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Chapter 8

CRISPR/Cas-Mediated Site-Specific Mutagenesis in *Arabidopsis thaliana* Using Cas9 Nucleases and Paired Nickases

Simon Schiml, Friedrich Fauser, and Holger Puchta

Abstract

The CRISPR/Cas system has recently become the most important tool for genome engineering due to its simple architecture that allows for rapidly changing the target sequence and its applicability to organisms throughout all kingdoms of life. The need for an easy-to-use and reliable nuclease is especially high in plant research, as precise genome modifications are almost impossible to achieve by *Agrobacterium*-mediated transformation and the regeneration of plants from protoplast cultures is very labor intensive. Here, we describe the application of the Cas9 nuclease to *Arabidopsis thaliana* for the induction of heritable targeted mutations, which may also be used for other plant species. To cover the concern for off-target activity, we also describe the generation of stable mutants using paired Cas9 nickases.

Key words Gene technology, Double-strand break repair, Engineered nucleases, Genome engineering, Targeted mutagenesis

1 Introduction

The molecular basis of a type II CRISPR (for clustered regularly interspaced short palindromic repeats) system from *Streptococcus pyogenes* was unraveled in 2012 [1]. The CRISPR-associated (Cas) protein Cas9 was shown to be a nuclease that interacts with two short RNAs, the CRISPR-RNA (crRNA) and the trans-activating crRNA (tracrRNA). The crRNA binds to the target site on the DNA by Watson–Crick pairing and Cas9 cleaves the DNA strands with two nuclease domains (termed RuvC-like domain and HNH motif), each cleaving one of the two DNA strands. It was also shown, that the two RNAs could be fused to form a chimeric single-guide RNA (sgRNA) without losing cleavage activity. Furthermore, it was demonstrated that, by mutating a single amino acid in the active site of one of the nuclease domains, (substitutions D10A or H840A) Cas9 could be converted to a nickase.

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The first demonstration of Cas9 as a programmable, engineered nuclease for the targeted induction of mutations in human cells [2, 3] marks one of the most important contributions to the field of genome engineering over the last years. Since then, the CRISPR/Cas system has successfully been applied to a wide range of organisms [4–8]. For plants, there has always been a tremendous need for tools to manipulate genomes in a site-specific manner, since transgenes transferred via *Agrobacterium tumefaciens* integrate completely haphazardly. Therefore, the CRISPR/Cas system was rapidly demonstrated to work in protoplast and callus cultures of *Arabidopsis thaliana*, tobacco, rice, and wheat [9–11]. Furthermore, it was reported by several groups that CRISPR-induced mutations in plants can be inherited, which is the most important step for reliable biotechnological applications [12–14].

However, despite the possibilities that CRISPR/Cas offers there are still enduring doubts about off-target activity. Although this has been intensively studied with different methods [15–19], it is still not completely clear whether the 23 nts target sequence guarantee sufficient specificity. To overcome this problem, a Cas9 nickase can be applied together with two sgRNAs, which guide the enzyme to cleave each of the two DNA strands. Thus, a double-strand break is formed and mutations are induced by nonhomologous end-joining [20, 21]. We have demonstrated that this approach can also be applied to plants, producing heritable mutations at a comparable frequency as for the Cas9 nuclease [22].

Here, we describe the design and cloning procedure of our CRISPR/Cas vector system as well as the generation of homozygously mutated lines for *A. thaliana* [13]. The target specificity of the sgRNA is determined by cloning a pair of annealed oligonucleotides into a respective plasmid harboring the sgRNA backbone. Subsequently, the complete sgRNA construct is transferred to a binary vector that contains the Cas9 expression cassette. The final vector can then be transformed into plants via *A. tumefaciens*. We also show the procedure to apply the paired Cas9 nickases to *A. thaliana* [22]. The modified sgRNA vector allows for parallel cloning of both sgRNA constructs. Two consecutive cloning steps combine both sgRNA sequences with the Cas9 expression system, again resulting in a binary vector for *Agrobacterium*-mediated transformation. This vector system may not only be used for the Cas9 nickase but also for the production of paired nucleases that create site- and sequence-specific deletions in the genome.

