Chapter 5

CRISPR/Cas9-Based Genome Editing Toolbox for *Arabidopsis thaliana*

Daisuke Miki, Gaurav Zinta, Wenxin Zhang, Fangnan Peng, Zhengyan Feng, and Jian-Kang Zhu

Abstract

CRISPR/Cas9 system has emerged as a powerful genome engineering tool to study gene function and improve plant traits. Genome editing is achieved at a specific genome sequence by Cas9 endonuclease to generate double standard breaks (DSBs) directed by short guide RNAs (sgRNAs). The DSB is repaired by error-prone nonhomologous end joining (NHEJ) or error-free homology-directed repair (HDR) pathways, resulting in gene mutation or sequence replacement, respectively. These cellular DSB repair pathways can be exploited to knock out or replace genes. Also, cytidine or adenine base editors (CBEs or ABEs) fused to catalytically dead Cas9 (dCas9) or nickase Cas9 (nCas9) are used to perform precise base editing without generating DSBs. In this chapter, we describe a detailed procedure to carry out single/multiple gene mutations and precise base editing in the *Arabidopsis* genome by using CRISPR/Cas9-based system. Specifically, the steps of target gene selection, sgRNA design, vector construction, transformation, and analysis of transgenic lines are described. The protocol is potentially adaptable to perform genome editing in other plant species such as rice.

Keywords CRISPR/Cas9, Sequence-specific nucleases, Targeted gene editing, Homologous recombination, Base editors, Genetic manipulation

1 Introduction

Genome sequencing technologies have revolutionized the biological sciences. With the availability of genome sequences of various living organisms including plants, the focus is now shifted to uncover gene function [1]. In this regard, both forward and reverse genetic approaches have contributed immensely to plant functional genomics [2]. Geneticists initially used natural mutants to elucidate the function of genes, but later on artificial mutants were created by using physical, chemical, and biological agents. However, these methods have some limitations. For instance,

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creating mutants by physical (radiation) and chemical (EMS, ethyl methanesulfonate) agents causes random mutations in the genome [3]. Thus, to establish a causal relationship between genotype and phenotype, large-scale genetic screening is required, which is labor intensive, costly, and time-consuming. The reverse genetics methods, including T-DNA insertion lines (e.g., SALK T-DNA insertion library), RNA interference (RNAi), and virus-induced gene silencing (VIGS), offer direct ways to elucidate gene function, which involve reduction of transcript levels of endogenous genes to generate knockdown mutants [4]. However, sometimes appropriate T-DNA insertion lines are not available in the libraries, the RNA silencing methods are not highly specific, and reductions achieved in the gene expression are variable and not stably inherited to next generation.

Sequence-specific nucleases (SSNs) generate target site-specific double strand breaks (DSBs) in the genome of numerous organisms [5–8]. Due to this property, SSNs including zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) have emerged as versatile tools for genome engineering. Particularly, CRISPR/Cas9 system is the most frequently used genome editing tool in plants due to its simplicity, high specificity, efficiency, and multiplexing capacity [9]. The Cas9 endonuclease is directed to specific genomic loci by a 20-nt single-guide RNA (sgRNA) (Fig. 1a). Type II *Streptococcus pyogenes* Cas9 (SpCas9) is the most widely used Cas9 that recognize NGG as a protospacer adjacent motif (PAM) sequence. The activity of Cas9 generates a double strand break (DSB) at 3–4 base pairs distal to the PAM sequence (Fig. 1b). DSBs are subsequently repaired by either error-prone nonhomologous end joining (NHEJ) or error-free homology-directed repair (HDR), if certain homologous DNA repair donor template is provided, resulting in gene mutations or knock-in/replacement, respectively (Fig. 1a).

The imprecise repair of DSBs by NHEJ causes random insertion or deletion of nucleotides at the target site. This results into generation of null mutants by frameshift mutation, which can be used for functional gene analysis. However, genetic redundancy is a formidable problem to understand gene function when members of a gene family perform the same function. The CRISPR/Cas9 system provides opportunities to overcome this issue by directing Cas9 to multiple genetic loci by co-expressing multiple sgRNAs, and facilitating multiplex gene targeting [10–12]. When targeting a single genetic locus in the genome, the expression sgRNA is driven by AtU6–26 Pol III promoter, whereas for targeting multiple genetic loci to obtain high-order mutants, a combination of other Pol III promoters such as AtU3b and At7SL-2 is used [10, 11]. This implies that multiple sgRNA expression cassettes

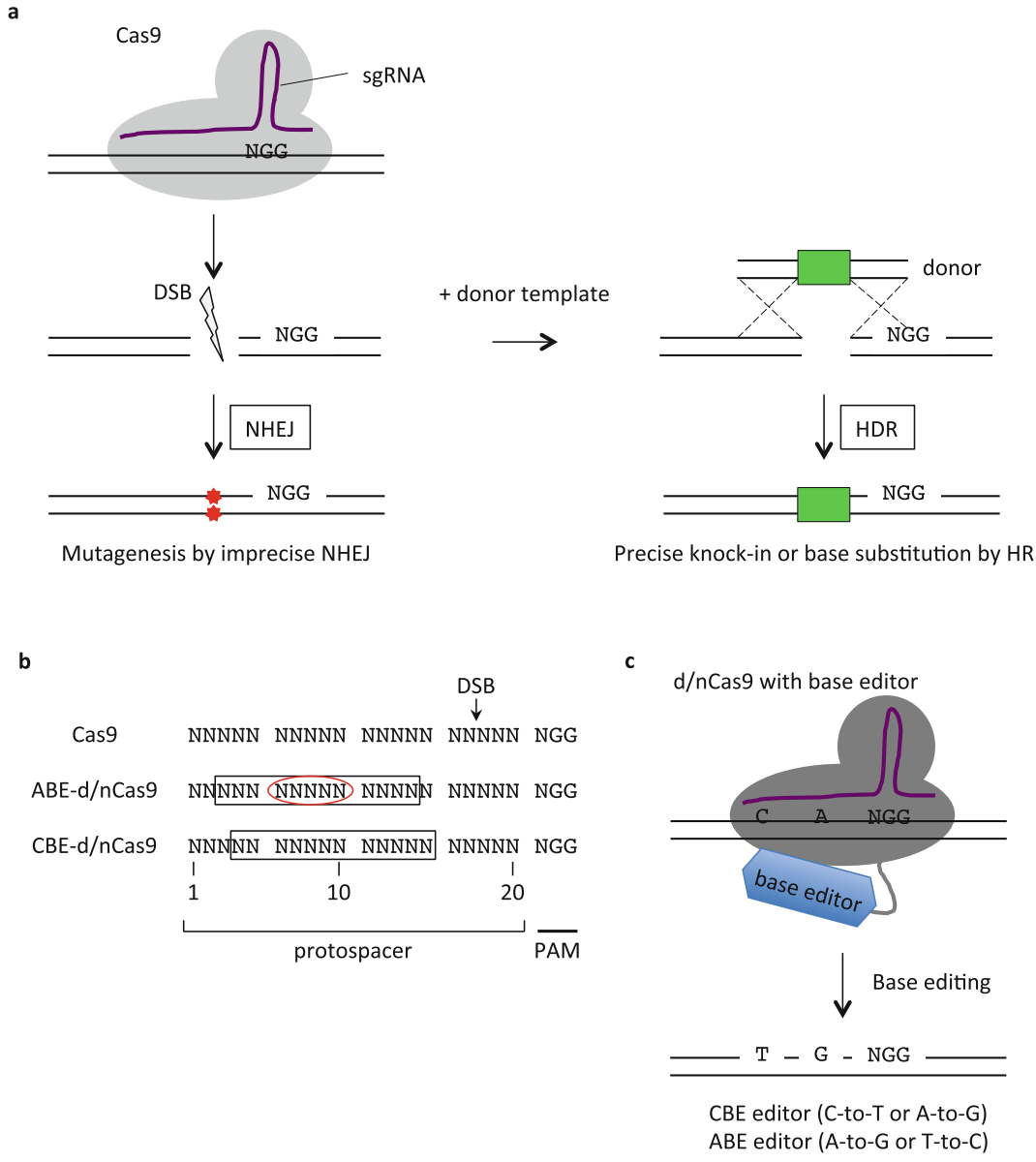

Fig. 1 Overview of CRISPR/Cas9 system induced mutagenesis, base editing, and gene targeting. (**a**) CRISPR/Cas9-induced DSB and cellular repair machinery. The sgRNA is assembled with Cas9 to form an RNA and protein complex. The sgRNA recognizes target sites by base-complementarity, and DSBs are generated. The DSBs are repaired via error-prone NHEJ that leads to random mutations, whereas error-free HDR creates precise sequence changes when a homologous DNA substrate is provided. (**b**) Possible DSB site generated by Cas9 and activity window of base editors. DSBs are generated by Cas9 nuclease activity at 3-bp upstream of the PAM (top). Bases in the squares represent the editing window of ABEs (middle) and CBEs (bottom). Red circle indicates hot spot of base conversion by ABEs. (**c**) d/nCas9 fused with the base editors to induce precise genome modifications. The cytosine base editor (CBE) can induce C-to-T or A-to-G base conversions, whereas adenine base editor (ABE) can induce A-to-G or T-to-C conversions

