

Use of the Cas9 Orthologs from *Streptococcus thermophilus* and *Staphylococcus aureus* for Non-Homologous End-Joining Mediated Site-Specific Mutagenesis in *Arabidopsis thaliana*

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Abstract

Since the discovery of the CRISPR/Cas system and its in vivo application for site-specific targeted mutagenesis, this technique is widely used in a great variety of organisms, such as many plant species. Commonly used for this application is the Cas9 enzyme from *Streptococcus pyogenes*. Here, we describe the application of two Cas9 orthologs from *Streptococcus thermophilus* and *Staphylococcus aureus* for targeted non-homologous end-joining mediated mutagenesis in *Arabidopsis thaliana*. With both orthologs, we could show efficient inheritance of the induced mutations. As both Cas9 orthologs are smaller in size than the enzyme of *S. pyogenes* and as the Protospacer adjacent motifs (PAMs) differ between all orthologs, they are attractive alternative tools for genome engineering in plants.

Key words Gene editing, Double-strand break repair, CRISPR/Cas, Genome engineering, Targeted mutagenesis

1 Introduction

In 2012, the molecular mechanism of the CRISPR/Cas system (for clustered regularly interspaced short palindromic repeats; CRISPR-associated) was discovered [1] and after the transfer of the molecular mechanism of the Cas9 enzyme to human cell culture [2–4] this system is now used for targeted induction of double-strand breaks (DSBs) in all kinds of organisms including plants [5].

The components needed for site-specific target recognition and subsequent cleavage are the Cas9 enzyme and its corresponding single guide RNA (sgRNA). The sgRNA consists of a CRISPR RNA (crRNA) and a trans-activating crRNA (tracrRNA) part, fused via a linker sequence to form this single RNA component interacting with the Cas9 enzyme. Target specificity is acquired through 20 nt of the crRNA part, the so-called spacer, which

Table 1

Comparison of the Cas9 orthologsOrthogous gene. Listed are the characteristics for the Cas9 orthologs SpyCas9, Sth1Cas9, and SauCas9 regarding their PAM sequence, size, and positions for creating nickase or dCas9 constructs

	<i>Streptococcus pyogenes</i> (Spy)	<i>Streptococcus thermophilus</i> 1 (Sth1)	<i>Staphylococcus aureus</i> (Sau)
PAM sequence	NGG NAG	NNAGAA NNGGAA	NNGRRT NNGRR(N)
Cas9 size	4.1 kb	3.4 kb	3.2 kb
point mutations for nickase/dCas9	D10A, H840A	D9A, H599A	D10A, N580A

binds complementary to the target sequence, where after the Cas9 nuclease induces a DSB. Next to the genomic target sequence, which is called protospacer, a protospacer-adjacent motif (PAM) is required for correct target recognition. Not only the PAM differs between Cas9 orthologs, but also the size of the Cas9 enzyme itself (see Table 1). The PAMs of here described Cas9 orthologs from *Streptococcus thermophilus* (Sth1Cas9) and *Staphylococcus aureus* (SauCas9) consist of five or six nucleotides compared to the only three nucleotides required for *Streptococcus pyogenes* Cas9 (Spy-Cas9) target recognition. This increment of specificity should lead to less off-target effects using these orthologs compared to Spy-Cas9, which was already demonstrated for SauCas9 in human cell culture [6, 7].

The Cas9 orthologs used here belong to the same class II type II-A CRISPR/Cas system, but are subdivided into different sub-clusters, in which the composition of the involved Cas proteins and the direction of the transcription of the CRISPR locus compared to the corresponding Cas operons differ [8–10]. Also the Cas9 proteins vary in their corresponding sgRNA sequences [7, 11, 12], at which structures like hairpins or additional stem loops are necessary for interaction with the correct Cas9. The requirements regarding the interaction between crRNA and tracrRNA and also the particular PAM sequences differ between these Cas9 orthologs, wherefore the systems should be able to function independently in a single cell.

