Protocol

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Publisher’s note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.
Protocol

Genome editing in *Penicillium funiculosum* using *in vitro* assembled CRISPR-Cas9 ribonucleoprotein complexes

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SUMMARY

The plasmid-free CRISPR-Cas9-based genome editing in fungi is a precise and time-saving approach. Here, we present a detailed protocol for genetic manipulation in *Penicillium funiculosum*, which includes design and synthesis of sgRNA, high-quality protoplast preparation, and PEG-mediated protoplast transformation of linear donor DNA along with *in vitro* synthesized RNP complex composed of sgRNA and host-specific Cas9. This technique is beneficial for researchers interested in functional analysis of genes as it improves reproducibility and replicability of the experiment.

For complete details on the use and execution of this protocol, please refer to Randhawa et al. (2021).

BEFORE YOU BEGIN

The protocol below describes the specific steps for deletion of the cbh1 gene in *P. funiculosum* NCIM1228 by transient introduction of purified Cas9 having NLS from *P. funiculosum*, pre-complexed with sgRNAs, together with donor DNA in the fungal protoplasts. PCR and Western blotting were done for confirmation of cbh1 gene deletion. Several other genes like nosA, atf21, and pacC were also deleted using this protocol, making it a well-established gene-editing technique for any gene deletion in *Penicillium* species.

Retrieve the sequences from the available genomic database

© Timing: 1 day

1. Identify the gene that needs to be deleted.

   Note: The genome database for the gene should be available either at the NCBI site or in the in-house database. For example, genome sequence database similar to the *P. funiculosum* NCIM1228 is available at https://www.ncbi.nlm.nih.gov/bioproject/?term=507506 on the NCBI website.

2. Derive the target gene sequence from the selected genome.
3. Remove all the white space and save the sequence in FASTA format.
4. If multiple FASTA files are there for the genome, then combine them into one big FASTA file.
5. Use this FASTA file as input genome sequence file for BiooTools software (www.biootools.com).
6. Fetch the well annotated gene sequence to be deleted, along with 1000 bases of 5’UTR and 1000 bases of 3’UTR region and save it as a FASTA format file.
7. Use this FASTA format file as an input file for BiooTools software.

Note: The annotated sequence should include the information about the ORFs, functional region (if applicable), promoter and terminator.

Preparation of buffers

@ Timing: 4–5 h

8. Prepare all the solutions, maintain pH and filter sterile them one day prior to the experiment. Make sure that there is enough of all solutions that are needed for protoplast formation.
9. Make sure that DEPC treated water is used for dilution of the sgRNAs to 1 μM.
10. Prepare approximately 20 μg of Donor DNA by PCR and purify in advance before the experiment. If needed, concentrate using Speedvac concentrator and bring the volume to 20 μL.

Preparation of the sporulated plates

@ Timing: 10–15 days

11. Prepare the Low Malt Peptone (LMP) agar plates, spot 10–20 μL of grown mycelia in the middle of the plate and incubate them at 28°C for 10–15 days.
12. When the plates are sporulated, flood them with 10 mL of sterile MilliQ water and scrap with sterile spreader to dislodge spores, filter the content with Mira cloth and use the spore suspension for inoculation in Potato Dextrose Broth (PDB) for protoplast preparation.

△ CRITICAL: Always use fresh sporulated plates for protoplast preparation. Healthy protoplasts are not formed when prepared with the stored spores.

Preparation of stocks for oligonucleotides and primers for polymerase chain reaction (PCR)

@ Timing: 1:30 h

13. Centrifuge the ordered lyophilized oligonucleotide vials at full speed for 20 min.
14. Resuspend the ordered oligonucleotide of 55 bases (Oligo_55) with sterile DEPC treated water to 100 μM for master stock and incubate the vials at 37°C for 1 h.
15. Resuspend all the required primers in 1× TE buffer to 100 μM for master stock, vortex for 1 min and incubate the vials at 37°C for 1 h.
16. Make working solution of 1 μM concentration of the Oligo_55 with sterile DEPC water.
17. Dilute the primers for PCR screening with MilliQ water up to 10 μM for working stock.
18. Store all the master and working stocks at −20°C until further use.

KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Bacterial and virus strains | | |
| Escherichia coli DH5 α cells | New England Biolabs | C2987H |
| Chemicals, peptides, and recombinant proteins | | |
| Lysing enzyme from Trichoderma harzianum | Sigma | L1412 |
| Potato Dextrose Broth, Granulated | HiMedia | GM403 |
| Magnesium sulphate, heptahydrate | HiMedia | GRM684 |

