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Research Article

Editing *Aspergillus terreus* using the CRISPR-Cas9 system

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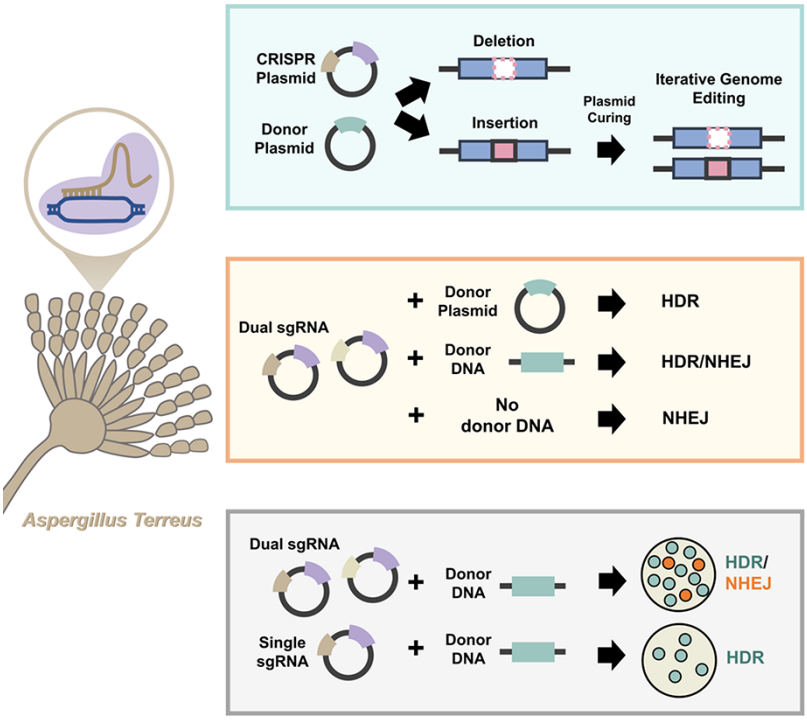
Abstract

CRISPR-Cas9 technology has been utilized in different organisms for targeted mutagenesis, offering a fast, precise and cheap approach to speed up molecular breeding and study of gene function. Until now, many researchers have established the demonstration of applying the CRISPR/Cas9 system to various fungal model species. However, there are very few guidelines available for CRISPR/Cas9 genome editing in *Aspergillus terreus*. In this study, we present CRISPR/Cas9 genome editing in *A. terreus*. To optimize the guide ribonucleic acid (gRNA) expression, we constructed a modified single-guide ribonucleic acid (sgRNA)/Cas9 expression plasmid. By co-transforming an sgRNA/Cas9 expression plasmid along with maker-free donor deoxyribonucleic acid (DNA), we precisely disrupted the *lovB* and *lovR* genes, respectively, and created targeted gene insertion (*lovF* gene) and iterative gene editing in *A. terreus* (*lovF* and *lovR* genes). Furthermore, co-delivering two sgRNA/Cas9 expression plasmids resulted in precise gene deletion (with donor DNA) in the *ku70* and *pyrG* genes, respectively, and efficient removal of the DNA between the two gRNA targeting sites (no donor DNA) in the *pyrG* gene. Our results showed that the CRISPR/Cas9 system is a powerful tool for precise genome editing in *A. terreus*, and our approach provides a great potential for manipulating targeted genes and contributions to gene functional study of *A. terreus*.

Key words: *Aspergillus terreus*; CRISPR-Cas9; genome-editing technology; marker-free donor DNA

Graphical Abstract



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1. Introduction

Natural products produced by fungi are extensively applied to medicine, food and agricultural areas, but the full potential is far from being unleashed. One serious obstacle toward this goal is the lack of tools allowing genetic analyses and manipulation of most fungi. The filamentous fungus *Aspergillus terreus* is a well-known producer of various secondary metabolites for pharmaceutical importance (1, 2). For example, it produces the polyketide-derived natural product lovastatin, which is the first cholesterol-lowering medicine approved by the US Food and Drug Administration for human use in hypercholesterolemia treatments (3). Moreover, abundant biologically relevant compounds, such as itaconic acid and terretonin, and enzymes, such as cellulases and xylanases, are also produced by *A. terreus*, and all of them are being produced by industries (4–6).

To optimize the production of valuable secondary metabolites produced by *A. terreus*, random mutagenesis using ultraviolet (UV) light or chemical mutagens has been conducted for decades (7). For instance, a high yield of lovastatin strain of *A. terreus* was obtained from UV-based mutagenesis (8), and a high β-glucosidase activity mutant strain of *A. terreus* was derived by ethyl methyl sulfonate treatment (9). However, random mutagenesis could create undesirable genetic changes in the host, and the process is laborious and time-consuming. Moreover, recombinant deoxyribonucleic acid (DNA) technologies including split-marker and fusion-polymerase chain reaction (PCR) approaches have been employed to manipulate the interest of genes in *A. terreus* (10). The drawbacks for this approach are a shortage of selectable markers and relatively low rates of site-specific recombination due to the fact that Non-homologous end joining (NHEJ) repair pathway is predominant in most fungi (11–13). Therefore, the development of highly efficient and precise approaches to perform genetic manipulation is desirable.

