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CRISPR/Cas9 Ribonucleoprotein-Mediated Mutagenesis in *Sporisorium reilianum*

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Abstract

Clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9 (CRISPR/Cas9) has become the state of the art for mutagenesis in filamentous fungi. Here, we describe a ribonucleoprotein complex (RNP)-mediated CRISPR/Cas9 for mutagenesis in *Sporisorium reilianum*. The efficiency of the method was tested in vitro with a cleavage assay as well as in vivo with a GFP-expressing *S. reilianum* strain. We applied this method to generate frameshift- and knock-out mutants in *S. reilianum* without a resistance marker by using an auto-replicating plasmid for selection. The RNP-mediated CRISPR/Cas9 increased the mutagenesis efficiency, can be applied for all kinds of mutations, and enables a marker-free genome editing in *S. reilianum.*

Key features

- First CRISPR/Cas9 application in *S. reilianum.*
- Generation of *S. reilianum* mutants without genomic integration of resistance marker.
- Allows the generation of multiple gene knockouts as well as deletion of large genomic regions.

Keywords: CRISPR/Cas9, Ribonucleoprotein, Knockout, Marker-free, *S. reilianum*, Smut fungi

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Background

The smut fungi consist of more than 1,500 species, being highly economically important due to their infection of relevant crops such as barley, sorghum, wheat, and maize [1]. The majority of smut fungi infect their host systemically through the roots and replace the inflorescences with teliospores without causing symptoms during early infection stages [2,3]. One example of this systemic infection is *Sporisorium reilianum f. sp. zeae*, which is the causal agent of maize head smut. *S. reilianum* is closely related to the intensively investigated model organism *Ustilago maydis*. However, they differ in their mode of infection as well as in the site of symptom development [4,5]. In 2010, a genome sequence of *S. reilianum f. sp. zeae* was published, which, together with the *U. maydis* genome, provided the foundation for systematic identification and genetic manipulation of effector genes contributing to virulence [6,7]. Genome comparison of *U. maydis* and *S. reilianum* revealed conserved effector genes even though they differ drastically in their pathogenesis on the same host, *Zea mays.* To characterize effector genes and their contribution to virulence, knock-out mutants are generated and compared to the wild type. In the past, *U. maydis* knock-out mutants were generated using PCR-amplified donor templates with resistance markers for gene replacements [8]. Importantly, it was shown that not only the genomic locus but also the integration of resistance markers can negatively influence the expression of reintegrated genes [9]. Recently, the mutagenesis of *U. maydis* was drastically improved with a marker-free approach using clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9 (CRISPR/Cas9) [10,11] and further developed for a seamless gene conversion approach [12]. In contrast to *U. maydis*, the generation of knock-out mutants in *S. reilianum* is still dependent on resistance markers, and multiple gene knockouts are hampered by the limited number (i.e., carboxine, hygromycin, nourseothricin, and phleomycin) of available resistance markers [8]. However, the plasmid-based CRISPR/Cas9 transformation as used in *U. maydis* has not been successful for *S. reilianum*.

CRISPR/Cas9, originating from the adaptive immune system of *Streptococcus pyogenes*, has been broadly adapted to many eukaryotic systems. It is a versatile tool for mutagenesis in various filamentous fungi [13]. The delivery strategies of CRISPR/Cas9 differ between fungal species: (i) stable genomic integration of *cas9*, (ii) transient delivery of Cas9 where the expression of Cas9 is dependent on selection pressure of a self-replicating plasmid or a telomere vector [10,14], or (iii) ribonucleoprotein complex (RNP)-mediated transformation [15,14]. Here, we describe CRISPR/Cas9 applications in *S. reilianum* using an RNP-mediated transformation approach. We demonstrate the generation of frameshifts as well as knock-out mutants mediated by RNPs, thereby generally improving the mutagenesis, and, for the first time, enable a marker-free editing in *S. reilianum*.

Material and reagents

Biological materials

S. reilianum strains were stored at -80 °C in 30% glycerol. For transformation, *S. reilianum* wildtype strains SRZ1 and SRZ2 [7] were used.

