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Chapter 12

Genome Editing in *Penicillium* chrysogenum Using Cas9 Ribonucleoprotein Particles

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Abstract

Several CRISPR/Cas9 tools have been recently established for precise genome editing in a wide range of filamentous fungi. This genome editing platform offers high flexibility in target selection and the possibility of introducing genetic deletions without the introduction of transgenic sequences. This chapter describes an approach for the transformation of *Penicillium chrysogenum* protoplasts with preassembled ribonucleoprotein particles (RNPs) consisting of purified Cas9 protein and in vitro transcribed single guide RNA (sgRNA) for the deletion of genome sequences or their replacement with alternative sequences. This method is potentially transferable to all fungal strains where protoplasts can be obtained from.

Key words Ribonucleoprotein particle, RNP, CRISPR/Cas9, Genome editing, NHEJ, HDR, *Penicillium chrysogenum*, Filamentous fungi

1 Introduction

The implementation of CRISPR/Cas9 tools in filamentous fungi has advanced its infancy and enables precise genome editing in at least 16 fungal species [1–16] up to date, and will promote the application of filamentous fungi as cell factories and facilitate basic research. The protocols outlined below describe methods developed for protoplast transformation of *Penicillium chrysogenum* (also termed *P. rubens*) using preassembled ribonucleoprotein particles of purified Cas9 protein conjugated with a single guide RNA (sgRNA) instead of expressing the Cas9 protein and the sgRNA from a plasmid or genetic locus, as described in multiple variations elsewhere. This approach makes the CRISPR/Cas9 tool potentially applicable in filamentous fungi where only limited knowledge on promoter performance exists or when rapid gene deletion is required for confirmation, for instance during the identification of genes involved in secondary metabolites formation.

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This chapter outlines a methodology for designing and verifying genome editing of filamentous fungi using RNPs. First, a selection pipeline for the identification of protospacers is described, followed by a protocol for in vitro synthesis of sgRNA using the T7-promoter and a discussion on design guidelines for donor DNAs. In the next section, a protocol for the preparation of protoplasts of *P. chrysogenum* is described, including the addition of Cas9 RNPs into the transformation mix and cryopreservation of protoplasts. Finally, a brief protocol for colony PCR of *P. chrysogenum* transformants to screen for the integration of dDNA or to obtain PCR products which can be sent for sequencing in order to, i.e., screen for gene disruption is described. The protocols can be adapted by the operator and transferred to other filamentous fungi where protoplasts can be obtained from. Some parts of the protocols presented below were previously described in another chapter [17].

2 Materials

2.1 sgRNA Design

2.1.1 Identification of Protospacers

1. Standard PC workstation with internet access.
2. Installed PERL package (e.g., DWIMperl via http://dwimperl.com/).
3. CasOT 1.0 or higher (download at http://eendb.zfgenetics.org/casot/).
4. Genome sequence for off-target search in FASTA-format.
5. FASTA file (maximum 1000 bp) containing the region to be searched for protospacers.

2.1.2 In Vitro Synthesis of sgRNA

1. Oligo sgRNA-tail-Reverse (5′-AAAAAAGCAC CGACTCGGTGCCACTTTTTTCAAGTTGAT AACGAACTAGTCTTATTTCAACTT GCTATGCT GTTTCCAGCATAGCT CTGAAAC-3′), 100 μM in nuclease-free water.
2. Oligo pT7-n20-sgRNA-FW (*see* Subheading 3).
3. Sterile pipette tips (10 and 100 μl).
4. Sterile PCR tubes.
5. Sterile, DNAse and RNAse-free water (fresh, autoclaved MQ).
6. KAPA HiFi HotStart ReadyMix (Kapa Biosystems, available from Roche, *see* **Note 1**).
7. PCR thermocycler.
8. 2.0% agarose gel with DNA stain added.
9. RNase Inhibitor (SUPERaseIn, Life Technologies, *see* **Note 2**).
10. In vitro T7 RNA polymerase Transcription Kit (AMBION MegaScript, available from Life Technologies, *see* **Note 2**).

2.2 Donor DNA Construction

2.2.1 Transient Selection with AMA1-Based Plasmids

1. Plasmid with AMA1 sequence that can be maintained in filamentous fungi (e.g., pMA171 or pMA172 [18], available via FGSC[1] (Fungal Genetics Stock Center) or pJAK-109 [6].

2.2.2 Donor DNA with Selection Marker

1. Selection marker cassette to be used, containing a suitable promoter.
2. Forward primer, adding 100 bp homology to target site to the selection marker cassette.
3. Reverse primer, adding 100 bp homology to target site to the selection marker cassette.
4. PCR thermocycler.
5. Sterile pipette tips (10 and 100 µl).
6. Sterile PCR tubes.
7. Sterile, DNAse and RNAse-free water (fresh, autoclaved MQ).
8. KAPA HiFi HotStart ReadyMix (Kapa Biosystems, available from Roche).
9. PCR clean-up kit of choice.

2.2.3 Marker-Free Donor DNA

1. Software for DNA viewing and editing of choice.
2. MoClo Toolbox (available from Addgene) or materials for other combinatorial cloning.
3. Transformation competent *Escherichia coli* cells.
4. Sterile pipette tips (10 and 100 µl).
5. Sterile PCR tubes.
6. Sterile, DNAse and RNAse-free water (fresh, autoclaved MQ).
7. PCR clean-up kit of choice.
8. Liquid LB (Luria–Bertani) medium containing appropriate antibiotics for plasmid maintenance.
9. Solid LB medium, containing appropriate antibiotics for plasmid selection.
10. Suitable antibiotics to select assembled plasmids.
11. Suitable restriction enzymes for plasmid linearization (e.g., XhoI for pMA171/pMA172 or KpnI and HindIII for pJAK-109).
12. Selection marker cassette to be used, containing a suitable promoter.
13. Forward primer, adding 100 bp homology with linearized AMA1-plasmid to selection marker cassette.

