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Efficient genome editing in *Aspergillus niger* with an improved recyclable CRISPR-HDR toolbox and its application in introducing multiple copies of heterologous genes

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ABSTRACT

Aspergillus niger is an important industrial producer of enzymes due to its high capacity for producing exocellular secretory proteins. The CRISPR/Cas9 system has been developed as a genetic manipulation tool in *A. niger*. However, only the basic functions of the CRISPR/Cas9 system, such as codon optimization of Cas9 nucleases and promoter screening of guide RNA (gRNA) expression, have been developed in *A. niger*. The CRISPR/Cas9 system for manipulating large genomic fragments and multiple gene knock-ins still needs to be established. Here, we improved the CRISPR/Cas9 homologous direct repair (CRISPR-HDR) tool box based on donor DNAs (dDNAs) and plasmid harboring AMA1 and the *pyrG* marker, allowing recycling of *pyrG* and Cas9 components. Furthermore, we used the CRISPR-HDR tool box to knock out the 0 kb (protospacer only), 2 kb, 10 kb and even 50 kb gene fragments. This CRISPR-HDR tool box could also be used to simultaneously knock in multiple genes at the loci of two highly expressed extracellular secreted proteins, glucoamylase A (*glaA*) and alpha-amylase (*amyA*, two copies). In our study, two or three copies of glucose oxidase (*goxC*) were precisely knocked in at the loci of *amyA* and *glaA*, resulting in 4-fold increased enzyme activity (869.86 U/mL). This CRISPR-HDR tool box can be easily manipulated, and the AMA1-based plasmid can be easily removed under selective pressure of 5-fluoroorotic acid and uridine.

1. Introduction

Aspergillus niger, an organism that is generally recognized as safe, has been widely used in the food industry (Fleissner and Dersch, 2010). To optimize enzyme fermentation and investigate the regulatory mechanisms of secondary metabolite biosynthesis, classical genome editing methods are required to construct mutants (Meyer, 2008). However, there are several difficulties associated with constructing classical knock-out cassettes and recycling selection markers (Niu et al., 2016). Currently, CRISPR/Cas9 is considered the most effective tool for gene editing and has been broadly applied across species. The CRISPR/Cas9 system is used to insert and delete genes with repair of non-homologous end joining (NHEJ) or donor DNA (dDNA)-harboring homologies near the double-stranded break that it creates (Peters et al., 2016; Pohl et al., 2016; Ran et al., 2013; Zeng et al., 2015).

The CRISPR/Cas9 system has been developed for filamentous fungi such as *Trichoderma. reesei*, *A. oryzae*, *A. fumigatus*, and *A. carbonarius* (Fuller et al., 2015; Katayama et al., 2016; Liu et al., 2015; Weyda et al., 2017; Zhang et al., 2016). In *A. niger*, based on the CRISPR/Cas9 technique, Nodvig successfully mutated the polyketide synthase gene *fwnA* using a series of pFC plasmids and then flanking transfer RNAs (tRNAs) to express multiple single-guide RNAs (sgRNAs) in one expression cassette to edit multiple sites (Nodvig et al., 2015). Zheng found that the 5S rRNA can be used as an sgRNA promoter and used the CRISPR/Cas9 system to delete a 48-kb gene cluster (Zheng et al., 2018). Song tested 37 tRNA promoters to drive the transcript of sgRNA and finally concluded that 36 of these tRNAs, including a tRNA gene plus 100 base pairs of upstream sequence, were reliable and efficient in the CRISPR/Cas9 system (up to 97% efficiency) (Song et al., 2018). Sarkari used CRISPR/Cas9 together with the Golden Gate cloning technique to integrate an aconitic acid expression cassette into the genome of *A. niger* (Sarkari et al., 2017).

In the present study, we developed a tool box called the CRISPR/Cas9 homologous direct repair (CRISPR-HDR) system and tested this

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system with dDNAs and *in vitro* synthesized sgRNAs in *A. niger*. Using the developed CRISPR-HDR tool box, we deleted 0-, 2-, 10-, and even two 50-kb fragments of secondary metabolism gene clusters separately. We then modified the plasmid pFC330 by adding a tandem sgRNA expression cassette to transcribe two sgRNAs (*amyA* site and *glaA* site). By co-transforming the modified pFC330 with dDNAs to *A. niger*, we obtained single-, double-, and triple-site deleted strains with efficiencies of 31.8%, 54.5%, and 13.6%, respectively. Using this optimized CRISPR-HDR tool box, we even successfully inserted the glucose oxidase gene *goxC* at the loci of *amyA* and *glaA* simultaneously and increased the production of GoxC to 869.86 U/mL, which was four-fold higher than that of the control strain.