2 Materials

2.1 Plant Material

Transformable plant material according to your standard transformation protocol (e.g., *A. thaliana* plants as previously described [23]).

2.2 Vectors

All vectors described can be obtained upon request to the authors or from the Arabidopsis Biological Resource Center (ABRC, donations CD3-1927 to CD3-1932). Sequence information on all plasmids is obtainable at www.botanik.kit.edu/crispr.

1. pChimera

   Cloning vector that was synthesized by GeneArt (Life Technologies Inc., Carlsbad, CA, USA). Contains the sgRNA expression system flanked by *Avr*II restriction sites and can be used for classical cloning into the binary Cas9 expression vector pCAS9-TPC. Confers resistance to ampicillin.

2. pEn-Chimera

   Based on the pGEM®-T-Easy backbone (Promega Corp., Fitchburg, WI, USA); contains the sgRNA expression system flanked by attR sites for Gateway cloning into the binary Cas9 expression vector pDe-CAS9. Confers resistance to ampicillin.

3. pEn-C1.1

   Identical to pEn-Chimera except for additional *Bsu*36I and *Mlu*I restriction sites on each side between the sgRNA and the attR site. Confers ampicillin resistance.

4. pCAS9-TPC

   Based on the backbone of pPZP201 [24], a binary plasmid for *Agrobacterium*-mediated transformation. Harbors the Cas9 expression cassette, the sgRNA from pChimera can be added by classical cloning with *Avr*II. Confers spectinomycin resistance in bacteria, the T-DNA contains a resistance gene against phosphinothricin (PPT) (*see* **Note 1**).

5. pDe-CAS9

   Resembles pCAS9-TPC, a Gateway destination sequence was added that contains a *ccdB* gene, to allow addition of the sgRNA from pEn-Chimera or pEn-C1.1 by Gateway cloning. Confers spectinomycin resistance in bacteria, the T-DNA contains a resistance gene against PPT has to be propagated in *ccdB*-resistant *E. coli* strain.

6. pDe-CAS9-D10A

   Identical to pDe-CAS9, the D10A exchange was accomplished by PCR-based site-directed mutagenesis. The plant resistance was changed to kanamycin.

2.3 Reagents

1. DNA-oligonucleotides

   All oligonucleotides were ordered in desalted purity and were used at a concentration of 50 pmol/µl. Table 1 lists all primers that are described in the Subheading 3.

2. Restriction enzymes *Bbs*I and *Avr*II with buffers as supplied (*see* **Note 2**).
3. Taq DNA Polymerase for colony-PCRs (*see* **Note 3**).
4. T4 DNA ligase with buffer as supplied.
5. Alkaline phosphatase with buffer as supplied.
6. LR Clonase II Enzyme mix (includes Proteinase K).
7. TE-buffer pH 8: 10 mM Tris–HCl, 1 mM EDTA.
8. LB-agar plates: 10 g/L pepton, 5 g/L yeast extract, 10 g/L NaCl, 7.5 g/L agar.
9. GM-agar plates: 4.9 g/L Murashige & Skoog, 10 g/L sucrose, adjust to pH 5.7, 8 g/L agar.
10. Antibiotics/herbicides: ampicillin, spectinomycin, kanamycin, and PPT.
11. Bacterial strains:

For propagation of *ccdB* encoding vectors (pDe-CAS9, pDe-CAS9-D10A), use a *ccdB*-resistant *E. coli* strain, e.g., DB3.1 (Life Technologies Inc.). All cloning steps are performed with NEB5α (New England Biolabs), a derivate of DH5α. For plant transformation, use *A. tumefaciens* strain GV3101 (*see* **Note 2**).