must be harbored simultaneously with Cas9 expression cassette on a same T-DNA construct. The efficiency and heritability of CRISPR/Cas9-mediated genome editing depends on the expression of Cas9 and sgRNAs in the cells. Driving the expression of Cas9 by germline-specific promoters increases the efficiency of heritable gene mutations in *Arabidopsis* as previously shown for egg cell-specific DD45 [13, 14], meiosis-specific MGE1 [15], male gametocyte-specific SPL [14], pollen-specific Lat52 [14], cell division-specific YAO [16] and CDC45 [11], and all developmental stage-specific RPS5A [17].

On the other hand, homology-directed repair (HDR) is exploited for gene targeting (GT) and makes precise modifications in genome such as DNA knock-in and gene replacement (Fig. 1a). However, HDR-mediated GT is extremely inefficient in plants, thus limiting its widespread application. We recently developed a simple, efficient, and precise method of gene targeting in *Arabidopsis* by using a combination of germline and early embryo-specific promoter DD45 to drive Cas9 expression and sequential transformation strategy [18]. By using this method we achieved a heritable GT efficiency of ~9%, and GT plants were easily identified by regular PCR.

Another alternative approach that enables precise base editing without DSB is the use of "base editors" allowing the direct conversion of target bases into different ones [19]. Two members of base editors are reported in plants, which include cytidine base editors (CBEs) and adenine base editors (ABEs). CBEs efficiently induce cytosine to thymine (C-to-T) or guanine to adenine (G-to-A) conversions, while adenine base editors (ABEs) convert A-to-G or T-to-C (Fig. 1c). The vector construct is prepared by fusing base editors with enzymatically dead Cas9 (dCas9, mutations at D10A and H840A domains) or nickase Cas9 (nCas9, mutation at D10A) and sgRNA expression cassette that enable binding to DNA in a precise manner [20–23]. These base editors can be utilized to edit one or a few single nucleotide(s) with high accuracy and efficiency (Fig. 1b), and have applicability where off-target editing of adjacent nucleotides is not tolerable and phenotype is controlled by a single nucleotide variation.

In this chapter, we discuss the applicability of CRISPR/Cas9 system to generate single/multiple knockout mutants, and perform precise base edits (including knock-in and replacement) in the genome of *Arabidopsis thaliana* (Fig. 2). The protocol is easy to follow and practical steps of target gene selection, sgRNA design, vector construction, transformation, and analysis of transgenic lines are described in detail. The principles of this protocol can potentially be adapted to perform genome editing in other plant species such as rice.

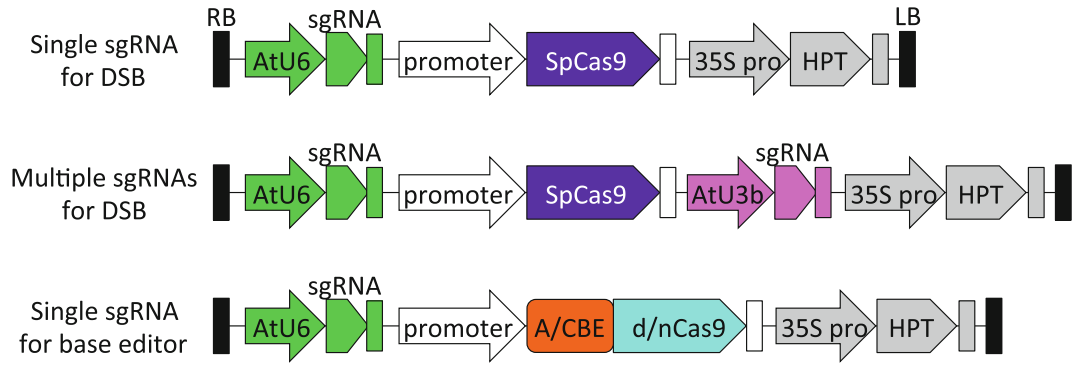

Fig. 2 CRISPR/Cas9 constructs. Schematic view of T-DNA constructs containing single (top) or multiple (middle) sgRNAs, and base editing system (bottom). The expression of single sgRNA is driven by AtU6-26 Pol III promoter. The expression of multiple sgRNAs is driven by a combination of other promoters such as AtU3b and At7SL-2 harbored on a same T-DNA construct with Cas9 expression cassette. The base editing construct consists of d/nCas9 fused with the base editors (CBE or ABE)

2 Materials

2.1 Vector Construction for Single/Multiple Gene Mutations and Base Editing

1. AtU6 sgRNA cassette plasmid (pBluescript SK background or binary vector background for one plasmid strategy).
2. Cas9 nuclease expression cassette harboring binary vector (we preferentially use YAO or CDC45 promoter to drive Cas9 expression in pCAMBIA1300 binary vector (Fig. 2) [11, 14].
3. Forward and reverse oligo of sgRNA (100 μmol/L) (Fig. 3, *see* Table 1).
4. Restriction enzymes BbsI, HindIII, and SalI.
5. T4 DNA Ligase and reaction buffer.
6. DH5α *E. coli* chemical competent cells.
7. Luria Bertani (LB) liquid medium (1% tryptone, 0.5% yeast extract, 1% sodium chloride) and solid medium (with 1.5% agar).
8. Antibiotics: carbenicillin (or ampicillin) and kanamycin.
9. QIAquick Gel Extraction Kit (Qiagen, #28704).
10. QIAprep Spin Miniprep Kit (Qiagen, #27104).
11. Thermocycler (e.g., Applied BiosytemsTM).
12. TaKaRa Taq DNA Polymerase (TaKaRa, #R001A).
13. Incubators (37 and 16 °C).
14. Zero Blunt TOPO PCR Cloning kit (Thermo Fisher Scientific, #450245).
15. Absorption spectrometer (e.g., NanoDrop).