Reagents

A. Single-guide RNA (sgRNA) synthesis

1. T4 DNA polymerase (New England Biolabs, catalog number: M0203S), storage: -20 °C
2. NEBuffer™ r2.1 (50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, 100 µg/mL BSA, pH 7.9), storage: -20 °C (New England Biolabs, catalog number: B7202S)
3. dNTPs (DNA) (Carl Roth, catalog number: K039.1), storage: -20 °C
4. NucleoSpin® Gel and PCR clean up (Machery and Nagel, catalog number: 740.609.250), storage: RT
5. HiScribe® T7 High Yield RNA Synthesis kit (New England Biolabs, catalog number: E2040S), storage: -20 °C
6. DNase I (Thermo Fisher, catalog number: EN0521), storage: -20 °C
7. DNase I buffer (Thermo Fisher, catalog number: EN0521), storage: -20 °C

8. RNA Clean & Concentrator 25 kit (Zymo Research, catalog number: R1017 and R1018), storage: RT
9. Purple loading dye (New England Biolabs, catalog number: B7024S); ingredients: 2.5% Ficoll®-400, 10 mM EDTA, 0.08% SDS, 0.02% Dye 1, 0.02% Dye 2, pH 8; storage: RT
10. Nuclease-free water, storage: RT

B. Formation of RNP and in vitro cleavage assay

1. EnGen® Spy Cas9 NLS + NEB buffer r3.1 (New England Biolabs, catalog number: M0667)
2. 500 mM Ethylenediaminetetraacetic acid (EDTA) (Carl Roth, catalog number: 8043.2)
3. Proteinase K (Thermo Fisher, catalog number: EO0491)
4. 100 bp ladder (New England Biolabs, catalog number: N3231S)
5. Universal agarose (Bio-Budget, catalog number: 10-35-1020)
6. 1% Ethidium bromide solution (Carl Roth, catalog number: 2218.2)
7. Potato dextrose agar (PDA) plates (39 g/L) (BD, Difco™, catalog number: 213400)
8. Tris base (Sigma, catalog number: 102262896)
9. Acetic acid (VWR, catalog number: 20103.330)
10. EDTA 0.5 M pH 8.0 (Carl Roth, catalog number: 8043.2)

C. Protoplasting and transformation of *S. reilianum*

1. Novozym 234 [Novo Nordisc; Denmark, not available anymore; alternative: lysing enzyme from *Trichoderma harzianum* (Sigma, catalog number: SLBJ0553V)]
2. Sodium citrate (Carl Roth, catalog number. 3580.1)
3. Sorbitol (Sigma, catalog number: 102466217)
4. Citrate acid (Carl Roth, catalog number: X863.2)
5. Sorbitol (Sigma, catalog number: 102466217)
6. Tris-HCl (Carl Roth, catalog number: 9090.3)
7. $CaCl_2$ (Sigma, catalog number: 1002825086)
8. Poly(ethylene glycol) PEG MW3350 (Sigma, P4338, catalog number: 102604683)
9. Bacto™-Yeast-Extract (Thermo Fisher, Gibco, catalog number: 212720)
10. Bacto™-Peptone (BD, Difco, catalog number: 211820)
11. Sucrose (Carl Roth, catalog number: 4621.2)
12. Sorbitol (Sigma, catalog number: 102466217)
13. Bacto™-Agar (BD, catalog number: 214030)
14. Potato dextrose agar (PDA) plates (BD, Difco™, catalog number: 213400)
15. Carboxine (5 mg/mL) (Sigma, catalog number: 102085144)
16. Heparin sodium salt from porcine intestinal mucosa (15 mg/mL) (Sigma, catalog number: 1001937695)

Solutions

1. 50× TAE buffer (see Recipes)
2. 1× TAE buffer (see Recipes)
3. SCS buffer (see Recipes)
4. STC buffer (see Recipes)
5. STC/40% PEG (see Recipes)
6. Regeneration agar light (see Recipes)

Recipes

1. 50× TAE buffer

Reagent	Final concentration	Quantity or Volume
Tris base	2 M (v/v)	242.0 g
Acetic acid	2 M (v/v)	57.1 mL

EDTA 0.5 M pH 8,0	10% (v/v)	100.0 mL

2. **1× TAE buffer**

Reagent	Final concentration	Quantity or Volume
50× TAE buffer	2% (v/v)	20.0 mL
Deionized water	98% (v/v)	980.0 mL

3. **SCS buffer**

Reagent	Final concentration	Quantity or Volume
Solution 1:		
Sodium citrate, pH 5	0.6% (w/v)	5.9 ml
Sorbitol	18.2% (w/v)	182.0 g
Solution 2:		
Citrate acid	0.4% (w/v)	4.2 g
Sorbitol	18.2% (w/v)	182.0 g

Solution 1 and 2 are mixed until pH 5.8 is reached (ratio ~5:1) and autoclaved.