Table 1
Sequences of all primers

Oligo name	Sequence 5′–3′	Orientation
M13 rev	CACAGGAAACAGCTATGAC	rv
SS42	TCCCAGGATTAGAATGATTAGG	fw
SS43	CGACTAAGGGTTTCTTATATGC	rv
SS102	CACCATGTTATCACATCAATCC	rv
SS61	GAGCTCCAGGCCTCCCAGCTTTCG	fw
SS72	CTTCTATCGCCTTCTTGACG	rv
SS143	CAAGAAAGCTGGGTCCTCAG	rv
SS144	GTCCGGACGTCTTAATTAACC	rv

3 Methods

The basic principle of our cloning system is made up by two cloning steps. The first step involves the specification of the sgRNA for the target sequence. This is achieved by annealing and subsequent cloning of two complementary oligonucleotides into a linearized plasmid. After confirmation of the cloning products, the sgRNA scaffold is then transferred into a binary vector that contains the Cas9 expression cassette. This second cloning step can either be performed by conventional, restriction-based cloning or via Gateway cloning. For the paired nickases, the sgRNAs have to be transferred in two subsequent cloning steps.

3.1 Cloning of sgRNAs

1. Digest the sgRNA vector (pChimera, pEn-Chimera, pEn-C1.1, *see* **Note 4**) with *Bbs*I. Mix 1 μg of plasmid miniprep with 2 μl restriction enzyme buffer and 1 μl *Bbs*I, add ddH_2O to a total volume of 20 μl. Incubate at 37 °C for at least 2 h or overnight.
2. Purify the digested vector with a PCR purification kit, there is no need to purify it from a gel. Elute in 30 μl ddH_2O and adjust the final concentration to 5 ng/μl (*see* **Note 5**).
3. Pick your 20 nt CRISPR target sequence upstream of an NGG PAM (protospacer adjacent motif, *see* **Notes 6–8**). Order the following oligonucleotides (Fig. 1):

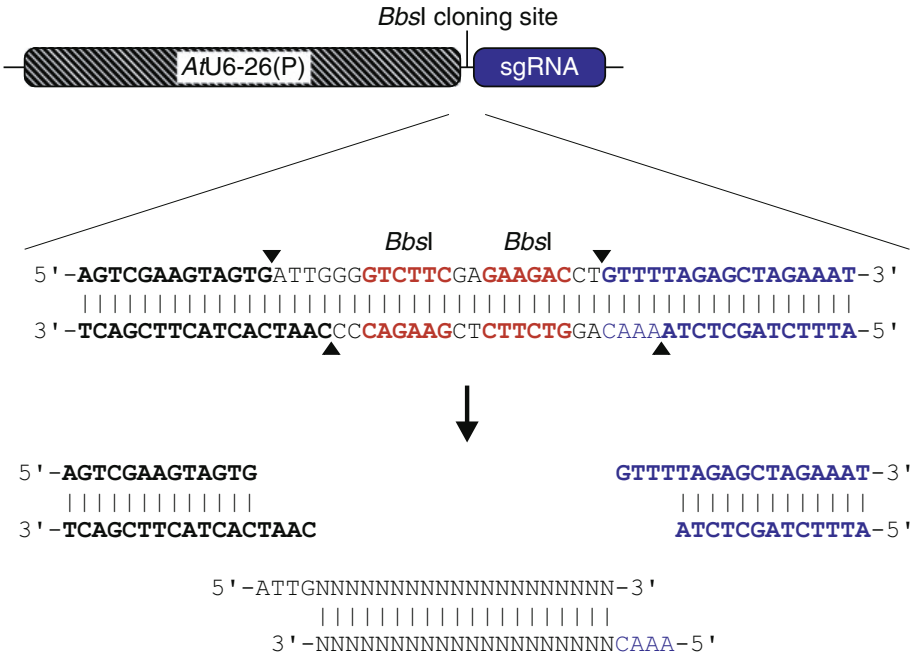

Fig. 1 Cloning principle for the specification of sgRNAs. The *Bbs*I recognition sites are cleaved out of the vector, generating defined sticky ends. Two complementary oligonucleotides with appropriate overhangs are cloned into the cutting site

Forward: 5′-ATTG-20 nts protospacer-3′

Reverse: 5′-AAAC-20 nts reverse complement of your protospacer-3′

4. Mix 2 µl of each oligo (50 pmol/µl) with 46 µl of ddH_2O. Incubate for 5 min at 95 °C, cool at room temperature for 10 min.
5. Mix 2 µl of digested sgRNA vector with 3 µl of the annealed oligos. Add 1 µl T4 ligase buffer, 3 µl ddH_2O, and 1 µl T4 ligase. Incubate for at least 2 h at room temperature. Transform 5 µl into *E. coli* NEB5α and plate on LB with ampicillin (100 mg/l).
6. Check colonies by colony-PCR (*see* **Note 3**). Use your forward protospacer oligo together with M13 rev, anneal at 56 °C. The expected band is at approx. 370 bp; you will find at least 70% of the clones to be correct, so checking 4–6 colonies is sufficient.
7. Purify plasmid DNA of one or two clones and sequence the plasmids with primer SS42 to verify correct integration of your spacer.

