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Short Communication

Highly efficient single base editing in *Aspergillus niger* with CRISPR/Cas9 cytidine deaminase fusion

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ABSTRACT

Classic genome editing tools including ZFN, TALEN, and CRISPR/Cas9 rely on DNA double-strand breaks for genome editing. To prevent the potential hazard caused by double-strand breaks (DSBs), a series of single base editing tools that convert cytidine (C) to thymine (T) without DSBs have been developed extensively in multiple species. Herein, we report for the first time that C was converted to T with a high frequency in the filamentous fungi *Aspergillus niger* by fusing cytidine deaminase and Cas9 nickase. Using the CRISPR/Cas9-dependent base editor and inducing nonsense mutations via single base editing, we inactivated the uridine auxotroph gene *pyrG* and the pigment gene *fwnA* with an efficiency of 47.36%–100% in *A.niger*. At the same time, the single-base editing results of the non-phenotypic gene *prtT* showed an efficiency of 60%. The editable window reached 8 bases (from C2 to C9 in the protospacer) in *A. niger*. Overall, we successfully constructed a single base editing system in *A. niger*. This system provides a more convenient tool for investigating gene function in *A. niger*, and provides a new tool for genetic modification in filamentous fungi.

1. Introduction

The ability to precisely edit genomic DNA using the Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR associated protein (CRISPR/Cas) system has revolutionized the field of genome engineering. Under the direction of a single chimeric guide RNA (sgRNA), the endonuclease Cas9 is guided to a specific locus where it recognizes and cleaves specific DNA sequences in a targeted manner, producing double-strand breaks (DSBs) in the genome (Jinek et al., 2012). DSBs are primarily repaired by non-homologous end-joining (NHEJ), resulting in small fragment mutations such as base-pair deletion, insertion (indels), or substitution (Rodgers and McVey, 2016). In the presence of template donor DNA, DSBs can also activate homologous recombination repair (HDR) for accurate genetic modification with less efficiency than NHEJ in higher eukaryotic cells (Gaj et al., 2013). Simultaneously, this system can often induce in-frame indels in a protein-coding gene that can still produce functional proteins and frame-shifting indels, leading to the translation of out-of-frame polypeptide sequences that can be immunogenic and may have unknown effects in cells (Komor et al., 2016). Thus, approaches to precisely edit the genome while avoiding DSBs are needed.

To avoid DNA damage during gene editing and eliminate the need to deliver an HDR donor template, a new approach called “Base Editor” (BE) has been developed in mammalian cells (Komor et al., 2016). In this base-editing system, a Cas9 variant (D10A nickase (nCas9) or catalytically deficient Cas9 (dCas9)) is engineered with a rat cytidine deaminase (rAPOBEC1) and uracil glycosylase inhibitor (UGI) to convert cytidine (C) to uridine (U) at the targeted sites without generating DSBs; this process yields a high frequency of C→T (or G→A) substitutions in human cells. This genome editing approach has the advantage that it does not require double-stranded DNA breaks or the donor DNA template; instead, it directly edits single nucleotides in the editing window (Komor et al., 2016). The CRISPR/Cas9 base editing system has recently been applied for genome editing in mammalian cells (Billon

Abbreviations: CRISPR/Cas, Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR associated protein; DSBs, double-strand breaks; NHEJ, non-homologous end-joining; HDR, homologous recombination repair; BE, Base Editor; BEC, base editing complex; 5-FOA, 5-fluoroorotic acid; U, uridine; CD, Czapek–Dox medium; DPY, dextrose-peptone-yeast extract medium; UGI, uracil glycosylase inhibitor

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et al., 2017; Kim et al., 2017a; Komor et al., 2016; Liu et al., 2018; Ma et al., 2018), zebrafish (Qin et al., 2018; Zhang et al., 2017), plants (Shimatani et al., 2017; Tian et al., 2018; Zong et al., 2017) and bacteria (Banno et al., 2018; Gu et al., 2018; Wang et al., 2018b) due to its simplicity, high efficiency, low off-target rate and reduced genome damage. However, no reports have described the performance of the CRISPR/Cas9 single base editing system in filamentous fungi. This system potentially offers an alternative to the HDR-mediated base replacement approach in filamentous fungi, and if it works, it will greatly facilitate precise molecular breeding.

A. niger is a well-known model organism for studying filamentous fungi biology (Pel et al., 2007), and its naturally high secretion capacity has long been exploited in industrial biotechnology for the production of homologous and heterologous proteins (Lubertozzi and Keasling, 2009; Punt et al., 2002; Ward, 2012). The development of microbial genetic engineering has enabled microbial breeding to be freed from traditional methods such as UV mutagenesis and chemical reagent mutagenesis. The genome-editing tool CRISPR/Cas9 applied in filamentous fungi accelerated research on gene function and genetic engineering (Nodvig et al., 2015; Zheng et al., 2018). However, in genetic engineering, the common problems (DSBs and donor DNA) of the CRISPR/Cas9 system still exist. Whether a single base editing system would generate inheritable point mutations in filamentous fungi urgently requires testing, as this new tool has great potential for filamentous fungi functional genomics research by inducing desired point mutations. Here, we developed a single base editing system in *A. niger* using rat APOBEC1, and provide a simple and highly efficient base conversion method for *A. niger*.