(Continued on next page)
| REAGENT or RESOURCE                             | SOURCE          | IDENTIFIER |
|------------------------------------------------|-----------------|------------|
| Disodium hydrogen phosphate heptahydrate       | VWR             | 0348       |
| Potassium dihydrogen phosphate anhydrous       | HiMedia         | TC011      |
| D (-)-Sorbitol                                  | HiMedia         | GRM109     |
| Tris (hydroxyethyl) aminomethane               | HiMedia         | TC072      |
| Polyethylene Glycol 3350                       | Sigma           | 2S322-68-3 |
| Sucrose                                        | HiMedia         | GRM3063    |
| Yeast extract powder                           | HiMedia         | RM027      |
| Tryptone                                       | HiMedia         | CR014      |
| Agar powder, Bacteriological grade             | HiMedia         | GRM026     |
| Malt Extract                                   | HiMedia         | RM004      |
| Soy Peptone                                     | HiMedia         | RM007      |
| DEPC                                           | HiMedia         | MB076      |
| Calcium chloride dihydrate                      | HiMedia         | RM3906     |
| Glycerol                                       | HiMedia         | GRM1027    |
| AatII restriction enzyme                        | Fermentas       | FD0994     |
| BstBI restriction enzyme                        | Fermentas       | FD0124     |
| Ahdl/ Eam1105I                                  | New England Biolabs | RO8545 |
| Alel/Ooll                                      | New England Biolabs | RO8585 |
| Phusion High-Fidelity DNA Polymerase            | Thermo Fisher Scientific | FS305 |
| Recombinant Cas9 protein (Having PfH2B NLS at N-terminus and SV40NLS at C-terminus of Cas9 along with N-terminus His-tag) | In-house | Custom synthetic product |
| Critical commercial assays                      |                 |            |
| EnGen® sgRNA Synthesis Kit, S. pyogenes        | New England Biolabs | E3322S |
| RNA Clean & Concentrator Kits                   | Zymo Research   | R1017      |
| PCR purification kit                            | QIAGEN          | 28104      |
| Quick- DNA/Fungal/Bacterial Miniprep kit        | Zymo Research   | D6005      |
| Experimental models: Organisms/strains          |                 |            |
| Penicillium funiculosum NCIM1228                | National Collection of Industrial Microorganism, Pune | NCIM1228 |
| Oligonucleotides                                |                 |            |
| cbh1_S_1 guide RNA oligonucleotide              | Sigma           | N/A        |
| GTACAAGAGCCGCCCCTCATCT                          |                 |            |
| cbh1_S_102 guide RNA oligonucleotide            | Sigma           | N/A        |
| GGTCCCTAACCTGTGTAG                              |                 |            |
| HygromycinF, Hygromycin Forward Primer          | Sigma           | N/A        |
| AATCGACAACATGTCCAGTAATCAATCTCTAATCTCCAATGGGG   |                 |            |
| HygromycinR                                     | Sigma           | N/A        |
| GGTCCACGAGGTGAACCCTAGTACGCCTGGATCC             |                 |            |
| CBH1_300bp_up_F                                | Sigma           | N/A        |
| GGACGTATAGATCGGGACTGTGAGG                      |                 |            |
| CBH1_300bp_ds_R                                | Sigma           | N/A        |
| TACGGCTTGGAGTTTGGCC                            |                 |            |
| CBH1_1124bp_up_F                               | Sigma           | N/A        |
| ATCTCGAATATCCTCAACAGGTGATCC                    |                 |            |
| Hph_int_R                                      | Sigma           | N/A        |
| CACTCCACCTAGTGCCTGGAACCTTT                    |                 |            |
| CBH1_718bp_dn_R                               | Sigma           | N/A        |
| GCCTGTAGATCGCTGGAG                            |                 |            |
| Hph_int_F                                      | Sigma           | N/A        |
| ATCGAAAGCTGAAAGCGAG                            |                 |            |
| Software and algorithms                         | www.biootools.com | N/A      |
| Other                                          |                 |            |
| Pasteur pipette                                | Tarsons         | 520063     |
| Mira cloth                                     | Merck           | 475855     |
| Neubauer counting chamber                      | Laboratory deal | Lab_9855  |

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### MATERIALS AND EQUIPMENT

#### Low Malt Peptone (LMP) agar

| Reagents                              | Final concentration | Amount |
|---------------------------------------|---------------------|--------|
| Malt extract                          | 1%                  | 10 g   |
| Soy peptone                           | 0.05%               | 0.5 g  |
| Agar                                  | 1.5%                | 15 g   |
| ddH₂O                                 | N/A                 | Make volume up to 1 L |
| Total                                 | N/A                 | 1 L    |

**Note:** Prepare four flasks of 25 mL each to yield 10⁷ protoplasts/mL. Make up fresh buffer for each experiment and filter sterile with 0.25 μm membrane and store at 4°C.

#### Protoplasts solution (pH-7)

| Reagents                              | Final concentration | Amount |
|---------------------------------------|---------------------|--------|
| Lysing enzyme from *Trichoderma harzianum* | 5 mg/mL             | 125 mg |
| 2 M MgSO₄                              | 1.2 M               | 15 mL  |
| 1 M Sodium Phosphate Buffer (pH-7)     | 10 mM               | 250 μL |
| http://cshprotocols.cshlp.org/content/2006/1/pdb.rec8303 |                     |        |
| ddH₂O                                 | N/A                 | 9.75 mL |
| Total                                 | N/A                 | 25 mL  |

**Note:** Prepare four flasks of 25 mL each to yield 10⁷ protoplasts/mL. Make up fresh buffer for each experiment and filter sterile with 0.25 μm membrane and store at 4°C.

