

Development of an Efficient and Stable Transformation System for *Aspergillus Oryzae* Based on the pyrG Gene

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Research

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Abstract

Background *Aspergillus oryzae* is an ideal host for expressing heterologous and homologous genes. An efficient and stable transformation system is the key to the successful expression of the gene of interest in *A. oryzae*.

Results To improve the expression efficiency of the gene of interest in *A. oryzae*, we constructed the uridine/uracil auxotrophic strains *A. oryzae* RIB40 Δ *pyrG* by Ultraviolet (UV) mutagenesis of *pyrG* gene deletion which would be used as a host for further transformation. In addition, a novel and efficient expression vector pBC-hygro.4 was constructed, including the *pyrG* cassette gene, His-Tag, *amyB* promoter and terminator, and green fluorescent protein *GFP* marker. pBC-hygro.4 transformed *A. oryzae* RIB40 Δ *pyrG* efficiently via the PEG-CaCl₂-mediated transformation method, and the stability of pBC-hygro.4 was tested by detecting the expression of the *GFP* reporter gene. Through phenotyping and sequencing verification, we successfully obtained a uridine/uracil auxotrophic strains *A. oryzae* RIB40 Δ *pyrG*. At the same time, the developed vectors are fully functional for heterologous expression of the *GFP* fluorescent proteins in the *A. oryzae* RIB40 Δ *pyrG*.

Conclusion Our work provides a new method that can be applied to other filamentous fungi to develop similar fungal transformation systems based on auxotrophic/nutritional markers for food-grade recombination applications.

Background

Aspergillus oryzae is an important industrial microorganism has been widely used in the fermentation industry and food processing^[1-3]. It was recognized as a safe strain by the US Food and Drug Administration (FDA)^[4, 5]. *A. oryzae* is often used as an ideal host cell for heterologous gene expression, protein secretion and metabolite production^[6]. Because it has the ability to secrete the target protein into the culture medium, which makes the purification process of products become simpler and more efficient^[7, 8].

The selection of appropriate selectable markers is necessary for the transformation process, which can make the screening of positive transformants more effective. Studies have shown that *A. oryzae* is to be inherently resistant to most common antibiotics such as hygromycin B, geneticin(G418) and bleomycin^[9, 10]. Thus, it is difficult to obtain transformants by selection with commonly used antibiotics. Therefore, it is necessary to develop a transformation system based on nutritional markers. The *pyrG* gene, ending orotidine-5'-monophosphate(OMP) decarboxylase participates in uridine biosynthesis and is also a target for the antimetabolite 5-fluoroorotic acid(5-FOA), which is as a selective marker and its auxotrophic strain as a genetic transformation host^[11-14]. OMP decarboxylase is able to convert the non-toxic 5-fluoroorotic acid (5-FOA) into the toxic product as 5-fluorouracil, which consequently inhibits the growth of wild-type fungi^[15, 16]. However, the corresponding *pyrG* mutants can grow normally on the medium containing 5-

FOA. Therefore, 5-FOA was often used to identify uridine/uracil auxotrophic mutant strains in different filamentous fungi^[17].

Currently the most commonly used methods for filamentous fungi transformation including PEG (polyethylene glycol)-mediated protoplast transformation (PMPT) and *Agrobacterium tumefaciens*-mediated transformation (ATMT)^[18, 19]. In comparison with the ATMT method, PEG-CaCl₂-mediated transformation has some disadvantages, such as low transformation efficiency, difficulty in obtaining high concentrations of viable protoplasts, high percentages of transient transformants^[20, 21]. However, due to its simplicity in technical operation and equipment required, the PEG-CaCl₂-mediated transformation remains the most commonly used method to conduct transformation in filamentous fungi^[22].