4. **STC buffer**

Reagent	Final concentration	Quantity or Volume
Sorbitol	50% (v/v)	500.0 mL
Tris-HCl, 1 M pH 7.5	1% (v/v)	5.0 mL
$CaCl_2$, 1 M, sterile-filtrated (100 mL total volume is enough)	10% (v/v)	50.0 mL

5. **STC/40% PEG**

Reagent	Final concentration	Quantity or Volume
STC buffer	60% (v/v)	600.0 mL
Poly(ethylene glycol) PEG, MW3350; sterile-filtrated, (50 mL total volume is enough)	40% (w/v)	400.0 g

6. **Regeneration agar light**

Reagent	Final concentration	Quantity or Volume
Bacto™ yeast extract	1% (w/v)	10.0 g
Bacto™ peptone	0.4% (w/v)	20.0 g
Sucrose	0.4% (w/v)	20.0 g
Sorbitol	18.2% (w/v)	182.2 g
Bacto™ agar	1.5% (w/v)	15.0 g

Laboratory supplies

1. PCR machine (Bio-Rad, model: T100™ Thermal Cycler)
2. Microfuge for PCR tubes (VWR, model: Ministar)
3. Tabletop centrifuge (VWR, model: Microstar 17)
4. 37 °C incubator (Memmert, model: UN110)
5. 28 °C incubator/room
6. Optional: Polyacrylamide gel electrophoresis (SDS-PAGE) equipment (Bio-Rad, model: PowerPac™ Basic, Mini-Protean® Tetra System)
7. Agarose gel electrophoresis equipment

8. Nanodrop (Thermo Scientific, model: Nanodrop 2000c)
9. ChemiDoc™ MP imaging system (or equivalent imaging system), with GFP filter (Bio-Rad, model: Universal Hood III)
10. Geldoc: visualization of DNA by UV radiation using a gel documentation unit (Peqlab/VWR, model: EBOX VX5)

Equipment

1. PCR tubes and 1.5 ml Eppendorf tubes
2. Sterile cut tips (1,000 µL and 20 µL)

Procedure

A. In vitro transcription of sgRNA

1. Design protospacer in CHOPCHOP sgRNA designer (https://chopchop.cbu.uib.no/) using *S. reilianum* as target organism. Choose the protospacer sequence starting with a **G**, which is needed for initiating the transcription by T7 RNA polymerase. If there is no desired protospacer starting with G, add an additional G upstream of the chosen protospacer sequence (21 nt).
2. Add T7 RNA polymerase promoter sequence and overlapping scaffold sequence upstream and downstream of the chosen protospacer sequence, respectively, and order the gene-specific oligonucleotide (Table 1). In addition, a reverse complementary constant oligonucleotide is needed, which harbors the scaffold and terminator sequence and a 20 nt overlap to the scaffold sequence of the gene-specific oligonucleotide (Table 1).

Table 1. Sequences of oligonucleotides for sgRNA synthesis

Oligo	Sequence
Gene-specific	CAAAATTCCATTCTACAAC-**G**NNNNNNNNNNNNNNNNNNN-GTTTTAGAGCTAGAAATAGCAAG
Constant	AAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAAC

Note: The underlined sequence of the constant oligo depicts the overlapping part with the gene-specific oligo.

3. Mix both oligonucleotides in a 1:1 ratio as follows:
   1 µL of protospacer oligo 100 µM stock
   1 µL of constant oligo 100 µM stock
   8 µL of H_2O
   10 µL total
4. Anneal the oligos using the following program in PCR machine:
   95 °C for 5 min
   95 °C to 85 °C at -2 °C/s
   85 °C to 25 °C at -0.1 °C/s
   4 °C pause
5. Add T4 DNA polymerase to fill in the overhangs:
   2.5 µL of dNTPs (10 mM)
   2 µL of NEBuffer™ r2.1(10×)
   5 µL of H_2O
   0.5 µL of T4 DNA polymerase
   10 µL total