[1] http://www.fgsc.net/

14. Reverse primer, adding 100 bp homology with linearized AMA1-plasmid to selection marker cassette.
15. KAPA HiFi HotStart ReadyMix (Kapa Biosystems, available from Roche).
16. PCR thermocycler.
17. PCR clean-up kit of choice.

2.3 Preparation of Protoplasts

1. KCl-Glucose solution: potassium chloride, glucose monohydrate.
2. 5×YNB: Yeast Nitrogen Base without amino acids, citric acid monohydrate, dipotassium hydrogen phosphate.
3. Yeast extract 10% (w/v), autoclaved.
4. YGG-broth: KCl-glucose solution, 5×YNB, yeast extract 10%, optional: 10,000 U/ml Penicillin/Streptomycin solution.
5. 0.22 μm sterile syringe filters.
6. 50-ml syringe.
7. Laminar flow bench (for medium preparation).
8. Clean bench (for inoculation).
9. Spore stock of *P. chrysogenum* (immobilized on rice grains or harvested from plates).
10. Shaking incubator with clamps for 100-ml shake flasks.
11. 100-ml shake flasks with cotton plugs, autoclaved.
12. Serological pipettes (10 and 25 ml).
13. 50-ml falcon tubes, sterile.
14. Lysing Enzyme from *Trichoderma harzianum* (Glucanex, Sigma Aldrich, *see* **Note 3**).
15. Cold (2–8 °C) KC-Buffer: potassium chloride, citric acid monohydrate, 1 M HCl, 1 M KOH.
16. Cold (2–8 °C) Sorbitol Tris calcium chloride buffer (STC): calcium chloride dihydrate, sorbitol, 1 M Tris–HCl buffer (pH 7.5).
17. Sterile inoculation loops.
18. Centrifuge with swinging bucket rotor and cooling option, cooled to 4 °C.
19. Cell counting chamber (0.1 mm chamber depth).
20. Light microscope with 40× magnification.

2.4 Cryopreservation of Protoplasts

1. Protoplasts in STC.
2. Cryopreservation Buffer (STC-PVP): PVP-40, STC-Buffer.
3. Sterile 2-ml cryotubes.

4. Freezing container, suitable for fitting 2-ml cryotubes, filled with isopropanol to manufacturer instructions, cooled to 2–8 °C.
5. Isopropanol.

2.5 Transformation of Protoplasts

1. 1.2 M Sorbitol solution: Sorbitol.
2. 60% PEG-4000 solution: PEG-4000, 1 M Tris–HCl (pH 7.5), 1.0 M calcium chloride solution.
3. 20% PEG-4000 solution: 60% PEG-4000 solution, SCT buffer.
4. 2× concentrated STC buffer (2×STC buffer): calcium chloride dihydrate, sorbitol, 1 M Tris–HCl buffer (pH 7.5).
5. 10× concentrated Cas9 activity buffer (10×Cas9 buffer): HEPES, potassium chloride, magnesium sulfate heptahydrate, 0.25 M EDTA in MQ, 1 M KOH, 1 M DTT in MQ.
6. Cas9 protein, 27 μg per transformation (*see* **Note 4**).
7. 1% (w/v) Iron (II) sulfate heptahydrate solution, filter sterilized.
8. Agar base; without nitrogen source: glucose monohydrate, magnesium sulfate heptahydrate, sodium chloride, sucrose, agar-agar.
9. Acetamide solution: 10% (w/v) in water, filter sterilized.
10. Sodium nitrate solution: 33% (w/v) in water, filter sterilized.
11. Terbinafine solution: 10 mg/ml terbinafine hydrochloride in methanol, store at −20 °C.
12. Phleomycin solution: 50 mg/ml phleomycin sodium salt in water, store at −20 °C.
13. 1 M Phosphate buffer (pH 6.8), autoclaved.
14. 500× trace element stock solution: (EDTA, 4 M NaOH, trisodium citrate dihydrate, iron(II) sulfate heptahydrate, magnesium sulfate heptahydrate, boric acid, sodium molybdate dihydrate, copper(II) sulfate pentahydrate, zinc sulfate heptahydrate, cobalt(II) sulfate heptahydrate, manganese(II) sulfate monohydrate, calcium chloride dihydrate.
15. Selection agar: agar base, 500× trace element stock solution, 1 M phosphate buffer (pH 6.8), acetamide solution, sodium nitrate solution, terbinafine solution, phleomycin solution.
16. 12-ml Greiner tubes or similar tubes with round-shaped bottom.
17. Incubator, set to 25 °C.
18. Microwave oven.
19. Centrifuge with swinging-bucket rotor, cooled to 4 °C.
20. Drigalski spatula, single or multi-use, sterile.

2.6 Colony PCR of P. chrysogenum

1. Phire Plant Direct PCR Kit (Thermo Fisher Scientific, *see* **Note 5**).
2. Forward primer to screen for editing event.
3. Reverse primer to screen for editing event.
4. PCR tubes, sterile.
5. Pipette tips (10 and 100 μl), sterile.
6. PCR thermocycler.
7. 0.8% agarose gel with DNA stain added.

3 Methods

3.1 sgRNA Design

In the following two subsections, protospacers with low potential off-target sites and a high cutting efficiency will be selected using freely available tools (*see* **Note 6**).