2. Material and methods

2.1. Strains and culture conditions

Escherichia coli strain Match1 T1 was used for general cloning and cultivated aerobically at 37°C in Luria–Bertani (LB) broth. All *A. niger* strains used in this study were cultivated in PDA medium or modified minimal medium (Czapek–Dox (CD) medium) (Wang et al., 2010) containing 2% (w/v) glucose as a carbon source, 0.3% (w/v) $NaNO_3$, 0.1% (w/v) K_2HPO_4, 0.2% KCl, 0.05% (w/v) $MgSO_4{\cdot}7H_2O$, and 0.001% (w/v) $FeSO_4{\cdot}7H_2O$, pH 5.5, or in complete medium (dextrose-peptone-yeast extract (DPY) medium) (Zhou et al., 2016) containing 2% (w/v) glucose, 1% (w/v) peptone, 0.5% (w/v) yeast extract, 0.1% (w/v) K_2HPO_4, and 0.05% (w/v) $MgSO_4{\cdot}7H_2O$. When required, 1 mg/ml 5-fluoroorotic acid (5-FOA), 10 mM uridine or 100 ug/ml hygromycin was added.

2.2. Codon optimization of Cas9 and plasmid construction

DNAs encoding the rAPOBEC1-XTEN linker, Cas9 with a D10A mutation (nCas9), and UGI were *A. niger* codon-optimized (Supplementary Table S1) and synthesized by GenScript Biotech Corp. (Nan Jing, China). PCR primer sets Ptef-F/R, rAPOBEC1-F/R, nCas9-F/R, SGGS-UGI-F/R, and Ttef-F/R were used to amplify the *tef* promoter (Ptef), rAPOBEC1-XTEN, nCas9, UGI and ter terminator (Ttef). The complete pFC332-BEC vector was constructed by inserting these fragments into the plasmid pFC332 (Nodvig et al., 2015), which was linearized with *Pac*I and *Pml*I using a NEBuilder HiFi Assembly Kit (New England Biolabs (NEB)). All sgRNAs were synthesized by IGE Biotechnology LTD (Guang Zhou, China). For *pyrG* gene editing, the sgRNAs amplified by the primer sets pyrGsgRNA-F/R were introduced into the plasmid pFC332-BEC, which was digested by *Blg*II with NEbuilder. For *fwnA* gene editing, the sgRNAs amplified by the primer sets fwnAsgRNA-F/R were inserted into the plasmid pFC330 (Nodvig et al., 2015), which was linearized with *Pac*I and *Pml*I using NEbuilder. For *prtT* gene editing, the sgRNA amplified by the primer sets prtT-sgRNA-F/R were inserted into the *BglII* digested plasmid pFC332. All the primers used in this study are listed in Supplementary Table S2.

2.3. RNA isolation and quantitative real-time PCR (qRT-PCR) analysis

Total RNA was isolated using RNAisoPlus (TaKaRa) according to the manufacturer's instructions. Reverse transcription was performed with the PrimeScript RT-PCR Kit (TaKaRa). Gene specific primers for gpdA, nCas9 and rAPOBEC1 expression analysis were listed in Supplementary Table S2.Quantitative real-time PCR (qRT-PCR) was performed using the ABI 7500 Fast Real-Time PCR System. The results were analyzed using the $2^{-\Delta\Delta CT}$ method.

2.4. DNA extraction and Sanger sequencing

Genomic DNA (gDNA) from edited mutants was isolated via standard molecular biology methods (Sambrook and Russell, 2006). The genomic regions of interest were amplified using site-specific primers. PCR products were generated with PrimerSTAR Max DNA polymerase according to the manufacturer's instructions, and then separated on a 1% agarose gel. The PCR extraction products were then subjected to Sanger sequencing.