CRISPR-Cas9 is a natural defense mechanism discovered in bacteria and archaea (14), which has subsequently been developed as a powerful genome-editing tool. The method is widely used in many organisms, including animals, filamentous fungi and plants. Due to its simplicity and specificity, CRISPR-Cas9 technology has been applied to different aspects of fungal research. For instance, CRISPR/Cas9 system has been utilized to study fungal pathogenicity (15), to facilitate genetic engineering for secondary metabolite production (12) such as increased gibberellic acid production in *Fusarium fujikuroi* (11) and boost pneumocandin B0 productivity in *Glarea lozoyensis* (16).

Since DiCarlo *et al.* (2013) demonstrated that CRISPR/Cas9-mediated genome editing could be applied to *Saccharomyces cerevisiae* (17), the approach was quickly introduced into a number of filamentous fungi including *Trichoderma reesei* (18), *Neurospora crassa* (19), a number of *Aspergillus* species (20) and *Penicillium chrysogenum* (21). Up to date, the CRISPR-Cas9 genome-editing methods have been established in >40 fungal species (12, 22, 23), and most of them were conducted using a single-guide ribonucleic acid (sgRNA) and Cas9 to introduce a DNA double-strand break into the target sequence and donor DNA with homology arms to introduce the desired genetic change (12). In contrast to other *Aspergillus* species, relatively few genetic manipulation methods have been established for *A. terreus* research. Various strategies have been considered for CRISPR genome editing in filamentous fungi, including (i) introducing the donor DNA containing a selectable marker, (ii) performing genome editing in a non-homologous end-joining–deficient strain, (iii) applying repair templates to different formats including circular, linear, single-stranded DNA and double-stranded DNA (dsDNA) (24) and (iv) using two guide ribonucleic acids (gRNAs) flanking the target site to achieve targeted genome editing at a high possibility (25). In the present study, we described CRISPR/Cas9 genome editing in *A. terreus* based on the method developed by Nødvig *et al.* (20). The genome-editing system used in this study is depicted in Figure 1, and the plasmids used to produce mutants are listed in Table 1. Using our procedure, we successfully performed targeted gene insertion and gene deletion in wild-type *A. terreus*, and auxotrophic and non-homologous end-joining–deficient strains were generated. We expect that our methods will facilitate future research in *A. terreus*.

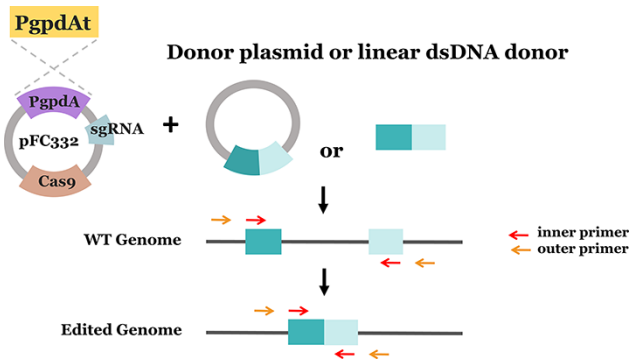

Figure 1. The genome-editing system in *A. terreus*. Gene editing in *A. terreus* was performed using a modified pFC332 plasmid, and *A. nidulans gpdA* promoter of pFC332 plasmid was replaced by *A. terreus gpdA* promoter to express sgRNA. Circular and linear donor DNAs were applied to enhance the precise genome editing. The inner primers were used to detect the donor DNA, and the combined inner primers with outer primers were used to validate the gene editing.

2. Materials and methods

2.1 Strains and growth conditions

Aspergillus terreus American Type Culture Collection (ATCC) 20542 used in this study was retrieved from the ATCC (Manassas, VA, USA). The strain was routinely cultured on potato dextrose agar (PDA) at 28°C. *Escherichia coli* DH5a was grown in Luria-Bertani medium (5 g yeast extract, 10 g tryptone and 10 g NaCl per liter) at 37°C. Transformed cells were screened in their corresponding medium supplemented with antibiotics: 100 μg/ml hygromycin B for *A. terreus* ATCC 20542 and 100 μg/ml ampicillin for *E. coli* DH5a. The *pyrG* deletion mutants were tested on PDA medium containing 10 mM uracil, 100 μg/ml hygromycin B and 1 mg/ml 5-fluoroorotic acid (FOA).

2.2 Plasmid construction

Plasmids pFC332 (Plasmid #87845) and pFC334 (Plasmid #87846) were obtained from Addgene (https://www.addgene.org/). To reduce off-target mutations, the gRNA oligos were designed by using GTScan (https://gt-scan.csiro.au/) and blasted with the whole genome sequence data of *A. terreus* ATCC 20542. The sgRNA/Cas9 expression plasmids were generated as described by Nødvig *et al.* (20), with slight modifications. In detail, *Aspergillus nidulans gpdA* promoter was replaced with the endogenous *A. terreus gpdA* promoter (26) to create a gRNA expression cassette (Figure 1). The donor DNA fragments containing 500–1000 bp of homology arms on each side flanking the cut site were