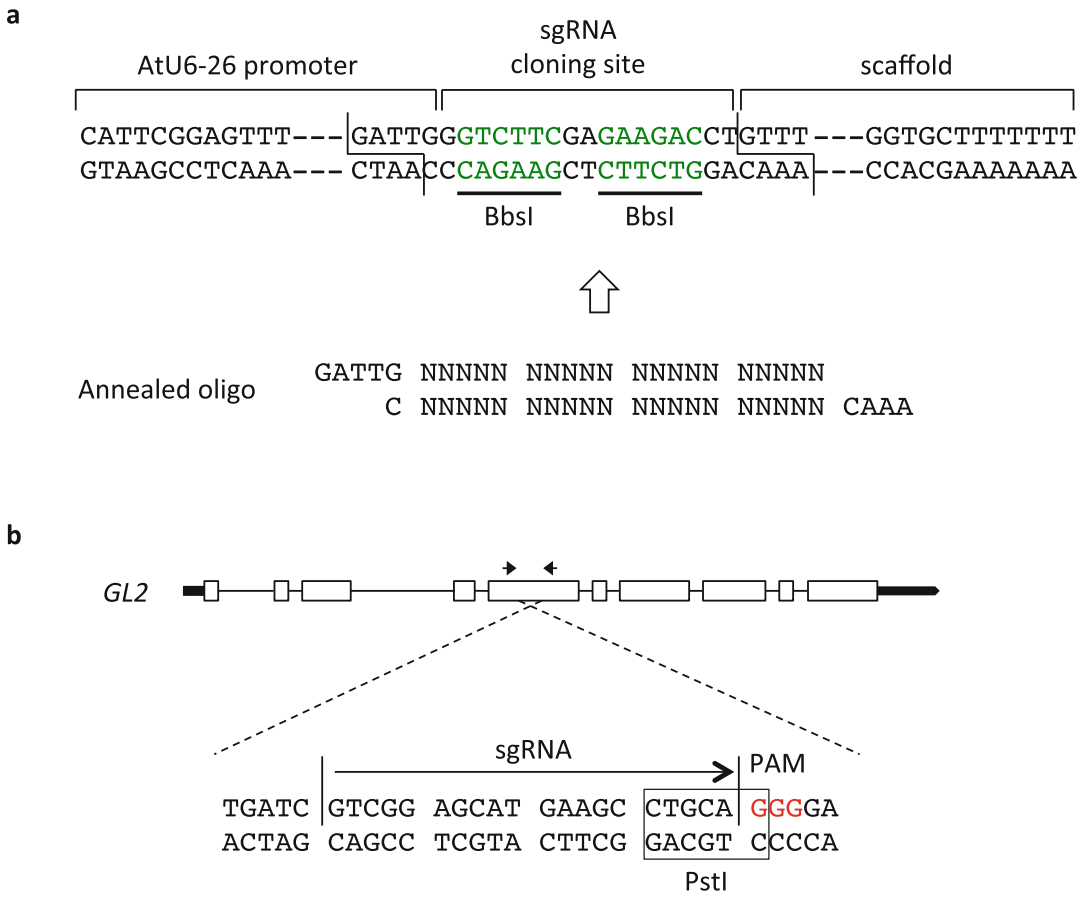

Fig. 3 Detailed sgRNA design. (**a**) Partial sequence of AtU6-26 promoter, sgRNA cloning site, and scaffold. BbsI restriction enzyme recognition sites are represented in green letters. The annealed oligo is cloned into the restriction sites. (**b**) *Arabidopsis* endogenous *GLABRA 2* (*GL2*; At1g79840) gene and targeting sgRNA sequence. Upper schematic represents genomic sequence structure of *GL2* gene, and two arrows indicate PCR primers used for genotyping (*see* Fig. 8b, Table 1). Red bases indicate PAM sequence of designed sgRNA, horizontal arrow indicates sgRNA target site, and square represents PstI site used for mutation screening in RFLP assay (*see* Fig. 8a, b). (**c**) Designed sgRNA oligomer to target *GL2* gene

16. Agarose, loading buffer and TAE (40 mM Tris (pH 7.6), 20 mM acetic acid, 1 mM EDTA).
17. Agarose gel electrophoresis tank and power supply.

2.2 Agrobacterium Transformation

1. *Agrobacterium tumefaciens* strain GV3101 competent cells.
2. T-DNA plasmid (Fig. 2).
3. LB liquid and solid medium with antibiotics.

Table 1
Sequence of primers used in this study

Primer name	Primer sequence (5′ - 3′)
Forward oligo of sgRNA	GATTGNNNNNNNNNNNNNNNNNN
Reverse oligo of sgRNA	AAACNNNNNNNNNNNNNNNNNNC
M13-F	TGTAAAACGACGGCCAGT
M13-R	CAGGAAACAGCTATGACC
pCAMBIA1300-P1	CCAGGCTTTACACTTTATGC
pCAMBIA1300-P2	GCGATTAAGTTGGGTAACGC
AtU6-26-sgRNA forward	CGTTGAACAACGGAAACTCGAC
AtU6-26-sgRNA reverse	AAAAAAAGCACCGACTCGGT
GL2 genotyping forward	AGTTAGGGTTCAGTTGCATG
GL2 genotyping reverse	AGTTATAGTAGCTGGTAACAG
ROS1-GFP-5 arm forward	GAATTCGTTATTCAACAGGTGAGACGGCTGATTC
ROS1-GFP-5 arm reverse	TGCTCACCATGGCGAGGTTAGCTTGTTGTCC
ROS1-GFP-GFP forward	TAACCTCGCCATGGTGAGCAAGGGCGAGGAG
ROS1-GFP-GFP reverse	TTTGCTTGCCTTACTTGTACAGCTCGTCCATGCCGT
ROS1-GFP-3 arm forward	GTACAAGTAAGGCAAGCAAACAAATACAAGCTTATG
ROS1-GFP-3 arm reverse	GACAACAATAGAATTCTTTGGTCCGGTTGAACTATC
DME-D1633A-5 arm forward	GGAACTTGTATTGATGATATGAAGGTTGACACG
DME-D1633A-5 arm reverse	GACATGCATTACAgTTagcTCTACTCTTTGTGC
DME-D1633A-3 arm forward	GCACAAAGAGTAGAgctAAcTGTAATGCATGTC
DME-D1633A-3 arm reverse	CCGTAGAGTCATTCCAAACTGTTCAATGTTG
pCAMBIA3301-foward	CTTCCGGCTCGTATGTTGTG
pCAMBIA3301-reverse	CTCTTCGCTATTACGCCAGC
ROS1-GFP-specific forward	GCAGTTGGAAAAGAGAGAACCTGATGATCC
ROS1-GFP-specific reverse	CTGAACTTGTGGCCGTTCACGTC
ROS1-GFP external forward	ACCTGATGATCCATGTTCTTATTTG
ROS1-GFP external reverse	CCTTGTACAACTCTAGGACTGTT

4. Antibiotics: kanamycin and rifampicin.
5. Liquid nitrogen.
6. Incubators (37 and 28 °C).

2.3 Arabidopsis Transformation

1. T-DNA transformed *Agrobacterium*.
2. LB liquid medium.

3. Freshly prepared floral dip transformation solution (5% sucrose, 0.03% Silwet L-77).
4. Antibiotics: kanamycin, hygromycin, carbenicillin, and rifampicin.
5. Centrifuge, 500 mL centrifuge tubes.
6. 5% Sodium hypochlorite solution.
7. 0.05% Agar (autoclaved).
8. Transgenic plant selection solid medium: 0.5× Murashige and Skoog (MS) with vitamins (Phyto Technology Laboratories, #M524), 0.8% agar, 30 mg/L hygromycin B, 50 mg/L carbenicillin.
9. Plant soils, pots, and trays.
10. Growth chambers and greenhouse.

2.4 Screening of Mutations or Base Editings

1. CTAB buffer: 2% cetyltrimethylammonium bromide (CTAB), 1.4 M NaCl, 20 mM EDTA, 100 mM Tris.
2. Chloroform/isoamyl alcohol (24:1).
3. 75% and absolute ethanol.
4. Ultra-pure and sterile water (ddH_2O).
5. Liquid nitrogen.
6. 1.5 mL Microcentrifuge tubes.
7. Steel balls.
8. Homogenizer (e.g., Retsch, Mixer Mill MM 400).
9. 65 °C Incubator.
10. Centrifuge.
11. TaKaRa Taq DNA Polymerase.
12. PCR primer sets (10 μM) (Fig. 3b, *see* Table 1).
13. Thermocycler.
14. Restriction enzyme and reaction buffer (Fig. 3b).
15. Agarose, loading buffer, and TAE.
16. Agarose gel electrophoresis tank and power supply.

2.5 Generation of Gene Targeting Constructs

1. DD45 pro::Cas9 parental line (CS69955 and CS69956 from Arabidopsis Resource Center (ABRC)).
2. Binary T-DNA vector pCAMBIA3301 (alternatively other T-DNA vector harboring Basta resistance gene).
3. High-fidelity polymerase (e.g., KOD DNA polymerase, EMB Millipore, #71085), reaction buffer, and dNTPs.