3.1.1 Identification of Protospacers Using CasOT

1. A genome sequence to be searched for protospacers is selected (maximum sequence length for CasOT: 1000 bp) and saved as FASTA (.fa) file (called TARGET.fa in the following).
2. The option string for CasOT is generated with the following settings:
   (a) [-mode]: select target-and-off-target mode
   (b) [-target]: the path to TARGET.fa
   (c) [-genome]: a FASTA file (single .fa or mutliple .fna are accepted) containing the entire genome
   (d) [-exon]: leave unfilled
   (e) [-output]: .csv (leave default)
   (f) [-seed]: 0
   (g) [-nonseed]: 0
   (h) [-pam]: A: -NGG only (default)
   (i) [-distance]: 0
   (j) [require5g]: required a 5′-G (default)
   (k) [-length]: 20 nt
3. The option string is copied to the CasOT/perl command window and executed.
4. The CasOT output results will be stored in the folder containing TARGET.fa after processing is finished.
5. The protospacer sequences and their locations are imported into a spreadsheet program of choice.

3.1.2 Predicting Cutting Efficiency Using sgRNA Scorer 2.0

1. TARGET.fa is submitted to sgRNA Scorer 2.0 [19] with the following settings:
   (a) [CRISPR system]: *S. pyogenes* (PAM: NGG)
   (b) [Spacer length (14–24)]: 20
   (c) [PAM sequence (e.g., NGG)]: NGG
   (d) [PAM orientation]: 3′
2. Users of sgRNA scorer 2.0 will obtain a message containing link to sgRNA Scorer 2.0 results.
3. Protospacers are imported into spreadsheet containing output of CasOT.
4. Lists are compared using spreadsheet functions (e.g., VLOOKUP function in MS Excel) and protospacers not identified by CasOT are excluded.
5. Protospacers with larger score are preferred (*see* **Note 7**).

3.1.3 In Vitro Synthesis of sgRNA

1. The protospacer sequence (20 bp, NGG removed) is pasted into template oligo pT7-n20-sgRNA-FW (5′-TGTAATACG ACTCACTATAnnnnnnnnnnnnnnnnnnnnGTTTCAGA GCTATGCTGGAAA-3′).
2. For each sgRNA, a separate forward oligo (60 bp length) is ordered in standard desalt purification quality (*see* **Note 8**).
3. Oligos are reconstituted to 100 μM with nuclease-free water and mixed briefly by vortexing (*see* **Note 9**).
4. For each sgRNA, a separate 200 μl PCR tube containing the following mixture is prepared: 15.0 μl 2× KAPA HiFi master mix, 14.5 μl nuclease-free water, 0.25 μl 100 μM pT7-n20-sgRNA-FW, 0.25 μl 100 μM sgRNA-tail_Reverse (*see* **Note 10**).
5. The reagents are mixed by tapping the tube and briefly centrifuged down before overlap PCR is performed by heating to 95 °C for 5 min, followed by 40 cycles of (98 °C for 15 s, 66 °C for 10 s, 72 °C for 15 s), and 72 °C for 1 min.
6. 4 μl of the overlap PCR reaction mix are electrophoresed on a 2.0% agarose gel for 15–20 min to confirm successful amplification.
7. 26 μl nuclease-free water are added to the remaining PCR reaction mix and samples are frozen to −20 °C if sgRNA synthesis is not performed directly afterward (*see* **Note 11**).
8. For sgRNA synthesis the ingredients of the MEGAScript Kit are thawed on ice and added to a sterile 200 μl PCR tube: 4.0 μl nuclease-free water, 0.3 μl SuperAse RNAse inhibitor, 1.0 μl 10× RNA synthesis buffer, 1.0 μl UTP, 1.0 μl ATP, 1.0 μl CTP, 1.0 μl GTP, 1.0 μl T7 RNA polymerase, 1.5 μl overlap PCR reaction mix (*see* **Note 12**).

9. Reagents are mixed by flicking the tube and collected by brief centrifugation.
10. sgRNA synthesis is performed at 37 °C in a PCR thermocycler/any 37 °C incubator without agitation for at least 6 h, preferably overnight.
11. An aliquot of 2.0 µl synthesis mixture is analyzed on a 2.0% agarose gel to confirm successful synthesis.
12. The remaining sgRNA synthesis reaction is mixed with 8.0 µl nuclease-free water and subjected to denaturing and refolding using a PCR thermocycler with the following conditions: 98 °C for 2 min, then 68 cycles of 10 s with 1 °C lower temperature per cycle (*see* **Note 13**).
13. Obtained sgRNA can be stored for several days at −80 °C, if not used immediately, however degradation can be expected to occur.

3.2 Preparation of Donor DNA for Transformation

In the following section, the design of donor DNA parts for the transformation will be discussed in a very general manner, so operators understand the underlying principle and can apply them to their own toolboxes.

3.2.1 Transient Selection with AMA1-Based Plasmids

1. The utilization of plasmids bearing the AMA1 region allows plasmids to be maintained in filamentous fungi transiently with limited risk of genomic integration [20]. Specifically, the expression of a dominant selection marker from such a plasmid enables a pre-selection of protoplasts that were competent in taking up DNA and thus gained resistance under selection conditions. The ratio of AMA1 plasmid relative to other transformed elements should be low, thus leveraging the chance that resistant colonies display the desired phenotype that is achieved upon integration of the donor DNA or the delivery of multiple sgRNAs for the creation of larger Indels. An application example of this strategy would be the disruption of genes involved in non-homologous end joining (NHEJ), to improve the overall success rate of correct integration of donor DNA.
2. This strategy can be extended further in strains that were rendered deficient in NHEJ by supplying a linearized plasmid and a repair template containing a selectable marker to circularize the plasmid by *in vivo* homologous recombination. In NHEJ-deficient *P. chrysogenum* strains, 100 bp homology towards the plasmid backbone were found to be sufficient for efficient recombination at a very low rate of untargeted integration events [6] into the host genome. This makes it possible to choose a selection marker cassette from any available template

and to amplify the cassette with primers giving homology toward the linearized AMA1 plasmid (*see* **Note 14**).