6. Incubate at 12 °C for 20 min in a PCR machine.
7. Purify the product with a PCR clean-up kit, measure the concentration with Nanodrop, and verify the PCR product on a 2%–3% TAE agarose gel.
8. Use 2 µg of the resulting DNA from above as template and the HiScribe T7 High Yield RNA Synthesis kit for the following reaction (NEB, protocol for small RNAs):
   6 µL of NTPs (25 mM each in stock)
   2 µL of 10× T7 buffer
   1.5 µL of T7 RNA polymerase mix
   X µL of Template (2 µg DNA template, step 6)
   Y µL of nuclease-free H_2O (add to 20 µL)
   20 µL total
9. Flip the tube, vortex shortly, and incubate at 37 °C overnight.
10. The next day, add 14 µL of nuclease-free H_2O, 4 µL of DNase I buffer (10×), and 2 µL of DNase I and incubate at 37 °C for 15 min.
    Caution: Small RNA is easy degradable; work in a RNase-free space and use gloves and a lab coat for all following steps!
11. Purify the resulting sgRNA with the RNA Clean & Concentrator 25 kit and use the manufacturer's protocol (manual, page 5).
    Optional: Check the quality of the RNA on 10% denaturing PAA gel using TBE buffer (89 mM Tris base, 89 mM boric acid, and 2 mM sodium EDTA) and 8 M urea and TBE as running buffer [14].
12. Measure the concentration by Nanodrop and proceed with in vitro cleavage assay (section B).
    Pause point: You can freeze the sgRNA at -80 °C and continue the next day; long-term storage of sgRNA is also possible at -80 °C. See Troubleshooting 1.

B. In vitro cleavage assay

1. To test the in vitro efficiency of the designed sgRNA, mix 1.5 µL of Cas9 (NEB) and ~1.5 µg of the sgRNA (1:1 molar ratio) and incubate it for 10 min at RT (Figure 1B).

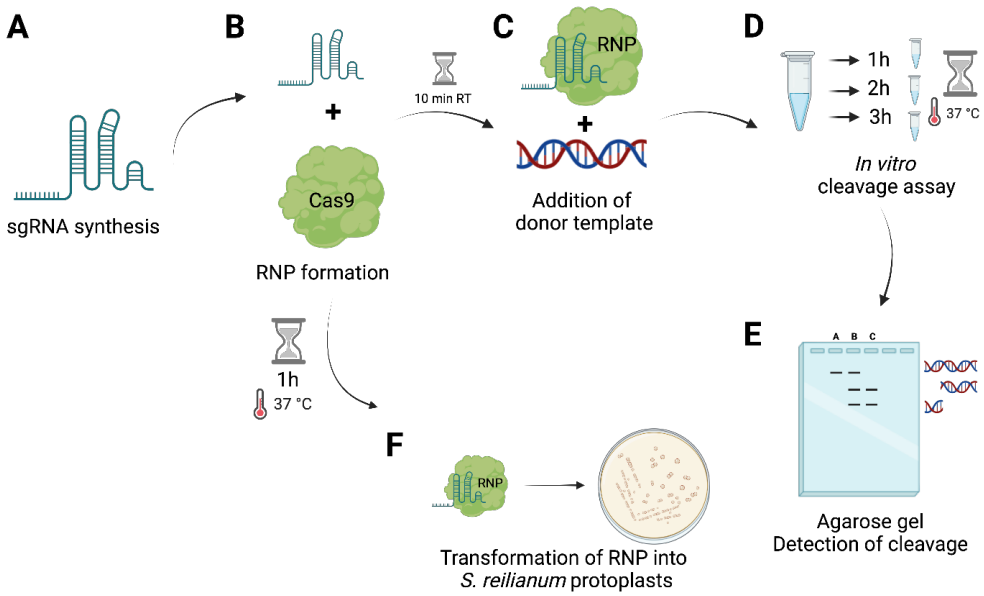

Figure 1. Graphical overview of the workflow of ribonucleoprotein complex (RNP)-mediated transformation in *S. reilianum*. (A) In vitro synthesis of sgRNA using T7 HiScribe kit. (B) RNP formation of in vitro–transcribed sgRNA with *Sp*Cas9. (C) Alternatively: To perform an in vitro cleavage

assay, incubation at room temperature (RT) for 10 min and subsequent addition of a donor template (amplification of the gene of interest region) and incubation at 37 °C for 3 h is conducted. (D) Sampling of 10 µL of reaction mix after 1, 2, and 3 h (or alternatively overnight). (E) Visualization of in vitro cleavage on a 1.5% agarose gel using 100 bp ladder. (F) RNP incubation for 1 h at 37 °C prior to transformation into *S. reilianum* protoplasts. Figure was created with biorender.com.