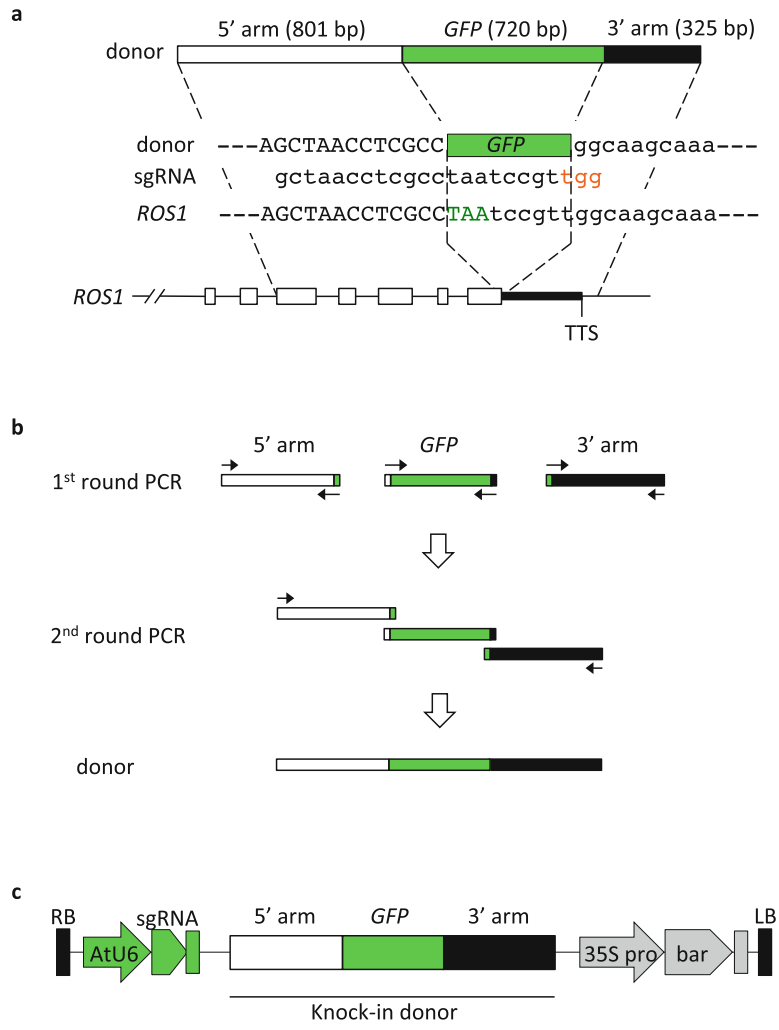

Fig. 4 Knock-in donor construct for gene targeting. (**a**) Schematic representation endogenous *ROS1* genomic sequence structure, showing *ROS1-GFP* donor and endogenous *ROS1* knock-in site. Red letters in sgRNA sequence indicate PAM, and green letters in *ROS1* partial sequence indicate stop codon. (**b**) A flow chart for knock-in donor vector construction. First round of PCR involves amplification of homology arms and knock-in sequence with specific primers, followed by second round of PCR to fuse them. (**c**) Knock-in donor vector construct harbors AtU6-sgRNA cassette, knock-in donor, and selection marker (bar) gene

4. PCR primer sets (10 μM) (Figs. 4 and 5, *see* Table 1).
5. Thermocycler.
6. Agarose, loading buffer, and TAE.
7. Agarose gel electrophoresis tank and power supply.

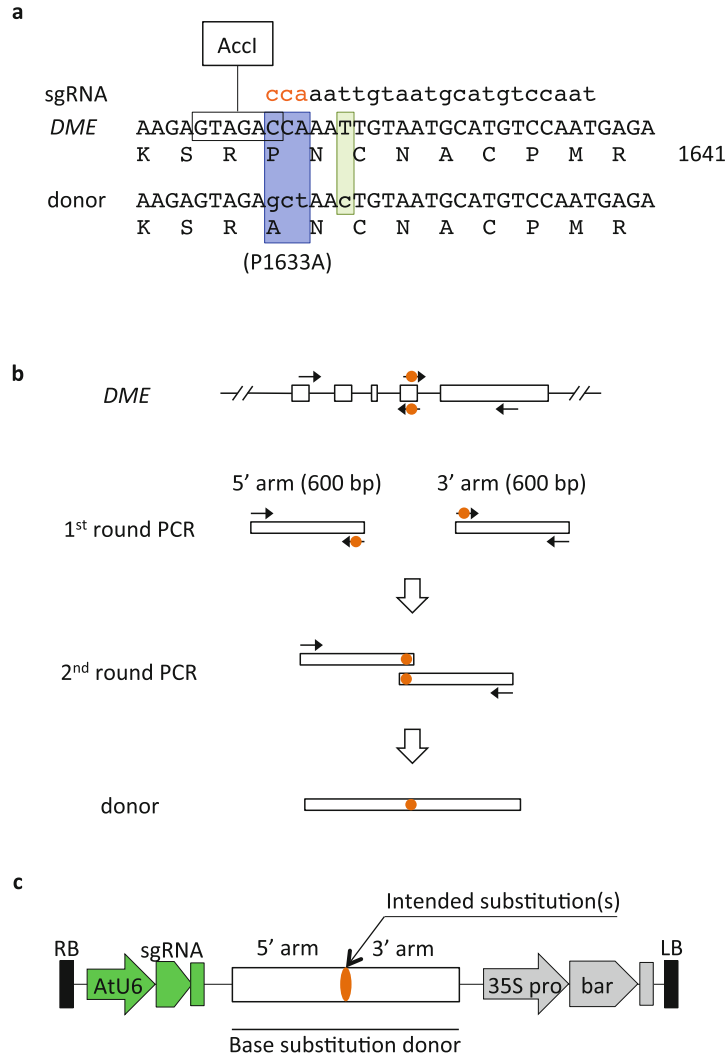

Fig. 5 Base substitution donor construct for gene targeting. (**a**) Target endogenous *DME*, sgRNA and P1633A substitution donor sequence. Red letters indicate PAM sequence, blue highlight indicates P1633A amino acid substitution site, green highlight indicates silent mutation, and black square indicates AccI site that is used for genotyping of GT plants. (**b**) A flow chart for construction of base substitution donor construct. First PCR round involves amplification of both homology arms with primers harboring the intended mutations (orange circle). In the second PCR round both these homology arms are fused together. (**c**) Base substitution donor T-DNA construct harbors AtU6-sgRNA cassette, base substitution donor, and selection marker (bar) gene

3 Methods

3.1 Single/Multiple Gene Mutations and Base Editing

3.1.1 Design of sgRNA

sgRNAs design is crucial to achieve the specificity and efficiency of the CRISPR/Cas9 system. Nowadays, a number of web-based tools are available that can help with quick sgRNA design. These tools are very useful for predicting the efficiency of sgRNAs and potential off-target sites. sgRNA design tools described below generate a list of candidate sgRNAs from the input sequence (*see* **Note 1**):

1. CRISPR Primer Designer: http://plantsignal.cn/CRISPR/crispr_primer_designer.html
2. CRISPR-PLANT: https://www.genome.arizona.edu/crispr/index.html
3. CRISPOR: http://crispor.tefor.net/
4. CHOPCHOP: http://chopchop.cbu.uib.no/
5. DESKGEN: https://www.deskgen.com/landing/cloud.html
6. CRISPR tool ATUM: https://www.atum.bio/eCommerce/cas9/input

Alternatively, sgRNAs can be designed manually by identifying the 20-bp sequence directly upstream of any 5′-NGG. A pair of DNA oligos are synthesized as follows (Fig. 3):

Forward oligo: 5′-gattGNNNNNNNNNNNNNNNNNNN-3′

Reverse oligo: 5′-aaacNNNNNNNNNNNNNNNNNNNC-3′

The "N"s in the forward oligo correspond to the 5′ 20-nt preceding the PAM (5′-NGG-3′) and those in the reverse oligo are just the reverse complementary sequence (Fig. 3c) (*see* **Note 2**).

To target multiple genes simultaneously, a single or multiple sgRNAs can be designed. When target genes share a conserved DNA sequence, designing an sgRNA from this sequence will be enough to generate multiple gene mutations, otherwise gene-specific multiple sgRNAs are designed (Fig. 2).

3.1.2 sgRNA Order and Dilution

Order the designed oligomers for sgRNA. Dissolve and dilute the sgRNA oligomers in ddH_2O to get a final concentration of 100 μM and store them at −20 °C.

3.1.3 Preparation of sgRNA Construct

1. Anneal each pair of DNA oligomers (Fig. 3a, c):
   1 μL Forward oligo (100 μM)
   1 μL Reverse oligo (100 μM)
   Add ddH_2O to 10 μL total reaction volume.

2. Anneal each pair of oligomers in a thermocycler under the following conditions: 95 °C 5 min, and ramp down to 25 °C at 0.2 °C/s.
3. Dilute annealed oligo 250 times with ddH_2O.
4. Digest AtU6 sgRNA cassette plasmid with BbsI (Figs. 3a and 6a, b):
   0.5 μg Plasmid.
   1 μL BbsI (10 U/μL).
   2 μL 10× Reaction buffer.
   Add ddH_2O to 20 μL.
   Incubate at least 1 h at 37 °C.