With these orthologous Cas9 enzymes, not only new targets could be aimed on due to the different PAM requirements, but also new genome engineering approaches are possible combining various catalytic activities of several orthologs. One requirement for this simultaneous application of these Cas9 orthologs is the independent and specific activity of each enzyme with each own but not a “foreign” sgRNA. This was already demonstrated in plants for SpyCas9 and SauCas9, so the simultaneous application of different

Cas9 orthologs in one single cell should be possible without any cross-interaction [13], allowing to develop applications for new genome and also transcriptome engineering approaches in plants. In human cell culture, different chromosomal loci were labeled using orthologous dead Cas9 enzymes (dCas9, catalytically inactive Cas9), leading to multicolor detection of these targeted loci [14, 15]. As these Cas9 orthologs have been shown to also work in plants [13, 16, 17], similar applications using more than one Cas9 ortholog at once should be possible in plants, too [18].

Here, we describe the design as well as the cloning procedure of the orthologous RNA-guided Cas9 nucleases from *S. thermophilus* and *S. aureus* for their application in *A. thaliana* to generate heritable homozygously mutated plant lines [13]. By adjusting 20 nt of the sgRNA by annealing two oligonucleotides and subsequent cloning into a plasmid containing the sgRNA backbone, targeting of the desired sequence can be achieved. This adapted sgRNA construct is then transferred into a binary vector containing the Cas9 expression cassette, where after the final construct can be transferred via *A. tumefaciens* transformation into plants.

2 Materials

2.1 Antibiotics and Herbicides

1. 100 mg/mL Ampicillin (stock solution).
2. 100 mg/mL Spectinomycin (stock solution).
3. 30 mg/mL Kanamycin (stock solution).

2.2 Bacterial Strains

1. NEB5 α (New England Biolabs) *E. coli* strain for conventional cloning steps.
2. GV3101 pMP90 *A. tumefaciens* strain for plant transformation.
3. DB3.1 (Life Technologies Inc.) *E. coli* strain for *ccdB* encoding vectors.

2.3 Plant Material, Buffer, Media, and DNA Oligonucleotides

1. Germination medium (GM): 4.9 g/L Murashige & Skoog, 10 g/L sucrose, adjust to pH 5.7 with KOH, 8 g/L agar.
2. Lysogeny broth (LB): 10 g/L pepton, 5 g/L yeast extract, 10 g/L NaCl, (17.5 g/L agar for solid plates).
3. Yeast extract broth (YEB): 5 g/L beef extract, 5 g/L pepton, 1 g/L yeast extract, 5 g/L sucrose, 493 mg/L MgSO₄, (12 g/L agar for solid plates).
4. TE buffer: 10 mM Tris-HCl, 1 mM EDTA (2,2',2'',2'''-(Ethane-1,2-diyl)dinitrilo)tetraacetic acid), adjust to pH 8.

Table 2
Sequences of all primers

Oligo name	Sequence 5'-3'	Orientation
CS 32	GTCATAGCTGTTTCCTTGC	rv
SS42	TCCCAGGATTAGAATGATTAGG	fw
SS102	CACCATGTTATCACATCAATCC	rv
SS61	GAGCTCCAGGCCTCCCAGCTTTCG	fw
SS72	CTTCTATCGCCTTCTTGACG	rv
SS143	CAAGAAAGCTGGGTCTCTCAG	rv

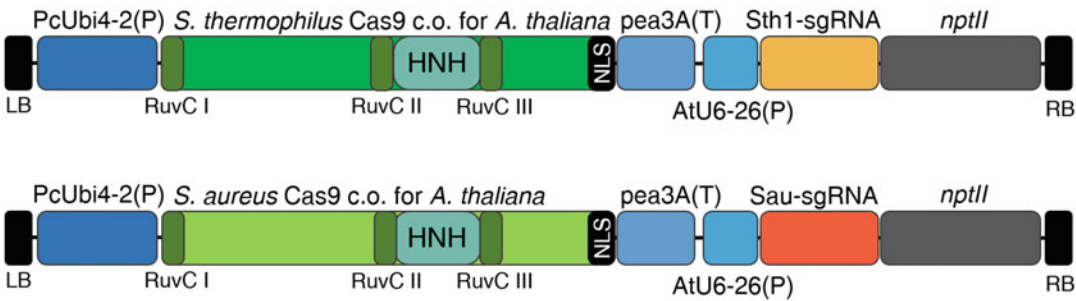


Fig. 1 Final T-DNA constructs for Sth1Cas9 nuclease and SauCas9 nuclease. The final expression cassettes include the orthologous Cas9 enzyme as well as the corresponding sgRNA on a T-DNA construct that is ready to use for plant transformation

5. 50 pmol DNA oligonucleotides, desalted purity. In Table 2, all primers described in the methods section are listed.
6. In conformity with your general transformation protocol every type of transformable plant material can be used (*see Note 1*).