3. Plasmids containing AMA1 sequences can be retrieved from the Fungal Genetics Stock Center (FGSC) or at request from published experiments.
4. For linearization of AMA1 plasmids, a suitable restriction enzyme is selected by the operator and the primer design for the marker cassette is planned accordingly, adding 90–100 bp homology toward the plasmid at the 5′- and 3′-end of the resistance marker cassette.

3.2.2 Donor DNA with Selection Marker

1. Due to the limited basal activity of homology-directed repair (HDR) pathways in many filamentous fungi, such as *P. chrysogenum*, a donor DNA for homologous recombination (HR) typically requires a minimum homology of 750 bp to observe a targeted integration of the donor DNA carrying a selection marker at sufficient rates for medium-throughput screening workflows. However, upon the creation of a double-stranded break by Cas9 in NHEJ-deficient protoplasts, the homology length required for targeted HR was drastically reduced [6] (*see* **Note 15**). This finding can be utilized to reduce the cloning effort in marker cassette generation to a single PCR with long oligos (120 bp) adding 90–100 bp homology to the target site. The generated marker cassettes can be utilized for any application that does not require marker-free genome editing, e.g., phenotype screen libraries or protospacer scoring libraries, substantially reducing the effort in donor DNA building.

3.2.3 Marker-Free Donor DNA

1. Combinatorial cloning techniques such as GATEWAY Cloning, MoClo [21], USER-Cloning [22], GoldenBraid [23], or other methods the operator is familiar with can be easily used to construct a deletion cassette that removes the entire open reading frame of the targeted gene.
2. To maximize efficiencies and because all the aforementioned techniques require sub-cloning steps into bacterial hosts that cannot be omitted, it is advised to design the donor DNA with the standard homology length used for donor DNA integration into the operators' fungal strains. For *P. chrysogenum*, 0.75–1.5 kb flanks are recommended.

3.3 Preparation of Protoplasts

1. Prepare KCl-Glucose: 10 g potassium chloride and 20 g glucose monohydrate are dissolved in deionized water, filled up to 1.0 L and autoclaved for 15 min at 121 °C.
2. Prepare 100-ml 5×YNB: 3.33 g Yeast Nitrogen Base without amino acids, 0.75 g citric acid monohydrate, and 3.0 g dipotassium

hydrogen phosphate are dissolved in 80 ml water and filled up to a final volume of 100 ml.

3. YGG-broth (Yeast Extract Glucose for Germination): in a sterile 500-ml bottle, mix 400 ml KCl-glucose, 100 ml sterile-filtrated 5×NBB, and 10 ml 10% (w/v) yeast extract; optional: to prevent contamination with bacteria, 5 ml of a 10,000 U/ml Pen/Strep solution can be added.
4. Prepare KC-Buffer: 60.0 g potassium chloride and 2.0 g citric acid are dissolved in 950-ml deionized water, pH is set to 6.2 with 1 M HCl/1 M KOH and volume is brought to 1.0 L with deionized water. 250-ml aliquots are autoclaved for 15 min at 121 °C, cool down to 2–8 °C, and use for transformations.
5. Sorbitol Tris calcium chloride buffer (STC): 5.5 g calcium chloride dihydrate, 219.0 g sorbitol, and 10 ml 1 M Tris–HCl buffer (pH 7.5) are dissolved in a final volume of 1.0 L, 250 ml aliquots are autoclaved for 15 min at 121 °C, cool down to 2–8 °C, and use for transformations.
6. In a sterile 100-ml shake flask, 25 ml YGG broth is inoculated with 1×10^6 conidia per ml medium or 25 grains of freeze-dried *P. chrysogenum* rice inoculum and incubated at 25 °C and 200 rpm for 24 h.
7. After 24 h, 25 ml YGG medium is added to the shake flask and the cultivation is continued for 24 h at 25 °C and 200 rpm. In case of rice inoculum, the rice grains should contain no freeze-dried inoculum anymore and can be removed by carefully decanting the broth into a new sterile 100-ml shake flask.
8. After 48 h post inoculum, the culture broth is transferred to a 50-ml falcon tube and mycelium is collected by centrifugation at 4000 g^{-1} for 8 min at 4 °C.
9. The culture medium is decanted and the mycelium pellet is resuspended in 50 ml KC solution by vigorous shaking, following centrifugation at 4000 g^{-1} for 8 min at 4 °C.
10. The supernatant is decanted and the pellet is resuspended in a volume of 10 ml KC solution by shaking. After complete resuspension, the volume is made up to 18 ml with KC solution.
11. In a sterile 100-ml shake flask, 400 mg Glucanex/Lysing Enzyme from *T. harzianum* (*see* **Note 16**) is weighed and the mycelium solution is transferred to the shake flask.
12. The mycelium is incubated at 25 °C and 120 rpm for 90 min (*see* **Note 17**). Shake flasks are shaken manually after 30 and 60 min to aid with separation of mycelium clumps or pellets, ensuring an even protoplast formation. Progression of protoplast formation is monitored by microscopy.