3. Results

3.1. Construction of the CRISPR/Cas9-rAPOBEC1 base editing system

A single base editing system requires two components: a single base editing complex (BEC) and sgRNA. Inspired by the successful establishment of the CRISPR/Cas9 system in *A. niger* (Nodvig et al., 2015), the plasmid pFC332 was used as the backbone to construct the BEC expression cassette. We designed and constructed the plasmid pFC332-BEC (Fig. 1A). In the pFC332-BEC plasmid, the *tef* promoter was used to drive the expression of the fusion protein of a deaminase (rat APOBEC1), Cas9 nickase (Streptococcus pyogenes Cas9D10A) and uracil glycosylase inhibitor (UGI). The rAPOBEC1 deaminase was linked to the N-terminus of the Cas9 protein via a 16-aa XTEN linker, and UGI was linked to the C-terminus of the Cas9 protein via the SGGS linker. For sgRNA transcription, the U6 promoter from *Aspergillus oryzae* was selected as a potent sgRNA transcriptional control element (Fig. 1B). The resulting plasmid pFC332-BEC was transformed into the industrial strain *A. niger* SH2 (Yin et al., 2014) and the model strain *A. niger* FGSC A1279 (Wang et al., 2018a). The nCas9 and deaminase (rAPOBEC1) genes were detected by qRT-PCR in the transformant carrying pFC332-BEC but not in the wild-type strain (Fig. 1C). Strains with BEC did not exhibit any growth defects (Fig. 1D), indicating that the expression of BEC is not detrimental to the growth of *A. niger*.

3.2. Inactivation of the uridine auxotrophic gene pyrG in A. niger via the BEC system

To test whether the BEC nuclease complex could catalyze site-specific base conversion in vivo in the *A. niger* genome, we selected the *pyrG* gene as a target. *pyrG* encodes an orotidine 5′-phosphate decarboxylase, and the deletion mutant is auxotrophic for uridine (Melin et al., 2008). To assess the feasibility of the BEC nuclease complex in *A. niger*, one target site of the endogenous gene *pyrG* (An*pyrG*/An12g03570) was selected (Fig. 2A). The sgRNA expression cassette of An*pyrG* was linked to the pFC332-BEC vector in the *Bgl*II restriction endonuclease site (Fig. 2B) and transformed into the host strain SH2. Thirteen transformants were selected randomly and genomic DNA was extracted. Genomic regions spanning the target sites were amplified by PCR and subsequently subjected to sequencing. Sanger sequencing chromatograms for An*pyrG* PCR products showed a set of T peaks in the target sites, indicating that the intended base substitutions did occur (Fig. 2C).