2. Materials and methods

2.1. Strains, media and culture conditions

A. niger strains were grown at 30 °C on potato dextrose agar (PDA) medium for sporulation or in dextrose peptone yeast extract (DPY) liquid medium for DNA extraction. High osmotic pressure Czapek–Dox (HCD) medium with sucrose (1 M) was used for protoplast transformation. Appropriate drugs and antibiotics (1 mg/mL 5-fluoroorotic acid, 10 mM uridine, 0.1 mg/mL hygromycin B and 10 mM acetamide) were added if required. *Escherichia coli* Mach1T1 was used for gene cloning. *E. coli* strains were maintained in Luria–Bertani (LB) medium at 37 °C with appropriate antibiotic (100 μg/mL ampicillin). Medium ingredients are shown in Table S1.

2.2. Plasmid construction

The Cas9 expression cassette was reconstructed based on the pFC330 plasmid (Nodvig et al., 2015). The gRNA expression cassette pAfU6-fwnA consisted of the pAfU6 promoter, 20 bp of *fwnA* protospacer, a gRNA scaffold (76 bp), and the *A. oryzae* U6 terminator (138 bp)(Katayama et al., 2016). The pFC330 plasmid was linearized by restriction endonucleases *Pac*I and Nt. *Bbvc*II. The linearized pFC330 and pAfU6-fwnA were purified using a HiPure PCR Pure Maxi Kit (Magen, Guangzhou, China) and assembled into the pFC330-fwnA plasmid using a NEBuilder® HiFi DNA Assembly Master Mix (NEB, Ipswich, USA). The pFC330-amyA-glaA plasmid, which contained two protospacers for the *amyA* and *glaA* genes for multiple-site gene deletions, was constructed with the same method. The primers used for PCR amplification are listed in Table S2.

2.3. In vitro synthesis of gRNA and amplification of dDNAs

For the *in vitro* synthesis of gRNA, we used a minimal T7 promoter (18 bp), a variable protospacer (20 bp), and a gRNA scaffold (76 bp) as templates. The DNA template for *in vitro* transcription of gRNAs was amplified using the F-gRNA-fwnA/R-gRNA primers. Purification was performed using a HiPure PCR Pure Maxi Kit. The templates were transcribed into RNA *in vitro* using a HiScribe™ T7 Quick High Yield RNA Synthesis Kit (NEB, Ipswich, USA). Transcribed RNAs were purified using phenol/chloroform extraction with ethanol precipitation and then dissolved in RNase-free water.

The dDNA of *fwnA* (dDNA-fwnA1, dDNA-fwnA2 and dDNA-fwnA3) contained three components in order: 39 bp upstream of *fwnA*1, the hygromycin B-resistance gene (*hygB*) and 39 bp downstream of *fwnA*1 (39 bp are shown in Table S3). F-dDNA1-fwnA/R-dDNA1-fwnA, F-dDNA2-fwnA/R-dDNA2-fwnA, and F-dDNA3-fwnA /R-dDNA3-fwnA were used as primers. The dDNA of cluster1 (dDNA-C1) and cluster2 (dDNA-C2) contained 3 components in order: 500 bp upstream of the cluster, *hygB* and 500 bp downstream of the cluster. The dDNA-amyA was constructed by overlap-extension PCR (OE-PCR), consisting of 574 bp upstream of PAM, the selection marker of *hygB* and 530 bp downstream of PAM. dDNA-glaA was constructed by OE-PCR as follows: 500 bp upstream of PAM, the selection marker of *amdS* and 500 bp downstream of PAM (Fig. 2B). The dDNA-goxC-amyA was constructed by OE-PCR, consisting of 574 bp upstream of PAM, the *goxC* expression cassette (containing Pna2/TPI, *goxC* ORF, and Ttef1), the selection marker of *hygB* and 500 bp downstream of PAM. The dDNA-goxC-glaA was constructed by OE-PCR as follows: 500 bp upstream of PAM, the *goxC* expression cassette (containing Pna2/TPI, *goxC* ORF, and Ttef1), the selection marker of *amdS* and 500 bp downstream of PAM (Fig. 3A).

2.4. Transformation

A. niger was transformed using the polyethylene glycol/CaCl$_2$-protoplast transformation method (Hinnen et al., 1978). The plasmids used in this study are listed in Table S4. The transformants were cultivated on CD (or selective CD) agar plates. Spores of positive transformants were inoculated into liquid DPY medium. Genomic DNA from colonies was extracted using a DNA-Pure Kit (GBCBIO, Guangzhou, China). PCR amplification was subsequently performed to verify mutants that were inoculated into PDA agar medium for high-quality spore collection. The strains used in this study are listed in Table S5.

2.5. Secondary metabolite analysis by HPLC-TOF-ESI/MS

Secondary metabolites were extracted as described (Wang et al., 2018) and analysed by HPLC-TOF-ESI/MS using a previously described method (Wang et al., 2018). The HPLC system comprised an Agilent 1200 HPLC system equipped with a column (Luna 5u C18 250*4.60 mm 5-μm Phenomenex, USA) and a Diode Array Detector (DAD, Agilent) and an ultra-high resolution TOF mass detector (Bruker maXis, Germany). Formic acid solution (0.1%, *v*/v, solution A) and acetonitrile solution (solution B) were used as the mobile phases in the gradient elution at 0.8 mL/min with a time-course of increasing solution B of 10–100% B for 0–15 min and 100–10% B for 15–30 min. Mass spectra in positive ion mode were recorded in 30 min.