3.2 Transfer of sgRNA to Binary Vector by Conventional Cloning

1. Assemble your sgRNA in pChimera as described in Subheading 3.1.
2. Digest your sgRNA vector and pCAS9-TPC with *Avr*II by mixing 1–2 µg plasmid DNA with 2 µl restriction enzyme buffer and 1 µl *Avr*II (*see* **Note 9**), add ddH_2O to a total volume of 20 µl. Incubate for at least 2 h at 37 °C.
3. Separate sgRNA from vector backbone in a 1 or 2% agarose gel. The desired band is at approx. 530 bps. Purify with a gel purification kit, elute in ddH_2O.
4. Purify digested pCAS9-TPC backbone directly from the reaction, elute in 30 µl ddH_2O. Dephosphorylate the backbone by adding 3.5 µl phosphatase buffer and 1.5 µl alkaline phosphatase. Incubate for 20 min at 37 °C and heat inactivate the enzyme as recommended.
5. Prepare ligation by mixing 5 µl of sgRNA insert with 5 µl dephosphorylated vector, 2 µl T4 ligase buffer, 7 µl ddH_2O, and 1 µl T4 ligase (*see* **Note 10**). Incubate for at least 3 h (or overnight) at RT. Transform 10 µl of the reaction into NEB5α and plate on LB with spectinomycin (100 mg/l).
6. Check colonies by colony-PCR. Use primers SS42/SS43, anneal at 60 °C. The expected band is at approx. 1 kb. Check 5–10 clones or more if necessary.
7. Purify plasmid DNA of one or two clones. Check the plasmids by control digestion with *Afl*II and *Nhe*I, expected bands are at approx. 5.9, 5, and 3.8 kb.
8. Correct vectors are ready for transformation into *A. tumefaciens* and subsequent plant transformation.

3.3 Transfer of sgRNA by Gateway Cloning

1. Assemble your sgRNA in pEn-Chimera as described in Subheading 3.1.
2. Adjust your sgRNA vector to 50 ng/μl and the destination vector to 100 ng/μl.
3. Prepare Gateway-reaction: Mix 2 μl of your specified pEn-Chimera plasmid with 3 μl of pDe-CAS9. Add 4 μl TE-buffer and 1 μl of LR Clonase II. Vortex and centrifuge the reaction and incubate for at least 2 h at room temperature.
4. Add 1 μl Proteinase K and incubate for 10 min at 37 °C (*see* **Note 11**).
5. Transform completely into NEB 5α and plate on LB with spectinomycin (100 mg/l).
6. Check by colony-PCR with primers SS42/SS43. Anneal at 60 °C, the expected band is at about 1 kb.
7. Purify plasmid DNA of one or two clones. Control by restriction digestion with *Afl*II and *Nhe*I, expected bands are at approx. 5.9, 5, and 3.8 kb.
8. Correct vectors are ready for transformation into *A. tumefaciens* for plant transformation.