2.4 Enzymes

1. Restriction enzymes 20,000 U/mL *AflII*, 10,000 U/mL *BbsI* and 10,000 U/mL *NheI* with 10× buffer as supplied.
2. 1000 U/mL T4 DNA Ligase with 10× buffer as supplied.
3. LR Clonase II Enzyme mix and 800 U/mL Proteinase K.
4. Dream 5000 U/mL Taq DNA Polymerase.

2.5 Vectors

The sequence information of all described vectors can be obtained at www.botanik.kit.edu/crispr. All vectors are obtainable upon request from the authors. The final T-DNA constructs are shown in Fig. 1.

1. pEn-Sau Chimera: The sequence coding for the specific Sau sgRNA is combined with the *A. thaliana* U6-26 promotor specific for the expression of small RNAs and a termination

signal. The sgRNA is flanked by attL sites necessary for gateway cloning. Between the sgRNA and the attL sites, two recognition sites for *Bsu36I* and *MluI* have been added for inserting up to three sgRNAs according to the pEnC1.1 vector established for Spy sgRNA [19]. The vector has been synthesized by GeneArt® (Life Technologies Inc.) based on the pMA-RQ vector backbone including an ampicillin resistance cassette.

2. pEn-Sth1 Chimera: Identical to the pEn-Sau Chimera except for the exchange of Sau sgRNA into specific sgRNA coding sequence of *Streptococcus thermophilus*. This vector also features an ampicillin resistance.
3. pDe-SauCAS9: Codon optimized for *A. thaliana* SauCas9 open reading frame (ORF) synthesized by GeneArt® (Life Technologies Inc.) based on the published sequence data [7]. Afterward, the SauCas9 ORF has been integrated in the pDe-CAS9 by classical cloning using *AscI* restriction sites. pDe-CAS9 harbors the plant-specific PcUbi4-2-promotor, pea3A-terminator and a *ccdB* gene, flanked by attR sites [20] and is located on a T-DNA-based pPZP201 backbone for *Agrobacterium*-mediated transformation [21]. Att sites are necessary for Gateway cloning for correct integration of sgRNA from pEn-Sau Chimera into the pDe-SauCAS9. The vector contains a spectinomycin resistance cassette for bacterial selection and the T-DNA includes an *nptII* gene conferring plant resistance to kanamycin.
4. pDe-Sth1CAS9: Codon optimized for *A. thaliana* Sth1Cas9 ORF synthesized by GeneArt® (Life Technologies Inc.), based on the published sequence data [22]. Beside the Cas9-ORF, pDe-Sth1CAS9 is identical to the pDe-SauCAS9 and also harbors a spectinomycin resistance cassette for bacterial selection and an *nptII* gene conferring plant resistance to kanamycin.

3 Methods

For a simple and reliable combination of Cas9 protein and specific adapted sgRNA, a cloning system containing the Gateway-based entry and destination vector has been designed for use. For both Cas9 orthologs from *S. thermophilus* and *S. aureus* the same cloning system is used, based on the previously established system designed for plant-specific expression of *S. pyogenes* Cas9 [19, 20]. Generally, this system is made up by two consecutive steps. In the first step, two complementary oligonucleotides, based on the target sequence, are integrated into the linearized entry vector, containing the sgRNA expression system. This cloning product is afterward transferred to the binary destination vector via a Gateway LR reaction (*see* Fig. 2 for an overview of the cloning procedure and Fig. 1 for the final T-DNA expression constructs).



pEn-Sau/Sth1

BbsI
digestion

Oligo Annealing

[illegible]

Spacer Sequence

Ligation

B)

pDe-Sau/Sth1

Destination vector

**Specified
entry vector**

Gateway reaction

By-product

C)

Expression Clone

■ PcUbi4-2 (P)

Sau/Sth1CA2

■ pcc3A(T)

peach (1)

F AtI16-26(P)

[illegible]