To further evaluate the efficiency of BEC-based genome editing in *A.*

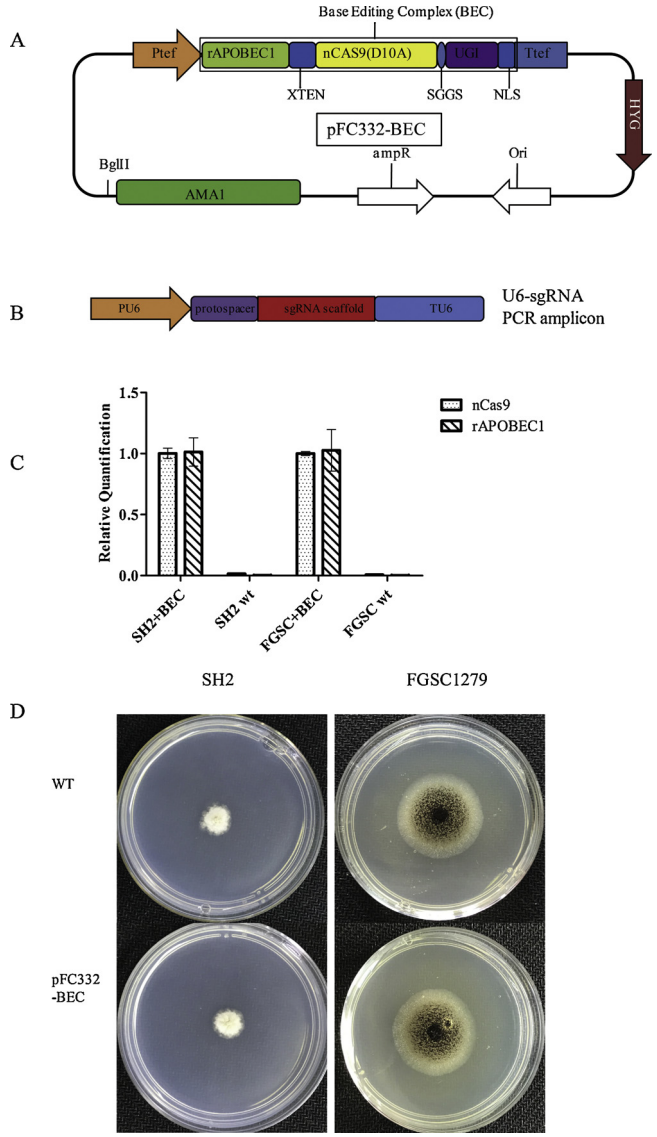

Fig. 1. Construction of the CRISPR/Cas9 base editing system. (A) A schematic map of the base editing plasmid pFC332-BEC. APOBEC1, a rat cytidine deaminase, is linked at the N terminus with nickase Cas9D10A (nCas9) by the XTEN linker. UGI (uracil DNA glycosylase inhibitor) is linked at the C terminus with nCas9 by the SGGS linker. The *tef* promoter, an *A. nidulans* constitutive promoter used to drive the expression of BEC; (B) the sgRNA expression cassette. The U6 promoter of *A. oryzae* used to drive the expression of sgRNA; (C) the growth of strains transformed with pFC332-BEC. The first column shows the presence of the *A. niger* SH2 wild-type strain and pFC332-BEC transformant on CD medium cultivated at 30 °C for 5 days. The second column shows 1-μl conidial suspensions (104 conidia/μl) of the *A. niger* FGSC1279 wild-type strain and pFC332-BEC transformant that were inoculated onto PDA medium and incubated for 4 days at 30 °C.

niger, we constructed two additional sgRNAs for targeting AN*pyrG* and introduced them into the ΔprtT strain (not published). The ΔprtT strain is a transformant that has been subjected to *prtT* gene knockout by HDR with the *Aspergillus nidulans pyrG* gene (AN*pyrG*/AN6157). Nine and twenty-four transformants were selected randomly by hygromycin and 5-FOA dual selection. The Sanger sequencing chromatograms for 9 ANpyrG1 PCR products revealed a cytosine substitution (C→T) at position 5 in all transformants (Fig. 2D). In addition, the sequencing results of 24 ANpyrG2 PCR products showed that the cytosine at position 6 in 23 mutants was mutated to thymine, and one of them had double mutations at positions 3 and 6 (Fig. 2E and F). The *pyrG* point mutation mutants exhibited uridine auxotrophy (Fig. 2G). The inactivation of the *pyrG* marker by BEC was carried out, and the results accurately proved that the BEC system works well in *A. niger*.

3.3. Evaluation of the editing efficiency of BEC system in A. niger by inactivating pigment gene fwnA

The results of editing the *pyrG* gene demonstrated that the BEC system could work well in *A. niger* and that the editing efficiency reached 100%. However, this was obtained by editing the auxotroph marker (*pyrG*) with another selection marker (*hygB*), which does not show the real editing efficiency of BEC in *A. niger*. To further investigate the editing efficiency of the BEC system, we evaluated BEC as a knockout tool via inducing premature stop codons (TAA, TGA, or TAG) by converting C:G base pairs to T:A base pairs for four codons (CAA, CGA, CAG, TGG) in the coding strand of the target gene *fwnA*. The *A. niger fwnA* gene is an ortholog of *Aspergillus oryzae* wA, which encodes a polyketide synthase (PKS) required for conidial pigmentation (Jorgensen et al., 2011; Nodvig et al., 2015). The *fwnA* mutant forms white conidia. Three target sites with the potential to generate stop codons in the endogenous gene *fwnA* were designed (Fig. 3A). We removed the Cas9 expression cassette in pFC330 (Nodvig et al., 2015) by double digestion with *Pac*I and *Pml*I and then fused the three sgRNA expression frameworks for *fwnA* base editing into pFC330 via NEbuilder (Fig. 3B). These three pFC330-sgRNAs were individually co-transformed with pFC332-BEC into *A. niger* FGSC A1279 for *fwnA* mutagenesis.

The examination of 19 pFC332-BEC/pFC330-fwnA1sgRNA transformants revealed that 9 harbored at least one C to T substitution in the target region (mutation efficiency of 47.36%). Substitution at positions 4, 5, and 8 of the protospacer was found (Fig. 3C). Three of the mutants were homozygous (C4, C5 and C8 to T4, T5 and T8) (Fig. 3C). Among the 6 heterozygotes were one single base substitution, three double-base substitutions and two triple-base substitutions (Supplementary Fig. S1). For fwnA2, 21 of the 31 colonies (67.74%) carried a C→T conversion, and the remaining 10 maintained the wild-type sequence (Supplementary Fig. S1). Of the 21 mutants, 18 had double mutations from C6 to T6 and C7 to T7, 14 of which were homozygous (Fig. 3D). In addition, a single point mutation occurred in three transformants, including two homozygous C7 to T7 mutations (Fig. 3D) and one heterozygous C6 to T6 mutation (Supplementary Fig. S1). Likewise, in the protospacer of fwnA3, 33 of the 35 mutants had a C to T conversion, and the editing efficiency reached 94.29%; 24 of these mutations were homozygous (Fig. 3E), and 9 were heterozygous (Supplementary Fig. S1). In the C→T mutants, a stop codon was introduced inside the gene that inactivated the *fwnA* gene, thereby yielding a phenotype of white spores (Fig. 3F).