3.4 Cloning of Constructs with Paired Nickases

1. Assemble both sgRNAs in pEn-C1.1 as described in Subheading 3.1.
2. Digest your first sgRNA vector and pDe-CAS9-D10A with *Bsu*36I and *Mlu*I (*see* **Notes 12** and **13**). Use 1–2 μg of plasmid along with 2 μl restriction enzyme buffer and 1 μl of each enzyme, add ddH_2O to a total volume of 20 μl. Incubate for at least 1 h at 37 °C.
3. Purify your sgRNA fragment from a gel, to separate it from its vector backbone. The fragment size is about 530 bp. The digested pDe-CAS9 can be purified directly from the reaction.
4. Prepare ligation using 5 μl of digested pDe-CAS9-D10A and 5 μl of digested pEn-C1.1, 2 μl T4 buffer, 6 μl ddH_2O, and 1 μl T4 ligase (*see* **Note 10**). Incubate for at least 3 h or overnight at room temperature.
5. Transform 10 μl of the reaction into a *ccdB*-resistant *E. coli* strain (e.g., DB3.1), plate on LB containing 100 mg/l spectinomycin.
6. Check clones by colony-PCR. Use primers SS42/SS102 (*see* **Note 13**), anneal at 60 °C, expected band is at approx. 1 kb.
7. Purify plasmid DNA of one or two clones. Control by restriction digestion with *Afl*II and *Nhe*I. Expected bands are at 7.5, 5.7, and 3.8 kb.

8. Adjust to 50 ng/µl. Adjust your second sgRNA-plasmid to 100 ng/µl.
9. Prepare Gateway-reaction: mix 2 µl sgRNA-plasmid with 3 µl destination vector (already containing the first sgRNA construct). Add 4 µl TE-buffer and 1 µl LR Clonase II. Vortex and centrifuge; incubate for at least 2 h at room temperature.
10. Add 1 µl Proteinase K and incubate for 10 min at 37 °C.
11. Transform completely into NEB 5α and plate on LB with 100 mg/l spectinomycin.
12. Check clones by colony-PCR using primers SS61/SS143. Anneal at 60 °C, the expected band is at approx. 1 kb.
13. Purify plasmid DNA of one or two clones. Control by digesting with *Afl*II and *Nhe*I. Expected bands are at 5.8, 5.7, 3.8, and 0.6 kb. Confirm by sequencing with primer SS144 (*see* **Note 14**).
14. Correct vectors are ready for *A. tumefaciens* mediated transformation.

3.5 Mutant Screening in A. thaliana

1. Transform your vector into *A. tumefaciens*.
2. Perform stable Agrobacterium-mediated transformation into *A. thaliana* (e.g., floral dip transformation as described in [23]).
3. Select for primary transformants on GM plates by using 7 mg/l PPT (nuclease) or 30 mg/l Kanamycin (nickase).
4. Cultivate 20 or more primary transformants to obtain progeny seeds (T2 generation, *see* **Notes 15** and **16** and Fig. 2).
5. Check T2 lines for correct Mendelian segregation on the respective selection medium to identify single-locus lines (25% should not be able to germinate).
6. For ten correctly segregating lines sow at least ten seeds each on medium or soil without selection marker (*see* **Note 17**).
7. After 10–14 days, extract DNA from your plants with a rapid extraction protocol [25].
8. Test samples for mutagenesis events. This can be done in numerous ways, the most common methods are T7 endonuclease assay, restriction digestion assay, or high-resolution melting analysis.
9. Confirm positively tested samples by Sanger sequencing (*see* **Note 18**).
10. Cultivate mutated plants to obtain T3 seeds.
11. For each T3 line, sow 20 seeds on respective selection medium to validate absence of the T-DNA (*see* **Note 19**).
12. For transgene-free T3 lines, test up to ten plants for the presence of the mutation by Sanger sequencing.