■ spacer sequence

Fig. 2 Simplified overview of the general cloning procedure. **(A)** For the specification the orthologous sgRNAs, *BbsI* recognition sites are cleaved, which are located between the AtU6-26 promoter and the sgRNA sequence, leading to non-complementary sticky ends. Annealed complementary target-specific oligonucleotides (orange), with matching overhangs to the sticky ends created by *BbsI* can be inserted into the orthologous entry vector pEn-Sth1 Chimera or pEn-Sau Chimera by ligation. **(B)** The following gateway reaction is performed via att sites present in the specified entry vector and the destination vector (pDe-Sth1CAS9 or pDe-SauCAS9 respectively). LR Clonase II performs the exchange of the regions between att sites resulting in the corresponding expression clone and a by-product **(C)**

3.1 Determination of an Appropriate PAM Sequence for Each Cas9 Ortholog and Oligo Annealing

1. Dependent on your final application you can choose between the different orthologs and their associated PAM requirements. In contrast to the PAMs needed for SpyCas9 target recognition, variable PAM sequences are available for both described orthologs.
2. Choose your PAM sequence suitable for your experimental set up (*see Note 2*). For the PAM sequences SauCas9 use “NNGRR(T)”; recommended SauCas9 PAM sequences are “NNGGGT” and “NNGAA”; for PAM sequences Sth1Cas9 use “NNAGAA” or “NNGGAA.”
3. Pick 20 nt protospacer, targeting your sequence of interest, upstream of the selected PAM (*see Notes 3 and 4*).
4. Design and order your oligonucleotides with corresponding overhangs fitting to *Bbs*I restriction sites (Fig. 2): Forward: 5'-ATTG – 20 nt protospacer sequence-3' and Reverse: 5'-AAAC – 20 nt reverse complementary protospacer sequence-3'.
5. Adjust oligonucleotides to 50 pmol and mix 2 μ L of each oligonucleotide with 46 μ L ddH₂O.
6. Perform denaturation at 95 °C for 5 min. No cooling is necessary, just incubate at room temperature for 10 min.
7. The so annealed oligos can be stored at –20 °C or can be transferred directly to the entry vector.

3.2 Transfer of Target-Specific Spacer Sequence into Entry Vector

1. To integrate the annealed oligos into the entry vector (pEn-Sau Chimera, pEn-Sth1 Chimera), linearize the vector by digestion with *Bbs*I. Therefore, mix 1 μ g of plasmid DNA (isolated by Miniprep with columns) with 2 μ L restriction enzyme buffer, 1 μ L *Bbs*I and add ddH₂O to a total volume of 20 μ L. Incubate for at least 2 h by 37 °C.
2. Use a PCR purification kit to purify the digested entry vector and adjust to a final concentration of 5 ng/ μ L. The purified vector can be stored at –20 °C for further reactions.
3. Ligation is performed by mixing 2 μ L of linearized vector with 3 μ L of annealed oligos, 1 μ L ligase buffer, 1 μ L T4 Ligase, and 3 μ L ddH₂O. Incubate at room temperature for about 2 h or at 4 °C overnight.
4. Transform 5 μ L of the ligation in *E. coli* (NEB5 α) according to your standard transformation protocol and plate on LB agar plates containing 100 μ g/mL ampicillin.
5. Test 5–10 colonies by colony PCR, usually at least 70% of colonies should be correct (*see Note 5*). As forward primer the forward protospacer oligo can be used and CS 32 as reverse primer. Recommended annealing temperature is 56 °C and fragments are about 310 bp for Sau sgRNA and 390 bp for Sth1 sgRNA.

6. Inoculate LB media with 100 µg/mL ampicillin with 1 or 2 colonies and incubate overnight. Purify plasmids and sequence them with SS42 to confirm correct spacer integration.