Table 1. Plasmids and donor DNA used to create mutations in this study

Targeted gene	First sgRNA	Second sgRNA	Donor DNA form	Edited size (bp)	
lovB	p*lovB*-sg1/Cas9 plasmid	–	Circular	−219	–
lovR	–	p*lovR*-sg2/Cas9 plasmid	Circular	−231	–
lovF	p*lovF*-sg1/Cas9 plasmid	–	Circular	+600	–
ku70	p*ku70*-sg1/Cas9 plasmid	p*ku70*-sg2/Cas9 plasmid	Circular	−943	–
pyrG	p*pyrG*-sg1/Cas9 plasmid	p*pyrG*-sg2/Cas9 plasmid	Circular	−834	–
pyrG	p*pyrG*-sg1/Cas9 plasmid	p*pyrG*-sg2/Cas9 plasmid	Linear	−834	−575
pyrG	p*pyrG*-sg1/Cas9 plasmid	p*pyrG*-sg2/Cas9 plasmid	–	−575	–

cloned into the pJET1.2 vector (Thermo Fisher Scientific, USA). The primers used in this study are given in Table S1.

2.3 Preparation of protoplasts

Protoplasts of *A. terreus* were produced from 7-day-old cultures on PDA. The conidia were collected from three plate cultures with 5 ml of sterile distilled water containing 0.2% Tween 20 and then filtered through Miracloth. The resulting conidial suspensions were incubated in 100 ml of Yeast Extract–Peptone–Dextrose medium (10 g yeast extract, 20 g peptone and 20 g dextrose per liter) at 28°C and 200 rpm for 16 h. The culture was harvested on gauze and digested in 25 ml of lysis buffer (1.2 M $MgSO_4$, 10 mM sodium phosphate and pH 5.8) containing 35 mg/ml lysing enzymes (Sigma, L1412) at 28°C with shaking at 100 rpm for 3 h. After filtration through Miracloth, the protoplasts were spun down at 1100 g for 5 min and adjusted to a final concentration of 1×10^7 cells/ml in Sorbitol, Tris-HCl, CaCl2 buffer (1.2 M Sorbitol, 10 mM Tris-HCl and pH 7.5, 10 mM CaCl2).

2.4 Polyethylene glycol (PEG)-mediated transformation

For transformation, 1 μg of sgRNA/Cas9 expression plasmid DNA and 1 μg of donor plasmid DNA were added to 50 μl of protoplasts and incubated on ice for 15 min. Subsequently, 1 ml of polyethylene glycol solution (60% PEG 4000, 50 mM $CaCl_2$, 50 mM Tris-HCl and pH 7.5) was added to the mixture and incubated at room temperature for 15 min. The PEG mixture was suspended in a molten osmotically stabilized medium (33% sucrose, Potato Dextrose Broth and 0.75% agar) containing 100 μg/ml hygromycin B and then poured into a Petri dish. After 72 h, the transformants were verified by PCR. For *pyrG* deletion, 10 mM uracil was added to the osmotically stabilized medium.

2.5 Validate transformants

To evaluate the transformation of donor DNA, the colony PCR was performed. Inner primers located within the homology region were used to detect the donor DNA. Outer primers located outside the homology region of donor DNA were applied to validate the gene editing (Figure 1). The mycelium was transferred into 20 μl of Tris-HCl buffer (pH 8.5) in a PCR tube and incubated at 98°C for 30 min. As PCR template, 2 μl of the supernatant was used. The editing efficiency was estimated using PCR on genomic DNA samples. The fungal genomic DNA was extracted as described previously (27). The resulting DNA pellets were dissolved with 500 μl of 10 mM Tris-HCl, pH 8.5, 1 M NaCl and 2 mM ethylenediaminetetraacetic acid, and the extraction procedure was repeated. The PCR was conducted under the following conditions: 95°C for 3 min, 30 cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 1 min per 1 kb, with a final extension at 72°C for 5 min. PCR products were visualized by agarose gel electrophoresis and subjected to Sanger sequencing.

3. Results

3.1 Strategies for CRISPR-mediated genome editing in *A. terreus*

Only limited CRISPR-Cas9 genome-editing tools are available for *A. terreus* (28). We therefore decided to investigate whether autonomous maintenance in Aspergillus-based plasmids encoding both Cas9 and sgRNAs developed by Nødvig *et al.* (20) for gene editing in a number of different *Aspergilli* could also be applied to *A. terreus*. With this aim, we firstly attempted to eliminate exon 5 of the *lovB* gene by CRISPR/Cas9-mediated DNA deletion. Two sgRNAs targeting the exon 5 of the *lovB* gene were designed and cloned into pFC332 plasmids. Transformants generated by co-transformation of the two plasmids into *A. terreus* protoplasts were selected on PDA medium supplemented with hygromycin B. If the chromosomal DNA Double-Strand Break (DSB) end upstream of exon 5 is joined to the chromosomal end downstream of exon by NHEJ, then exon 5 should be excised. To investigate this possibility, the genomic DNA of five transformants were extracted and analyzed by PCR. However, despite the repeated attempts, we were unable to obtain this deletion. We therefore examined the expression levels of *cas9* in the transformants and in wild-type strains using reverse transcriptase PCR. As expected, expression of *cas9* was only detected in the transformants containing the CRISPR plasmid (Supplementary Figure S1). Importantly, the results demonstrated that *cas9* could be expressed in *A. terreus* using our CRISPR/Cas9 vectors. To address the failure of genome editing in *A. terreus*, we pursued three strategies: (i) we optimized sgRNA expression in *A. terreus* by replacing the *A. nidulans gpdA* promoter with the endogenous *A. terreus gpdA* promoter to drive expression of the sgRNA genes on the CRISPR plasmids; (ii) to enhance homologous direct repair (HDR)–mediated genome editing, we included a donor repair template in the co-transformation reactions to facilitate HR-mediated repair of the Cas9-induced DNA DSBs; and (iii) to attain targeted genome editing at a high possibility, we also considered using two sgRNAs against the targeted gene.

