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Efficient gene deletion and replacement in *Aspergillus niger* by modified in vivo CRISPR/Cas9 systems

Yuan Zhang, Liming Ouyang* , Yilin Nan and Ju Chu

Abstract

Aspergillus niger, as an important industrial strain, is widely used in the production of a variety of organic acids and industrial enzymes. To excavate the greater potential of *A. niger* as a cell factory, the development of highly efficient genome editing techniques is crucial. Here, we developed a modified CRISPR/Cas9 system for *A. niger* highlighted in two aspects: (1) construction of a single and easy-to-use CRISPR/Cas9 tool plasmid derived from pAN7-1 which is widely used in filamentous fungi; (2) redesign of the easy-to-switch "ribozyme–gRNA–ribozyme (RGR)" element in the tool plasmid. We examined the gene inactivation efficiency without repair fragment and the gene replacement efficiency with repair fragment utilizing the modified system, respectively, and both of them reach the efficiency as high as over 90%. Especially, the co-transformation of the tool plasmid and the specific repair fragment can easily realize one-step knock-out/knock-in of target genes, even with the length of homologous arms as only 100 bp. The establishment of this system will lay a solid foundation for the gene function research and rational design of cell factory in *A. niger* or broader filamentous fungi hosts.

Keywords: *Aspergillus niger*, CRISPR/Cas9, Filamentous fungi, Genome editing, Ribozyme, pAN7-1, Glucoamylase

Introduction

Aspergillus niger, as a member of filamentous fungi, is widely used to produce and secrete a variety of bioactive substance including organic acids, like citric acid (Wang et al. 2017), oxalic acid (Lee et al. 2018), gallic acid (Mata-Gomez et al. 2015) as well as many carbohydrate-active enzyme (CAzymes), such as glucoamylase (Suyama et al. 2017), glucose oxidase (Zhu et al. 2018), and amylase (Varalakshmi et al. 2009), which are valuable in industry especially food industry. And it is generally recognized as safe (GRAS) by the United States Food and Drug Administration. So, *A. niger* is considered to be one of the most important cell factories for industrial enzymes and organic acids production. Despite the importance of *A. niger*, the gene function and synthetic biology research have fallen behind because of the dominance of non-homologous DNA end joining (NHEJ) over homology directed repair (HDR) in filamentous fungi (Carvalho et al. 2010; Meyer et al. 2007).

In 1990s, deletion of *Ku70/Ku80*-protein complex was utilized to inactivate the NHEJ pathway, which was reported to strongly reduce the random integration of DNA fragments and lead to high homologous recombination efficiency in filamentous fungi. In *A. niger*, the deletion of *kusA* gene which encodes the ortholog of *Ku70* protein strongly improved homologous recombination efficiency up to over 80% and did not influence the growth of *A. niger* (Weld et al. 2006). However, Δ*ku70* was not easy to generate from wild type (WT) strain by traditional ways, and the mutants were further found to be more sensitive to some chemicals such as methyl mesylate, ethyl methane sulfonate and bleomycin (Meyer et al. 2007) so its application is still limited.

Recently, the clustered regularly interspaced short palindromic repeats (CRISPR) system has been discovered as an efficient gene editing technology in many organisms, such as plants (Liu et al. 2017; Shan et al. 2013), mice (Shen et al. 2013) and rice (Li et al. 2013). The

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nuclease Cas9 can cause a double-strand break (DSB) in the target deoxyribonucleic acid (DNA) which is formed by a 20-bp DNA sequence of target gene with a 5′-NGG nucleotide sequence in the subsequent downstream which is called the protospacer-adjacent motif (PAM) (Mei et al. 2016). Recently, the CRISPR/Cas9 system has been successfully applied in many filamentous fungi, such as *Trichoderma reesei* (Liu et al. 2015), *Aspergillus oryzae* (Katayama et al. 2016), *Aspergillus fumigatus* (Fuller et al. 2015), *Neurospora crassa* (Matsu-Ura et al. 2015), and *Penicillium chrysogenum* (Pohl et al. 2016). What is more, Nødvig et al. (2015) established a versatile CRISPR–Cas9 system with a special ribozyme–gRNA–ribozyme (RGR) structure for filamentous fungi which was benefit from the study in plant (Gao and Zhao 2014) and applied successfully in six different filamentous fungi consisting of *A. niger*. This structure effectively solved the problem of lacking promoters recognized by RNA polymerase III for the transcription of gRNA in *A. niger*. But the construction of the CRISPR–Cas9 vector depending on USER cloning or USER fusion (New England Biolabs, USA) raised the cost of the process. And the backbone plasmid pFC330 series in the paper is not common for most researchers. So, techniques based on common plasmid and easy-to-assemble elements still need to be further investigated.

In this study, we constructed a CRISPR/Cas9 tool plasmid based on general expression plasmid pAN7-1 for filamentous fungi and designed an RGR element in which the middle gRNA sequence can be easily changed by simple molecular cloning. The efficiency of using it to inactivate a pigment gene of *olvA* in *A. niger* was firstly investigated.

