutant colonies/number of total sequenced colonies) × 100%.
12. After evaluation of the editing efficacy mediated by several different pairs of sgRNAs for the target gene of interest in *Arabidopsis* and tobacco protoplasts, the most efficient sgRNA pair can be further used for generating targeted modifications in the desired genes in *Arabidopsis* and tobacco plants to obtain inheritable mutations (Fauser, Schiml, &

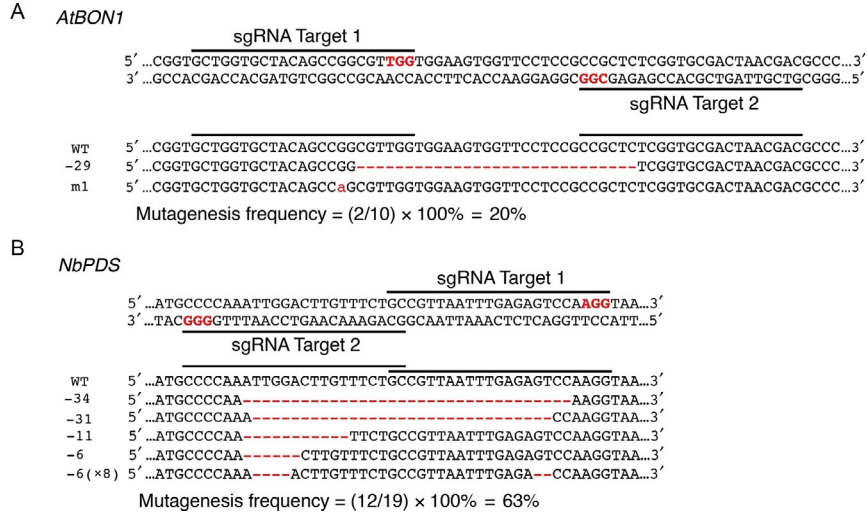

Figure 21.3 Representative results of dual sgRNA/Cas9-mediated genome editing in protoplasts. Dual sgRNA-induced mutagenesis in the *AtBON1* and *NbPDS* genes in *Arabidopsis* and tobacco protoplasts, respectively. A black line marks each target sequence in the *AtBON1* and *NbPDS* genes. The protospacer adjacent motif "NGG" is in red (gray in the print version). Nucleotide deletions and substitution are shown in red (gray in the print version) as dashes and lower case letter, respectively.

Puchta, 2014; Feng et al., 2014; Nekrasov, Staskawwicz, Weigel, Jones, & Kamoun, 2013). A commonly used strategy is to clone the Cas9 and sgRNA expression cassettes into a single binary vector and then generate transgenic *Arabidopsis* plants stably expressing Cas9 and two sgRNAs using the *Agrobacterium*-mediated floral-dip transformation method (Fauser et al., 2014; Feng et al., 2014). The T1 transgenic *Arabidopsis* will express Cas9 and two sgRNAs 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* (Fauser et al., 2014; Feng et al., 2014). A DNA repair donor with homology to the target region can also be codelivered into transgenic *Arabidopsis* via the same binary plasmid (De Pater, Pinas, Hooykaas, & van der Zaal, 2013) to facilitate homologous recombination-mediated genome modifications in transgenic *Arabidopsis*. Currently, the entire procedure to generate and screen targeted homozygous mutatants is time and labor consuming. Integration of Cas9 and sgRNA expression cassettes into the *Arabidopsis*

genome and constant production of these genome-editing reagents, even after the generation of intended site-specific mutagenesis, may increase risk of off targets but could be genetically segregated.

4. PERSPECTIVES

Rapid advances in less than a year have demonstrated that the CRISPR/Cas9 technology is applicable in protoplasts, callus tissues and intact plants in diverse plant species (Baltes, Gil-Humanes, Cermak, Atkins, & Voytas, 2014; Fauser et al., 2014; Feng et al., 2014; Li et al., 2013; Miao et al., 2013; Nekrasov et al., 2013; Shan et al., 2013; Sugano et al., 2014; Xie et al., 2014). It is conceivable that the new genetic engineering tools could be established in all plant species amenable to transient or stable gene expression manipulations. The available data suggest that mutagenesis rates appear to be much higher in tobacco and rice protoplasts with higher deletion events than in *Arabidopsis* protoplasts using similar pcoCas9 and sgRNA designs (Li et al., 2013; Shan et al., 2013). Although homozygous mutants have been obtained in transgenic *Arabidopsis* plants (Fauser et al., 2014; Feng et al., 2014), it is possible to further enhance the mutagenesis rates using dual sgRNAs demonstrated here (20% in *Arabidopsis* and 63% in tobacco; Fig. 21.3; Li et al., 2013). Manipulation of DNA repair pathways (Qi et al., 2013) and the introduction of geminivirus-based DNA replicons expressing Cas9, sgRNAs, and donor DNA templates offer promising strategies to further enhance mutagenesis rates and HDR-based gene replacement (Baltes et al., 2014). Recent improvement in tissue culture methods is promising in converting *Arabidopsis* protoplasts harboring targeted genome modifications into plants through regeneration (Chupeau et al., 2013). Coexpression of sgRNA and Cas9 by DNA or RNA bombardment and agroinfiltration in regenerating tissues, meristems, embryos or germ cells may potentially broaden the plant range accessible to genome editing.