In recent years, *A. oryzae* as an expression host for heterologous gene expression has attracted a lot of interest, so the construction of highly efficient *A. oryzae* expression vectors is particularly important. Many previous studies have shown that vectors constructed with plasmid pBC-hygro as the backbone can be effectively transformed in various filamentous fungi, such as *Aspergillus fumigatus*^[23], *Aspergillus sydowii*^[24], *Thermomyces lanuginosus*^[25], and *Podospira anserine*^[26]. Taka-amylase (amyB) promoters and terminators were widely used for the efficient expression of many genes. In this study, a novel vectors pBC-hygro.4 carrying *A. oryzae pyrG* marker was constructed for the transformation of uridine/uracil auxotrophic *A. oryzae* via an adapted PEG-CaCl₂-mediated transformation method. The construction of the *A. oryzae* expression system will lay the foundation for the successful expression of foreign genes in *A. oryzae* and provide a new method that can be applied to other filamentous fungi to develop similar fungal transformation systems based on auxotrophic / nutrition markers.

Results

Determination of 5-FOA minimum use concentration

The resistance of wild-type *A. oryzae* RIB40 to different concentrations 5-FOA was analyzed. To isolate a selection agent suitable for *pyrG* deletion mutants screening, the growth of wild-type *A. oryzae* RIB40 on CD plates containing 5-FOA at different concentrations (0.5, 0.75, 1.0, 1.25, 1.5 mg/mL) was observed. The result shown that 1.0 mg/mL 5-FOA inhibited the growth of wild-type *A. oryzae* RIB40 for the longest time (Fig. 1). Therefore, 1.0 mg/mL 5-FOA was chosen as the selection agent for *pyrG* deletion mutants screening.

Screening and characterization of *pyrG* deletion mutants

We obtained many mutant strains by UV mutagenesis. After preliminary screening through 5-FOA plates, we succeeded to gain five resistant strains that can grow on 5-FOA plates, named P1-P5. Then, mutant strains to resistant to 5-FOA were transferred simultaneously to the CD, CD + Uri/Ura and CD + Uri/Ura + 5-

FOA (1 mg/mL) to examine their growth (Fig. 2). The result showed that the wild type was unable to grow on CD + Uri/Ura + 5-FOA plates and grew well on CD, CD + Uri/Ura. However, the *pyrG* deletion mutants P1-P5 were unable to grow on CD plates without uridine and uracil and grew well on CD + Uri/Ura, CD + Uri/Ura + 5-FOA plates (Fig. 2), indicating that strains P1-P5 were uridine auxotrophs. These uridine auxotrophs were detected by further PCR to identify *pyrG* deletion.

For the five uridine auxotrophs, the predicted 1.8 kb *pyrG* cassette fragments (Fig. 3) were amplified from the genomic DNA of the mutants using the primer pair *pyrGF/pyrGR*. The P1-P5 strain amplified a specific band of about 1.8 kb, which was consistent with the wild-type *A. oryzae* RIB40 *pyrG* cassette sequence size. We selected strain P1 and purified its PCR product by gel recovery kit and sent it to invitrogen for sequencing. The sequencing results were compared with the wild type *A. oryzae* RIB40 *pyrG* cassette sequence, nucleotide sequencing revealed that strain containing *pyrG* mutations were successfully obtained. Strain P1 contain 1 bp deletion and 1 bp insertion at the target sequence of the *pyrG* gene (Fig. 4). These mutations all cause frameshifts, which suggests that the *pgrG* gene does not function in the strains.

Dna Manipulation And Plasmid Construction

First, we verified the resistance of *A. oryzae* RIB40 $\Delta pyrG$ to hygromycin B. The spore suspension was cultivated in CD medium containing 0, 0.5, 1.0, 1.5, 2.0 mg/mL for 4 days at 28 °C. We found that hygromycin B has no inhibitory effect on *A. oryzae* RIB40 $\Delta pyrG$, so we need to delete the hygromycin B gene sequence. Analysis of the plasmid pBC-Hygro's sequence revealed that it contained three *Nde* I restriction sites, which were located at sequences 3861 bp, 4539 bp, and 4991 bp, and the sites at 4539 bp and 4991 bp were located in hygromycin B gene sequence. Therefore, we can choose to delete the *Nde* I restriction sites at 3861 bp and use *Nde* I digestion to destroy the hygromycin B resistance gene so that it loses its activity. We use site-directed mutagenesis to mutate base C at 3861 bp to base T. For specific methods, refer to the site-directed mutation kit. Design mutant primers TbndeF/TbndeR and sequencing primers TucxF/TucxR. The primer sequences are shown in Table 2.