α-Glucosidase is a by-product of glucoamylase fermentation and its expression will reduce the activity and yield in the glucoamylase fermentation process of *A. niger*. So, disruption of α-glucosidase coding gene may benefit the production of glucoamylase. We chose one of the α-glucosidase family members *agdF* as the target gene for disruption because its expression level is the highest in the family during enzyme production process according to our previous transcriptome sequencing data (Lu et al. 2018). Replacement of *agdF* by glucoamylase gene using the CRISPR/Cas9 tool plasmid and a repair fragment was further tested.

Materials and methods

Strains and media

Escherichia coli strain DH5α was used to propagate all plasmids. The glucoamylase production *A. niger* strain CBS513.88 was preserved in our laboratory and used for genetic manipulation. PDA media (20% potato, 2% glucose, 2% agar) were used for sporulation of *A. niger* strains. CM medium (0.1% cosamino acids, 0.5% yeast extract, 2% 50% glucose mother liquor, 2% Asp + N, 0.2% 1 M $MgSO_4$, 0.1% trace elements) was used for germination of spore. The transformants were grown on the following double layer medium. The upper media contained 0.95 M sucrose, 0.6% agar, 2% Asp + N, 0.2% 1 M $MgSO_4$, 0.1% trace elements stock solution. The lower media contained 0.95 M sucrose, 1.2% agar, 2% Asp + N, 0.2% 1 M $MgSO_4$, 0.1% trace elements. Asp + N (50 × stock solution): 3.5 M $NaNO_3$, 0.35 M KCl, 0.55 M KH_2PO_4. Trace elements (1000 × stock solution): per liter, 10 g EDTA, 4.4 g $ZnSO_4 \cdot 7H_2O$, 1.01 g $MnCl_2 \cdot 4H_2O$, 0.32 g $CoCl_2 \cdot 6H_2O$, 0.315 g $CuSO_4 \cdot 5H_2O$, 0.22 g $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 1.11 g $CaCl_2$, 1 g $FeSO_4 \cdot 7H_2O$).

Protoplast transformation of *A. niger*

Fungal spores were inoculated from glycerol stock on PDA culture plates and cultured at 30 °C for 4–5 days for the mycelium growth and spore generation. The fresh spores were eluted with 0.85% NaCl and 0.02% Tween 80. Spore suspension was inoculated into 250 ml liquid CM culture medium at a concentration of 2×10^8/ml for overnight cultivation (16–18 h) at 30 °C, 50 rpm. The germinated spores were harvested by filtering through three layers of miracloth (Millipore, cut and sterilized in advance), washed with SMC solution (1.33 M sorbitol, 50 mM $CaCl_2$, 20 mM MES buffer, pH 5.8) and digested with enzyme solution (200 mg lysing enzyme with 10 ml SMC solution) for 3 h with rotatory shaking at speed of 75 rpm at 30 °C. Protoplast quality and quantity could be checked by microscopy during the digestion process. Before collecting protoplasts, 10 ml STC solution (1.33 M sorbitol, 50 mM $CaCl_2$, 10 mM Tris/HCl, pH 7.5) was added to increase the release of protoplasts. Digested protoplasts were separated from cell wall debris by filtering through three layers of miracloth and the filtrate was precipitated at 3500 rpm for 10 min at 10 °C. The supernatant was discarded and the protoplasts were then washed 1-2 times with 1 ml STC solution at 4500 rpm for 5 min at 10 °C and resuspended in STC solution. The volume of STC solution was set according to the quantity of transformations (100 μl/transformation) and the final concentration of protoplast should be over 7×10^6/ml. Several 50 ml sterile centrifuge tubes were prepared, depending on the number of transformation experiments. For each transformation, 100 μl protoplast suspension, 8–10 μg of plasmid DNA and 25 μl PEG 6000 (25% polyethylene glycol 6000, dissolved at 65 °C in water bath for 10 min) were added to an empty 50 ml centrifuge tube and mixed softly. Subsequently, 1 ml PEG 6000 was added and evenly mixed into the mixture. Then, the tubes were incubated on ice for 5 min. 2 ml additional STC solution was added to the suspension and mixed

slightly. Then, 10 ml upper medium containing hygromycin (100 μg/ml) was added into the suspension and mixed uniformly. The mixture was overlaid on the lower medium containing hygromycin (100 μg/ml), and the plates were put at 30 °C for 5–7 days allowing the growth of transformants.

Tool plasmid construction

The CRISPR/Cas9 expression vector pAN-olvA was constructed as follows. The Cas9 endonuclease from the *Streptococcus pyogenes* Type II CRISPR/Cas system with a sequence encoding SV40 nuclear localization signal (SV40 NLS) at both 3′ and 5′-end was amplified from the plasmid pX458 (purchased from Addgene) by polymerase chain reaction (PCR) using primers Cas9-F and Cas9-R. The amplified Cas9 was inserted between the gpdA promoter (P_{gpdA}) and trpC terminator (T_{trpC}) in pAN7-1 (purchased from Addgene) which was an expression plasmid widely used in filamentous fungi, yielding plasmid pANCas9. The pre-sgRNA fragment containing a hammerhead (HH) ribozyme at the 5′-end, the sequence-specific gRNA portion in the middle and a hepatitis delta virus (HDV) ribozyme at the 3′-end was obtained by synthesis (BGI, Shanghai). To facilitate the replacement of different sgRNA element targeting different genes in future, we added two unique endonuclease sites (*Hin*dIII and *Spe*I) at both ends of HH-sgRNA-HDV element by fusion PCR using primers (*Hin*dIII-sgRNA-F and sgRNA-*Spe*I-R). The CRISPR/Cas9 vector was assembled by One-Step Cloning Kit (Vazyme Biotech, China).