3.4. Evaluation of the editing efficiency through editing the non-phenotypic gene prtT using the BEC system

The *pyrG* and *fwnA* are two phenotype genes, and their inactivation can be directly determined from the phenotype plate. In order to avoid relying on phenotypes for transformant screening, the protease regulator *prtT* was selected for non-stop codon mutations. Within the gene, we chose TTTGACCAAAAGCATCATCA as the protospacer, in which there are two Cs at position 6 and 7. The 6th C mutated to T resulted in an amino acid mutation from threonine (T) to isoleucine (I) at position 142. The 7th C mutated to T resulted in a synonymous mutation. The synthetic prtT-sgRNA was ligated to the *BglII* digested pFC332-BEC vector by ligase, and the constructed pFC332-BEC-prtTsgRNA was transferred into *A.niger* CBS513.88 (Fig. 4A). Fifteen transformants were randomly picked for sequencing. The sequencing results showed that 9 transformants were edited, among which 6 mutants had double base mutation, and synonymous mutations occurred at position 7 in three transformants (Fig. 4B). The editing efficiency of non-phenotypic

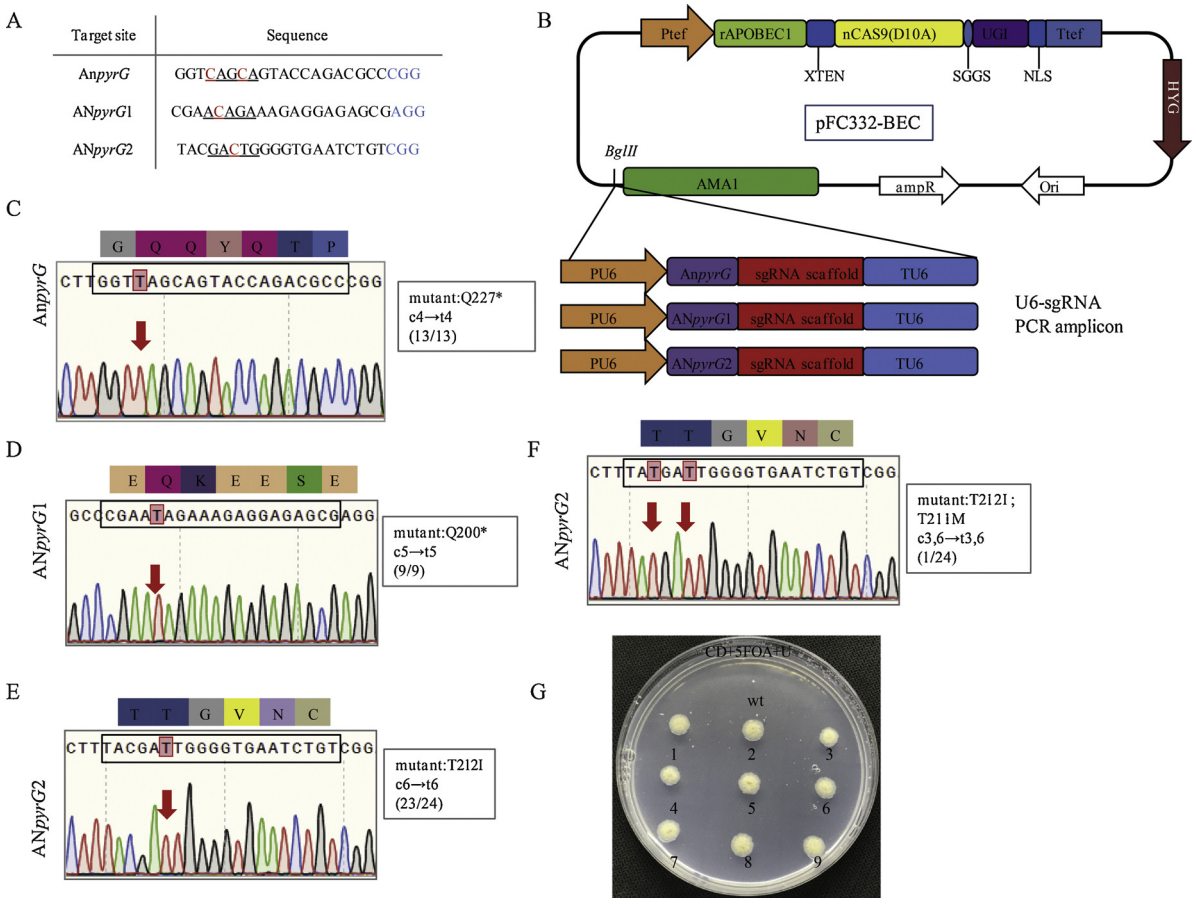

Fig. 2. The pFC332-BEC system enables highly efficient C → T conversion in aconidial *A. niger* SH2. (A) Target sites chosen for the uridine auxotrophy gene *pyrG*. The PAM sites are colored blue. The potential editable window is underlined, and Cs are colored red. (B) BEC and sgRNA co-expression plasmid. Each vector for *pyrG* base editing was linked to the pFC332 plasmid via a *Bgl*II site. (C and D) Q227 of the An*pyrG* gene (C) and Q200 of the AN*pyrG* gene (D) in the *A. niger* SH2 strain were successfully mutated to a stop codon (*) with 100% efficiency by the base editing system. The black box indicates the protospacer. The red arrow indicates the mutated base. The small gray box on the right indicates the proportion of this mutant of the total. (E and F) Single amino acid (E) and double amino acid (F) mutations of the AN*pyrG* gene. (G) The wild-type (wt) and base editing mutant strains were spot cultivated for 5 days at 30 °C on CD agar media with 5-FOA and uridine (U). wt: wild-type strain of *A. niger* SH2; An*pyrG* (1,2,3); AN*pyrG*1 (4,5,6); AN*pyrG*2 (7,8,9). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

gene *prtT* reached 60%. The above results indicated that even if a non-phenotypic gene is selected for single base editing, mutants in which a base mutation occurs can be easily obtained in *A. niger*.

4. Discussion