2.6. Glucose oxidase enzyme activity assay

Glucose oxidase enzyme activity assays were performed as previously described (Karmali et al., 2004). One unit (U) of glucose oxidase activity was defined as the amount of enzyme that oxidizes 1 μmol of glucose per min at 37 °C and a pH of 6.2.

3. Results

3.1. CRISPR/Cas9 homologous direct repair (CRISPR-HDR)-mediated large gene fragment deletion in Aspergillus niger

To detect the feasibility of the CRISPR/Cas9 homologous direct repair (CRISPR-HDR) system in *A. niger*, the polyketide synthase gene *fwnA*, whose deletion results in white spores (Fig. 1A), was chosen as the target gene. A series of donor DNAs (dDNAs) was designed with various distances from the 20 bp protospacer (with high efficiency, as proven in Nodvig's research) and 3 bp PAM (Fig. 1B). Plasmid pFC330-fwnA was constructed as shown in Fig. S1. *A. niger* CBS-CT (*kusA* deleted) was used as the host strain. The constructed plasmid pFC330-fwnA (Fig. S1) was co-transformed respectively with dDNA-fwnA1(0 kb), dDNA-fwnA2 (2 kb), and dDNA-fwnA3 (10 kb) into the host strain (CBS-CT). Consequently, several *albino* colonies were obtained with the selection of hygromycin B after 4 days. Ten transformants were randomly chosen from each transformation for PCR and sequencing. The sequencing results showed that the *hygB* gene was precisely integrated into the locus with efficiencies of 80%, 50% and 10% (Fig. S2). To remove the pFC330-fwnA plasmid and recycle the *pyrG* marker, reverse screening was performed using 5-fluoroorotic acid and uridine (van Hartingsveldt et al., 1987). After 4 days of incubation