To inactivate the pigment gene *olvA*, we constructed the sgRNA-olvA expression cassette. The HH-sgRNA-*olvA*-HDV fragment was inserted between the P_{gpdA} and T_{trpC}, forming P_{gpdA}-*olvA*-T_{trpC} cassette. The cassette was then cloned into the vector pANCas9, generating the plasmid pANCas9-*olvA*. As a selection marker, the 2.7-kb hygromycin gene with promoter and terminator from the plasmid pBC-hygro (purchased from Addgene) was amplified by PCR using the primers *hyg*-F and *hyg*-R. The *hyg* expression cassette was cloned into the vector pANCas9-*olvA*, yielding the final plasmid pAN-*olvA* (Fig. 1) ready for the inactivation of *olvA* gene. And it was also served as a template plasmid ready for the switch of different sgRNA elements in our further study.

Over-expression of *glaA* gene and down-regulating *agdF* in one step by CRISPR

To reduce the expression of α-glucosidase and increase the expression of glucoamylase in the same time, we designed the one-step replacement strategy by CRISPR which utilized a plasmid with sgRNA targeting to *agdF* gene (pAN-*agdF*), a repair fragment containing

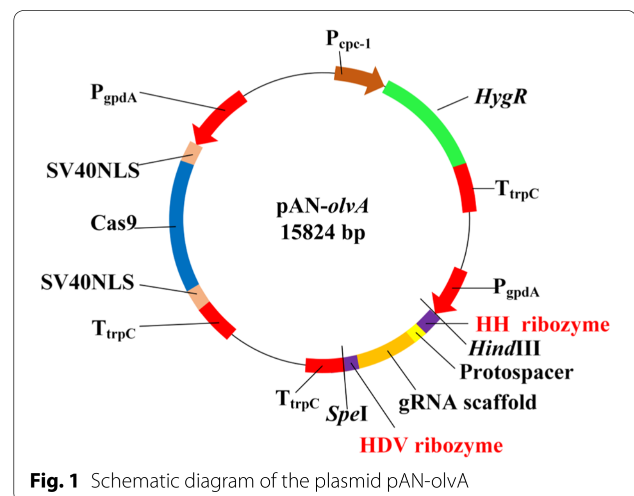

Fig. 1 Schematic diagram of the plasmid pAN-olvA

over-expression cassette of glucoamylase encoding gene *glaA* and two 1 kb homologous arms flanking the *agdF* gene.

Because a 6 bp DNA which was the inverted repeat of the 5′-end of the protospacer is necessary to be inserted into the 5′-end of the HH ribozyme region to form special secondary RNA structure for the self-cleavage, the pre-sgRNA element had to be constructed by a two-step PCR. The up and down parts of the insertion DNA were, respectively, amplified by PCR using primers P1 and P2, P3 and P4. The whole pre-sgRNA cassette was obtained by fusion PCR using primers P1 and P4 (Fig. 2). Then by simple digestion and ligation, the HH-*olvA*-HDV element in pAN-*olvA* was changed into HH-*agdF*-HDV, forming the plasmid pAN-*agdF*. *E. coli* transformation and plasmid extraction were done according to standard protocols. Primers used for plasmid construction and transformants identification are listed in Table 1.

Then, the repair fragment and the plasmid pAN2 (targeting *agdF* site) was co-transformed into *A. niger* CBS513.88 by protoplast transformation. After co-transformation, the transformants were firstly screened on medium with hygromycin. To obtain homozygotes, the spores of primary transformants were isolated and spread on the screening medium again. Then, the genome DNA of the single homozygote colony was extracted for the PCR identification (Fig. 3a, b). One of the positive mutants (named as *agdF::glaA* mutant) identified by PCR was further cultured in shake flasks to examine the enzyme activity of glucoamylase and α-glucosidase. For the contrast, we also measured the enzyme activities of glucoamylase and α-glucosidase of WT strain CBS513.88 at the same time. Gene replacement using homologous arms of reduced length (500 bp and 100 bp) was also performed.