3.2 Gene deletion in *A. terreus*

By implementing the first two improvements of the CRISPR/Cas9 method to create a deletion in *A. terreus*, the sgRNA/Cas9 plasmid, carrying an sgRNA targeting exon 5 of the *lovB* gene, was co-transformed with a donor plasmid into *A. terreus*. The circular donor plasmid with 550–650 bp homology arms was designed to introduce a 219-bp deletion into the *lovB* gene. After hygromycin B selection, we first examined the transformants for the presence of the donor plasmid by colony PCR. Specifically, the amplification of a 281-bp fragment demonstrated that the presence of the donor plasmid was confirmed in 11 of the 16

transformants. Genomic DNA was purified from the 11 colonies and then analyzed for the presence of the deletion by PCR. Among the 11 clones, 4 clones were homokaryotic containing the desired deletion mutants, and 4 clones were heterokaryotic mutants containing wild-type and deletion alleles. Subsequently, Sanger sequencing demonstrated that the four homokaryotic mutants contained the expected targeted 219-bp deletion in the *lovB* gene (Figure 2).

We then attempted to generate a 231-bp deletion in the *lovR* gene. Two sgRNAs designed to target different sites of the *lovR* gene exon 1 were inserted into sgRNA/Cas9 expression vectors, respectively, to produce p*lovR*-sg1/Cas9 and p*lovR*-sg2/Cas9 plasmids. The *lovR* donor plasmid contained a fusion of sequences upstream (593 bp) and downstream (764 bp) of exon1 designed to introduce the desirable 231-bp deletion. Next, *A. terreus* protoplasts were co-transformed with the *lovR* donor plasmid and either the p*lovR*-sg1/Cas9 plasmid or the p*lovR*-sg2/Cas9 plasmid. Hygromycin B–resistant transformants were selected, and diagnostic colony PCR analyses were applied to identify those that also contained the donor plasmid. Sixteen colonies were identified in this manner, and three clones contained the donor plasmid. For each of these transformants, genomic DNA was examined for the presence of the 231-bp deletion by PCR. These analyses revealed a single colony that appeared to be a heterokaryon containing wild-type and mutant *lovR* gene copies. To obtain a pure strain, we therefore streak purified the colony and repeated the PCR test. After a first round of re-streaking, the colony showing the most intense mutant PCR fragment was selected for another round of re-streaking. Finally, a clean *lovR* gene mutant strain was obtained after several rounds of re-streaking (Figure 3), and the deletion was confirmed by Sanger sequencing.

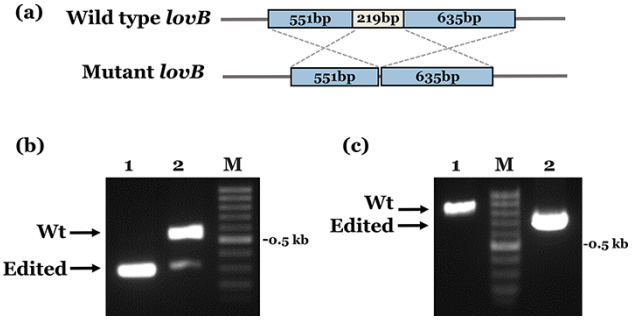

Figure 2. Generation of *lovB* deletion mutant. (a) Diagram showing *lovB* gene deletion. (b) The 281-bp band represents the colony PCR product amplified from the donor plasmid of *lovB* gene. (c) Genome PCR analysis revealed that *lovB* gene was deleted, resulting in an expected band of 695 bp. Lane 1: wild-type (Wt) strain, lane 2: edited strain and lane M: 100-bp DNA marker.

3.3 DNA insertion in *A. terreus*

In order to access whether this approach can be used to insert a DNA fragment into a target gene, we constructed a donor plasmid containing an insert DNA cassette containing the *lovF* gene. The DNA cassette was generated by fusing three fragments together in a single PCR reaction: a fragment containing 960 bp of sequence upstream of *lovF*, a 600-bp fragment containing the 5′-untranslated regions of the glucoamylase gene and a fragment containing 900 bp of the *lovF* gene. The resulting PCR product was inserted into the pJET1.2 vector to produce *lovF* donor plasmid. *Aspergillus terreus* protoplasts were co-transformed with the donor plasmid and either the p*lovF*-sg1/Cas9 plasmid or the p*lovF*-sg2/Cas9 plasmid. Eighteen transformants obtained from the selective medium were analyzed by colony PCR using primers targeting the donor plasmid, and 16 transformants showing positive bands for the donor plasmid were identified. We then examined the efficiency of DNA insertion into the *lovF* gene via genomic DNA PCR. These analyses identified 12 transformants containing edited and unedited DNA, indicating that they were heterokaryons. These transformants were therefore purified by re-streaking on the selective medium. After three rounds of streak purification, pure *lovF* DNA insertion mutants were obtained (Figure 4). The mutants were also verified by Sanger sequencing.