#### Separation Buffer (pH-7)

| Reagents                              | Final concentration | Amount |
|---------------------------------------|---------------------|--------|
| 2 M Sorbitol                          | 0.6 M               | 7.5 mL |
| 1 M Tris (pH-7)                       | 100 mM              | 2.5 mL |
| ddH₂O                                 | N/A                 | 15 mL  |
| Total                                 | N/A                 | 25 mL  |

**Note:** Prepare the separation buffer one day before the protoplasts preparation, filter the solution and store at 4°C.

#### Storage Buffer (pH-7.5)

| Reagents                              | Final concentration | Amount |
|---------------------------------------|---------------------|--------|
| 2 M Sorbitol                          | 1.2 M               | 15 mL  |
| 100 mM Tris (pH-5)                    | 10 mM               | 2.5 mL |
| ddH₂O                                 | N/A                 | 7.5 mL |
| Total                                 | N/A                 | 25 mL  |
Note: Store the storage buffer at 4°C.

| 1 M CaCl₂ | Final concentration | Amount |
|-----------|---------------------|--------|
| CaCl₂.2H₂O| 14.7 mg             | N/A    |
| ddH₂O     | N/A                 | 100 mL |
| Total     | N/A                 | 100 mL |

Note: Filter sterile and store at 4°C.

50% PEG solution

| Reagents       | Final concentration | Amount |
|----------------|---------------------|--------|
| PEG 3350       | N/A                 | 50 g   |

Add 50 mL of ddH₂O to a 200 mL beaker, weigh PEG 3350 and heat it to speed dissolving

| Reagents       | Final concentration | Amount |
|----------------|---------------------|--------|
| 1 M CaCl₂      | 10 mM               | 1 mL   |
| 1 M Tris (pH 7.5) | 10 mM            | 1 mL   |
| Volume make up to 100 mL |
| Total          | N/A                 | 100 mL |

Note: The PEG solution is viscous, sterile by vacuum filtration through a 0.45 µm using Cellulose Acetate membrane. Store the sterilized PEG solution at 25°C.

STC solution

| Reagents       | Final concentration | Amount |
|----------------|---------------------|--------|
| 2 M Sorbitol   | 1.2 M               | 30 mL  |
| 1 M CaCl₂      | 10 mM               | 0.5 mL |
| 1 M Tris       | 10 mM               | 0.5 mL |
| ddH₂O          | N/A                 | 19 mL  |
| Total          | N/A                 | 100 mL |

Note: Prepare a day before protoplasts preparation and store at 25°C.

1 x Nuclease Buffer (pH 7.9)

| Reagents       | Final concentration | Amount |
|----------------|---------------------|--------|
| 1000 mM NaCl   | 100 mM NaCl         | 10 mL  |
| 500 mM Tris-HCl| 50 mM Tris-HCl      | 10 mL  |
| 100 mM MgCl₂   | 10 mM MgCl₂         | 10 mL  |
| 2 mg/mL BSA    | 100 µg/mL BSA       | 5 mL   |
| ddH₂O          | N/A                 | 65 mL  |
| Total          | N/A                 | 100 mL |

Note: Store at –20°C.

STEP-BY-STEP METHOD DETAILS

CRISPR/Cas9 cleavage target sequence identification

© Timing: 1 h
Identify the potential target sequences for DNA cleavage within the genomic region of interest by using the sgRNAcas9 software (V3.0), as shown in Figure 1. sgRNAcas9 software package is publicly available at BiooTools website (www.biootools.com) under the terms of the GNU General Public License.

Note: Detail about the software is described by Xie et al. (Xie et al., 2014).

1. Enter the gene sequence for knockout in the target sequence and the fungal genome sequence in the genome sequence, and then run the program (Figure 1A).
2. The software generates a report file that highlights CRISPR target sites, potential off-target sites and risk of evaluation (Figure 1B).
3. Carefully choose the nucleotide sequence of 20 bases that are shown best in the risk of evaluation column generated by the software based on number of off-targets.

Note: The oligonucleotides should not include PAM sequence.

4. Add ‘G’ at the 5’-end if not present in the selected nucleotide of 20 bases (Figure 1C).
**Note:** Addition of ‘G’ at the 5’-end helps T7 RNA polymerase by providing the extra stability required in transcription (Kuzmine et al., 2003).

5. Append T7 promoter sequence: TTCTAATACGACTCATA to the 5’ end and 14 nucleotides overlap sequence: GTTTTAGAGCTAGA to the 3’ end of the target specific oligonucleotide(s).

6. Check complete oligo sequence of 55 bases long: 5’TCTAATACGACTCATA(N)20GTTTTAGAGCTAGA 3’ before ordering/synthesizing (Figure 1C).