Table 2
Primers and their sequences used for PCR in this study.

Primers	sequences(5'-3')
pyrGF	GGGAATTCATGCGAAGGTAAGTGCTTCT
pyrGR	GGACTAGTTGGCTAGGCTCTGACTCG
TbndeF	TACAGAATAGCGCGGCATCTATATGTTAGT
TbndeR	AGATGCGCGCGCTATTCTGTAGAGCTCTGGG
TucxF	GTTGGAGGCCCTGTCTCCG
TucxR	TTCTGAGTGTTCAAGATTGAAGCT
pyrgbdhF	GGAATTCCATATGTGAAAGACTGCTGCAAAGCC
pyrgbdhR	GGAATTCCATATGAAGCAGTCGTACATACATGG
GFPf	CACTAGCTAGCATGAGTAAAGGAGAAGAAC
GFPPr	CCCAAGCTTTTATTTGTATAGTTCATCCATG
amybtF	ATAAGAATGCGGCCGCGGGTGGAGAGTATATG
amybtR	ATAAGAATGCGGCCGCAATTCTTGAGGACCATTAC

Mutagenesis reactions were performed in a 50 μ L volume containing 10 μ L 5 \times FastAlteration Buffer, 2 μ L template plasmid DNA, 2 μ L of each TbndeF/TbndeR, 1 μ L FastAlteration DNA Polymerase, and 33 μ L RNase-Free ddH₂O. Reactions were thermal cycled: 95°C for 2 min, followed by 18 cycles of 94°C for 20 sec, 60°C for 10 sec, and 68°C for 210 sec, then a final incubation of 68°C for 5 min. Reactions were cooled on ice and digested with 5 units of *Dpn* I for 1 h at 37°C to cleave methylated and hemimethylated parental DNA, but not the newly synthesized mutant DNA molecules. The mutant plasmid was verified by DNA-sequencing using the sequencing primers TucxF/TucxR (Table 2), Name the correct mutant plasmid pBC-Hygro.1.

The primers used in this study are listed in Table 2. The novel protein expression vector pBC-Hygro.4 consisted of Three fragments (One, Two, Three). Fragment One, the *pyrG* cassette as the auxotrophic marker was amplified from the genome of the wild-type *A. oryzae* RIB40 strain using primers pyrgbdhF/pyrgbdhR. Fragment Two includes the promoter *amyB* amplified from wild-type *A. oryzae* RIB40 strain with primers amybF/amybR, and a green fluorescent protein *GFP* marker amplified from plasmid pHBT-GFP-NOS using primers GFPf/GFPPr, which is fused by SOE-PCR. The fused fragment amgfp was inserted into pET30b at the restriction enzyme sites *Sal*I/*Xho*I, resulting in the pET-amgfp expression vector. Meanwhile, the amgfp + His-Tag amplified from plasmid pET-amgfp with primers amygfpHTf/amygfpHTr. Fragment Three includes terminator amyB amplified from the genome of the wild-type *A. oryzae* RIB40 strain with primers amybtF/ amybtR. All of the three DNA fragments were

ligated with ClonExpress MultiS One Step Cloning Kit and T₄DNA ligase to generate pBC-Hygro.4. All the constructed plasmids were confirmed by PCR and DNA-sequencing.

A. oryzae RIB40ΔpyrG protoplast preparation, transformation

A. oryzae RIB40ΔpyrG protoplasts were prepared with reference to the BestBio company Filamentous Fungal Protoplast Preparation Kit. The transformation was performed by the PEG-CaCl₂ transformation method as described by Unkles *et al*^[49].

GFP reporter gene expression

DNA of the plasmid pBC-Hygro.4 20 μL was mixed with 200 μL of protoplasts. The transformation was performed by the PEG-CaCl₂ transformation method as mentioned above. The transformants were verified by PCR with the primers amygfpHTf and amygfpHTr. Then, the positive colony was cultured in fermentation medium at 30 °C for 72 h to obtain the positive recombinants. The fermentation broth was transferred to a glass slide and observed with a fluorescence microscope. The exposure time was 0.1 s, the excitation light was 488 nm, and the emission light was 597 nm.