Currently, the CRISPR/Cas9 system has been established in multiple filamentous fungi, including *A. oryzae* (Katayama et al., 2016), *A. niger* (Nodvig et al., 2015; Zheng et al., 2018), *A. nidulans* (Nodvig et al., 2015), *A. fumigatus* (Fuller et al., 2015), and *Trichoderma reesei* (Liu et al., 2015). CRISPR/Cas9 has been widely used for gene insertion, DNA fragment deletion and multi-site mutation, but single base editing without DSBs has not been reported in filamentous fungi. In this study, we successfully used the BE3 single base editing system established by Liu et al.(Komor et al., 2016) to edit the uridine synthesis gene *pyrG*, the pigment gene *fwnA* and the non-phenotypic gene *prtT* in *A. niger*.

In the CRISPR/Cas9 system, the transcription of sgRNA requires a special promoter. Previously, various promoters have been used for sgRNA expression in the process of constructing the CRISPR/Cas9 platform in filamentous fungi, including the hammerhead enzyme (Nodvig et al., 2015; Zheng et al., 2018) and the U6 promoter (Katayama et al., 2016; Zheng et al., 2018), which were genetically edited in different hosts. Recent studies have reported that sgRNA transcription levels can be increased by using 5sRNA from *A. niger* as a promoter (Zheng et al., 2018), and the transcription of sgRNA could be driven by endogenous tRNA promoters in *A. niger* (Song et al., 2018). In this study, we tried to edit a target gene with 5sRNA as a promoter but did not obtain the correct transformants. Finally, we used the 568-bp U6 promoter and the 138-bp U6 terminator from *Aspergillus oryzae* (Katayama et al., 2016) to control sgRNA transcription (Fig. 1B).

According to the target sites we selected, the efficiency of single base editing was between 47% and 100%, which is more efficient than the editing efficiency in animals (Komor et al., 2016) and plants (Zong et al., 2017) but lower than that in prokaryotic microorganisms (Gu et al., 2018; Wang et al., 2018b). In terms of gene deletion, the efficiency in filamentous fungi (including *A.niger, A.oryzae, A.fumigatus* and *A.nidulans*) using the traditional active CRISPR/Cas9 system was over 95% in the *kusA*-deficient strains (Nodvig et al., 2018; Song et al., 2018; Zhang et al., 2016a), which was more efficient than BEC system. To improve the base editing efficiency, the system has been upgraded from the original BE1 (rAPOBEC1-16aa XTEN linker-dCAS9) (Komor et al., 2016) to BE4 (rAPOBEC1-32 aa XTEN linker-nCAS9-double UGI) (Komor et al., 2017). We chose nCas9 instead of dCas9 for base editing because Liu et al. reported that the base editing efficiency of BE3 (rAPOBEC1-16aa XTEN linker-nCAS9-UGI) was increased by an additional two- to six-fold compared to that of BE2 (rAPOBEC1-16aa XTEN linker-dCAS9-UGI) (Komor et al., 2016) and the process of constructing BE4 is more complicated. Recently, it was reported that the use of Cpf1 instead