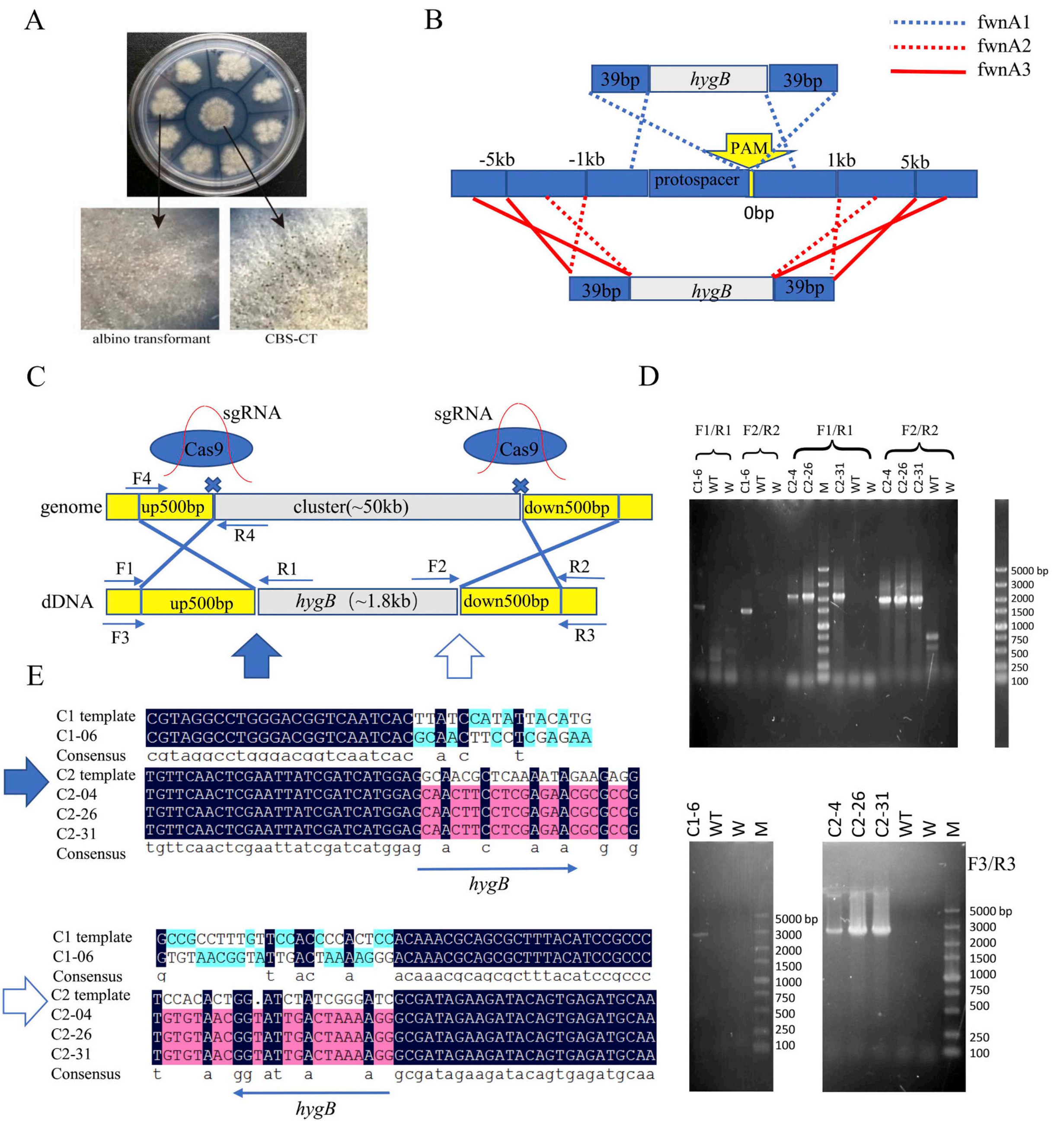

Fig. 1. CRISPR-HDR-mediated large gene fragment mutation in *A. niger*.

(A) Phenotype of the *fwnA* mutants. The spore of CBS-CT is black, while the mutant is white. (B) Schematic of the design of *fwnA*1, *fwnA*2 and *fwnA*3 represented separately as the 0 kb, 2 kb, and 10 kb knock-out, respectively. (C) Schematic of the 50-kb gene cluster knock-out and design of PCR verification primers. (D) PCR amplification results using primer sets F1/R1, F2/R2 and F3/R3 for verification. Primers F1/R1 (1589 bp for C1, 2231 bp for C2) and F2/R2 (1506 bp for C1, 1999 bp for C2) were designed for the location of the dDNAs, and primers R1 and F2 were designed in the fragment of *hygB*. The primer set F3/R3 (2886 bp for C1, 2724 bp for C2) was designed for amplification of the entire dDNA fragment, and C1-6 is the transformant of C1. C2-4, C2-26, and C2-31 are the 3 transformants of C2. WT is the wild-type host strain. W is water used as the template. M DL5000 is the ladder maker with bands representing 5,000 bp, 3,000 bp, 2,000 bp, 1,500 bp, 1,000 bp, 750 bp, 500 bp, 250 bp, and 100 bp from top to bottom. (E) Sequence alignment of PCR products using the F3/R3 primers. Dark-highlighted sequences are homologous sequences. Solid and hollow arrows indicate the sequence alignment position, and thin blue arrows indicate the start of *hygB*. The template was the wild-type genome sequence of C1 or C2. C1-06, C2-04, C2-26 and C2-31 were the four transformants.to the web version of this article.)

on a CD plate with 5-fluoroorotic acid and uridine (CD-5FOAU), colonies grew and were inoculated on a new CD-5FOAU plate. Fig. S3 shows that the progeny can grow on CD-5FOAU but not on the CD plate. PCR amplification for verification was performed using primer sets of F-AfpyrG/R-AfpyrG and F-Cas9/R-Cas9. The results showed that the auxotroph markers *pyrG* and *Cas9* had disappeared (Fig. S4). Therefore, genome fragment deletion can be accomplished within 5 kb upstream and 5 kb downstream flanking the PAM site of the target gene *via* the CRISPR-HDR system. The CRISPR-HDR system using dDNAs could be packaged as a tool box to proceed with genome editing in *A. niger*.

To test whether the CRISPR-HDR tool box could be used to delete large genomic fragments, two *A. niger* secondary metabolite biosynthetic gene clusters were chosen as the target fragments: cluster 1 (C1, for the biosynthetic of pestalamideA, An09g01790–An09g01970) and cluster 2 (C2, for the biosynthesis of 8-amino-2,5,7-trihydroxynaphthalene-1,4-dione, An11g07160–An11g07350). Pestalamide A is a type of secondary metabolite produced by *A. niger* that can inhibit the replication of HIV-1 in C8166 cells and has potential antifungal activity against *A. fumigatus* (Ding et al., 2008) (Fig. S5). The dDNAs were designed as shown in Fig. 1C. The pFC330 plasmid, dDNAs, and *in vitro* transcribed sgRNAs were co-transformed into *A. niger* CBS-CT. Several colonies grew with selection of hygromycin B after 4 days. PCR amplification (using primer sets F1/R1, F2/R2, F3/R3 and F4/R4) and sequencing results of F3 and R3 indicated that the *hygB* gene was successfully inserted into the genome of transformants C1–6, C2–04, C2–26 and C2–31 (Fig. 1D, Fig. 1E and Fig. S6). To test whether pestalamide A disappeared in C1–6, the secondary metabolites from CBS-CT and C1–6 were extracted and identified by comparing the HPLC retention time, UV absorption, and *m/z* fragmentation patterns of quasi-molecular ions. The results (Fig. S7) showed that pestalamide A ($C_{18}H_{17}NO_6$, exact mass: 343.1056) was detected in the CBS-CT strain but not in the C1–6 strain. These findings demonstrated that the 50-kb fragment of transformants C1 and C2 was successfully knocked out using the CRISPR-HDR tool box.