Discussion

It is well-known that *A. oryzae* is an excellent host used to express homologous and heterologous proteins^[29]. In recent years, it has been widely used in the production of heterologous proteins and has received increasing attention^[8]. *A. oryzae* has many advantages as an expression host, such as a strong ability to produce and secrete proteins, and strong post-translational modification^[30, 31]. Therefore, it is important to develop an effective *A. oryzae* transformation system.

There are three kinds of selective markers commonly used in filamentous fungi transformation: auxotrophic complementary genes, drug resistance genes, and genes that can make the host use some unusual carbon or nitrogen sources^[22]. The transformation systems, which were based on drug resistance genes as main selectable markers have the advantage of the availability of a wild type strain as a host and easy operation^[32]. During the transformation of filamentous fungi, most choose to use some genes encoding resistance as selection markers, such as hygromycin and phleomycin^[33, 34]. However, studies have shown that *A. oryzae* is insensitive to most the common antibiotics such as hygromycin B, geneticin(G418) and bleomycin^[9, 10]. Moreover, in this study, we found that hygromycin B cannot inhibit *A. oryzae* RIB 40 ΔpyrG growth. Compared with the other systems, the auxotrophic complementary genetic transformation system has proved to be more efficient.

As a result, transformation systems for *A. oryzae* have been developed mainly based on nutritional markers. The *pyrG* gene, ending orotidine-5'-monophosphate (OMP) decarboxylase participates in uridine biosynthesis but is also a target for the antimetabolite 5-fluoroorotic acid (5-FOA). Thus, *pyrG* genes in filamentous fungi have been widely used as nutritional/auxotrophic markers for fungal transformation^[35]. In the present study, the *pyrG* deletion mutants were successfully obtained by UV mutagenesis. Mutants were selected with 5-FOA, which selectively allows the growth of *pyrG* deletion strains. As expected, these strains exhibited uridine/uracil auxotrophy and resistance to 5-FOA. Meanwhile, the deletion of the *pyrG* gene was further confirmed by genome PCR and DNA sequencing. UV mutagenesis is a simple method, we successfully obtained *A. oryzae* RIB 40 $\Delta pyrG$ through this method. However, mutations induced by UV mutagenesis also have disadvantages such as unstable and uncertain direction of mutation^[36].

With the widespread use of *A. oryzae* in the expression of heterologous proteins, it is particularly important to construct a safe and efficient expression vector for *A. oryzae*. In this study, a novel expression-stable vectors pBC-hygro.4 was constructed and stably expressed in *A. oryzae* RIB 40 $\Delta pyrG$ strain. These vectors possess the changeable components including the *pyrG* cassette gene, His-Tag, amyB promoter, and terminator amyB. The *pyrG* transformation system has a great advantage of a lower false-positive background in transformation experiments (Hao *et al.* 2008). Promoters and terminators are important expression elements that play a key role in the efficient expression of genes. Studies have shown that different promoters have different efficiency, and strong promoters can effectively improve the stability and transcription level of mRNA^[37]. There are some strong promoters for gene expression in *A. oryzae*, such as *amyB*, *melO*, *glaA*, *gpdA* and *tef1*^[38-41]. Based on previous reports, the *amyB* promoter is much better than the *gpdA* promoter in the regulation of gene expression in *A. oryzae*^[42]. Therefore, in this study, the *amyB* promoter and terminator have been considered as the strongest elements in the construction of expression vectors for *A. oryzae* to produce homologous and heterologous proteins. GFP is the most common fluorescent proteins used for tagging filamentous fungi, which can as a reporter to test the stability of pBC-Hygro.4 in *A. oryzae* RIB 40 $\Delta pyrG$ ^[43]. In the present study, we could directly detect the expression of the GFP reporter gene by recombinant *A. oryzae* RIB 40 $\Delta pyrG$ cultures presented remarkable green fluorescence in the mycelia. These results suggest that the *gfp* gene was successfully expressed in *A. oryzae* RIB 40 $\Delta pyrG$, which demonstrated that vector pBC-Hygro.4 can be used for the expression of foreign genes in *A. oryzae*.