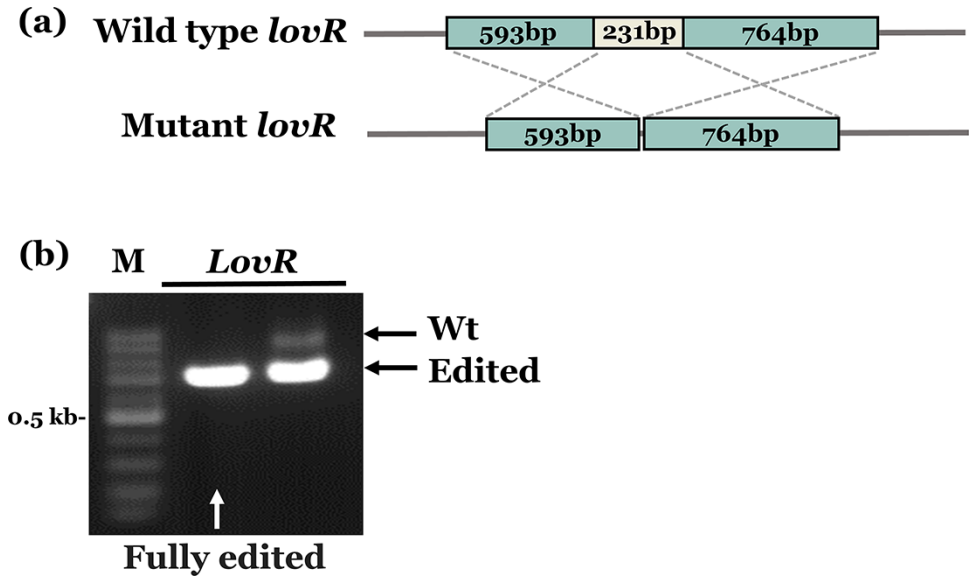

Figure 3. Isolation of fully edited strains by streaking method. Before streaking, bands of both edited and unedited strains can be seen in transformants. After applying for streaking, only the edited band was observed in the fully edited strain. (a) Diagram representing *lovR* gene deletion. (b) Generation of homokaryotic mutants of the *lovR* gene. Wt: wild-type and M: 100-bp DNA marker.

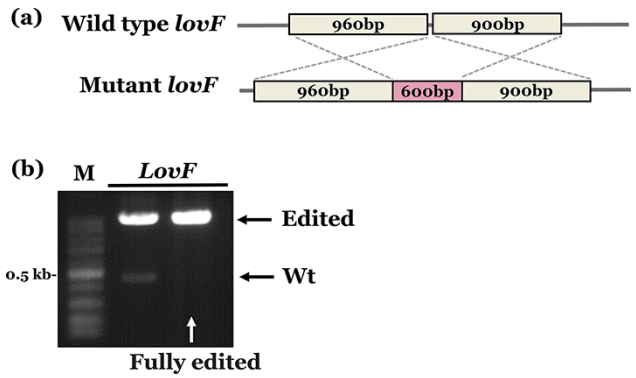

Figure 4. Isolation of fully edited strains by streaking method. (a) Diagram showing *lovF* gene insertion. (b) Generation of DNA-inserted homokaryotic strain. Wt: wild-type and M: 100-bp DNA marker.

3.4 Iterative genome editing in *A. terreus*

To perform iterative genome editing, we set out to generate a *lovR* and *lovF* double mutant strain using the strain containing the *lovF* insertion mutation as a starting point. First, p*lovF*-sg1/Cas9 plasmid and the donor DNA used for obtaining the *lovF* mutant were lost from the *lovF* strain by repeated culturing of the strain in antibiotic-free PDA medium. To ensure complete plasmid loss, the potential plasmid-free strains were tested for sensitivity to hygromycin B and finally checked by colony PCR. Next, plasmid-free strains identified in this manner were then protoplasted and used in a second round of genome editing. Specifically, to create a double mutant strain, the *lovF* mutant protoplasts were co-transformed with the p*lovR*-sg2/Cas9 plasmid and the donor plasmid containing the *lovR* mutation. Transformants were easily obtained, and homokaryotic mutants were obtained after three rounds of re-streaking on the selective medium. Genomic DNA extracted from the mutants were checked by PCR using the validation primers detecting *lovF* and *lovR* (Figure 5) and confirmed by Sanger sequencing. This analysis demonstrated that four double mutants were successfully produced by this approach.

3.5 Construction of a *ku70* and *pyrG* deletion strain for improved gene editing

Encouraged by the successes of our initial CRISPR efforts, we next aimed to construct a strain, which is more amiable for future genetic engineering of *A. terreus*. Specifically, we decided to delete *ku70*, which generally increases template-directed gene-editing efficiencies by eliminating repair by NHEJ, and *pyrG*, which can then be used as a counter-selectable marker. Although CRISPR/Cas9 genome editing can be achieved by using an sgRNA, validating sgRNAs located in different regions can be a time- and labor-consuming process as different sgRNAs may have various efficiencies. Many studies revealed that high genome-editing frequencies were accomplished by applying two sgRNAs (29–31). Therefore, we performed double sgRNAs to delete *ku70* and *pyrG*, respectively. Accordingly, we made two pairs of CRISPR plasmids, which encode sgRNAs targeting *ku70* and *pyrG*, respectively. To direct gene deletion, two donor plasmids were constructed. For *ku70* deletion, a donor plasmid containing a fusion of two ~800-bp regions up- and downstream of the *ku70* gene was constructed with the aim of introducing a 943-bp deletion in the *ku70* gene (Figure 6). Similarly, a donor plasmid containing 700–850 bp of up- and downstream sequences of *pyrG* gene was constructed to facilitate an 834-bp deletion of *pyrG* deletion (Figure 7).