3.2. Multiple gene knock-in using the CRISPR-HDR toolbox in A. niger is feasible

To test the feasibility of multiple gene knock-in using the CRISPR-HDR tool box designed above, we selected two major extracellular protein genes (alpha-amylase A gene *amyA* and glucoamylase gene *glaA*) as the targets (Lu et al., 2010; Yuan et al., 2008). To express the sgRNA continuously and stably, sgRNA was expressed using the U6 promoter and terminator. The sgRNA expression cassette of *glaA* was constructed using the *A. oryzae* U6 (AoU6) promoter and the AoU6 terminator (Katayama et al., 2016), and the sgRNA expression cassette of *amyA* was constructed using the *Aspergillus fumigatus* U6–2 (AfU6–2) promoter (Zhang et al., 2016) and the AoU6 terminator. A tandem sgRNA expression cassette was constructed as described above and ligated with the pFC330 framework. The resulting plasmid was called pFC330-amyA-glaA (Fig. 2A). The dDNAs were constructed using overlap-extension PCR (OE-PCR) as shown in Fig. 2B and C. The plasmid pFC330-amyA-glaA was co-transformed with dDNAs into the host strain *A. niger* CBS-CT (*kusA* deleted). Control experiments were simultaneously performed with the same host strain and two dDNAs only (without the plasmid pFC330-amyA-glaA). Several colonies grew with the selection of hygromycin B and acetamide after 4 days. Twenty-two transformants in the experimental group and all three transformants in the control group were selected. PCR amplification was performed to detect the location of dDNAs (using primer sets F-pyrG-an05g02100-up/R-pyrG-an05g02100-up, F-pyrG-an05g02100-down/R-pyrG-an05g02100-down, F-pyrG-an12g06930-up/R-pyrG-an12g06930-up, F-pyrG- an12g06930-down/R-pyrG- an12g06930-down, F-amdS-glaA-up/R-amdS-glaA-up and F-amdS-glaA-down/R-amdS-glaA-down). The results showed that the frequency of single insertion was 31.8%, double insertion was 54.5% and triple insertion was 13.6% (Table S6). The fermentation products of the 13 selected transformants were chosen for SDS-PAGE and MALDI-TOF analysis and compared with controls, which revealed that the two extracellular proteins AmyA (red box) and GlaA (yellow box) disappeared in all 13 transformants in the experimental group (Fig. 2D, Table. S7).

3.3. Multiple gene knock-in using the CRISPR-HDR toolbox can improve the secretion of glucose oxidase (GoxC) in A. niger

The *A. niger* glucose oxidase gene *goxC* was chosen to test whether the CRISPR-HDR tool box could be used for multi-copy expression. The *goxC* expression cassette was constructed using promoter Pna2/TPI (Moore, 2009) and the terminator of translation elongation factor *tef1* (derived from *A. nidulans*). The *goxC* expression cassette was inserted into the dDNA between the upstream homologous arm and the selection marker (Fig. 3A). The newly constructed goxC-expressing dDNAs were named dDNA-goxC-amyA and dDNA-goxC-glaA (Fig. 3A). The plasmid pFC330-amyA-glaA was co-transformed with dDNA-goxC-amyA and dDNA-goxC-glaA into the host strain *A. niger* HL-1 (*kusA* deleted, named Project 1). Several colonies grew with the selection of hygromycin B and acetamide after 6 days of incubation. PCR amplification using the primer set F-goxC275/R-goxC1735 showed that almost all transformants contained the *goxC* cassette (Fig. S8). To confirm whether the *goxC* cassettes were precisely located in the loci of *amyA* (*an05g02100* and *an12g06930*) or *glaA*, PCR amplification was performed (using primer sets F-goxC-an05g02100-up/R-goxC-an05g02100-up, F-goxC-an05g02100-down/R-goxC-an05g02100-down, F-goxC-an12g06930-up/R-goxC-an12g06930-up, F-goxC-an12g06930-down/R-goxC-an12g06930-down, F-goxC-glaA-up/R-goxC-glaA-up and F-goxC-glaA-down/R-goxC-glaA-down), and the results are shown in Table S8. The other project was performed with the same host strain but with only one dDNA (dDNA-goxC-amyA) and pFC330-amyA-glaA (called Project 2). PCR amplification of the transformants was performed (using the primer sets F-goxC-an05g02100-up/R-goxC-an05g02100-up, F-goxC-an05g02100-down/R-goxC-an05g02100-down, F-goxC-an12g06930-up/R-goxC-an12g06930-up and F-goxC-an12g06930-down/R-goxC-an12g06930-down). PCR amplification results showed that the single insertion rate was 41.7% in Project 1 and the same in Project 2. The double insertion rate was 25% in Project 1 and 29.2% in Project 2, and there were 2 transformants with 3 copies of *goxC*.