In addition, *A. oryzae* has the ability to express large amounts of various enzymes, such as α -amylase^[44], glucoamylase^[45, 46], and α -glucosidase^[47]. Compared with eukaryotic expression systems based on *Pichia pastoris* and *Saccharomyces cerevisiae*, *A. oryzae* expression has higher safety, so it can be widely used in food industry. Therefore, the *A. oryzae* expression system constructed in this study provides a prerequisite for the expression of more foreign genes in the future. In future studies we can use this system to express more heterologous and homologous proteins, such as xylanase, cellulose Enzymes, proteases, etc.

Conclusions

In summary, in the present study, we successfully obtained *A. oryzae* RIB 40 Δ *pyrG* strain by UV mutagenesis which would be used as a host for further transformation. In addition, we have constructed a new type of safe and efficient *A. oryzae* expression vector, which can stably express in *A. oryzae* RIB 40 Δ *pyrG*. This will be a new method that can be applied to other filamentous fungi to develop similar fungal transformation systems based on auxotrophic/nutritional markers for food-grade recombination applications.

Materials And Methods

Bacterial strains and plasmids

All bacterial strains and plasmids used in this work are listed in Table 1. *A. oryzae* RIB40, a wild-type strain, was stored previously in our laboratory. *Escherichia coli* DH5 α was used for routine plasmid construction and maintenance, which grown in Luria-Bertani (LB) medium.

Table 1
Strains and plasmids used in this study.

Strains or plasmids	Description	Source
Strains		
<i>A. oryzae</i> RIB40	The wild-type strain	This lab
<i>A. oryzae</i> RIB40 Δ <i>pyrG</i>	The <i>pyrG</i> deletion mutant generated from the RIB40 strain	This study
RIB40 Δ <i>pyrG</i> ::GFP	The auxotrophic RIB40 Δ <i>pyrG</i> strain transformed with pEX2B carrying the <i>pyrG</i> marker and the GFP reporter gene under control of the inducible amyB promoter	This study
<i>E. coli</i> DH5 α	<i>E. coli</i> expression host	This lab
plasmids		
pBC-Hygro	Filamentous fungal expression vector; Hyg ^R	This lab
pHBT-GFP-NOS	pHBT carrying gfp gene	This lab
pBC-Hygro1.0	Hygromycin antibiotic gene deleted in pBC-Hygro	This study
pBC-Hygro4.0	pBC-Hygro1.0 + <i>pyrG</i> marker + GFP reporter gene + His-tag + Promoter amyB + Terminator amyBt	This study

Media And Culture Conditions

The *A. oryzae* wild strain RIB40 was cultivated on Czapek-Dox (CD) medium (2% sucrose, 0.3% NaNO₃, 0.1% KH₂PO₄, 0.05% MgSO₄●7H₂O, 0.2% KCl, 0.05% NaCl, 0.002% FeSO₄●7H₂O, 2.0% agar, pH5.5). 5-fluoroorotic acid (5-FOA) medium containing 0.6% NaNO₃, 0.05% KCl, 0.08% KH₂PO₄, 0.104% K₂HPO₄, 0.05% MgSO₄●7H₂O, 1% glucose, 0.122% uridine 2.0% agar, 0.5–1.5 mg/mL 5-FOA. CD + Uri/Ura medium containing 0.5% uridine and 0.2% uracil was used for the selection of *pyrG* deletion mutants. CD + 5-FOA + Uri/Ura medium containing 1 mg/mL 5-FOA, 0.5% uridine and 0.2% uracil was used for the growth of *pyrG* mutants. *E. coli* DH5a was used for the construction, propagation, and amplification of hybrid plasmids.

Spore Preparation And Determination Of 5-foa Minimum Use Concentration

The wild-type *A. oryzae* RIB40 was grown on (CD) medium for 3–5 days until the spores are mature. Sterile distilled water was added to the culture plate and fungal spores were released from the mycelium by scraping the agar surface with a sterile glass spreader under a laminar flow hood. The resulting mixture was collected with a micropipette and filtered through 500 mesh nylon cloth before centrifugation at 4000 rpm for 10 min. The spore pellet was washed twice with sterile distilled water and resuspended in sterile distilled water to obtain the spore suspension. The spore concentration was examined using a thoma counting chamber and adjusted to 1.0×10^7 spores/ml for fungal transformation. The prepared spore suspension was pipetted into 200 μ L of 5-FOA medium, which is containing 5-FOA at different concentrations, and allowed to stand for 4–6 days at 28 °C to observe the growth of *A. oryzae*.