7. Place order or synthesize the customized 55 base-long oligonucleotide (Oligo55).

8. The sgRNA is synthesized with the help of EnGen® sgRNA Synthesis Kit (NEB #E3322). The customized 55-base-long oligonucleotide (from Step 6) is used as template according to the manufacturer’s instructions.

**Note:** For knockouts, to study the loss of function, a pair of sgRNAs is preferred with DNA cleavage sites near to start and stop codon of the gene for complete removal of ORF (Figure 1D).

**Note:** If the report file is not generated by the software or target cleavage site risk column shows discard then check for the input sequences. The software does not allow any white space in the file name or file path.

**Donor DNA design and preparation**

© Timing: 1 month

The double strand breaks (DSB) generated by Cas9 at the cleavage site is repaired by either by Non-Homologous end joining (NHEJ) or homologous recombination when donor DNA construct is provided for repair of the genome (Chen et al., 2020). We prepared Donor DNA for cbh1 deletion having antibiotic resistance marker, hygromycin, flanked by the 300 bp homologous region of the cbh1 ORF to be knocked out.

9. Check the target sequence and the upstream / downstream genomic sequence flanking the target cleavage sites.

**Note:** We have found for NCIM1228 that donor DNA with 300 bp–500 bp homologous region is efficient for the recombination.

10. A two-step cloning method for cbh1 deletion cassette preparation is mentioned below-a. The 3432-bp genomic region of NCIM1228 containing cbh1 ORF of 1590-bp to be amplified using CBH1_1124bp_up_F and CBH1_718bp_dn_R primers and cloned in pCambia1302 vector at BstBI and AatII restriction enzymes sites to obtain pCBH1 construct and transform in DH5 alpha cells (Figure 2A).

| PCR reaction master mix for cbh1 amplification |
|-----------------------------------------------|
| Reagent                                      | Amount            |
| DNA template                                 | 1 µL (100 ng)     |
| (2 U/µL) Phusion                             | 0.5 µL            |
| 10 µM CBH1_1124bp_up_F                       | 5 µL              |
| 10 µM CBH1_718bp_dn_R                       | 5 µL              |
| 10× GC Buffer                               | 10 µL             |
| 2.5 mM dNTPs                                | 5 µL              |
| ddH2O                                       | 23.5 µL           |
b. In the second step, in place of cbh1 ORF, hygromycin selection marker to be sub-cloned at AhdI and AleI restriction enzymes using Hygromycin F and Hygromycin R primers to obtain pHygro construct (Figure 2B).

**Figure 2. Cloning strategy for the Donor DNA construction**

(A) cbh1 is PCR amplified from genomic DNA and digested with restriction enzymes, the pCAMBIA backbone is linearized and ligated with the cbh1 fragment to obtain pCBH1.

(B) The resulting pCBH1 plasmid is linearized by restriction digest and the Hygromycin cassette is ligated into the linearized vector to yield pHygro.

(C) The linear dsDNA donor for CRISPR engineering is obtained from the final pHygro using CBH1_300bp_up_F and CBH1_300bp_ds_R.

**PCR cycling conditions for cbh1 amplification**

| Steps         | Temperature | Time  | Cycles |
|---------------|-------------|-------|--------|
| Initial Denaturation | 98°C        | 30 s  | 1      |
| Denaturation   | 98°C        | 10 s  | 30 cycles |
| Annealing     | 61°C        | 30 s  |        |
| Extension     | 72°C        | 10 min|        |
| Final extension | 72°C      | 10 min|        |
| Hold          | 4°C         | forever|        |
c. Using cbh1_300bp_US_F forward and cbh1_300bp_DS_R reverse primers, the donor DNA is to be amplified (Figure 2).

| PCR reaction master mix for Donor DNA amplification |
|-----------------------------------------------------|--------|
| Reagent                                            | Amount |
| DNA template                                       | 1 µL (100 ng) |
| (2 U/µL) Phusion                                   | 0.5 µL |
| 10 µM cbh1_300US_F                                 | 5 µL |
| 10 µM cbh1_300bp_DS_R                              | 5 µL |
| 10× GC Buffer                                      | 10 µL |
| 2.5 mM dNTPs                                       | 5 µL |
| ddH₂O                                              | 23.5 µL |

**Alternatives:** Donor DNA can be synthesized by overlapping PCR or by chemical synthesis.

**Note:** While cloning of selection marker cassette, restriction enzymes must be selected near to start and stop codon of the ORF so that most portion of the gene is removed.

11. Amplify the linear Donor DNA through PCR using forward and reverse primers binding at 300 bp upstream of cleavage site at promoter region and 300 bp downstream of another cleavage site at the terminator region (Figure 2C) and purify the PCR product using QIAGEN PCR purification kit.

a. Use 20 µg of linear PCR product for transformation. Generally, six aliquots of 50 µL PCR reaction yields 20 µg of DNA.

b. Reprecipitate the DNA using sodium acetate/100% ethanol or use Speedvac concentrator for evaporation of the solution.

c. Resuspend the DNA pellet in a solution of STC/PEG with the volume ratio at 4:1 to obtain the concentration of ~1 mg/mL for transformation.

d. Store at −20°C until use. Use linear donor DNA along with RNP complex on the day of transformation.