   Alternatively, AtU6 sgRNA cassette harboring binary vectors can be used as one plasmid system (Fig. 6b) [17]. If the one plasmid system is applied, annealed oligo is directly cloned into the binary vector. The use of restriction enzyme (s) depends on the type of plasmid.
5. Run digested plasmid on a 0.8% TAE agarose gel and purify the digested plasmid with QIAquick Gel Extraction Kit; elute the digested plasmid into 30 μL elution buffer.
6. Ligate annealed oligo into BbsI-digested plasmid (Figs. 3a and 6a, b):
   10 ng BbsI-digested plasmid.
   1 μL Annealed oligo.
   1 μL 10× T4 DNA Ligase buffer.
   0.5 μL T4 DNA Ligase.
   Add ddH_2O to 10 μL.
   Incubate at 16 °C overnight or room temperature for 10 min.
7. Transform 100 μL of *E. coli* DH5α chemically competent cells with 5 μL of ligation product using a heat shock at 42 °C for 45 s.
8. Recover transformed *E. coli* with 200 μL LB liquid medium and incubate at 37 °C for 1 h.
9. Spread 100 μL of transformed *E. coli* onto LB plate with 50 mg/L carbenicillin (or ampicillin in case of pBluescript SK (Fig. 6a), otherwise suitable antibiotics should be applied). Incubate at 37 °C for overnight.
10. Screen for correct clones by colony PCR using primers AtU6–26-sgRNA forward and reverse oligo for sgRNA (*see* Table 1):
    2 μL 10× Taq buffer:
    1.6 μL dNTP (2.5 mM)

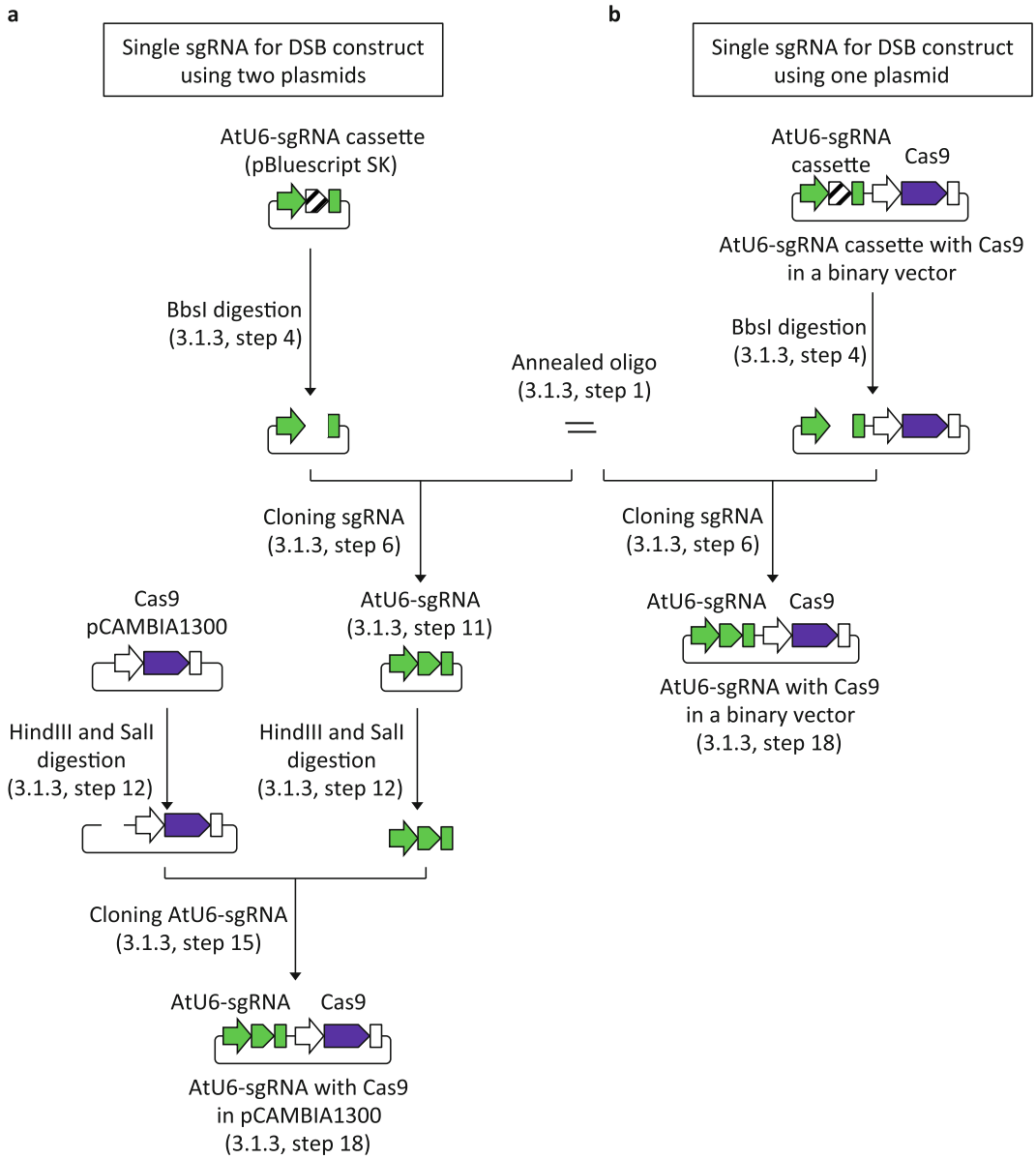

Fig. 6 Preparation of CRISPR/Cas9 vector containing single sgRNA. The flow charts show preparation of CRISPR/Cas9 T-DNA construct containing single sgRNA by using (**a**) two plasmids or (**b**) one plasmid (*see* Fig. 2, top panel). To prepare single sgRNA containing construct from two plasmids AtU6-sgRNA cassette is digested with restriction enzyme, followed by ligation with annealed oligo, digestion of binary vector, and lastly cloning into Cas9 harboring pCAMBIA1300 vector. However, for one plasmid system, annealed oligo is directly cloned into the AtU6 sgRNA cassette harboring binary vector by using suitable restriction enzymes

0.05 μL Taq Polymerase

0.4 μL Forward primer (Plasmid Specific; e.g., AtU6–26-sgRNA Forward) (10 μM)

0.4 μL Reverse primer (Reverse Oligo) (10 μM)

Add ddH_2O to 20 μL.

Perform PCR: 95 °C for 5 min, 35 cycles of 94 °C for 30 sec, 55 °C for 30 sec, and 72 °C for 1 min, and additional 5 min to 72 °C.

Correct clones will give PCR products of approximately 470 bp.

11. Miniprep 2–4 clones using the QIAprep Spin Miniprep Kit and confirm the AtU6-sgRNA cassette in pBluescript SK by DNA sequencing.
12. Digest AtU6-sgRNA cassette and Cas9 harboring pCAMBIA1300 binary vector with restriction enzymes (Fig. 6a):

    10 μL AtU6-sgRNA in pBluescript SK.

    1 μL HindIII.

    1 μL SalI.

    5 μL CutSmart Buffer.

    Add ddH_2O to 50 μL.

    10 μL Cas9 harboring pCAMBIA1300.

    1 μL HindIII.

    1 μL SalI.

    5 μL CutSmart Buffer.

    Add ddH_2O to 50 μL.

    Incubate at 37 °C for 1 h to overnight.

13. Run digested plasmids on a 1.2% agarose gel. Gel-purify the insert AtU6-sgRNA and linearized Cas9 harboring pCMABIA1300 by using QIAquick Gel Extraction Kit and elute the digested plasmid into 30 μL elution buffer.
14. Determine the concentration of digested insert and vector by spectrometer.
15. Clone AtU6-sgRNA cassette into the Cas9 harboring pCAMBIA1300 (Fig. 6a):

    X μL AtU6-sgRNA cassette (insert).

    10 ng Cas9 harboring pCAMBIA1300.

    1 μL 10× T4 DNA Ligase buffer.

    0.5 μL T4 DNA Ligase

    Add ddH_2O to 10 μL.