Screening and characterization of *pyrG* deletion mutants

Pipette 3 mL of the 1.0×10^7 spores/ml spore suspension prepared above into a Petri dish, place it under UV light (30 W) and irradiated for 5 minutes. The prepared spore suspension was pipetted into 500 μ L of 5-FOA medium, the above plate was wrapped with tin foil and allowed to stand for 3 to 4 days at 28 °C. Mutant strains to resistant to 5-FOA were transferred simultaneously to the CD, CD + Uri/Ura and CD + Uri/Ura + 5-FOA (1 mg/mL) to examine their growth. The mutants, which were unable to grow on the CD minimal medium, but could grow normally on CD + Uri/Ura as well as on CD + Uri/Ura + 5-FOA, were selected for single spore isolation. These mutant strains were tested for mitotic stability for five successive generations on CD + Uri/Ura + 5-FOA before re-growing on CD medium.

The wild-type strains and the uridine/uracil auxotrophic mutants were cultivated in CD medium or CD + Uri/Ura for 4 days, at 28 °C. The obtained mycelia were used for genomic DNA extraction, and the primer *pyrGF/pyrGR* were used for PCR screening of *pyrG* deletion mutants.

Abbreviations

PyrG

orotidine-5'-monophosphate decarboxylase

UV mutagenesis

Ultraviolet mutagenesis

GFP

Green Fluorescent Protein

PMPT

polyethylene glycol-mediated protoplast transformation

ATMT

Agrobacterium tumefaciens-mediated transformation

FDA

Food and Drug Administration

OMP

orotidine-5'-monophosphate

5-FOA

5-fluoroorotic acid

PDA medium

potato dextrose agar medium

Uri/Ura

uridine/uracil

CD medium

Czapek-Dox (CD) medium

Declarations

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Not applicable.

Author Contributions

- Caixia Zhou performed the experiments, authored or reviewed drafts of the paper.
- Yujun Wan and Huipeng Yao performed the experiments.
- Hui Chen authored or reviewed drafts of the paper.
- Zizhong Tang conceived and designed the experiments, approved the final draft.
- Yirong Xiao and Zhi Shan analyzed the data.
- Tongliang Bu and Hong Chen prepared the figures and/or tables.
- Gang Wang contributed reagents/materials/analysis tools.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article and its additional files.

Ethics approval and consent to participation

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no conflicts of interest.

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Figures

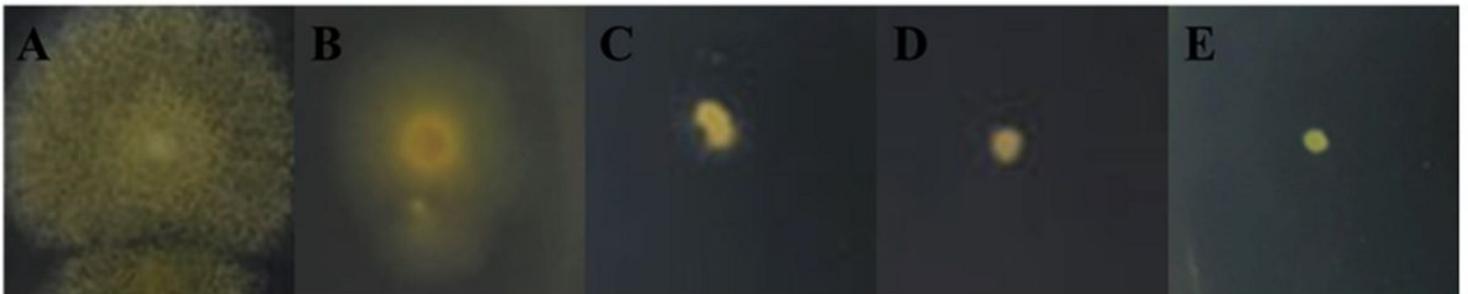


Figure 1

Effects of 5-FOA on the growth of *A. oryzae* RIB40 A-E: Growth of *A. oryzae* RIB40 in 0.5, 0.75, 1.0, 1.25, 1.5 mg/mL 5-FOA plates