3.3 Integration of Orthologous sgRNAs into Corresponding Destination Vectors by Gateway Cloning

1. For subsequent Gateway reactions combining Sth1 sgRNA with Sth1Cas9 or Sau sgRNA with SauCas9, adjust your destination vector (pDe-SauCAS9, pDe-Sth1CAS9) to 50 ng/µL and your previously prepared entry vector (pEn-Sth1 Chimera or pEn-Sau Chimera with integrated sgRNA) to 100 ng/µL.
2. The Gateway reaction can be performed by mixing 2 µL of target specific entry vector with 3 µL destination vector, 4 µL TE buffer and 1 µL LR Clonase II. Vortex and centrifuge the whole reaction and incubate afterward for about 2 h at room temperature.
3. To inactivate the LR Clonase II, add 1 µL Proteinase K and incubate at 37 °C for 10 min (*see Note 6*).
4. Transform the complete reaction into *E. coli* (NEB5α) and plate on LB agar plates containing 100 µg/mL spectinomycin.
5. Test 3–6 colonies by colony-PCR, almost all colonies should be correct. Use SS42 as forward primer and SS 102 as reverse primer. Annealing temperature is 60 °C and expected fragment lengths are about 860 bp for SauCas9 and 940 bp for Sth1Cas9.
6. Pick 1 or 2 colonies and inoculate LB medium containing spectinomycin. Incubate overnight and purify the plasmid DNA.
7. Test your plasmids by restriction digestion with *Afl*III and *Nhe*I. In case of pDe-SauCAS9 the expected bands are 5.4, 4.7, and 3.8 kb, for pDe-Sth1CAS9 the expected bands are 5.8, 4.1, 3.8, and 1 kb.
8. Sequence the plasmids with SS42 to confirm correct gateway reaction (*see Note 7*).
9. Verified vectors can be transformed in *A. tumefaciens* for subsequent plant transformation.

3.4 Detection of Orthologous Cas9-Induced Mutations in *A. thaliana*

1. Transform sequence checked vectors with specified sgRNAs into *A. tumefaciens*.
2. Stably transform *A. thaliana* plants via Agrobacterium-mediated transformation (e.g., floral dip transformation as described in [23]).
3. Perform primary transformant selection using GM plates containing 30 µg/mL kanamycin.
4. Propagate at least 20 primary transformants to acquire progeny seeds (T2 generation, *see Note 8*).