**Alternatives:** Donor DNA can be prepared using Gibson Assembly Reaction.

**List of primers and sgRNAs oligonucleotides**

| Primer                  | Oligonucleotides                          |
|-------------------------|-------------------------------------------|
| cbh1_5_1                | GTACAAGAGCGCCCTCATCT                      |
| cbh1_S_102              | GGTCCTACCACCTGTGTAG                      |
| HygromycinF             | AATCGACACAATGTCCTGCAGATAATCCTCAATGGG     |
| HygromycinR             | GGTTCAGCAGCGTGAAACCACGTAGTCACGCGTGATCC  |
| CBH1_300bp_up_F         | GGACGTATAGATCGGGACTGTGAGG                |
| CBH1_300bp_ds_R         | TACGCTTGGATGAAATTTTGGGCC                 |
| CBH1_1124bp_up_F        | ATCTCGAATATCCCAACAGGTCGATCC             |
| Hph_int_R               | CACTCCACTTAGGTTCGAACTCTT                 |
| CBH1_718bp_dn_R         | GCGTGAGTACGCGCTAGG                       |
| Hph_int_F               | ATCGAAGCTGAAAGCACGAG                    |

**sgRNA synthesis and purification**

© Timing: 3 h

The sgRNA is synthesized with the ordered oligonucleotides consists of 55 bases (Oligo_55) using EnGen® sgRNA Synthesis Kit, *S. pyogenes* (NEB #E3322) according to manufacturer’s instructions (https://international.neb.com/protocols/2016/05/11/engen-sgRNA-synthesis-kit-s-pyogenes-protocol-e3322) and purified with the Zymo Research RNA clean and concentrator.
1) The *S. pyogenes* Cas9 Scaffold Oligo is provided as one of the components of the 2x sgRNA Reaction mix which includes 14 bases at the 3'-end that are complementary to the 14 nucleotide overlap sequence of Oligo_55. Its sequence is as follows:

5' AAAAGCACCAGCCTGGTGCCACCTTTTTTCAAGTGAATAACGGACTAGCCTATTATTTAACCGTCTATTTACTGCTCTAAAC 3'

2) The overlapped oligos:

5' TTCTATACG ACTCATA GTACAAGGAGGCCCTCATC TGTTTAGAG CTAGA 3'
3' -------------------------- ----------------------------------- -CAAATCTC GATCTTTATC

GTCAATTTT ATTGAGATTAAGTTGACTTCTTCT ACCGTGGCTC AGCCACGAAA 5'

3) The DNA polymerase further fills the gap and RNA polymerase transcribes the final sgRNA. The final sgRNA sequence would be:

5' GUCAAAGAGGCCCCUAUC U GUUUAGAG CUAG
AAAAGCAAGUUAAUAAAGCGUAGUCGGUUAUCAC
UUGAAAAGUGGCACCGAGUCGCUGUUU 3'

Figure 3. An example of sgRNA synthesis using Engen sgRNA synthesis kit

kit (https://www.zymoresearch.com/collections/rna-clean-concentrator-kits-rcc/products/rna-clean-concentrator-25). The EnGen 2x sgRNA Reaction Mix contains 80 bases-long scaffold oligo of *S. pyogenes* for Cas9 recognition, which includes 14 bases at the 3'-end that are complementary to the 14 nucleotide overlap sequence of Oligo_55. The final sgRNA would contain 20 bases of target nucleotide and 80 bases of scaffold sequence (Figure 3).

12. Prepare sgRNA on the day of protoplasts preparation.
   a. Thaw all the reagents, mix and pulse spin.
   b. Assemble the reaction in microcentrifuge tube at room temperature as follows-

| Reagents                        | Amount |
|--------------------------------|--------|
| Nuclease-free water            | 2 µL   |
| EnGen 2x sgRNA Reaction Mix, *S. pyogenes* | 10 µL |
| DNA Oligo_55 (1 µM)            | 5 µL   |
| DTT (0.1 M)                    | 1 µL   |
| EnGen sgRNA Enzyme Mix         | 2 µL   |
| Total volume                   | 20 µL  |

13. Incubate at 37°C for 1 h 30 min in the thermo cycler.
14. Transfer reaction to ice and add 2 µL of DNase I provided in the kit and bring volume of the reaction to 50 µL by adding 30 µL of nuclease-free water.
15. Incubate at 37°C for 15 min.
16. Proceed with purification of RNA using RNA clean and concentrator kit (Zymo Research # R1017). Add binding buffer and ethanol to the sample, then bind, wash and elute ultra-pure RNA.
17. Measure the concentration of sgRNA using Nanodrop and use the required amount. Keep on ice until use.