13. The shake flasks with protoplast suspension are chilled on ice for 5 min and afterward transferred to a 50-ml falcon tube. Subsequently, the tube is filled up to 50 ml with KC-Buffer and protoplasts are pelleted by centrifugation for 5 min at 600 × *g* using a swinging bucket rotor centrifuge cooled to 4 °C. In all the following steps, protoplasts need to be stored on ice.
14. The supernatant is discarded and the protoplasts are resuspended in 25 ml KC-Buffer by carefully inverting the tube. Alternatively, a sterile inoculation loop can be used. After resuspension, the volume is made up to 50 ml with STC.
15. The protoplasts are washed a second time by spinning at 600 × *g* and 4 °C for 5 min. Following the aforementioned procedure, protoplasts are resuspended in 50 ml STC.
16. If required, the protoplasts can be counted by using a counting chamber before a final centrifugation step at 600 × *g* and 4 °C for 5 min.
17. The protoplasts are resuspended in 5 ml SCT buffer or the required volume to obtain a concentration of 2×10^7 protoplasts/ml and are kept on ice until transformation is performed (*see* **Note 18**) or protoplasts are stored at −80 °C.

3.4 Cryopreservation of Protoplasts

1. Prepare Cryopreservation Buffer (STC-PVP): dissolve 20% (w/v) PVP-40 in STC-Buffer, autoclave for 15 min at 121 °C, keep at 2–8 °C.
2. For 1 volume of protoplasts in STC, 1 Volume of STC-PVP is added to the tube containing the protoplasts and slowly inverted to mix.
3. Protoplasts in STC-PVP are aliquoted (1–2 ml per tube) into sterile cryotubes. Protoplasts should be kept on ice during aliquoting.
4. Cryotubes are placed in a cryotube freezing container, precooled to 4 °C, and stored in a −80 °C freezer for a minimum of 8 h. Frozen protoplasts are stable for approximately 6 month (*see* **Note 19**).
5. For preparation of frozen protoplasts for subsequent transformations, the cryotube containing the desired strain is thawed on ice for 15 min (*see* **Note 20**).
6. Using a wide-bore pipette tip, the protoplast STC-PVP suspension is transferred to a 12-ml Greiner tube and filled up to 10 ml with STC.
7. STC-PVP is removed by pelleting the protoplasts using a swinging bucket rotor at 1000 × *g* for 5 min and 4 °C following resuspension in half the initial volume STC.

3.5 Transformation of Protoplasts with Cas9 RNPs

1. Prepare 1.2 M Sorbitol solution: 218.6 g Sorbitol are dissolved in a final volume of 1 l. Aliquots of 250 ml are autoclaved for 15 min at 121 °C.
2. Prepare 60% PEG-4000: 60.0 g PEG-4000 are dissolved in 40 ml water by heating in a microwave, 1.0 ml 1 M Tris–HCl, pH 7.5 and 5.0 ml 1 M calcium chloride solution are added. The volume is brought to 100 ml with deionized water. Aliquots of 100 ml are autoclaved for 15 min at 121 °C. Storage at room temperature is required to circumvent precipitation.
3. Prepare 20% PEG-4000 solution: 33 ml 60% PEG-4000 are mixed with 67 ml SCT buffer, 25 ml aliquots are autoclaved for 15 min at 121 °C.
4. Prepare 2× STC buffer (2×STC): 2.5 g calcium chloride dihydrate, 109.5 g sorbitol, and 5.0 ml 1 M Tris–HCl buffer (pH 7.5) are dissolved in a final volume of 250 ml, 50 ml aliquots are autoclaved for 15 min at 121 °C.
5. Prepare 10× concentrated Cas9 activity buffer (10×Cas9): 0.476 g HEPES, 1.118 g potassium chloride, and 0.203 g magnesium sulfate heptahydrate are dissolved in deionized water. 40 µl 0.25 M EDTA solution is added and pH is set to 7.5 with KOH. Afterward, 50 µl of a 1 M DTT Stock solution is added and the solution is filter sterilized. Freeze aliquots of 1–2 ml at −20 °C.
6. Prepare agar base; without nitrogen source: 10.0 g glucose monohydrate, 0.5 g magnesium sulfate heptahydrate, 3.0 g sodium chloride, and 342.0 g sucrose are dissolved in 950 ml H_2O, 20.0 g agar-agar and 1.0 ml 1% (w/v) Iron(II) sulfate solution are added and the medium is autoclaved for 20 min at 121 °C.
7. Prepare 500× Trace Element Stock Solution: 7.81 g EDTA is added to 150 ml of deionized water, pH is set to 8.5 with 4 M NaOH. The following trace element salts are dissolved in the indicated order, each salt is added separately to allow for complete dissolution: 10.94 g trisodium citrate dihydrate, 6.21 g Iron(II) sulfate heptahydrate, 64.10 g magnesium sulfate heptahydrate, 0.0031 g boric acid, 0.0031 g sodium molybdate dihydrate, 0.16 g copper(II) sulfate pentahydrate, 0.63 g zinc sulfate heptahydrate, 0.16 g cobalt(II) sulfate heptahydrate, 0.76 manganese(II) sulfate monohydrate, 0.40 g calcium chloride dihydrate. The pH is set to 6.5 by dropwise adding small amounts of a 1 M NaOH solution. The solution is filled up to 250 ml with deionized water, 0.22 µm filter-sterilized and stored in 50 ml aliquots (*see* **Note 21**).
8. Prepare selection agar: to agar base add 2 ml trace elements solution, 10 ml 1 M phosphate buffer pH 6.8 and the following combinations for selection (A—10 ml acetamide solution;

B—10 ml sodium nitrate solution and 140 μl terbinafine solution; C—10 ml sodium nitrate solution and 1.0 ml phleomycin solution). Pour plates from 20 to 25 ml medium, let solidify and dry off moisture from surface for 15 min under laminar flow hood.