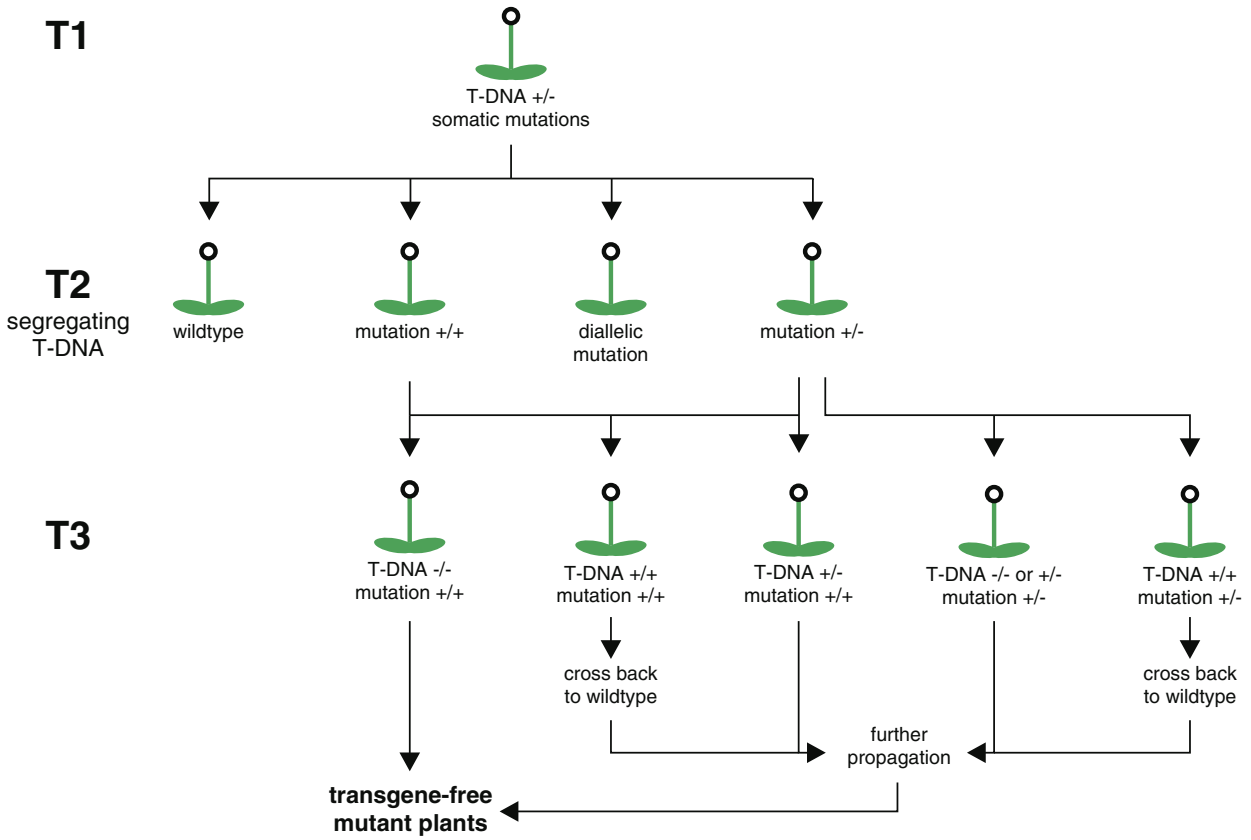

Fig. 2 Simplified overview of possible mutagenesis outcomes. Somatic mutagenesis events can occur in transgenic T1 plants. In T2, the T-DNA segregates in a Mendelian fashion for single-locus lines. Heritable mutations can be heterozygous, homozygous, or diallelic (transheterozygous). A transgene-free, homozygously mutated plant can occur in T2 as well as in later generations. T2 plants tested positively for a mutagenesis event should therefore be cultivated to T3 and the offspring should be tested for the presence of the T-DNA and for the mutation genotype. Note that in any heterozygous mutant or wild-type plant with at least one T-DNA allele, ongoing mutagenesis can create chimeric plants

4 Notes

1. Figure 3 summarizes the final constructs. Note that all elements can be exchanged to fit your specific needs (e.g., different promoter or plant resistance).
2. Enzymes and strains listed are according to what we use in the lab. Since the procedures are designed to be standard cloning steps, just use the normal material that your lab is used to.
3. Colony-PCR is used to identify correct colonies on your transformation plate. This is generally an optional but recommended step, as it reduces the number of plasmid isolations you have to perform. However, a robust *Taq*-polymerase is needed, that can cope with inhibiting substances in the reaction. We routinely use DreamTaq polymerase (ThermoFisher Scientific). Also, it is recommended to make 50 µl reactions, so that inhibitors are more diluted.