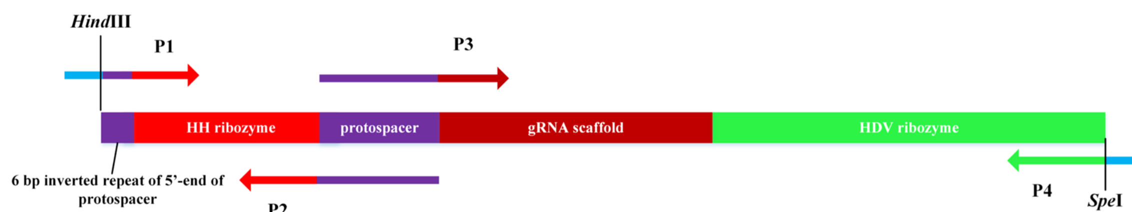

Fig. 2 The construction of HH-sgRNA-HDV element. The blue portions of P1 and P4 represented the homologous complementary of the inserting site of plasmid pAN-agdF

Table 1 Primers used in the study

Primer name	Sequence (5′–3′)
Cas9-F	GCCTGAACTCACCGCGACGTGACTATAAGGACCACG ACGG
Cas9-R	TCAGTAACGTTAAGTGGATCCTTTTTCTTTTTTGCCTGGC
*Hin*dIII-sgRNA-F	AACTCACCGCGACGTAAGCTTATGACCCAAT**ACACCG**CT
*Hin*dIII-sgRNA-R	CAGTAACGTTAAGTGACTAGTGACCCAATGTCCCATTCG
hyg-F	GTACTGTGTAAGCGCTCCGTCTCCATTGGCTCT
hyg-R	GTACTGAGAGTGCAGCAAGGAGATGGCGCCCAA
P1	AACTCACCGCGACGT**TTACAT**CTGATGAGTCCGTGAGGA
P2	**CTCAACGGACGCTGTTACAT**GACGAGCTTACTCGTTTC
P3	**ATGTAACAGCGTCCGTTGAG**GTTTTAGAGCTAGAA ATAGC
P4	CAGTAACGTTAAGTGTCGAGATGACCCAATGT
PU1	AATTCAACCTCAGCCCTA
PU2	TGGCTCTTAATGAGCTGG
PD1	CCTACAGGACACACATTC
PD2	CAAATTCCACAGTCTCGG

The bold letters indicate the protospacer sequences. The underline nucleotide sequences indicate restriction enzyme sites

Results

Efficient inactivation of pigment gene *olvA* in *A. niger* by CRISPR/Cas9 system embodied HH-sgRNA-HDV element

To test the feasibility and efficiency of the CRISPR/Cas9 system in *A. niger*, we choose to target the *olvA* gene which can be easily screened phenotypically after inactivation. *olvA* gene encodes the YWA1 hydrolase homolog and its deletion can form olive conidia on regeneration medium while wild strain shows black conidia. A 20 bp of the protospacer sequence with the 3′-PAM AGG for *olvA* was chosen at the start of the ORF (nucleotide −18 to −37). The resulting plasmids pAN1 was transformed into *A. niger* through protoplast transformation. Most transformants (24/26) grown on hygromycin plates (Fig. 4a) showed the desired phenotype of olive colonies, with the mutational rate of over 90%. Because conidia of *A. niger* contain two or more nuclei, the isolation of single mutant colony from the original transformants is necessary. We diluted the conidia of primary transformants to 10^4–10^5 times and streak the conidia diluent on medium with hygromycin for growth of 3 days to isolate the homozygotes (Fig. 4b). Afterwards, the genome DNA extracted from the single positive colony was used as the template for PCR and DNA identification. The sequencing results reflected either single nucleotide deletion or mutation in upstream of the PAM motif in *olvA* gene in the two transformants (Table 2).

Over-expression of *glaA* and down-regulation of *agdF* in one step by CRISPR

After transformed by plasmid pAN-*agdF* and repair fragment containing *glaA* expression cassette and 1 kb arms homologous to *agdF*, *A. niger* colonies with hygromycin resistance were obtained. PCR verification of the up and down parts of the insertion (Fig. 3b) in the genome of positive transformants was performed. The result shows (Fig. 3c) three out of five candidates possessing the insertion fragment. One of the three was selected as the *agdF::glaA* mutant for further enzyme activity assay. As it shows in Fig. 5, the activity of glucoamylase of *agdF::glaA* mutant was 25.9% higher than wild strain, while the activity of α-glucosidase of *agdF::glaA* mutant was 61.4% lower than wild strain.

Efficiency of gene replacement with different length of the homologous arm

To test the relation between the efficiency of gene replacement and the length of the homologous arms, we further designed the repair fragments with 2 sets of different arm lengths (500 and 100 bp) based on the above-mentioned experiment. We co-transformed the plasmid pAN-*agdF* and the repair fragments with different length of homologous arms. After co-transformation, we selected 3 or 5 transformants from each transformation and extracted their genome DNA for PCR identification. And we found all tested transformants (3/3 and 5/5, respectively) were positive. The results of PCR (Fig. 6) demonstrated that a pair of 100-bp homology arms is sufficient to obtain homologous integration stimulated by the CRISPR/Cas9 system in *A. niger* with high efficiency.