    The ratio of insert DNA mass (insert:vector = 3 ~ 5:1) can be calculated using a website (https://nebiocalculator.neb.com/#!/ligation). Incubate at 16 °C overnight or room temperature for 10 min.

16. Transform ligation product into *E. coli* as described above (Subheading 3.1.3, **steps 7–9**).
17. Screen correct clones by colony PCR using pCAMBIA1300-specific P2 primer and reverse oligo for sgRNA (*see* Table 1). Correct clones are expected to give PCR products of approximately 650 bp by following above procedure (Subheading 3.1.3, **step 10**).
18. Miniprep 2–4 clones using the QIAprep Spin Miniprep Kit and confirm the AtU6-sgRNA cassette with Cas9 in pCAMBIA1300 by DNA sequencing using P2 primer (*see* Table 1).

3.1.4 Preparation of T-DNA Construct for Multiple Targets (Figs. 2 and 7)

1. Construct a certain number of sgRNA cassettes for multiple target genes following the above procedure (*see* Subheading 3.1.3) (Figs. 3 and 6a).
2. If necessary, amplify some sgRNA cassettes to alter those restriction enzyme sites by PCR using high-fidelity polymerase (Fig. 3a, *see* Table 1):

   AtU6–26-sgRNA forward with restriction enzyme site: 5′-restriction enzyme site-CATTCGGAGTTTTTGTATC TTGTTTC-3'.

   AtU6–26-sgRNA reverse with restriction enzyme site: 5′-restriction enzyme site-AAAAAAAGCACCGACTC GGT-3′.

   0.5 μL Template sgRNA Plasmid

   PCR reaction buffer (final concentration 1×).

   1.6 μL dNTP (2.5 mM).

   0.1 μL High-Fidelity Polymerase (e.g., KOD DNA Polymerase)

   0.4 μL AtU6–26-sgRNA forward with restriction enzyme site primer (10 μM).

   0.4 μL AtU6–26-sgRNA Reverse with Restriction Enzyme Site Primer (10 μM)

   Add ddH_2O to 20 μL.

   Perform PCR: 95 °C for 5 min, 30 cycles of 94 °C for 30 sec, 60 °C for 30 s and 72 °C for 1 min, and additional 5 min of 72 °C.

3. Run a 1.2% agarose gel and gel-purify the PCR products by using QIAquick Gel Extraction Kit. Correct clones will give PCR products of approximately 550 bp. Elute the products into 30 μL elution buffer. If necessary, subclone the PCR products into Zero Blunt TOPO PCR Cloning according to manufacturer's instructions. Then, confirm the DNA sequence (*see* **Note 3**).

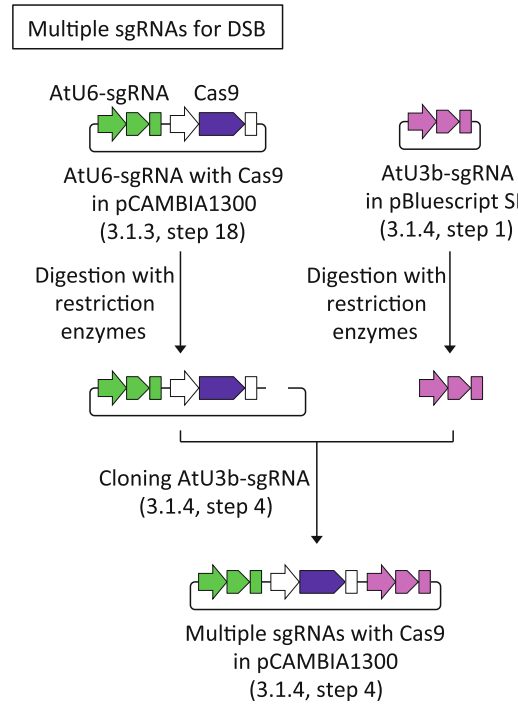

Fig. 7 Preparation of CRISPR/Cas9 vector containing multiple sgRNAs. The flow chart shows construction of CRISPR/Cas9 T-DNA construct for multiple sgRNA by using one plasmid (*see* Fig. 2, middle panel). One or some sgRNA expression cassette(s), AtU3b-sgRNA in this Figure, are cloned into the AtU6 sgRNA cassette and Cas9 harboring binary vector by using suitable restriction enzymes

4. Cloning sgRNA cassettes into Cas9 expression binary vector. Procedure is described above (*see* Subheading 3.1.3, **steps 15–18**) (Fig. 7) (*see* **Note 4**).

3.1.5 Agrobacterium-Mediated Arabidopsis Transformation

1. Transform sgRNA with Cas9 binary vector, which was constructed at Subheadings 3.1.3, **step 18** or Subheading 3.1.4, **step 4**, into *Agrobacterium tumefaciens* competent cells (strain GV3101). Add 1 μL T-DNA binary vector construct into 50 μL of *Agrobacterium* competent cells.
2. Incubate on ice for 5 min.
3. Incubate in liquid nitrogen for 5 min.
4. Incubate at 37 °C on heat block or water bath for 5 min.
5. Add 1 mL of LB, mix well, and incubate at 28 °C for 1 h.
6. Spread 100 μL on a LB plate with 50 mg/L kanamycin and 50 mg/L rifampicin (or gentamicin) at 28 °C for 2 days.
7. Pick a single colony of *Agrobacterium* and culture it in 8 mL LB liquid medium with 50 mg/L kanamycin and 50 mg/L rifampicin (or gentamicin) at 28 °C overnight.

8. Add 8 mL *Agrobacterium* culture into 100 mL LB liquid medium and culture at 28 °C for 8 h.
9. Spin down cultured *Agrobacterium* cells by centrifugation at 5000 *g* for 10 min at room temperature.
10. Prepare floral dip transformation solution during centrifuge.
11. Resuspend *Agrobacterium* pellet into the 100 mL floral dip transformation solution.
12. Transform *Arabidopsis* by the flower dipping method [24]. Briefly, soak *Arabidopsis* flowers in the resuspended *Agrobacterium* solution, and place under dark and humid conditions overnight.
13. Allow transformed *Arabidopsis* plants to grow and collect seeds.

3.1.6 Screening of Mutations or Base Editings

The modified target gene is detected by PCR and restriction enzyme digestion (restriction fragment length polymorphism: RFLP) (Figs. 3b and 7a) (*see* **Note 5**).

1. Sterilize 100 to 200 μL T1 *Arabidopsis* seeds (obtained in Subheading 3.1.5, **step 13**) with 5% sodium hypochlorite solution in 1.5 mL microcentrifuge tube by rotation for 10 min.
2. Wash sterilized seeds five times with 1 mL ddH_2O. Spin down the seeds and resuspend with ddH_2O, repeat five times.
3. Resuspend the washed seeds with 1 mL 0.05% agar, and store at 4 °C for 2 days.
4. Plate sterilized seeds on 0.5x MS medium containing 30 mg/L hygromycin with 50 mg/L carbenicillin to restrict *Agrobacterium* growth and screen antibiotic resistance seedlings. Seal the petri dishes with surgical tape.
5. Transplant antibiotic resistant T1 *Arabidopsis* plants in soil, and grow them in greenhouse under long day conditions (16 h light-8 h dark) at 22 °C.
6. Collect one or two rosette leaves from each T1 transgenic plants in 1.5 mL microcentrifuge tube with 2 or 3 steel balls, put them into liquid nitrogen, and homogenize leaf tissues by shaking or by using homogenizer.
7. Add 500 μL CTAB buffer to the microcentrifuge tubes, then mix well and thaw the frozen sample tissues.
8. Incubate at 65 °C for 30 min, invert tubes every 10 min.
9. Add 300 μL of chloroform/isoamyl alcohol, and vortex well.
10. Centrifuge at maximum speed (e.g., 13,000 × *g*) for 5 min at room temperature.
11. Transfer 400 μL of the upper layer aliquot to a new 1.5 mL microcentrifuge tube.

12. Add 300 μL isopropanol to each tube, invert tubes, and mix well.
13. Centrifuge at maximum speed for 10 min, and discard supernatant.
14. Add 500 μL of 75% ethanol.
15. Centrifuge at maximum speed for 5 min, and discard supernatant.
16. Centrifuge those tubes again to spin down all aliquot.
17. Remove all aliquots by using pipet.
18. Add 20–50 μL ddH_2O to solve the DNA.
19. Design PCR primers that amplify the on-target site (Figs. 3b and 8a). If you used a sgRNA design website such as CRISPOR (*see* Subheading 3.1.1), the website also designs genotyping primers and certain restriction enzyme sites.
20. Perform PCR by including a wild-type (WT) DNA sample (e.g., Col-0) as a control:

    0.5 μL Template extracted genome DNA (from Subheading 3.1.6, **step 18**).