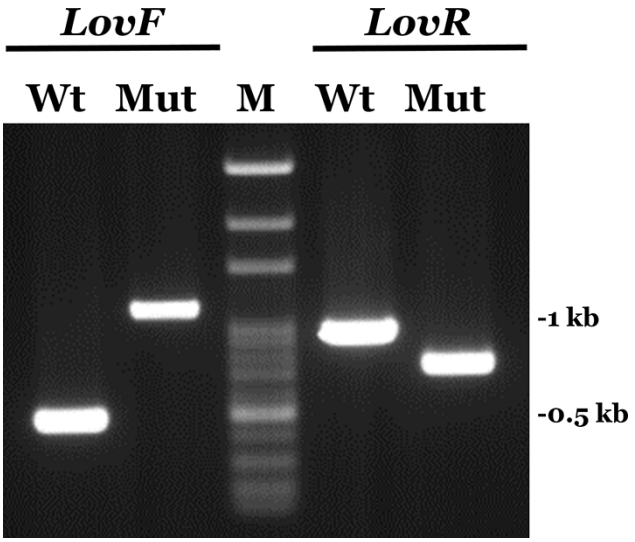

Figure 5. Iterative genome editing in *A. terreus*. After the plasmid removal process, the plasmid-free *lovF* mutant was used to apply the deletion of the *lovR* gene. The target mutations were detected by genome PCR using the corresponding primers. From left to right: the 414-bp band represented the unedited genome, and the 1014-bp band represented the inserted *lovF*. For the second genome editing, the 937-bp band represented the unedited genome, and the 724-bp band represented *lovR* deletion. Wt: wild-type, Mut: edited mutant and M: 100-bp DNA marker.

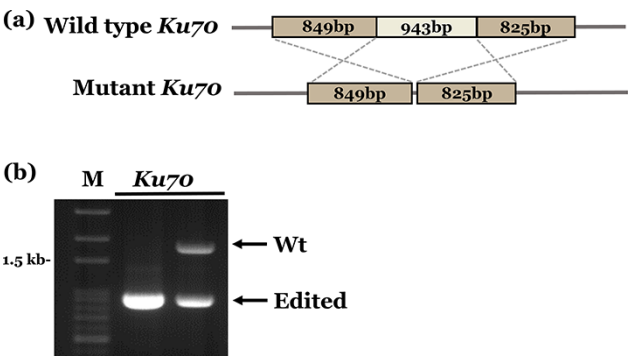

Figure 6. Generation of gene deletion with two sgRNAs. (a) Diagram representing *ku70* gene deletion. (b) An 833-bp band corresponding to the size of the deleted *ku70* was observed on the agarose gel. Wt: wild-type and M: 100-bp DNA marker.

After co-transforming protoplasts with the p*ku70*-sg1/Cas9 plasmid, the p*ku70*-sg2/Cas9 plasmid and the circular donor DNA, 20 transformants were selected for a further analysis. Hence, by colony PCR analyses, we identified eight transformants that contained the donor plasmid. Genomic DNA from the eight positive transformants were extracted and subjected to diagnostic PCR analysis. These PCR experiments indicated that all eight colonies contained the *ku70* deletion but that only two were homokaryons containing only the *ku70* deletion and no trace of the wild-type allele. Lastly, Sanger sequencing demonstrated that the homokaryotic strains contained the expected *ku70* deletion. To make a *pyrG* deletion mutant strain, protoplasts of a wild-type strain were transformed with the p*pyrG*-sg1/Cas9 plasmid, the p*pyrG*-sg2/Cas9 plasmid and the *pyrG*-donor plasmid. To detect the presence of a donor plasmid, 24 transformants were analyzed by colony PCR. Fifteen of the 24 transformants contained the donor plasmid, and genomic DNA was purified from these 15 transformants and checked by PCR. The result