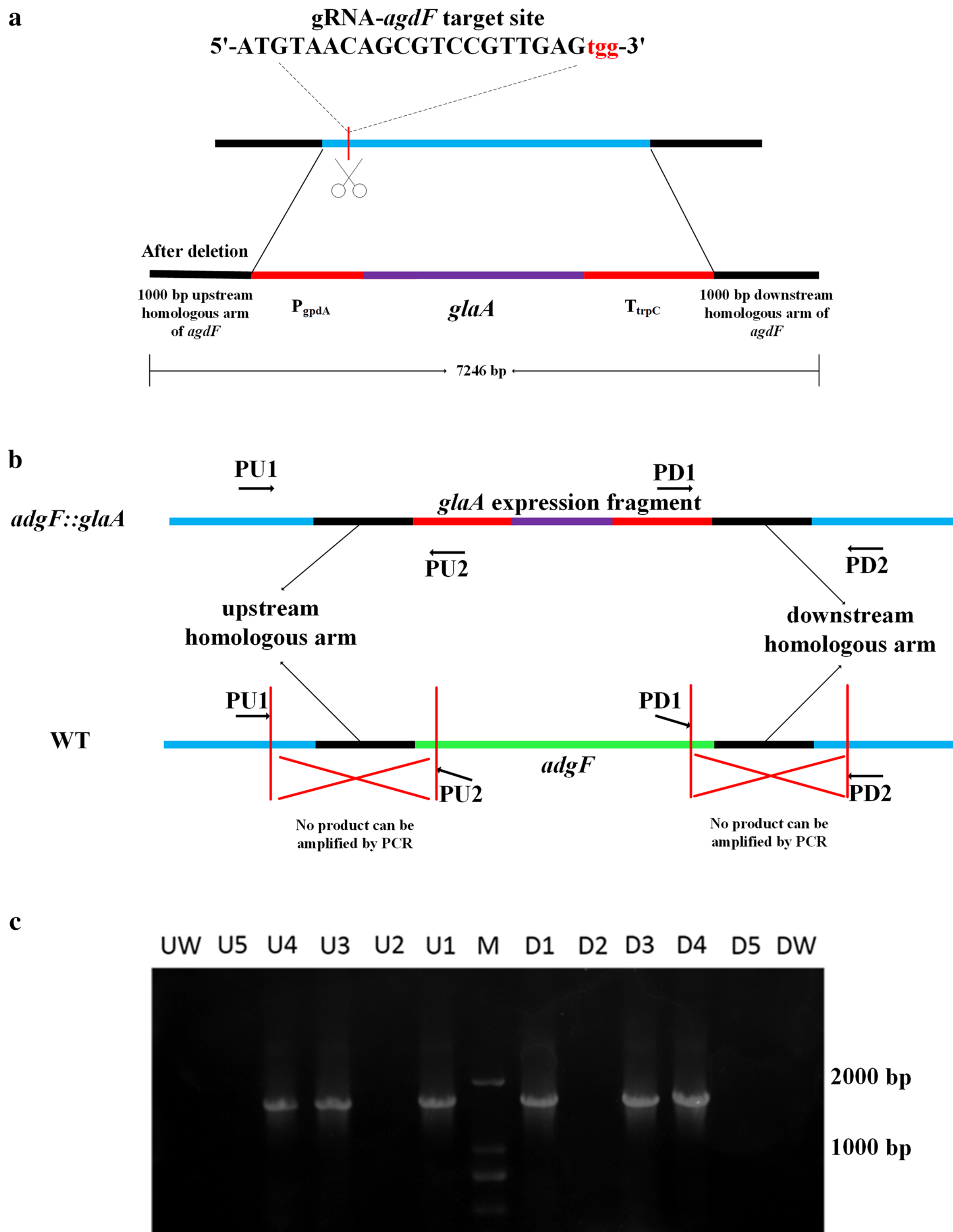

Fig. 3 Strategy and verification of gene replacement by CRISPR. **a** Schematic for replacement of *agdF* by *glaA* expression cassette mediated by CRISPR/Cas9 system. **b** Schematic for PCR verification of mutants. The expected length of fragments amplified from both up- and down-stream of *agdF* locus in genomes of mutants was about 1600 bp, while there is no product amplified from the genome template of WT strain CBS513.88. **c** The PCR results of mutants and WT strain. Five single colonies were separated from the primary transformant with the WT strain CBS513.88 as the negative control (U means results of upstream PCR and D means results of downstream PCR; 1–5 stands for the 5 mutant candidates and W stands for WT strain.) The results confirmed that expected gene replacement was arisen in 3 mutants (1, 3 and 4)