9. For each transformation, a separate Cas9 RNP is formed by mixing the following reagents: 30 μl 10× Cas9 activity buffer, 35 μl 2× STC, 27 μg Cas9 protein (*see* **Note 22**), 4 μl of synthetized sgRNA.
10. The mixture is homogenized by tapping the tube, briefly spun down, and incubated for 10 min at 37 °C.
11. A 12-ml Greiner tube is placed on ice and the following components are added in the indicated order: 200 μl 20% PEG, donor DNA in a maximum volume of 50 μl, the entire Cas9 RNP mixture (up to 90 μl), and 200 μl of protoplasts.
12. The components are mixed by gently tapping the tube several times, placed back on ice, and incubated 30 min.
13. The tubes are removed from the ice (*see* **Note 23**), incubated for 2 min at room temperature before 1.5 ml 60% PEG-4000 solution is added. The highly viscous mixture is homogenized completely by carefully rotating the tube at an angle of 45° for 2 min.
14. The tubes are placed in a 25 °C incubator for 25 min to allow uptake of RNPs (and DNA) into the protoplasts.
15. The volume is made up to 11 ml with 1.2 M Sorbitol and protoplasts are pelleted in a centrifuge with swinging bucket rotor at 2770 × g^{-1} for 5 min at 25 °C.
16. The supernatant (viscous) is decanted and the tubes are placed upside down without cap on a tissue to allow residual droplets of PEG-4000 containing buffer to run down (*see* **Note 24**).
17. The protoplasts are gently resuspended in 1 ml 1.2 M Sorbitol.
18. Aliquotes of 100–200 μl of protoplasts are plated on osmotic stabilized selection media by gently spreading with a Drigalski spatula without applying pressure, creating a homogenous layer of liquid on the plate surface.
19. Plates are allowed to dry at room temperature for 30 min and then placed in a 25 °C incubator upside down (*see* **Note 25**).
20. Plates are incubated 5–7 days until colonies appear.

3.6 Colony PCR of *P. chrysogenum*

1. A small piece of mycelium (approx. 1.5–2.0 mm diameter) is taken with the tip of a 10 μl pipette and transferred into 20 μl of DNA dilution buffer aliquoted in a PCR tube.
2. With the pipette tip used for transfer, the mycelium piece is slightly smashed, and incubated for 3 min at room temperature.

3. The mycelium is spun down with a table-top centrifuge for 1 min.
4. For each colony PCR reaction, a PCR mixture is prepared: 10.0 μl 2× Phire Plant PCR Buffer, 0.4 μl Phire Hot Start II DNA Polymerase, 9.4 μl nuclease-free water, 0.15 μl 100 μM Forward Primer, 0.15 μl 100 μM Reverse Primer (a master mix for multiple aliquots can be prepared in 1.5-ml reaction tube).
5. 20 μl aliquots are prepared in PCR tubes and 0.5 μl DNA dilution buffer containing no mycelium debris is added.
6. The following program is run on a PCR thermocycler: heating to 95 °C for 5 min, followed by 35 cycles of (98 °C for 15 s, 61 °C for 15 s, 72 °C for 20 s/kb product), and 72 °C for 5 min.
7. 5 μl of the PCR reaction mix are electrophoresed on a 0.8% agarose gel for 25–30 min to confirm successful amplification.

4 Notes

1. We highly recommend using the indicated polymerase as the PCR reactions frequently failed in our hands when other commercially available PCR Master Mixes were used.
2. Another RNAse inhibitor might be used by the operator if it is compatible with RNA synthesis reactions. The operator of this protocol can also select a T7 RNA transcription Kit of his choice; however, we want to state that the obtained yields of sgRNA can also depend on the used Kit.
3. Lysing enzyme from *Trichoderma harzianum* is the preferred enzyme cocktail for lysis of cell walls for several Penicillium and Aspergillus species. If the operator of this protocol wishes to prepare protoplasts of another fungal species, we recommend searching the literature for alternatives, avoiding failure to obtain good quality protoplasts.
4. For initial trials, Cas9 protein having a nuclear localization signal can be purchased from a vendor of choice. For routine usage, it is more economic to overexpress Cas9 from pET28(a) [24] in E. coli T7 express lysY (New England Biolabs) or a comparative T7 expression strains and purify it via affinity chromatography.
5. We highly recommend using the indicated colony PCR Kit for initial sample preparation trials as other methods had higher failure rates in our hands. If the operator has experience with colony PCR of filamentous fungi, he might choose another method himself.

6. Protospacers at target sequences might be identified with any tool the operator of this protocol is familiar with. However, several online protospacer identification tools such as CCTop [25], ChopChop v2.0 [26], or E-CRISP [27] have a limited set of available genomes that can only search protospacers in pre-selected list of genomes typically not including filamentous fungi. A good alternative to CasOT is CRISPy-web [28], accepting any genome in genbank file format and is able to perform an off-target prediction. Additionally, to ensure that off-target effects are avoided, we highly recommend performing an off-target analysis of identified protospacers. Further, the cleavage efficiency of protospacers should be accessed to exclude low-potency protospacers from the workflow. It is recommended to follow the literature [19, 29] on this topic to ensure that efficiencies can be increased in the future.
7. It is recommended to select two protospacers per target and if applicable, test them individually to select the most efficient. Otherwise, multiple sgRNAs targeting the same gene can be used without constraints in experiments.
8. Experience with primers ordered from Sigma Aldrich. Alternatively, primers can be obtained in other purity grades.
9. For easier handling of liquids, primers may be diluted to 10 μM. All liquid volumes for the PCR reaction in the following steps need to be adjusted accordingly.
10. It is strongly recommended to use KAPA HiFi Polymerase for the described protocol. If alternative PCR protocols need to be attempted by the operator, we advise to include a negative control to ensure amplification.
11. It is not required to purify the PCR reaction.
12. For all the steps working with RNA, gloves should be used to prevent RNAse contaminations. Master mixes for multiple reactions can be prepared. For each sgRNA, 4 μl will be required per transformation—a 10 μl reaction provides sufficient material for the experiment, gel analysis, and control experiments. A negative control containing no T7 polymerase can be prepared.
13. Denaturing of misfolded sgRNA secondary structures and re-annealing was shown to be beneficial for the sgRNA activity [30].
14. If little experience with PCR using long primers, it is recommended to perform initial PCR reactions using KAPA HiFi Polymerase at and annealing temperature of 67 °C or to perform a gradient with available polymerases at a low reaction volume.