2. Afterwards, add 333 ng of a DNA cleavage template (PCR product of the region of interest) (Figure 1C).
3. After 1, 2, and 3 h take 10 µL samples (Figure 1D) and stop the reaction by adding 1 µL of 500 mM EDTA, pH 8.
4. Subsequently, add 1 µL of proteinase K to the reaction and incubate the reaction mix for 30 min at 50 °C for degradation of Cas9.
5. Stop the reaction by the addition of 1× purple loading dye.
6. After the collection of all samples, check cleavage on an 1.5% agarose gel with 100 bp ladder (stained with ethidium bromide solution) visualized using a Gel-Doc (Figure 1E, see Troubleshooting 2).

C. Assembly of RNP for transformation into *S. reilianum*

1. Use 2 µg of the in vitro–transcribed sgRNA targeting the gene of interest and mix it with 6 µg of *Sp*Cas9.
2. Subsequently, add 1× NEBuffer™ 3.1 and water in a minimum volume (Figure 1B).
3. After mixing and centrifugation, incubate the reaction for 1 h at 37 °C prior to transformation (Figure 1B).

D. Transformation of *S. reilianum*

1. Prepare *S. reilianum* protoplasts using Novozym 234 as described previously [8].
2. For RNP transformation (Figure 1E), thaw the protoplasts for 5 min on ice.
3. Add a self-replicating plasmid with antibiotic resistance cassette [e.g., pNEBUC—Carboxine (Cbx); Brachmann et al. [8], replicating in *S. reilianum*], the RNP (formed in section C), 1 µL of 15 mg/mL heparin, and, optionally, 1.5 µg of a donor template to the protoplasts (Figure 2).
   Note: The self-replicating plasmid is lost when the selection for Cbx resistance is stopped. So far, we could not report an integration into the genome of S. reilianum.
4. Incubate the protoplasts for 10 min on ice.
5. Add 500 µL of STC/40% PEG and resuspend the protoplasts carefully with a tip-cut blue tip until the liquid looks homogenous without clumps (5–8 times pipetting up and down).
6. Incubate the protoplasts for another 15 min on ice.
7. Spread the protoplasts on a regeneration agar light plate with two layers [bottom layer: corresponding selective antibiotic (for pNEBUC—carboxin: 2.5 µg/mL), top layer: without antibiotic resistance].
8. The next day, flip the transformation plate upside down.
9. After four days, use a blue tip to single out transformants from regeneration agar to PDA + Carboxin (2.5 µg/mL) for 2–3 days.
10. Afterwards, transfer a single colony for two days to PDA plates to lose the resistance.
11. Subsequently, DNA is isolated [16] and used for further confirmation (see section E).

E. RNP-assisted homologous recombination to generate a knockout in *S. reilianum*

For the generation of an antibiotic-resistance-free knock-out mutant in *S. reilianum*, a CRISPR/Cas9-mediated homology-directed repair was exploited. To do this, a donor template is generated by cloning the 1 kb homology flanking regions of the target gene into a MOCLO vector TK#1_pAGM1311 by Gibson assembly (Figure 2).

1. For the transformation of *S. reilianum* protoplasts (see section D), add the donor template together with a self-replicating plasmid (pNEBUC), the RNP (with a sgRNA against the target region), and 1 µL of heparin.
   Note: The transformation efficiency is high > 100 colonies; if your efficiency is lower, repeat protoplasting and transformation.

2. Transfer obtained transformants as described above (see section C).
3. Isolate DNA of the transformants and the wild type.
4. Conduct a PCR using the forward primer of the left flank and the reverse primer of the right flank (Figure 2C) and compare the band sizes to the wild type (Figure 2D).
5. Putative positive mutants from PCR are selected for further verification via Southern blot [17,18] using the deletion construct (left flank + right flank), previously used as a donor template, as probe for hybridization.