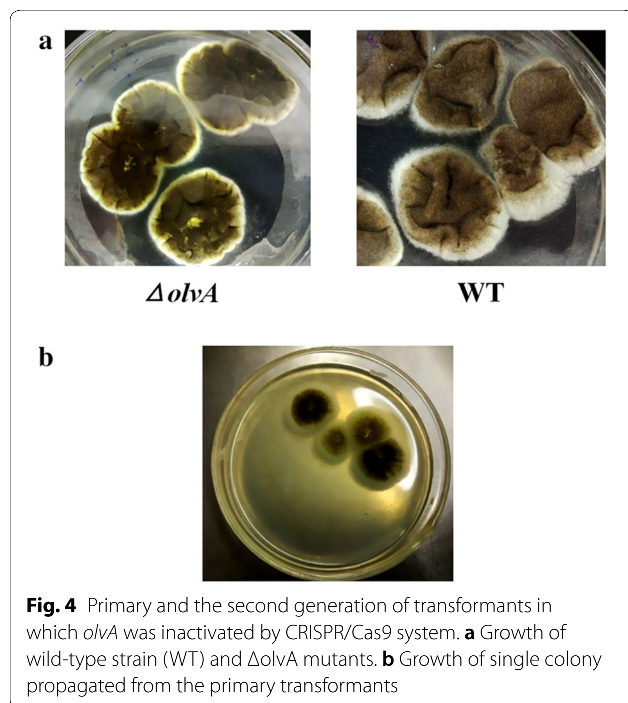

Fig. 4 Primary and the second generation of transformants in which *olvA* was inactivated by CRISPR/Cas9 system. **a** Growth of wild-type strain (WT) and ΔolvA mutants. **b** Growth of single colony propagated from the primary transformants

Discussion

In this study, the CRISPR/Cas9 system showed high efficiency for targeted gene inactivation and replacement in *A. niger*. Using this system, gene replacement can be introduced by homology arms as short as 100 bp. The results made the technique promising when compared to the reported efficiency of 80% in NHEJ-deficient *A. niger* using 500 bp homology arms (Meyer et al. 2007).

In this study, the Cas9 coding gene was from the *Streptococcus pyogenes*—without codon optimization, and the Cas9/sgRNA expression vector was derived from pAN7-1 which did not contain the AMA1 element necessary for extrachromosomal replication of a plasmid in filamentous fungi (Carvalho et al. 2010). It was undeniably that the copy numbers of Cas9 and sgRNA could be higher when carried by autonomously replicating plasmid. However, it has been considered that the amount of Cas9 protein and gRNA was not limiting factors for gene editing over many cases. Recently, Zheng et al. (2018) also reported their CRISPR/Cas9 gene editing system derived from pUC plasmid successfully worked in *Aspergillus*.

Table 2 Mutated sequence of *ΔolvA* mutants

Strain	Sequence alignment
WT	TGGGGGTAAACGGTGTTGAACTTCTCGCCGAGGATCCAAG
Δ*olvA*1	TGGGGGTAAACGGTGTTGAACTTCTC-CCGAGGATCCAAG
Δ*olvA*2	TGGGGGTAAGCGGTGTTGAACTTCTCGCCGAGGATCCAAG

The red letters indicate the PAM sequence, the blue letters indicate the protospacer sequences, the purple letter indicates mutational nucleotide, and the hyphen indicates deleted nucleotide

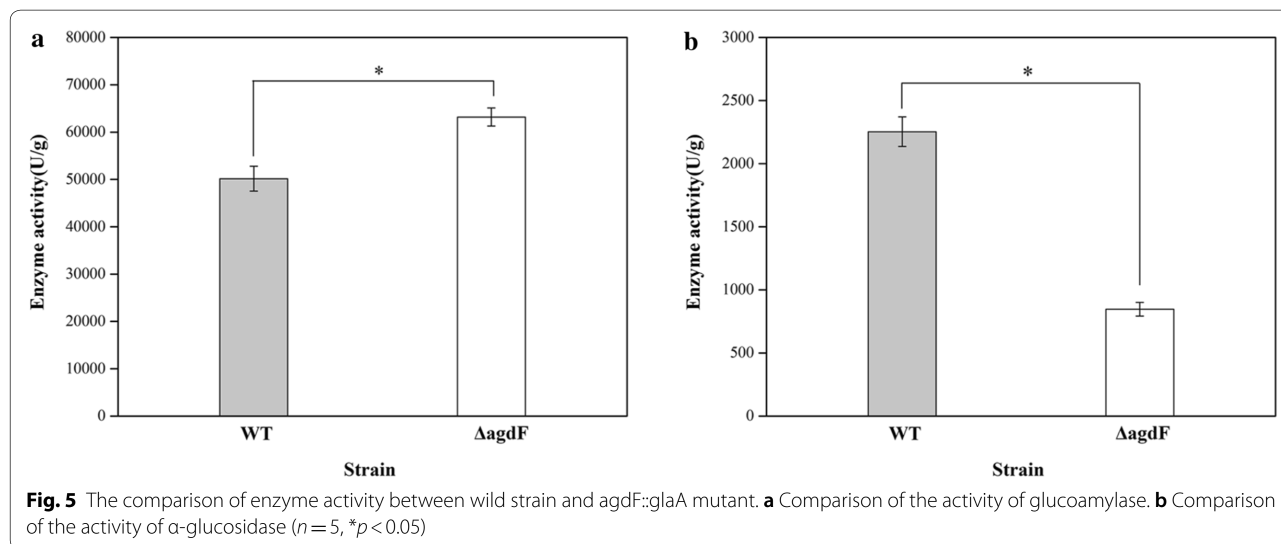

Fig. 5 The comparison of enzyme activity between wild strain and agdF::glaA mutant. **a** Comparison of the activity of glucoamylase. **b** Comparison of the activity of α-glucosidase ($n = 5$, $*p < 0.05$)