To detect the expression level of *goxC*, 7 transformants with different copy numbers of *goxC* were chosen: A and B contained 3 copies at all three loci, C and D contained two copies of *goxC* at two alpha-amylase genes (an05g02100 and an12g06930), E and F contained two copies at an05g02100 and *glaA*, and G only contained one copy at an05g02100, which was obtained before this experiment as a control (Fig. S9). Seven representative transformants were inoculated in fermentation media and cultivated at 30 °C and 250 rpm. Fermentation supernatants were collected at 2–8 days. SDS-PAGE results of GoxC (the GoxC band is approximately 90 kDa, shown as the blue arrow) showed that the GoxC bands of A~F were thicker than that of G. Enzyme activity of GoxC in these fermentation supernatants was measured, and the results are shown in Fig. 3C. The enzyme activity of A–F reached twice that of G after day 6, especially in F, which reached 869.39 U/mL at 4 times that of G on day 8. These results indicated that multiple copies of *goxC* could be inserted into the genome of host cells *via* the CRISPR-HDR tool box, thereby increasing product and enzyme activity.

4. Discussion

The efficiency of gene modification in filamentous fungi was very low using traditional genetic manipulation. CRISPR/Cas9 is a new genetic tool that has been used in filamentous fungi. For the application of the CRISPR/Cas9 system in filamentous fungi, Nodvig first used an AMA1-based plasmid to express Cas9 protein and the “HH scaffold” to express gRNA in *A. niger* (Nodvig et al., 2015). Then, they designed a vector system with the capacity of delivering Cas9 and multiple sgRNAs based on RNA polymerase III promoters and tRNA spacer templates of specific Cas9/sgRNA-induced DNA DSBs in filamentous fungi (Nodvig et al., 2018). Kuivanen used CRISPR/Cas9 with *in vitro* synthesized gRNA to disrupt catabolism of galactaric acid in *A. niger*, which highlighted the usefulness of *in vitro* synthesized gRNA in the CRISPR/Cas9 system in *Aspergillus* (Kuivanen et al., 2016).

For long fragment deletion in filamentous fungi, Pohl used the marker-free method to delete a 26-kb gene fragment in *Penicillium chrysogenum* (Pohl et al., 2016). Nodvig knocked out a 9-kb wA gene by introducing two sgRNAs at a time (Nodvig et al., 2018). Xiaomei also used the two sgRNA methods and successfully knocked out a 48-kb gene cluster (Zheng et al., 2018). In this study, we used the CRISPR/Cas9 homologous direct repair (CRISPR-HDR) system to knock out fragments of different lengths in *A. niger*. By knocking out *fwnA* and two secondary metabolic gene clusters, we believed that gene deletion could be achieved within a range of 0 to 50 kb flanking the PAM sites, and the