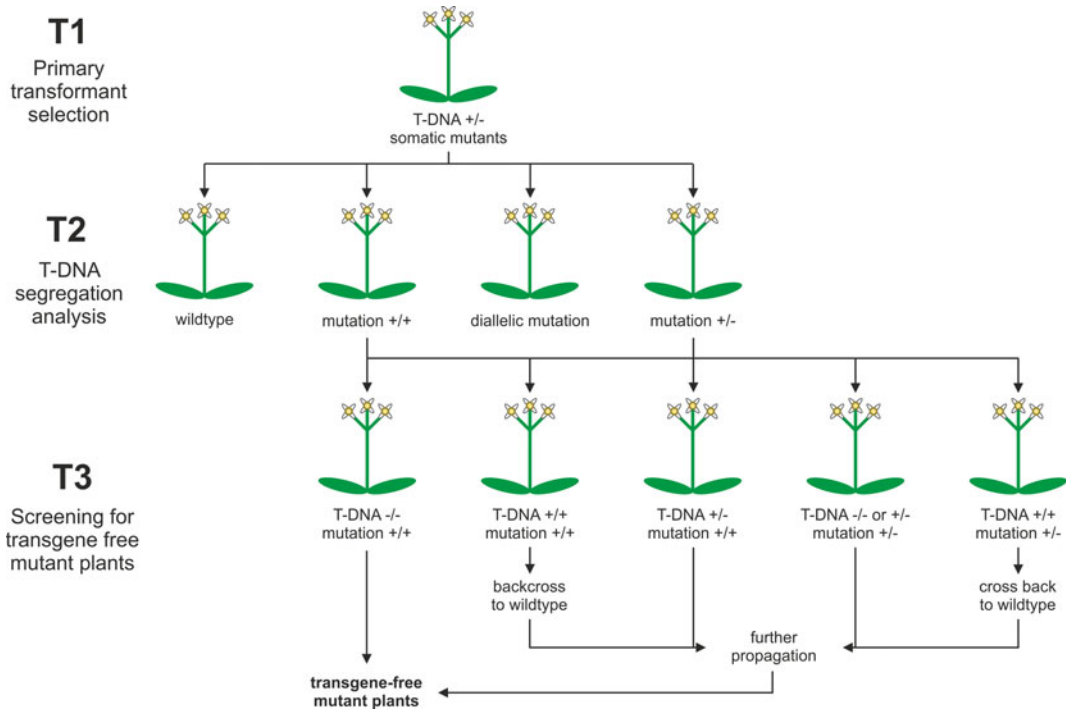


Fig. 3 Overview of the line establishment to detect transgene free mutants. In T1, transformed plants can be identified by selection. In T2, single locus lines for the T-DNA can be identified by their Mendelian segregation pattern. In this generation, heritable mutations can either be heterozygous, homozygous, or diallelic (trans-heterozygous). Already in the T2 generation, transgene-free, homozygously mutated plants might be identified. Otherwise, such plants will be found in the next generation (T3). It is important to be aware that as long as the T-DNA is expressing the nuclease, while a wild-type allele is present in the respective plants, chimerically mutated plants might arise

- Identify correct Mendelian 3:1 segregating T2 plant lines by sowing the seeds on selection media. Correct segregation single locus lines show 75% antibiotic resistant and 25% sensitive seedlings (*see* Fig. 3).
- Use 10 correct segregating single locus lines and sow at least 10 seeds each on GM without selection marker or on soil (*see* Note 9).
- Extract DNA of 2-week-old plants. Fast protocols as described in [24] are sufficient.
- Test your DNA samples for targeted mutagenesis events via restriction digestion or high-resolution melting analysis.
- Confirm individuals with positive results by Sanger sequencing (*see* Note 10).
- Propagate the plants showing mutations to obtain T3 seeds.
- Sow these seeds (at least 20 seeds per line) on selection media to identify plant lines that have lost the T-DNA (*see* Note 11).

12. Confirm the obtained results in T2 plants by also Sanger sequencing the transgene-free T3 progeny for the presence of the mutation.

4 Notes

1. In case of *A. thaliana* transformation, the floral dip method is the recommended procedure, as previously described [23].
2. The mentioned PAM sequences were tested in *A. thaliana* for induction of targeted mutagenesis events and partially for induction of HR events [13]. For the RNA-guided Cas9 nuclease from *S. aureus*, different mutation patterns were obtained using the two here described PAM sequences. Intriguingly, the targeted mutagenesis data using the NNGGGT PAM showed a higher number of larger deletions while the data for the NNGAA PAM were leading predominantly to single nucleotide insertions. Dependent on the aim of your experiment it is recommendable to choose the PAM advisedly.
3. Of course you can always use PAM sequences as reverse complement. Therefore, your reverse-oligo should directly resemble your target sequence, downstream of the PAM, in combination with AAAC and the corresponding forward-oligo should be designed using the reverse complementary sequence.
4. On the majority, every target sequence can be used as a protospacer, there are only a few constraints you have to take heed of. Make sure that your selected sequence does not contain more than five consecutive Ts, as this can lead to an abortion of sgRNA expression. In case of the integration of more than one sgRNA into your destination vector (multiplexing) check for the absence of *Mlu*I and *Bsu*36I restriction sites.
5. Colony-PCR is an optional step to reduce the number of plasmid isolations you have to perform. For this purpose a robust *Taq*-polymerase is mandatory, that can handle inhibiting substances in the reaction. In our lab, the DreamTaq polymerase (ThermoFisher Scientific) is normally used for this application.
6. Proteinase K treatment is crucial, do not skip this step as it could inhibit the formation of correct clones.
7. Additionally, the vectors can be sequenced by SS61 binding in *pea*3A-terminator domain.
8. You could also check the T1 primary transformants for nuclease activity via T7 endonuclease assay or restriction digestion, but this is a mandatory step.

9. Testing 100 individual plants each should lead to the detection of a mutant. However, the exact number can vary. By testing more plants, the possibility of detecting the desired kind of mutation increases.
10. Plants carrying heterozygous mutations show double-peaked chromatograms. You can analyze the sequences using Poly Peak Parser [25]. In this case, repeat sequencing in the next generation to obtain homozygously mutated plants.
11. The optimal outcome are transgene-free T3 plants that carry the desired homozygous mutation. If the mutation or the transgene are still heterozygous in T3, the procedure can be repeated in the next generation or more plants could be tested. If no plants with outsegregated T-DNAs can be obtained (T-DNA is homozygous, all seeds germinate on selection medium), it is possible to cross these lines back with wild-type plants.

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