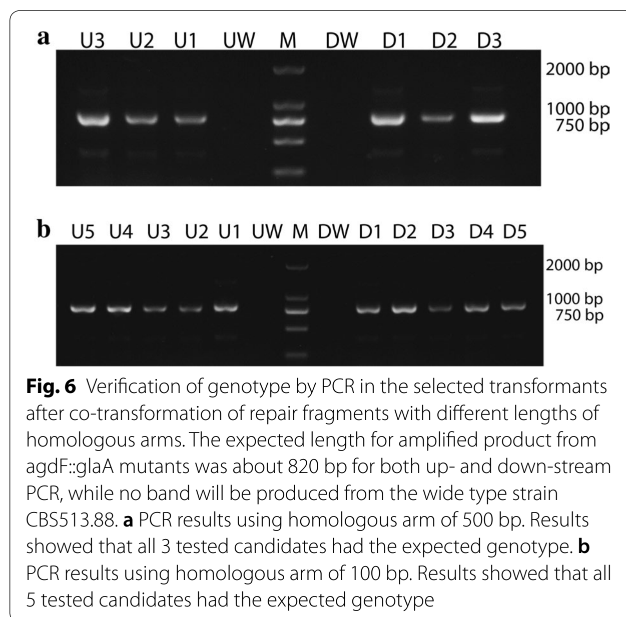

Fig. 6 Verification of genotype by PCR in the selected transformants after co-transformation of repair fragments with different lengths of homologous arms. The expected length for amplified product from agdF::glaA mutants was about 820 bp for both up- and down-stream PCR, while no band will be produced from the wide type strain CBS513.88. **a** PCR results using homologous arm of 500 bp. Results showed that all 3 tested candidates had the expected genotype. **b** PCR results using homologous arm of 100 bp. Results showed that all 5 tested candidates had the expected genotype

Although the RNA polymerase III promoters recently have been identified and used for expressing sgRNA in *A. niger* (Nødvig et al. 2018), RNA II promoters in RGR structure still have advantages. An important one is that some of them are inducible and can be regulated by environmental factors, such as carbon source and pH. This would benefit the expression of the RGR structure in special conditions and then control the function of the gene editing system in some extent.

The selection marker for plasmid construction and the length of repair fragment are among other issues that need to be considered when using this in vivo CRISPR system based on plasmid pAN7-1. In our study, there is no phenotype selection marker on the repair fragment, so the conidiophores germinated from primary positive colony on selective medium may have two types: correct mutants or off-targeted mutants in which the hygromycin resistant gene in plasmid integrated into another non-specific site. The two types can be further verified by PCR and DNA sequencing. For direct identification of gene knock-out, one antibiotic resistant gene for the Cas9/sgRNA expression plasmid and another antibiotic resistant gene or an auxotrophic marker gene for the repair fragment are recommended. However, a corresponding nutritional deficient host is needed for the latter condition.

Conclusion

In conclusion, we constructed a modified CRISPR/Cas9 system containing specific "RGR" structure and easy-to-switch element for a variety of target genes. The efficiency of the system was tested on the gene disruption of a pigment gene *olvA* in *A. niger*, and the mutation rate was up to 93%. In our gene replacement experiments targeting *agdF* genes for the enhanced expression of *glaA* gene, 3 secondary transformants out of 5 were confirmed to be the correct genotype and the activity of glucoamylase of *agdF::glaA* mutant was 25.9% higher than wild strain, while the activity of α-glucosidase of *agdF::glaA* mutant was 61.4% lower than wild strain. More interestingly, when the homologous arms of the repair fragment were shortened as 100 bp, the apparent recombination frequency is still high. The method may be applicable to more filamentous fungi that are difficult to genetically manipulate, such as *Cephalosporium capitis* (unpublished data), and so should have wide applications in genome editing of filamentous fungi.

Abbreviations

CAzymes: carbohydrate-active enzyme; CRISPR: clustered regularly interspaced short palindromic repeats; DNA: deoxyribonucleic acid; (DSB: double-strand break; HH: hammerhead; HDV: hepatitis delta virus; HDR: homology-directed repair; NHEJ: non-homologous DNA end joining; PAM: protospacer-adjacent motif; RGR: ribozyme–gRNA–ribozyme; WT: wild type.

Authors' contributions

YZ performed the experiments, analyzed data, and wrote the draft; LO designed the experiments and wrote the paper; YN and JC were involved in writing the manuscript. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

All materials are available in our lab.

Consent for publication

All the authors agree for publication.

Ethics approval and consent to participate

Not applicable.