Several issues remain to be addressed to achieve robustness, versatility and specificity in targeted genome editing and gene expression manipulation using the sgRNA/Cas9 system and its derivatives as transcription activators and repressors, chromosomal locators, and epigenome regulators. Although off-target mutations do not appear to be prevailing based on the limited cases examined in plant cells (Feng et al., 2014; Nekrasov et al., 2013; Shan et al., 2013) and can potentially be outcrossed, genome-wide sequencing in targeted mutants remains the most thorough and comprehensive option

to precisely detect and critically evaluate off-target sites in each plant species. To improve specificity, it is necessary to systematically evaluate the "seed" sequences of sgRNAs and test truncated sgRNA designs and paired nickases (Sander & Joung, 2014). The effects of sgRNA sequences and target sites, paired sgRNA configurations (Fig. 21.3), protospacer adjacent motif (PAM) numbers, distance and locations in the genome, alternative PAM sequences, as well as chromatin structures and modifications may all contribute to the efficiency and specificity. It is unexplored regarding the nuclear retention, stability and sgRNA/Cas9 efficacy in different cell-types, organs, developmental stages, and plant species.

One of the most exciting applications of the sgRNA/Cas9-based genome-editing tools is the realization of simple and efficient homologous recombination-based gene or sequence replacement, or creation of novel plant genome designs that was out of reach in most plant species in the past. As shown in tobacco protoplasts, short homologous sequences flanking the sgRNA target site enabled a relatively high rate of gene replacement specifically in the presence of a DNA donor template (Li et al., 2013). Further improvement and refinement of the sgRNA/Cas9 technology will promise unprecedented opportunities and innovations in plant research, breeding and agriculture.

5. NOTES

1. Although targeting an *Arabidopsis* gene with a single sgRNA may be sufficient in triggering loss-of-function mutagenesis in some cases, we generally recommend using two closely targeting sgRNAs for a single gene to trigger genomic deletion to ensure the disruption of target gene function. However, single sgRNA may generate different missense or dominant gain-of-function mutations. As different sgRNAs targeting to the same gene may work with variable efficiency due to unknown factors, it is most desirable to evaluate three to four pairs of sgRNAs for targeting the same gene using the simple and rapid protoplast transient expression system (Li et al., 2013; Yoo et al., 2007). An optimal pair of sgRNAs can be rapidly identified within a week for the target gene prior to the time- and labor-consuming endeavor of generating CRISPR/Cas-mediated mutagenesis in plants with inheritable and homozygous mutations. For targeted homologous recombination,

we recommend the use of a single sgRNA 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 sgRNA 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 sgRNA target sites in *N. benthamiana*. Genomic N_{20}NGG sequences can be manually identified from a tobacco gene of interest as the sgRNA 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., *Arabidopsis U6-1* promoter; Waibel & Filipowicz, 1990) is required to drive sgRNA transcription. Optimal transcription by the *Arabidopsis U6-1* promoter is initiated with "G". Therefore, if the selected sgRNA target sequence (N_{20}NGG) is not initiated with "G" (N_1 as "C", "A" or "T"), an additional "G" should be introduced behind the *Arabidopsis U6-1* promoter through the primer R1 using a sequence of 5′ the reverse complement of N_{20} + CAATCACTACTTCGTCTCT 3′ (Fig. 21.3B). The *Arabidopsis U6-26* promoter has been used successfully in transgenic plants to obtain inheritable homozygous mutations in T2 generation (Fauser et al., 2014; Feng et al., 2014).
4. Restriction sites of *Sac*I, *Pac*I, *Pst*I, *Kpn*I, *Sma*I, or *Hin*dIII in the *pUC119-MCS* vector as cloning sites for multiple sgRNAs flanked by two *Asc*I sites are highly recommended, as sgRNAs can be easily subcloned into the binary plasmid *pFGC-pcoCas9* through *Asc*I digestion and insertion (Fig. 21.2B). Avoid using *Stu*I in sgRNA cloning because the *Arabidopsis U6-1* promoter contains an internal *Stu*I site.
5. A sgRNA 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), despite with much increased time and cost. A more convenient *U6-26* promoter plasmid (*pChimera*) based on type II restriction enzyme BbsI cloning is recently published (Fauser et al., 2014).
6. One can also clone individual sgRNA expression cassettes into the *pUC119-MCS* vector to obtain separate sgRNA expression plasmids and then achieve sgRNA coexpression by protoplast cotransfection

with two different sgRNA expression plasmids. However, cloning a pair of sgRNA expression cassettes into the same *pUC119-MCS* vector better ensures coexpression of two sgRNAs 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 commercial DNA maxiprep kits is acceptable but may lead to lower protoplast transfection efficiency.
8. In the case of obtaining targeted homologous recombination in protoplasts, 20 μL of DNA transfection cocktail is composed of 8 μL of the *p35SPPDK-pcoCas9* plasmid (2 μg/μL), 8 μL of the *pU6-sgRNA* 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 (Li et al., 2013). Longer homology arms are likely to promote the efficiency of homologous recombination.
9. 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 will not be disturbed. Tobacco protoplasts tend to aggregate during incubation.
10. Design of genomic PCR amplicons with sizes around 300 bp (Fig. 21.1) 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.

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