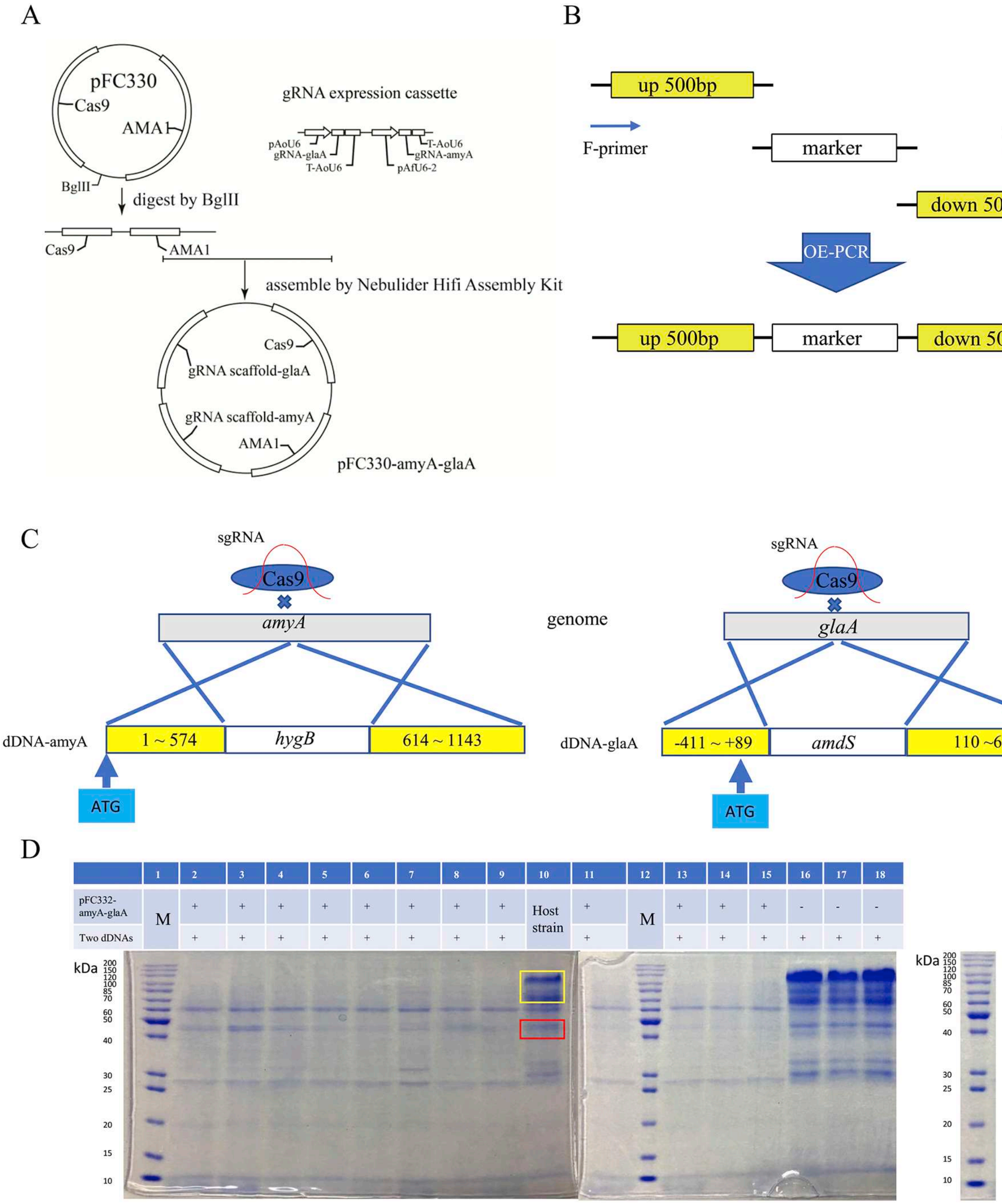

Fig. 2. Triple knock-in of *hygB* and *amdS* in the loci of *amyA* or *glaA* using the CRISPR-HDR system in *A. niger CBS 513.88*.
(A) Schematic of pFC330-amyA-glaA construction. (B) Schematic of the construction of dDNAs. (C) Schematic of the design of multiple gene knock-in using the CRISPR-HDR system. The blue arrows indicate the start codon of *amyA* or *glaA*. (D) SDS-PAGE electrophoresis results from multiple knock-in transformants. M, marker; lines 2–9, 11, 13, 14, and 15 were triple knock-in transformants; lines 16–18 were transformants of the control group. Bands in the yellow box indicate glucoamylase GlaA, and the red box indicates alpha-amylase AmyA, which disappeared in lines 2–9, 11, 13, 14, and 15. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

deletion efficiency decreased as the length of target fragments increased.

For editing multiple genes (knock-out or knock-in) *via* the CRISPR/Cas9 system in filamentous fungi, Laure et al. developed a simple Cas9-based gene targeting method of genomic deletions and insertions by leveraging locus-specific “pop-out” recombinations to suppress off-target integrations and achieved 100% gene editing efficiency across three loci (Leynaud-Kieffer et al., 2019). Nodvig used a vector containing Cas9 ORF and multiple sgRNAs based on RNA polymerase III promoters and tRNA spacers. The sgRNAs can be released by RNase P and RNase Z *in vivo*, which can be used to introduce two or more DSBs at once (Nodvig et al., 2018). Zhang knocked out two genes (*pksP* and *cnaA*) at multiple predicted sites at once in *A. fumigatus*. Liu used the *in vitro* transcribed sgRNA method and optimized the concentration ratios of sgRNA and dDNA. The frequency of gene knock-out approached 100% for single, 45% for double, and 4.2% for triple sites. Parveen successfully integrated several expression cassettes of aconitic acid using the CRISPR/Cas9 system and Golden Gate cloning strategy (Sarkari et al., 2017). Kulvanen used *in vitro* assembled RNP complexes and repair donor DNAs containing selection markers to insert several additional copies in *A. niger*