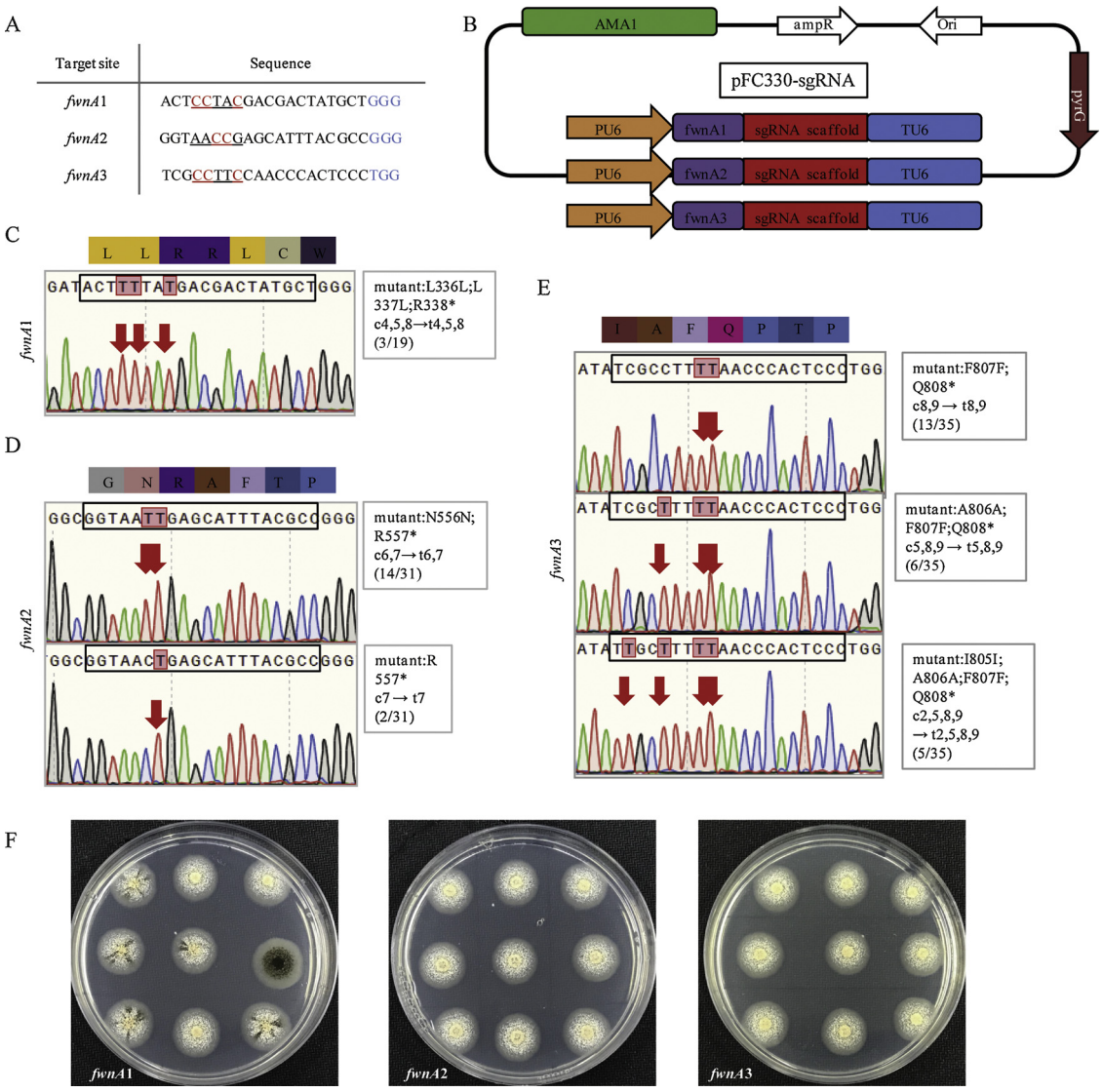

Fig. 3. Inactivation of the conidial pigmentation gene *fwnA* in *A. niger* FGSC1279 using the pFC332-BEC system. (A) Target sites chosen for the pigmentation gene *fwnA*. The PAM sites are colored blue. The potential editable window is underlined, and Cs are colored red. (B) A sketch map of the sgRNA expression plasmid for *fwnA* editing. The sgRNA cassette was linked to the pFC330 plasmid via NEbuilder. (C, D and E) Editing results for 3 different sites of the *fwnA* gene. (F) Phenotypes of the mutants obtained by the base editing system. Conidial suspensions (104 conidia/μl) of each mutant were inoculated onto PDA medium and incubated for 4 days at 30 ˚C. Homozygous mutants exhibited completely white spores. Heterozygous mutants showed black and white mixed spores. Synonymous mutants showed pure black spores. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