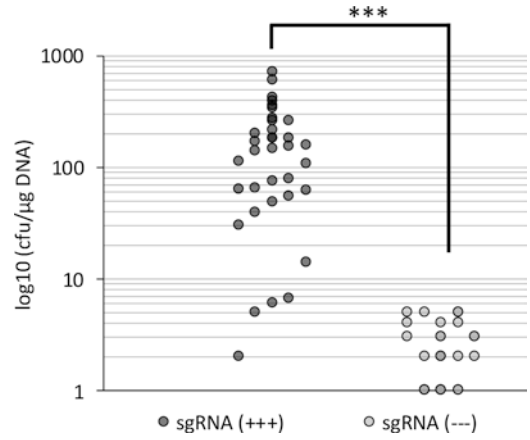

Fig. 1 Scatter plot of colony forming units (cfus) obtained with donor DNA (containing a p*gpdA-amdS*-t*amdS* selection marker cassette) having 100 bp homology toward the 5′and 3′-UTR of 12 different target genes (median = 174 cfu/µg DNA). Data was generated for testing a total of 35 individual protospacers targeting 12 different genes. Experiments were performed at different days with independent batches of fresh and frozen protoplasts of *P. chrysogenum* DS68530, by two operators. Difference reporter is relative to transformations of donor DNA without addition of sgRNA, two-tailed Students *t*-test assuming unequal variance: $p = 6.12 \times 10^{-7}$

15. The efficiency of the marker integration is depending on the sgRNA used for cutting (*see* Fig. 1).
16. This concentration of Glucanex was found to work for *P. chrysogenum* protoplasts of strain DS68530 [31]. If the operator is working with another filamentous fungus, it is suggested to rely on the established protocols. If no knowledge on the Glucanex dose exists for the fungus to be transformed, it is advised to start with a concentration of 25 mg/ml. and control the protoplast formation after 60 min. If less than 50% of mycelium was digested, the Glucanex concentration can be increased further.
17. Protoplast quality decreases considerably for *P. chrysogenum* when incubation in KC + Glucanex solution exceeds 90 min. Due to the delayed release of protoplasts from clumped mycelium, early protoplasts are exposed too long to Glucanex and show decreased vitality. To circumvent this, the Glucanex concentration can be increased stepwise by 5 mg/ml and incubation time can be reduced to 60 min, if protoplasts are of low quality.

18. Prolonged incubation (>3 h) of protoplasts without nutrients should be omitted as this could decrease the vitality.
19. The reproducibility of transformations of frozen protoplasts with sgRNA is sufficiently high to verify efficiency (*see* Fig. 2a and b).
20. Thawed protoplasts should not be frozen again. If operators wish to use that protocol for other filamentous fungi, it is advised to perform transformation trials with fresh and frozen protoplasts to compare efficiencies.
21. Calcium chloride dihydrate will dissolve very slowly in trace element solution, it can take up to 20 min, do not add 1 M NaOH before it has dissolved completely. Generally, add 1 M NaOH only in small amounts (1–2 drops) and wait until it is completely mixed before adding the next drop. After storage over several days, a color change to dark brown will take place which does not influence the quality of the trace element solution.
22. The amount of Cas9 added influences the number of colonies that will be obtained (*see* Fig. 3).
23. As the 60% PEG-400 solution easily crystalizes when cooled below 20 °C or placed back on ice, it is advised to incubate tubes 2 min at RT before adding the PEG to omit this.
24. Residual PEG-4000 can crystallize on the surface of the selection plates, strongly reducing transformant recovery. The operator has to ensure that only little (<100 µl) solution remains in the tube.
25. Osmotic stabilized plates tend to dry out quicker than normal plates. It is advised to keep a beaker with sterile water in the incubator to saturate atmosphere with water, which helps recovery of transformants.