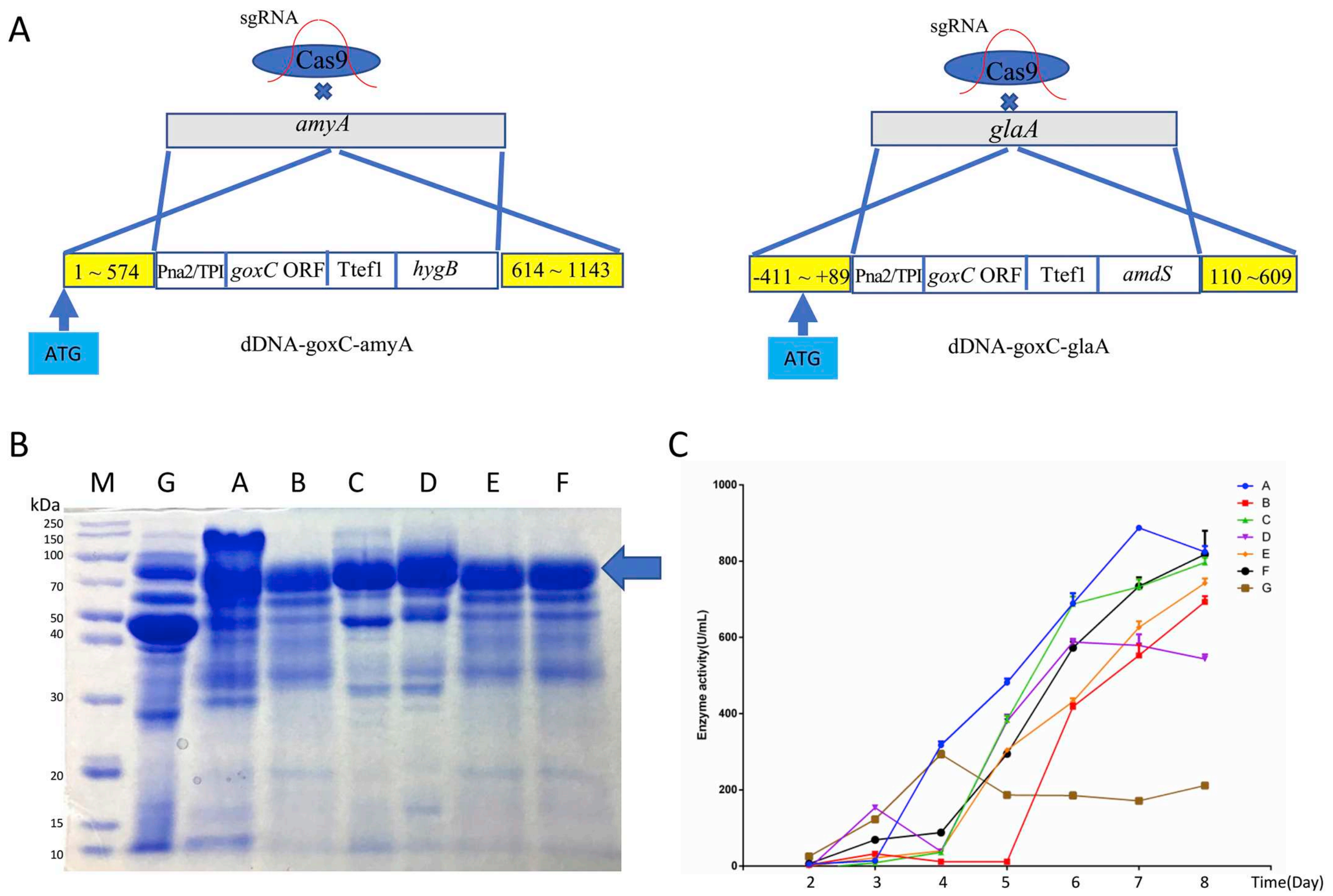

Fig. 3. Triple knock-in of *goxC* in the loci of *amyA* or *glaA* using the CRISPR-HDR system in *A. niger HL-1*.
(A) Schematic of the construction of dDNA-goxC-amyA and dDNA-goxC-glaA. The blue arrow indicates the start codon of *amyA* or *glaA*. (B) SDS-PAGE electrophoresis results from multiple knock-in of *goxC*. M, marker; A and B have 3 copies of *goxC* at all 3 loci, C and D have 2 copies of *goxC* at 2 loci of *amyA*, E and F have one copy at *amyA* and one copy in *glaA*, and G only has one copy of *goxC* at the *amyA* locus. (C) Enzyme activity trends of 7 transformants described in (B), 2–8 days. Enzyme activity was measured with a triplicate biological repetition. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(Kuivanen et al., 2019). For instance, Shuobo inserted a 24-kb fragment into the highest copy number of the δ site using the Di-CRISPR platform, resulting in an unprecedented 18-copy genomic integration in *Saccharomyces cerevisiae* (Shi et al., 2016). Leanne constructed a landing pad system that can facilitate multi-copy gene integration of one, two, three, or four DNA copies in a single transformation. However, multiple-gene knock-in for filamentous fungi remains underdeveloped compared with that in yeast. In this study, we constructed plasmid pFC330 with two gRNA expression cassettes by harboring two different U6 promoters derived from *Aspergilli*. First, we co-transformed this plasmid with dDNAs harboring selection markers to knock-in selection markers of *hygB* and *amdS* at the predicted sites (two copies of *amyA* and one copy of *glaA*) and obtained a knock-in rate of 57% at the *amyA* site and 100% at the *glaA* site. In addition, 57% of the transformants contained target genes knocked in at two sites. Next, we used CRISPR-HDR to improve the production and enzyme activity of GoxC. The *goxC* gene was knocked in at the predicted sites of *amyA* and *glaA*. The enzyme activities of GoxC were higher than those reported in *Aspergilli*.

5. Conclusions

Based on the co-transformation of dDNAs and the AMA1-based plasmid that allowed recycling of the selection marker *pyrG* and components of the CRISPR/Cas9 system, we constructed the CRISPR-HDR tool box to edit the genome of *A. niger*. Using this tool box, we successfully knocked out a 50-kb gene cluster and simultaneously knocked out selection markers at one, two and three gene loci with efficiencies of 31.8%, 54.5% and 13.6% in *A. niger*, respectively. Furthermore, using this tool box, we successfully knocked in two or three *goxC* expression cassettes into the precise loci of *glaA* and *amyA*, increasing the enzyme activity of GoxC to 869.39 U/mL, which was four times that of the control strain.

Declaration of interests

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mimet.2019.105655.

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