*Note:* Wearing of gloves and use of RNase free tubes and workbench is strongly recommended.
**P. funiculosum NCIM1228 Cas9 synthesis**

- **Timing: 2–3 months**

This step describes how to customize the Cas9 protein having host-specific nuclear localization signal (NLS) for the efficient entry inside the nucleus of the targeted background host, i.e., *P. funiculosum* NCIM1228. This step is necessary since the classical nuclear localization sequence (NLS) of SV40 is unable to efficiently translocate Cas9 into its nucleus (Wang et al., 2018).

18. Chemically synthesize the dual NLS Cas9 construct.
   a. Fuse the 5′ end of the *Streptococcus pyogenes* Cas9 gene with the host specific NLS sequence.

   **Note:** Nucleotide sequence (171 bases) encoding NLS of the *P. funiculosum* NCIM1228 from histone H2B protein was fused to 5′ end of the codon optimized gene for SpCas9. The sequence of the resulting PfNLS is as follows-

   \[
   \text{ATG}\text{CCTCCAAAGCTGCCGAGAAGAAGCCCAGCACTGGTGGCAAGGCCCCAGCTGGAAA}
   
   \text{GGCTCCTGCTGAGAAGAAGGAGGCTGGCAAGAAGACTGCCACCGCTGCCTCTGGCGAGA}
   
   \text{AGAAGAAGCGCGCAAGACTGCAAGGAGACCTACTCTTCCTACATCTACAAG.}
   \]

   **Note:** The SpCas9 sequence was considered from pHis-parallel1-NLS H2BCas9 vector (Wang et al., 2018).

   b. Add the nucleotide sequence encoding Simian Virus (SV40) NLS at the 3′-end of SpCas9.

   **Note:** NLS at both ends to obtain higher translocation efficiency inside the nucleus.

   c. Chemically synthesize the gene encoding customized Cas9 with dual NLS.

   d. Clone the dual NLS Cas9 construct in the expression vector for purification.

   **Note:** The construct was cloned in pET28a vector at Ndel and Xhol restriction sites such that the expressed protein would have 6×His tag at N-terminus to facilitate purification. The resultant vector pET-PfCas9 and the expressed Cas9 protein are shown in Figures 4A and 4B, respectively.

**RNP complex formation**

- **Timing: 20 min**

19. Take 1.6 μg of Cas9 and 1 μg of each sgRNAs in a microcentrifuge tube, add 1 x nuclease reaction buffer and DEPC treated water to make reaction volume to 5 μL.

| Reagent         | Amount      |
|-----------------|-------------|
| Cas9            | 1.6 μg      |
| sgRNA           | 1 μg        |
| 10X Nuclease Buffer | 0.5 μL   |
| DEPC water      | Volume upto 5 μL |
20. Incubate at 30°C for 20 min.
21. Store the tubes in ice, transform together with donor DNA when protoplast is ready. Before proceeding for transformation, perform in vitro cleavage assay to check the efficiency (Figure 5).
22. For in vitro cleavage assay,
   a. Dilute the purified Cas9 and take volume equal to 200 ng in three different microcentrifuge tubes, take approximately 100 ng of each sgRNA - add sgRNA-I in one tube, sgRNA-II in second tube, sgRNA-I & sgRNA-II together in the third tube and incubate at 30°C for 20 min for RNP complex formation.
   b. Amplify cbh1 sequence (i.e., the gene that has to be deleted) along with the sgRNA cleavage site, purify using the PCR purification kit and add 150 ng in each tube.
   c. Add 10X Nuclease Reaction Buffer and 10X BSA to the final concentration of 1X, make volume up to 15 μL with DEPC treated water.
   d. Incubate the tubes at 37°C for 1 h.
   e. Incubate at 80°C for 5 min to deactivate Cas9.
   f. Analyze the band shift of PCR product on 1.2% agarose gel (Figure 6).

**Note:** It’s important to dilute Cas9 to 200 ng as higher concentration of protein makes it difficult to analyze the band shift on agarose gel.
Protoplasts preparation protocol

© Timing: 3 days

23. Harvest the spores from the sporulated plate of the fungus with 10 mL of sterile MQ (Figure 6).
24. Inoculate four flasks containing 25 mL of PDB with $10^7$ spores in each flask.
25. Incubate the flask for 36 h at 28°C at 120 rpm.
26. After 36 h, harvest mycelia by filtering the PDB using Mira cloth.
27. Inoculate one full spatula of mycelia to the 25 mL of lysing solution and incubate for 3 h at 28°C at 120 rpm.
28. After 1 h, view under microscope using Neubar counting chamber for protoplasts formation.
29. After 3 h, filter the protoplasts using Mira cloth and collect the 25 mL filtrate in 50 mL Falcon.
30. Overlay the protoplasts solution with 25 mL of protoplasts separation buffer. Centrifuge the tube at 1500 rcf for 15 min.
31. Collect the protoplasts from the interface layer and dilute it with storage buffer. Keep on ice until use.
32. Pellet the protoplasts at 3000 rcf for 10 min and resuspend it in storage buffer and store at 4°C.
33. For transformation, resuspend protoplasts pellet in 500 μL of STC/PEG (4:1) and keep on ice.
34. Take 10 μL of the suspension and count the protoplasts using Neubar counting chamber at 100× magnification (Figure 6). Dilute in STC/PEG if required.