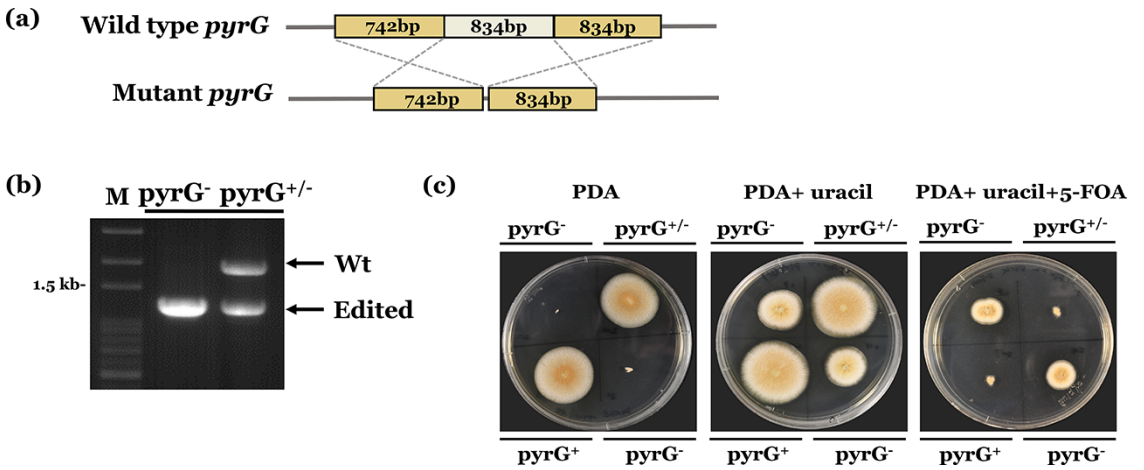

Figure 7. Generation of gene deletion with two sgRNAs. (a) Diagram showing *pyrG* gene deletion. (b) Genome PCR analysis showed that *pyrG* gene was deleted, resulting in *pyrG*$^{-}$ (homokaryotic deletion) and *pyrG*$^{\pm}$ mutants (heterokaryotic deletion). (c) Wild-type and mutant strains were checked on PDA medium, PDA medium with uracil and PDA medium containing uracil and 5-FOA. Wt: wild-type and M: 100-bp DNA marker.

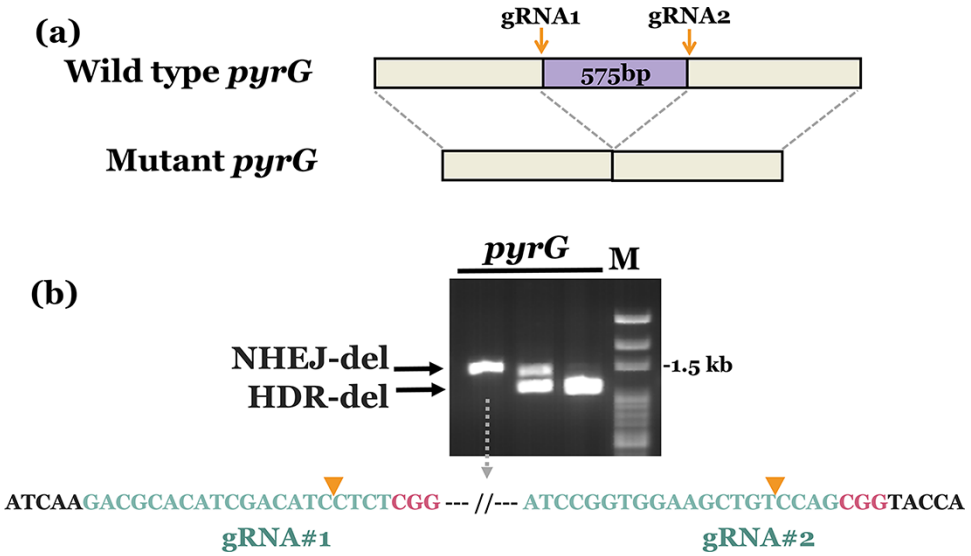

Figure 8. Targeted deletion of *pyrG* by NHEJ. (a) Diagram representing *pyrG* gene deletion via NHEJ. (b) *pyrG* mutations were determined by PCR. Sequence analysis of *pyrG* deletion, the sgRNA sequence was shown in green and the PAM region was highlighted in dark pink. Break sites were indicated by yellow arrowheads. M indicates100-bp DNA marker.

showed that all the 15 colonies contained the *pyrG* deletion. Ten were homokaryons, and Sanger sequencing demonstrated that they all contained the expected *pyrG* deletion. Importantly, when the *pyrG* mutants were tested on PDA medium, PDA medium with uracil and PDA medium containing uracil and 5-FOA, the *pyrG* mutants grew on PDA medium containing uracil and 5-FOA but not on PDA medium as expected (Figure 7). This contrasts with the phenotype of the wild-type strain, which grew on PDA medium, but not on PDA medium supplemented with 5-FOA and uracil.

3.6 Gene editing with linear donor DNA

To investigate the efficiency of using linear DNA in double sgRNA CRISPR experiment, we attempted to introduce the *pyrG* gene deletion into a wild-type *A. terreus* strain by using the p*pyrG*-sg1/Cas9 plasmid, the p*pyrG*-sg2/Cas9 plasmid and a PCR-amplified dsDNA donor generated from the *pyrG* donor plasmid.

Genome PCR analysis of 13 transformants showed that they contained two types of deletions, representing loss of 575 bp and 834 bp, respectively. Specifically, nine colonies contained an 834-bp deletion, three colonies contained a 575-bp deletion and one colony contained both the 834-bp and 575-bp deletions. The sequencing of PCR products covering the borders of the deletions demonstrated that the 834-bp deletion was introduced by HDR using the *pyrG*-based donor fragment as a repair template, and the 575-bp deletion was mediated by NHEJ. Notably, the NHEJ-based deletion was achieved by sealing sgRNAs/Cas9 breaks induced 4 bp from the Protospacer adjacent motif (PAM) sequences (Figure 8).