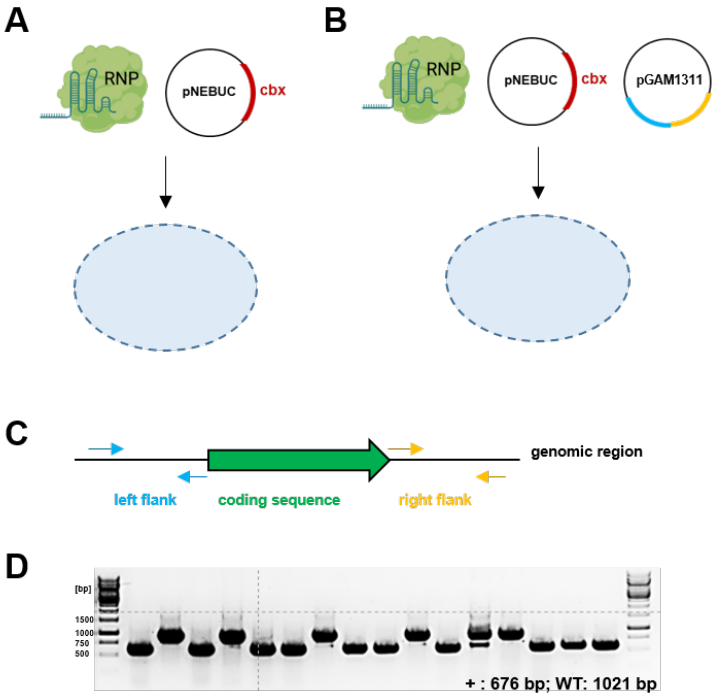

Figure 2. Transformation of *S. reilianum* using ribonucleoprotein complex (RNP) with and without a donor template. (A) Generation of frameshift mutants using RNP-mediated transformation. Generated frameshift mutants are screened by sequencing or as described in Figure 3 by the loss of fluorescence. (B) Generation of knock-out mutants using RNP-assisted homologous recombination with a donor template. (C) Donor template design for the generation of a knockout. 1 kb flanking regions of the coding sequence are amplified by PCR with overhangs for Gibson assembly into backbone TK#1_pAGM1311 (MOCLO backbone, Level -1). (D) Example of deletion mutant verification PCR using Left Flank (LF) forward primer and Right Flank (RF) reverse primer [expected sizes: WT, 1021 bp; mutant (+), 676 bp; efficiency: 62.5% (10/16)]. The efficiency can vary between different genomic loci. Figure 2A and B were created with biorender.com.

Validation of protocol

The efficiency of the RNP CRISPR/Cas9 can, for instance, be tested with GFP fluorescence as a readout (Figure 3). To test the efficiency in *S. reilianum*, a strain harboring a single integration of GFP controlled by pOTEF (constitutive promoter) was generated in the *ip* locus of SRZ2 strain and confirmed via Southern blot (Figure S1). For the transformation of *S. reilianum* protoplasts, a sgRNA against GFP together with the Cas9 in a RNP (see section C) and an auto-replicating plasmid (pNEBUC) for selection on regeneration agar was used. Transformants were singled out after four days of incubation at 28 °C on PDA + Cbx (2.5 mg/mL) and, after two days, were transferred to PDA plates and checked for their fluorescence using a Chemi-Doc.

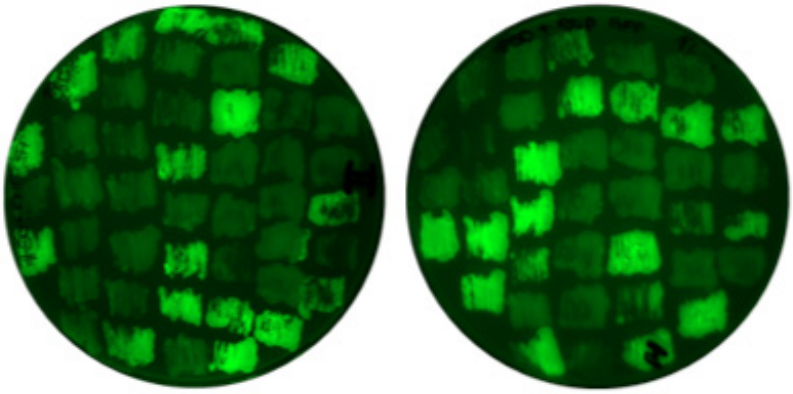

Figure 3. GFP as target for ribonucleoprotein complex (RNP)-mediated transformation in *S. reilianum*. An example shows the efficiency of RNP-mediated CRISPR/Cas9 transformation in *S. reilianum*. A *S. reilianum* SRZ2 strain expressing GFP under pOTEF promoter was generated. sgRNA+Cas9 targeting GFP coding sequence was transformed, and mutants with frameshift lose the GFP signal. Efficiency for GFP sgRNA: ~41% (34/83).

General notes and troubleshooting

Troubleshooting

No.	Step	Problem	Suggestion/solution
1	sgRNA synthesis	Low concentration (<500 ng/µL)	Do not proceed with transformation, repeat synthesis; high concentration in minimum volume is needed
2	In vitro cleavage assay	No bands after cleavage	1) Test functionality of Cas9 enzyme (use a control) 2) Design of new sgRNAs

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Supplementary information

The following supporting information can be downloaded here:

1. Figure S1. Southern blot of single integrated GFP into SRZ2 strain.