△CRITICAL: Protoplasts are very fragile, thus fast up and down pipetting or vigorous mixing will damage the protoplasts. Always mix by tapping the bottom of the tube or gentle up and down pipetting. Cut the end of the pipette tip to reduce the stress on the protoplasts while pipetting.

△CRITICAL: Overlaying of separation buffer should be done slowly and in around the rim of 50 mL centrifuge tube using Pasteur pipette.

Note: Always use swing bucket rotor for pellet formation.

PEG- mediated transformation

© Timing: 6 h
Liu et al. (Liu and Friesen, 2012) procedure was followed for PEG-mediated transformation with minor modifications.

35. Place 15 mL centrifuge tubes on ice and pipette 100 μL (10^7) of protoplasts suspension to each tubes (Figure 6).
36. Pipette 20 μL (11–20 μg) of linearized DNA and RNP complex into protoplasts suspension and mix gently by tapping the solution. Use equal volume of the STC/PEG (4:1) solution for control tubes. Incubate the tubes on ice for 20 min.
37. Place tube at room temperature and add 100 μL of PEG solution to each tube, mix well by tapping.
38. Add 300 μL of PEG solution to each tube and mix well by tapping.
39. Further add 600 μL of PEG solution to each tube and mix well by tapping.
40. Incubate at room temperature for 20 min.
41. Add 1 mL of STC to each tube, mix well by tapping.
42. Then add 3 mL of STC to each tube, mix well by tapping.
43. Then add 4 mL of STC to each tube, mix well by gently inverting the tubes at several times.
44. Centrifuge the tubes at room temperature for 10 min at 3000*g to pellet the treated protoplasts.
45. Carefully pour off the supernatant and resuspend the pellet in 0.8 mL regeneration media by gentle pipetting and add another 0.8 mL of the regeneration media.
46. Incubate the tubes at 28°C–30°C with shaking at 70 rpm for 3 h.
47. Mix protoplasts suspension in 50 mL microcentrifuge tube with 20 mL of regeneration media agar, add antibiotic and pour onto plates and incubate at 28°C–30°C for 3–5 days. Use 100 μg/mL concentration of hygromycin.

Figure 6. Overview of protoplasts preparation and PEG-mediated transformation

Liu et al. (Liu and Friesen, 2012) procedure was followed for PEG-mediated transformation with minor modifications.
48. Cut the emerged colonies and spot on sporulation media plate containing hygromycin.
49. Isolate genomic DNA of the transformants grown in PDB culture using Zymo Research Genomic Isolation Kit and screen them by PCR. For genomic isolation follow the link https://files.zymoresearch.com/protocols/_d6005_quick-dna_fungal-bacterial_miniprep_kit.pdf.

**Note:** We have experienced that variation in batches of PEG, sorbitol and sucrose can significantly affect transformation efficiency.

**Note:** Tap the tubes several times for proper mixing of PEG solution during the incubation.

**Note:** Add PEG and STC solution step-by-step into the tubes, not all at once and invert the tubes several times for proper mixing of the solution.

**Note:** The treated protoplasts need at least 1 h in the regeneration media for sufficient recovery of the cell wall.

**Note:** Always make control plate to check the protoplasts viability after the treatment. On control plates without antibiotics, protoplasts will germinate and grow quickly, covering the whole plate within 2 days.

**Note:** Check the sensitivity of hygromycin concentration of the specific fungal strain.

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### EXPECTED OUTCOMES

The expected outcome is the deletion of the target gene via in-locus homologous recombination of linear donor DNA at the double strand break created by the Cas9. The transient introduction of RNP complex into the fungal protoplasts makes it a rapid procedure and also fastens the appearance of the transformants. Some regions of the gene are resistant for targeting; they can also be deleted by making sgRNA against them. The targeted genome editing efficiency is also high (80%–100%) as compared to the conventional method.

Our protocol yielded an average of $10^7$/mL healthy protoplasts by sorbitol density gradient centrifugation. After transformation, almost 100 colonies grew on regeneration media plate having hygromycin, out of which 10 colonies were grown in PDB for genomic DNA isolation and screening. The genomic DNA was isolated using the Quick-DNA/Fungal/Bacterial Miniprep kit. The 50–100 ng of genomic DNA of each transformants were screened for gene deletion via PCR using gene specific primers \( CBH1\_1124bp\_up\_F \) and \( CBH1\_718bp\_dn\_R \) as well as hygromycin marker specific primers hph\_Int\_F and hph\_Int\_R. All of them were found to be positive for \( cbh1 \) deletion (Figure 7). The protein profile of the \( cbh1 \) deleted strains was checked on the SDS-PAGE gel. The 60 kDa band corresponding to the \( CBH1 \) protein is completely absent in the \( \Delta cbh1 \) strains. These knockouts were further confirmed by Western blot with the help of anti-\( CBH1 \) antibody confirming the absence of \( CBH1 \) protein in the enzyme supernatant (Figure 8). “TYPTNATGTPGARGTC” amino acid sequence of PINCIM1228 \( CBH1 \) corresponding to position 391–407 was used to raise the antibodies in New Zealand white rabbits and used for Western blotting (Ogunmolu et al., 2017).