We next set out to determine whether using two sgRNAs provides a better gene-editing performance than an sgRNA in a wild-type strain. We therefore edited *pyrG* gene by using an sgRNA and the linear *pyrG*-based dsDNA donor, and colony PCR was performed to determine the presence and type of the deletion. By applying p*pyrG*-sg1/Cas9 plasmid with the dsDNA donor, five out

of six transformants contained an 834-bp deletion, whereas when the p*pyrG*-sg2/Cas9 plasmid was used along with the linear donor fragment, four out of seven transformants contained an 834-bp deletion. As confirmed by PCR product sequencing, the deletion was achieved by HDR.

3.7 NHEJ-based deletion using dual sgRNAs

To determine the frequency of NHEJ-mediated gene deletion using two sgRNAs, we performed *pyrG* gene deletions using p*pyrG*-sg1/Cas9 plasmid and p*pyrG*-sg2/Cas9 plasmid in the absence of a repair template. The PCR results showed that 21 out of 24 colonies contained a 575-bp deletion, which was expected if the two Cas9-induced breaks were sealed by NHEJ. Three PCR fragments from three transformants were examined by DNA sequencing. The sequencing results revealed that the transformants all carried precise deletions mediated by NHEJ, cutting 4 bp upstream of the PAM sequences.

4. Discussion

By using endogenous *gpdA* promoter to drive gRNA expression and co-delivering the gRNA/Cas9 expression plasmids with donor plasmids as repair templates, we successfully applied CRISPR/Cas9 genome editing to *A. terreus*. Our results revealed that various gRNAs have different efficiencies. Previous studies indicated that the sequence and secondary structure of sgRNA play a key role in gene-editing efficiency and specificity (32–35). Therefore, it is important to design multiple sgRNAs to target different sites of candidate genes, and sgRNA design tools could help to choose highly active sgRNAs.

Additionally, repeated streaking is a time-consuming procedure to obtain homokaryotic mutants. Several methods have been developed for single spore isolation, such as isolating single spores under microscope, and using a capillary pipette or syringe to pick up single spores (36), which could accelerate the process of obtaining pure CRISPR-edited strains.

By removing the gRNA/Cas9 expression plasmids, we demonstrated that iterative genome editing in *A. terreus* is feasible. The protoplasts isolated from the plasmid-free mutant were applied to perform another gene editing. One of our CRISPR/Cas9 strategies was to apply a donor DNA with homology to the targeted region. Our results showed that donor DNA containing homologous arm sequence was essential to achieving precise genome editing in *A. terreus*. Typically, a repair template containing a selection marker is employed in CRISPR-mediated homology-directed repair to facilitate the screening process for many fungi. Instead, we used a marker-free donor plasmid in this CRISPR/Cas9 system, which provides a great potential for performing iterative gene editing in filamentous fungi, with a limited number of selectable markers such as *A. terreus*.

Moreover, *ku70* and *pyrG* deletion strains have been generated by using two sgRNAs. Although CRISPR/Cas9 genome editing can be achieved by using a single gRNA, validating gRNAs located in different regions can be a time- and labor-consuming process as different gRNAs may have various efficiencies. Many studies revealed that high genome-editing frequencies were accomplished by applying two sgRNAs (29–31). Therefore, we performed double gRNAs to delete *ku70* and *pyrG*, respectively. Employing two sgRNAs with circular donor DNA, pure ku70 and pyrG deletions were generated without streaking process. Furthermore, we tested linear donor DNA in *pyrG* deletion. While performing with a linear dsDNA donor, both HDR- and NHEJ-based deletions were obtained. However, all mutants were HDR-derived mutations by using circular donor DNA as the repair template.

Previous studies indicated that circular homologous donor DNA performed better gene targeting than linearized donor DNA due to the fact that NHEJ plays a dominant role in DSB repair in most filamentous fungi. Unlike circular donor DNA, linearized donor DNA with free ends can attract NHEJ proteins and create random integration into the genome (20). Additionally, circular donor DNA is highly resistant to exonucleases and stable in the cell, and it can bind HR proteins but not NHEJ proteins. Therefore, for precise genome editing, circular donor DNA may be a better option.

In contrast to the co-delivery of two sgRNAs with a linear dsDNA donor, applying a single gRNA with a linear dsDNA donor obtained fewer edited strains. The results suggested that applying two sgRNAs in gene editing could increase the chance to edit the target gene. Additionally, in the no-donor DNA assay, all mutations were NHEJ-based deletions. Surprisingly, most mutants were created by removing the fragment between two gRNA target sites, cutting 4 bp upstream of the PAM sequences.

In conclusion, we presented CRISPR/Cas9 gene editing that can be conducted in wild-type *A. terreus* in detail. In the present study, iterative genome editing was accomplished by using marker-free donor DNA, the efficiency of different types of donor DNAs was examined and high gene-editing frequency was achieved by using two sgRNAs. The optimized CRISPR gene-editing workflow enhances the possibility of molecular breeding and provides a better understanding of biosynthetic mechanisms and gene function in *A. terreus*.

Supplementary data

Supplementary data are available at SYNBIO Online.

Data availability

All the data described in this article are available within the article and in its online supplementary files.

Material availability

All plasmids used in this study are publicly available from Addgene (https://www.addgene.org/) with Plasmid #87845 and Plasmid #87846.

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Ethical statement. The experiments conducted on this study do not contain any studies with human participants or animals performed by any of the authors.

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