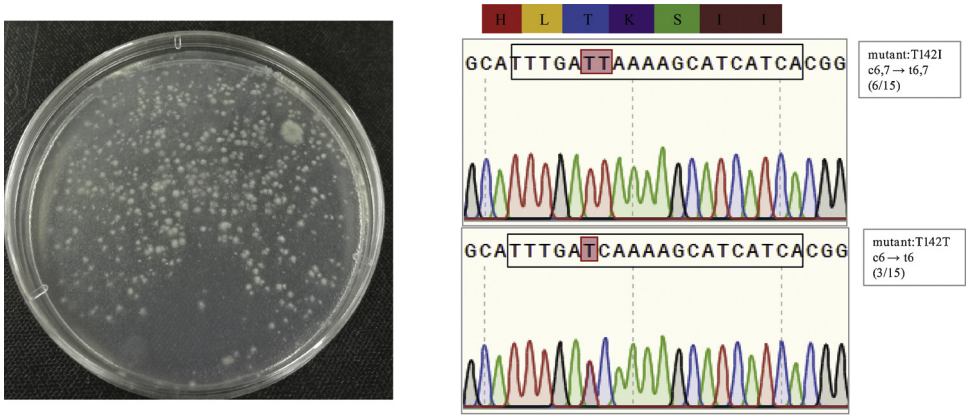

Fig. 4. Mutation of the protease regulator *prtT* using CRISPR/nCas9.(A) Transformation of the plasmid pFC332-BEC-prtTsgRNA containing nCas9 and *prtT*-sgRNA targeting prtT. (B) Sequence analysis of *prtT*-mutant strain revealed single base or double bases mutations.

of nCas9 could also achieve single base editing (Li et al., 2018). At the same time, some researchers have optimized the deaminase (Kim et al., 2017b) and improved the precision of gene editing. These attempts show that there is still much room to improve the efficiency of single base editing systems.

In human cells, a base-editing system was used to create C to T substitutions in a window from position 4–8 in the protospacer (Komor et al., 2016), counting from the distal end to the protospacer-adjacent motif (PAM). Subsequent applications of this system in plants found that the editable window is 7 bases (C3 to C9) (Shimatani et al., 2017; Zong et al., 2017). In *Staphylococcus aureus*, the Cs at positions 4–8 could be mutated to Ts with 100% efficiency, whereas the Cs at positions 2, 3, and 9–12 could not be mutated to Ts by the pnCasSA-BEC system (Gu et al., 2018). Our results showed that the editable window is 4–8 for fwnA1, 6–7 for fwnA2, 2–9 for fwnA3 and 6–7 for *prtT*. In general, the position of the editable window in *A. niger* is 2–9, which is wider than that in mammals. We suspect that the reason for this difference in editable windows is specific to the species and the different protospacer sequences. Interestingly, Wang et al. (Wang et al., 2018b) recently reported that base editing preferentially occurred at positions 1–5 when cytidine deaminase rAPOBEC1 was replaced by AID, indicating that deaminase changes can broaden the editing window and facilitate the selection of the appropriate deaminase based on the position of C in the protospacer sequence. Among the mutant strains we obtained, most were homozygous, but some were heterozygous. The reason might be that *A. niger* cells are multinuclear. Despite this, the probability of obtaining a homozygous strain is greatly increased compared to animal and plant cells.

The development of single-base editor provides an important tool for directed editing and correction of key nucleotide variations in the genome. Since this technology does not introduce DNA double-strand breaks, it demonstrated its potential application value in microbial field such as gene inactivation, mutation of key amino acid, and research on key cis-elements. However, it is worth noting that the risk of off-target has always been a concern (Zhang et al., 2016b). If CRISPR/Cas9 and its derivatives are used clinically, off-target effects may cause many side effects including cancer. Although a number of off-target detection schemes have been introduced before, the methods used in the past either relied on the computer software predictions (Singh et al., 2015) or relied on the high-throughput sequencing (Kim et al., 2015; Tsai et al., 2015) to detect DSB production. These methods have some limitations, and it is not possible to detect off-target mutations, especially single nucleotide mutations with high sensitivity. Therefore, the true off-target rate of CRISPR/Cas9 and its derivatives has been controversial. Recently, Yang et al. (Zuo et al., 2019) and Gao et al.(Jin et al., 2019) from the Chinese Academy of Sciences established methods for detecting single-base off-target efficiency in 2-cell embryo and rice, respectively. Their findings confirmed that gene editing technologies represented by BE3 have higher off-target risk compared to traditional active cas9, allowing the world to re-examine the risks of this emerging technology. Single-base editing technique has a long way to go before they can be applied widely and safely.

In conclusion, we successfully applied a single base editing system (derived from mammals) in *A. niger* that works well in both industrial host and standard model strains. Because nCAS9 is an inactivating nuclease, DNA double strands are not cleaved, thereby avoiding unnecessary deletion or insertion. Therefore, C to T conversion in a 20-bp protospacer can be accurately performed. Of course, this system also has similar limitations to the CRISPR/cas9 system, so it cannot be edited optionally on the genome due to the limitation of PAM sites. Meanwhile, this technique is not competitive with the previous published Cas9 system in *A. niger* for gene knock out and has higher off-target efficiency, but it is worthwhile to expect that single-base editing technology will provide another option for gene editing of filamentous fungi, such as mutations in key amino acids, mutations in key cis-acting elements, mutations in the upstream open reading frame (uORF) of the key regulatory genes. In general, this technology provides a toolkit for the genetic engineering of filamentous fungi and expands the methods for molecular breeding in *A. niger*.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.micres.2019.03.007.

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