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### LIMITATIONS

While using in vitro CRISPR/Cas9 system for fungal genome editing is efficient, its also tedious. The sgRNAs, RNP and protoplasts prepared need to be transformed the same day, since their storage reduces the efficiency of the transformation.

One of the major limitations is reproducibly producing protoplasts, making of high quality protoplasts and maintaining viability at every step of transformation. Maintenance of a constant external environment, like isotonic buffer, temperature and rpm of the shaker is indispensable for the protoplasts.
Off target effects are associated with the implementation of CRISPR/Cas9 for gene editing, which requires optimization of sgRNAs designs. Another limitation is the PAM sequence for recognition of Cas9; the sequence must be available near the target site. Sometimes, CRISPR–induced DSBs can be lethal to cells rather than gene editing.

**TROUBLESHOOTING**

**Problem 1**
The sgRNA Cas9 software search returned to no results or only generates discard Cas9 cleavage target sites.

**Potential solution**
Make sure that the input sequences format is according to the software guidelines. It does not support sequence with blank space, or with contig IDs. Genome sequence should be continuous and in FASTA format (step 1).

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**Figure 7. Test for integration of Donor DNA at the target site in the hygromycin-resistant transformants**
(A) Schematic representation of the donor DNA integration at the cbh1 locus and two different primer sets location.
(B) Amplification of 1,324 bp fragment amplified by primers CBH1_1124bp_up_F (P1) and Hph_int_R (P2). The amplicon refers to the region 1,124 bp upstream to cbh1 ORF and 200 bp of hph resistance cassette.
(C) Amplification of 1,468 bp fragment amplified by primers CBH1_718bp_dn_R (S2) and Hph_int_F (S1). The amplicon refers to the region 718 bp downstream to cbh1 ORF and 750 bp of hph resistance cassette.

Off target effects are associated with the implementation of CRISPR/Cas9 for gene editing, which requires optimization of sgRNAs designs. Another limitation is the PAM sequence for recognition of Cas9, the sequence must be available near the target site. Sometimes, CRISPR–induced DSBs can be lethal to cells rather than gene editing.

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**Figure 8. Confirmation of deletion of cbh1 gene**
SDS-PAGE gel of secretome of NCIM1228 and Δcbh1 mutant showing band corresponding to CBH1 protein missing in Δcbh1 secretome; second panel showing Western blot performed with anti-CBH1 antibody confirming the absence of CBH1.
Problem 2
No healthy protoplasts formed after 3 h of incubation.

Potential solution
Many times we observed that protoplasts burst during the incubation period, the main reason for this is sodium phosphate buffer, always prepare fresh buffer for the lysing enzyme solution. Incubation of 25 mL solution always be done in 250 mL flask not in 100 mL flask to increase the surface area, so that the protoplasts don’t collide with each other and burst. Inoculum size is also important, standardize the amount of mycelia to be inoculated in the protoplasts forming buffer. Overcrowding of inoculum does not allow lysing enzyme to act on it (step 28).

Problem 3
No transformants on plate.

Potential solution
Usually, we found $10^7$ protoplasts give approximately 100 colonies on selection plates. However, if no transformants are identified, make sure that the sgRNA prepared is not degraded by performing in vitro cleavage assay. Ensure the Donor DNA is of good quality before transforming into the protoplasts (step 23).

Problem 4
In vitro activity present but no in vivo activity observed for RNP complex.

Potential solution
It may happen that in vitro cleavage assay shows functional RNP complex formation, but its introduction inside the cell may not cause target gene editing or random integrations can occur.

Delivery of the RNP complex is always a challenging part of this protocol, immediate introduction of the complex inside the protoplasts can save it from degradation. Also, off target effects can led to random integrations rather than targeted gene editing. For this, sgRNA sequence needs to be optimized. Its potential off target sites need to be checked in the report file, and always best suited sgRNA should be selected (step 23).

Problem 5
Non-reproducible results.

Potential solution
The main reason for non-reproducibility is the RNP complex entry inside the nucleus. RNP complex must enter inside the nucleus for the Cas9 to recognize the target sequence. It is found that SV40 NLS is not suitable for efficient translocation of the Cas9 into the nucleus. The fungal NLS sequence should be added to the Cas9 sequence, and cloned, expressed and purified for the CRISPR/Cas9 gene editing (step 19).

RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Syed Shams Yazdani (shams@icgeb.res.in).

Materials availability
Customized PICas9 with dual NLS is available upon request from the lead contact.

Data and code availability
This study did not generate or analyze datasets or code.
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AUTHOR CONTRIBUTIONS
T.S. and S.S.Y. coordinated the study and designed the experiments. T.S. conducted the experiments and S.S.Y. generated resources for the experiments. T.S. and S.S.Y. analyzed the results and wrote the paper.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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