    2 μL 10× Taq buffer.

    1.6 μL dNTP (2.5 mM).

    0.05 μL Taq polymerase.

    0.4 μL Forward primer (10 μM).

    0.4 μL Reverse primer (10 μM)

    Add ddH_2O to 20 μL.

    Perform PCR: 95 °C for 5 min, 35 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 1 min, and additional 5 min of 72 °C.

21. Digest the PCR products (Fig. 8a):

    10 μL PCR product.

    2 μL 10× Reaction buffer.

    0.5 μL certain restriction enzyme (e.g. PstI, Fig. 3b)

    Add ddH_2O to 20 μL.

    Incubate at 37 °C at least 1 h.

22. Run digested products on a 1.5% agarose gel. PCR product form control WT, such as Col-0, must be completely digested while products from modified samples should be undigested band (Fig. 8a, b).
23. Purify the undigested band by using QIAquick Gel Extraction Kit, and identify the mutation by sequencing (Fig. 8b, c).

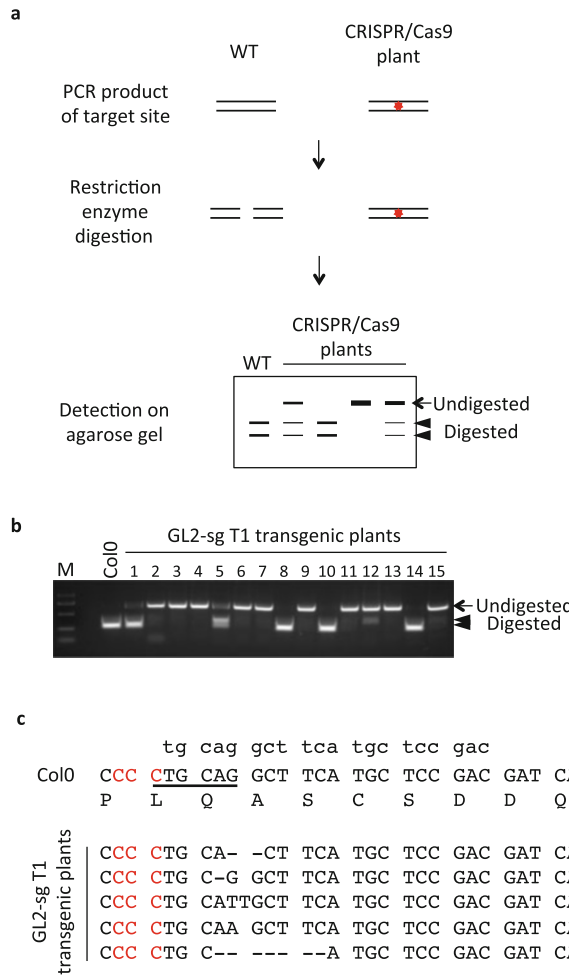

Fig. 8 Identification of gene modified plants. (**a**) Schematic representation of genotyping by PCR-restriction fragment length polymorphism (RFLP) assay. PCR is performed with primers flanking the CRISPR/Cas9 target site, and the PCR products are digested with a restriction enzyme. The Cas9-induced mutations can be easily detected by RFLP. Mutations introduced are resistant to restriction enzyme digestion because of the loss of the restriction sites, and result in an undigested band (arrow). (**b**) RFLP assay of individual T1 transgenic plants at *GL2* locus. PCR products were digested with PstI (see Fig. 3b). Two arrow heads indicate digested bands, an arrow indicates undigested band. (**c**) Identify mutations by DNA sequencing. The horizontal line represents PstI site in WT *Arabidopsis* (Col-0). Lower five sequences represent identified mutations in individual T1 transgenic plants

24. In the T1 generation, a variety of mutation levels will be detected (Fig. 8b). For instance, almost no mutations (#8, 10, 14), moderate chimeric mutations (#1, 5, 12, 15), and nearly 100% mutations (#4, 13) (Fig. 8b). Mutant lines such as #4 and 13 (Fig. 8b, c) are potentially heritable and must be

selected for the next generation. To confirm heritable gene mutations, an additional screening and genotyping should be performed in the T2 generation. Furthermore, if necessary, the obtained mutants should be backcrossed with WT to remove Cas9 transgene and to purify genetic background.

3.2 Gene Targeting

3.2.1 Design of sgRNA

1. Design sgRNA by following the instructions given above (*see* Subheading 3.1.1). The sgRNA should be designed close to the knock-in or amino acid substitution site (Figs. 4a and 5a) (*see* **Note 1**).
2. Prepare AtU6-sgRNA expression cassette in the pBluescript SK background, if it is necessary amplify it by using certain restriction enzyme harboring primers, as described above (*see* Subheadings 3.1.3 and 3.1.4).

3.2.2 Design of Donor DNA

Designing homology arms of 500–800 bp should be enough for efficient gene targeting (Figs. 4a and 5b) (*see* **Note 6**). Silent mutations must be incorporated at the PAM sequence to avoid additional DSBs [25]. If it is impossible, at least three silent mutations should be integrated at the protospacer target sequence (Fig. 5a).

3.2.3 Preparation of Donor T-DNA Construct for Agrobacterium Transformation

1. Prepare donor DNA using high-fidelity PCR. The donor DNA fragment is prepared by two rounds of PCR (Figs. 4b and 5b). Homology arms and knock-in sequence fragments are amplified in the first round and they are fused in the second round:
   0.5 μL Template genomic DNA or plasmid.
   PCR reaction buffer (final concentration 1×).
   1.6 μL dNTP (2.5 mM).
   0.5 μL High-fidelity polymerase (e.g., KOD DNA polymerase).
   0.4 μL Forward primer (10 μM).
   0.4 μL Reverse primer (10 μM)
   Add ddH_2O to 20 μL.
   Perform PCR: 95 °C for 5 min, 30 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 1 min, and additional 5 min of 72 °C.
2. Run 1.2% agarose gel and gel-purify the PCR product by using QIAquick Gel Extraction Kit; elute the PCR product into 30 μL elution buffer.
3. Carry out the second round PCR (Figs. 4b and 5b):
   0.5 μL Each purified template first round PCR products (*see* **Note 7**).
   PCR reaction buffer (final concentration 1×).

1.6 μL dNTP (2.5 mM).

0.1 μL High-fidelity polymerase (e.g., KOD DNA polymerase).

0.4 μL Forward primer (10 μM).

0.4 μL Reverse primer (10 μM)

Add ddH_2O to 20 μL.

Perform PCR: 95 °C for 5 min, 30 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 1 min, and additional 5 min of 72 °C.

4. Run PCR products on a 0.8% agarose gel and gel-purify them by using QIAquick Gel Extraction Kit; elute the PCR products into 30 μL elution buffer. If necessary, subclone the PCR products into Zero Blunt TOPO PCR Cloning according to manufacturer's instruction, then confirm DNA sequences (*see* **Note 3**).
5. Digest AtU6-sgRNA cassette, donor DNA, and pCAMBIA3301 binary vector with certain restriction enzymes:

   10 μL AtU6-sgRNA in pBluescript SK (see Subheading 3.2.1).

   1 μL HindIII.

   1 μL SalI.

   5 μL CutSmart Buffer.

   Add ddH_2O to 50 μL.

   10 μL donor DNA PCR product or subcloning in Zero Blunt TOPO (see Subheading 3.2.3, **step 4**).

   1 μL EcoRI.

   1 μL SalI.

   5 μL CutSmart Buffer.

   Add ddH_2O to 50 μL.

   10 μL pCAMBIA3301.

   1 μL HindIII.

   1 μL EcoRI.

   5 μL CutSmart Buffer.

   Add ddH_2O to 50 μL.

   Incubate reactions at 37 °C for 1 h to overnight.

6. Run digested plasmids on a 1.2% agarose gel. Gel-purify the insert AtU6-sgRNA, donor DNA, and linearized pCMABIA3301 by using QIAquick Gel Extraction Kit and elute the digested plasmid into 30 μL elution buffer.
7. Determine the concentration of digested inserts and vector by spectrometer.