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References

Carvalho NDSP, Mark A, Min JK, Vera M, Ram AFJ (2010) Expanding the ku70 toolbox for filamentous fungi: establishment of complementation vectors and recipient strains for advanced gene analyses. Appl Microbiol Biotechnol 87:1463–1473

Fuller KK, Chen S, Loros JJ, Dunlap JC (2015) Development of the CRISPR/Cas9 system for targeted gene disruption in *Aspergillus fumigatus*. Eukaryot Cell 14:1073–1080
Gao Y, Zhao Y (2014) Self-processing of ribozyme-flanked RNAs into guide RNAs in vitro and in vivo for CRISPR-mediated genome editing. J Integr Plant Biol 56:343–349
Katayama T, Tanaka Y, Okabe T, Nakamura H, Fujii W, Kitamoto K, Maruyama J (2016) Development of a genome editing technique using the CRISPR/Cas9 system in the industrial filamentous fungus *Aspergillus oryzae*. Biotechnol Lett 38:637–642
Lee SI, Lee KJ, Chun HH, Ha S, Gwak HJ, Kim HM, Lee JH, Choi HJ, Kim HH, Shin TS, Park HW, Kim JC (2018) Process development of oxalic acid production in submerged culture of *Aspergillus niger* F22 and its biocontrol efficacy against the root-knot nematode *Meloidogyne incognita*. Bioprocess Biosyst Eng 41:345–352
Li D, Qiu Z, Shao Y, Chen Y, Guan Y, Liu M et al (2013) Heritable gene targeting in the mouse and rat using a CRISPR–Cas system. Nat Biotechnol 31(8):681–683
Liu R, Chen L, Jiang Y, Zhou Z, Zou G (2015) Efficient genome editing in filamentous fungus *Trichoderma reesei* using the CRISPR/Cas9 system. Cell Discov 1:15007
Liu X, Xie C, Si H, Yang J (2017) CRISPR/Cas9-mediated genome editing in plants. Methods 121–122:94–102
Lu H, Cao W, Liu X, Sui Y, Ouyang L, Xia J, Huang M, Zhuang Y, Zhang S, Noorman H, Chu J (2018) Multi-omics integrative analysis with genome-scale metabolic model simulation reveals global cellular adaptation of *Aspergillus niger* under industrial enzyme production condition. Sci Rep 8:14404
Mata-Gomez M, Mussatto SI, Rodriguez R, Teixeira JA, Martinez JL, Hernandez A, Aguilar CN (2015) Gallic acid production with mouldy polyurethane particles obtained from solid state culture of *Aspergillus niger* GH1. Appl Biochem Biotechnol 176:1131–1140
Matsu-Ura T, Baek M, Kwon J, Hong C (2015) Efficient gene editing in *Neurospora crassa* with CRISPR technology. Fungal Biol Biotechnol 2:4
Mei Y, Wang Y, Chen H, Sun ZS, Ju XD (2016) Recent progress in CRISPR/Cas9 technology. J Genet Genomics 43:63–75
Meyer V, Arentshorst M, El-Ghezal A, Drews AC, Kooistra R, van den Hondel CA, Ram AF (2007) Highly efficient gene targeting in the *Aspergillus niger* kusA mutant. J Biotechnol 128:770–775
Nodvig CS, Nielsen JB, Kogle ME, Mortensen UH (2015) A CRISPR–Cas9 system for genetic engineering of filamentous fungi. PLoS ONE 10:e0133085
Nødvig CS, Hoof JB, Kogle ME, Jarczynska ZD, Lehmbeck J, Klitgaard DK, Mortensen UH (2018) Efficient oligo nucleotide mediated CRISPR–Cas9 gene editing in Aspergilli. Fungal Genet Biol 115:78–89
Pohl C, Kiel JA, Driessen AJ, Bovenberg RA, Nygård Y (2016) CRISPR/Cas9 based genome editing of *Penicillium chrysogenum*. Acs Synth Biol 5:754
Shan Q, Wang Y, Li J, Zhang Y, Chen K, Liang Z et al (2013) Targeted genome modification of crop plants using a CRISPR–Cas system. Nat Biotechnol 31(8):686–688
Shen B, Zhang J, Wu H, Wang J, Ma K, Li Z, Zhang X, Zhang P, Huang X (2013) Generation of gene-modified mice via Cas9/RNA-mediated gene targeting. Cell Res 23:720–723
Suyama Y, Muraki N, Kusunoki M, Miyake H (2017) Crystal structure of the starch-binding domain of glucoamylase from *Aspergillus niger*. Acta Crystallogr F Struct Biol Commun 73:550–554
Varalakshmi KN, Kumudini BS, Nandini BN, Solomon J, Suhas R, Mahesh B, Kavitha AP (2009) Production and characterization of alpha-amylase from *Aspergillus niger* JGI 24 isolated in Bangalore. Pol J Microbiol 58:29–36
Wang B, Chen J, Li H, Sun F, Li Y, Shi G (2017) Pellet-dispersion strategy to simplify the seed cultivation of *Aspergillus niger* and optimize citric acid production. Bioprocess Biosyst Eng 40:1–9
Weld RJ, Plummer KM, Carpenter MA, Ridgway HJ (2006) Approaches to functional genomics in filamentous fungi. Cell Res 16:31–44
Zheng X, Zheng P, Zhang K, Cairns TC, Meyer V, Sun J, Ma Y (2018) 5S rRNA promoter for guide RNA expression enabled highly efficient CRISPR/Cas9 genome editing in *Aspergillus niger*. ACS Synth Biol. https://doi.org/10.1021/acssynbio.7b00456
Zhu X, Sun J, Chu J (2018) High-content screening of *Aspergillus niger* with both increased production and high secretion rate of glucose oxidase. Biotechnol Lett 40:103–110

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