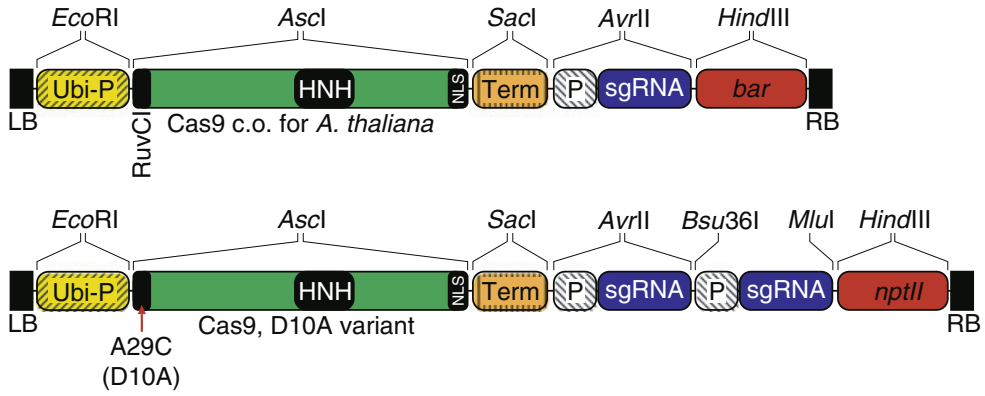

Fig. 3 Final T-DNA constructs for nuclease (*top*) and paired nickases (*bottom*). The shown restriction sites may be used to exchange any of the contained elements to fit your specific situation

4. Generally, pChimera is for conventional cloning, pEn-Chimera is for Gateway cloning, and pEn-C1.1 is for paired sgRNAs.
5. The digested vector can be stored at −20 °C, so there is no need to do a new *Bbs*I restriction reaction for every sgRNA.
6. The design of spacers can be done manually. There are only few limitations: avoid presence of an *Avr*II restriction site, if you want to do conventional cloning. For paired nickases, one of your spacers should be devoid of *Bsu*36I and/or *Mlu*I. Furthermore, avoid having more than five subsequent T's in your spacer sequence, as this can terminate expression of the sgRNA. The spacer sequence does not have to start with a G.
7. If you prefer using design tools, we recommend CCTop [26] or CRISPR-P [27]. The latter is especially useful, if you want to identify mutants via the disruption of a restriction site.
8. It is also possible to pick CCN as PAM if this fits better for your specific experiment. In this case, your forward-oligo (still starting with ATTG) should contain the reverse-complementary sequence of the 20 nts downstream of the PAM, while the reverse oligo directly resembles your target sequence.
9. Use undiluted plasmid miniprep for the digestion reaction, normally ranging in concentrations from 100 to 500 ng/μl.
10. Simply taking 5 μl of each ligation fragment without caring for concentrations proved to be working well. However, if you experience problems with your cloning, changing to a threefold molar excess of the insert fragment should improve ligation success.
11. Proteinase K treatment is crucial. There will be no correct clones, if this step is skipped.
12. Depending on your spacer design, it is also possible to use only one of the two enzymes. However, in this case, it is necessary to dephosphorylate the vector prior to ligation.

13. The described procedure can also be used for paired nucleases, to induce specific deletions. Just use pDe-CAS9 instead of pDe-CAS9-D10A. The reverse primer for the first colony-PCR changes to SS72 and the control digestion bands differ slightly.
14. Make sure to identify the 0.6 kb band, as it indicates the presence of both sgRNA cassettes. The sequencing covers both sgRNA cassettes in one run, so it confirms your sgRNAs are both present and intact.
15. The exact number may vary depending on the bacterial strain and the transformation method. Make sure to have at least ten individual single-locus lines.
16. It is possible, yet not necessary to check for nuclease activity in primary transformants. This can be done by T7 endonuclease or restriction digestion assay.
17. Again, the exact number can vary and is hard to predict. Testing 100 plants should be enough to identify a mutant; however, this number can be scaled up easily.
18. Plants are likely to be heterozygous in this stage. A double peaked chromatogram can be analyzed with Poly Peak Parser [28].
19. Transgene-free T3 offspring represents the optimal situation, indicating that the respective T2 mother plant was already transgene free. If the mutation and/or the T-DNA are still heterozygous in T3, the number of plants and lines can be scaled up or the procedure can be repeated in T4. If the T-DNA is homozygous (i.e., all plants grow on selection medium), it is possible to cross back to wild-type plants.

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