8. Clone AtU6-sgRNA cassette and donor DNA into the pCAMBIA3301:

   X μL AtU6-sgRNA cassette (insert).

   Y μL Donor DNA (insert).

   10 ng pCAMBIA3301.

   1 μL 10× T4 DNA Ligase buffer.

   0.5 μL T4 DNA Ligase

   Add ddH_2O to 10 μL.

   The ratio of insert DNA mass (insert:vector = 3 ~ 5:1) can be calculated from the website https://nebiocalculator.neb.com/#!/ligation. Incubate at 16 °C overnight or room temperature for 10 min.

9. Transform ligation product into *E. coli* as described above (*see* Subheading 3.1.3, **steps 7–9**).
10. Screen for correct clones by colony PCR using pCAMBIA3301-specific R primer and reverse oligo for sgRNA (see Table 1). Correct clones will give PCR products of approximately 650 bp. The procedure is described above (*see* Subheading 3.1.3, **step 10**).
11. Miniprep 2–4 clones using the QIAprep Spin Miniprep Kit and confirm the AtU6-sgRNA cassette and donor DNA in pCAMBIA3301 by DNA sequencing using pCAMBIA3301-specific F and R primers (Figs. 4c and 5c, *see* Table 1).
12. Transform sgRNA and donor DNA in pCAMBIA3301 binary vector into *Agrobacterium* competent cells (strain GV3101) as described above (*see* Subheading 3.1.5, **steps 1–6**).
13. Transform the donor construct into the DD45 pro::Cas9 harboring parental line (CS69955 or CS69956) as described above (*see* Subheadings 3.1.5, **steps 7–13**).

3.2.4 Screening of Gene-Targeted Arabidopsis Plants

1. Select T1 transgenic *Arabidopsis* plants by 50 mg/L glufosinate ammonium antibiotic containing 0.5× MS plate as described above (*see* Subheading 3.1.6, **steps 1–5**).
2. Extract genomic DNA from T1 transgenic *Arabidopsis* leaves as described above (*see* Subheadings 3.1.6, **steps 6–18**).
3. For knock-in, the positive gene targeting plants can be identified by PCR. False-positive bands would be detected when GT-specific primer sets are used, due to hypersensitivity of the primer sets. The external primer sets seem better option for screening of true GT-positive plants (Fig. 9a, b). Perform PCR including a WT DNA sample as a control:

   0.5 μL Template extracted genome DNA.

   2 μL 10× Taq buffer.

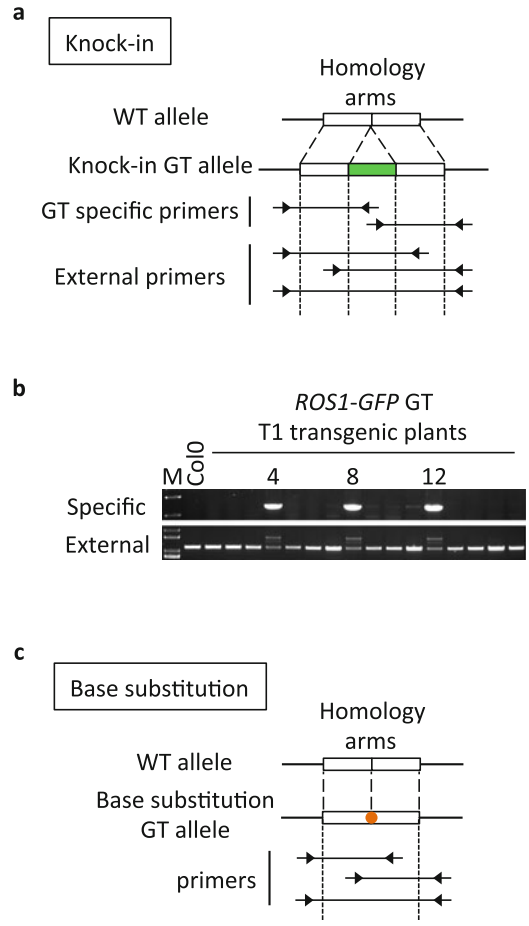

Fig. 9 Screening strategy for GT plants. (**a**) Primer design for screening of knock-in GT plants. GT-specific primers are specific to knock-in GT alleles, but external primers can amplify both endogenous and knock-in GT alleles. (**b**) Screening of *ROS1-GFP* GT. PCR screening of T1 individual *ROS1-GFP* GT samples show that some nonspecific bands (false positive) are detected when specific primers are used (upper panel). Three GT-positive plants (# 4, 8, 12) are detected by using external primer set (lower panel). Plants found positive in both PCR genotyping strategies are potentially true GT plants, and their heritability is tested in T2 generation. (**c**) Primer design for screening of base substitution GT plants. Orange circle indicates intended mutation(s)

1.6 μL dNTP (2.5 mM).

0.05 μL Taq polymerase.

0.4 μL Forward external primer (10 μM).

0.4 μL Reverse External primer (10 μM).

Add ddH_2O to 20 μL.

Perform PCR: 95 °C for 5 min, 25 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 1 min, and additional 5 min of 72 °C (see Note 8).

4. Run PCR products on a 0.8% agarose gel (Fig. 9b).
5. For amino acid substitution, the combination of PCR and restriction enzyme digestion (RFLP) is useful to detect gene targeting (Fig. 5a, 9c). The procedure is the same as the one described above (*see* Subheading 3.1.6, **steps 19–23**).
6. Both knock-in and substitution GTs are mainly established in the T1 generation such as line #4, 8, and 12 (Fig. 9b). Then, to identify heritable knock-in and substitution GTs, an additional screening and genotyping should be performed in the T2 generation [18].

4 Notes

1. Sometimes actual sgRNA activity might be different from the efficiency predicted by software. So to avoid the possibility that mutants will not be obtained due to low Cas9 nuclease activity, we usually design at least two independent sgRNAs for each target gene. This enables obtaining different mutant alleles, and could be a good option to minimize the risk of experiment failure.
2. The PAM sequence should be not included in the oligomers.
3. If PCR products could not be subcloned into the Zero Blunt TOPO PCR cloning vector, dilute the concentration of PCR products 1/5 to 1/20 (or more) with ddH_2O.
4. In this protocol, AtU6-26 PolIII promoter (At3g13855) was introduced for multiple targets, a combination of other PolIII promoters such as AtU3b and At7SL-2 are used to obtain higher order mutants [10, 11]. The protocol for AtU6-26 described in this chapter is adaptable to other PolIII promoters (*see* Subheading 3.1.3).
5. The traditional Cas9 generates DSB at 3 bp upstream of PAM sequence. Thus short in-del mutations ensue surrounding this DSB site (Figs. 1b and 8c). On the other hand, ABE and CBE base editors generate base conversions at positions 3–14 and 4–15 of the protospacer, respectively (Fig. 1b) [20–23]. Thus, certain restriction enzyme sites must overlap with those possible mutation regions, when the combination of PCR and restriction enzyme digestion (RFLP) is used for mutant screening. Alternatively, Cas9-induced mutations can be detected by derived cleaved amplified polymorphic sequences (dCAPS), T7 endonuclease I assay or deep sequencing. The activity window depends on the base editors and CRISPR/Cas [26]. Thus, confirmation of base editor activity window should be priorly done.

6. We have used 801 bp and 325 bp for 5′ and 3′ homology arms for *GFP* knock-in into the *ROS1* locus, respectively (Fig. 4a), and the donor construct worked well [18]. However now we use 800 bp homology arms each for donor.
7. If the products of the second round of PCR are not obtained, a gradient of annealing temperature PCR (55–70 °C) can be tried. Alternatively, the concentration of first round PCR products can be determined by using the absorption spectrometer, and an equal amount of PCR products can be used as template for the second round PCR.
8. As described in Subheading 3.2.4, **step 3**, the GT-specific primer set give rise to some false-positive signals that are not detected by the external primer set (Fig. 9b). However, sometimes it is difficult to amplify both the endogenous and the knock-in alleles with the external primer sets due to the size of PCR products and the PCR amplification efficiency. The PCR conditions for external primer sets should not exceed 25 cycles. When additional cycles are performed, the band corresponding to the knock-in allele will not be detected because the PCR product from the endogenous allele is preferentially amplified.

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