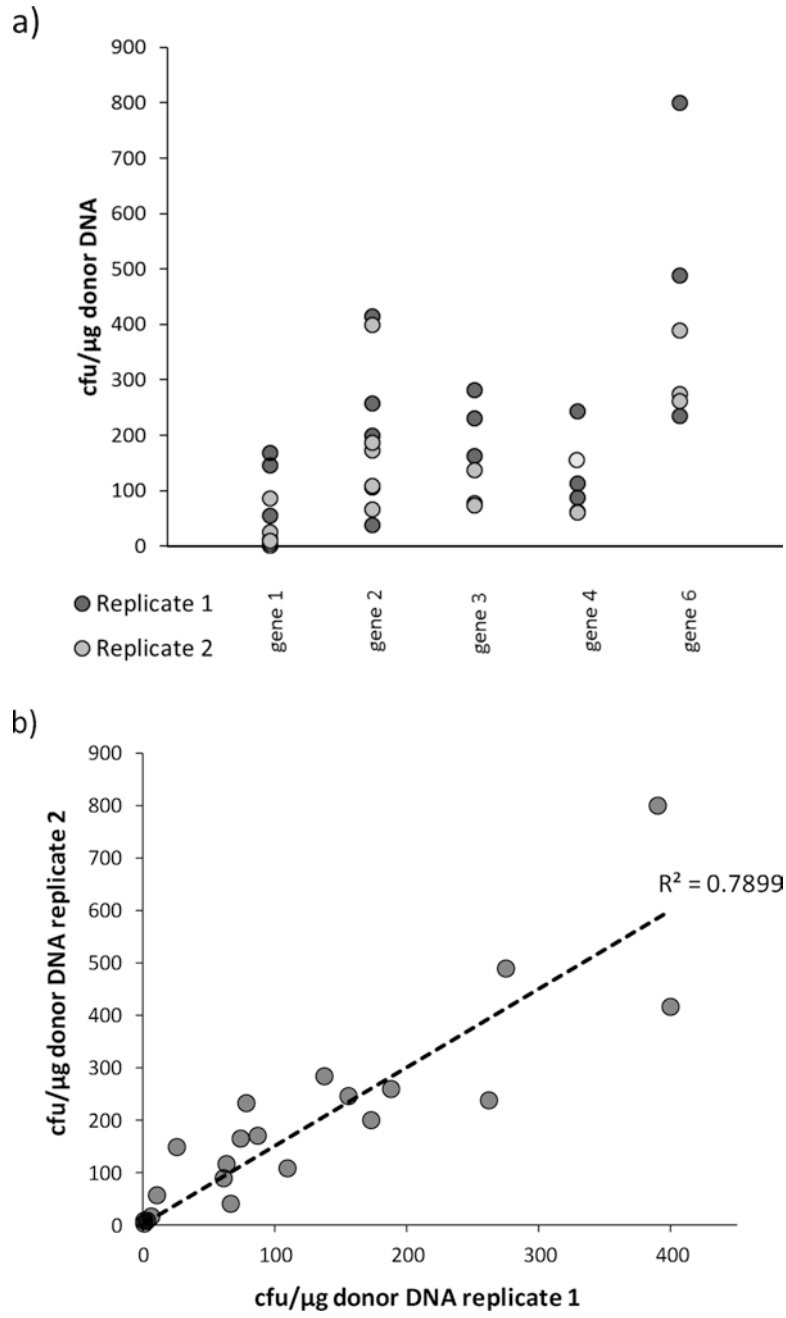

Fig. 2 Comparison of the (**a**) efficiency and (**b**) reproducibility of the targeting of six different genes in *P. chrysogenum* DS68530 using 24 different protospacers. Cryopreserved protoplasts were thawed and transformed at different days with independently synthetized sgRNAs by the same operator. Efficiency variation is related to the protospacer that was chosen and not the targeted gene. No essential genes were targeted. The reproducibility of colonies obtained with same sgRNAs in experiments performed at different days is sufficiently high ($R^2 = 0.78$)

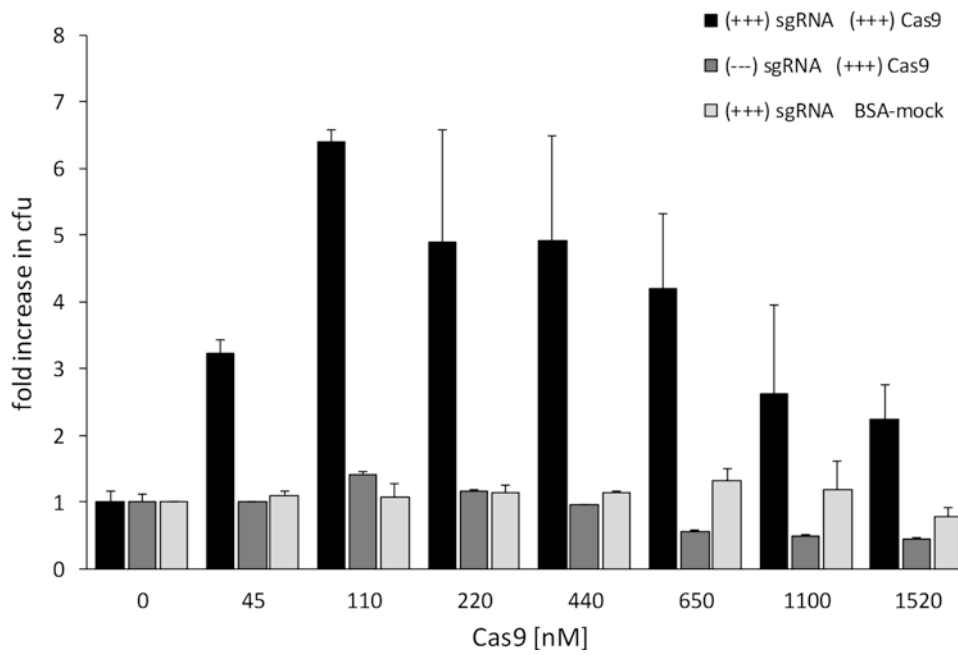

Fig. 3 Impact of Cas9 concentration on colony forming units (cfus) obtained after transformation. An optimal concentration is around 110–220 nM (equaling 27 μg per transformation) Cas9 in the transformation mixture. A p*gpdA-amdS-tamdS* selection marker cassette, having 1000 bp homology toward the 5′ and 3′-UTR of Pc21g16000 (PKS17, involved in YWA biosynthesis) was used for this experiment

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