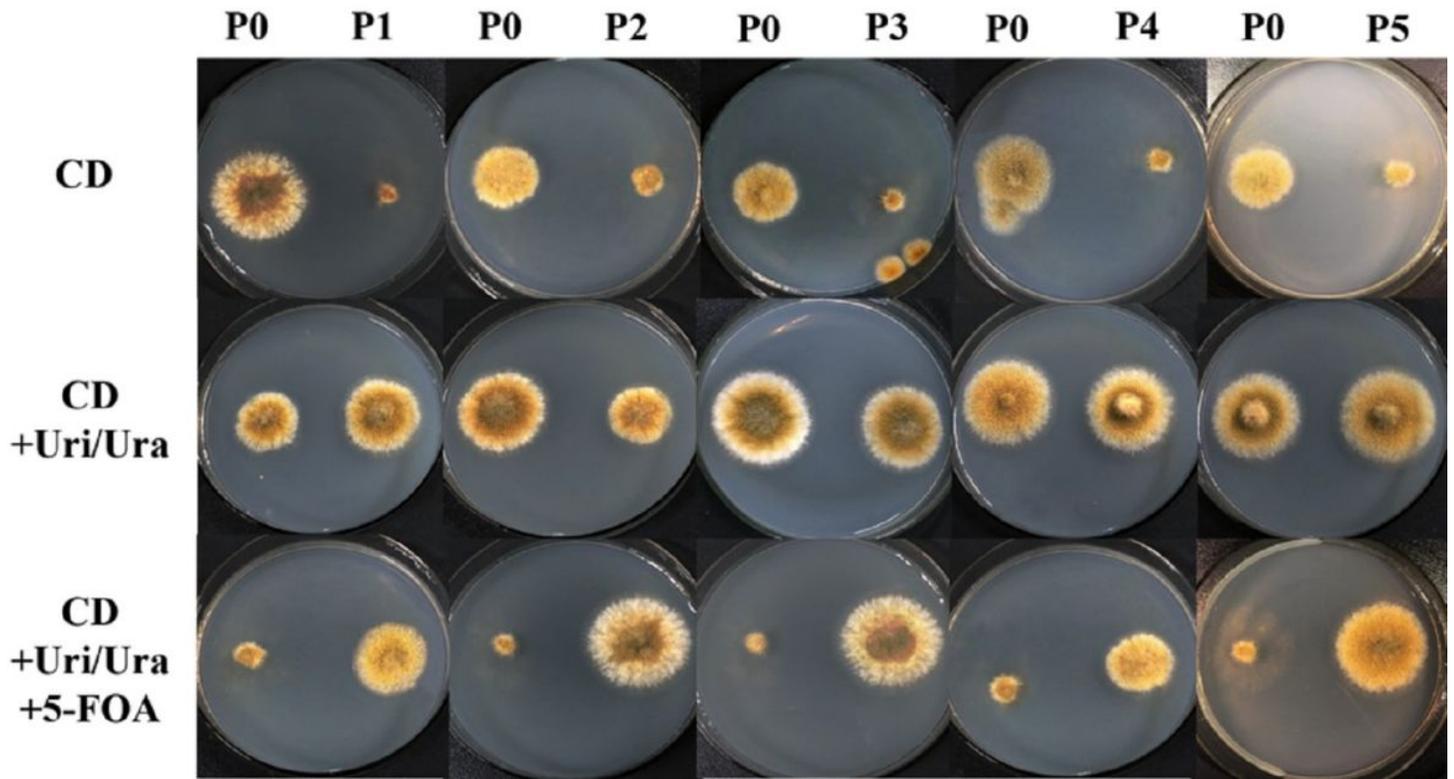


Figure 2

Characterization of *pyrG* deletion mutants generated from the *A. oryzae* RIB40 strain P0: wild type RIB40; P1-P5: the *pyrG* deletion strains.

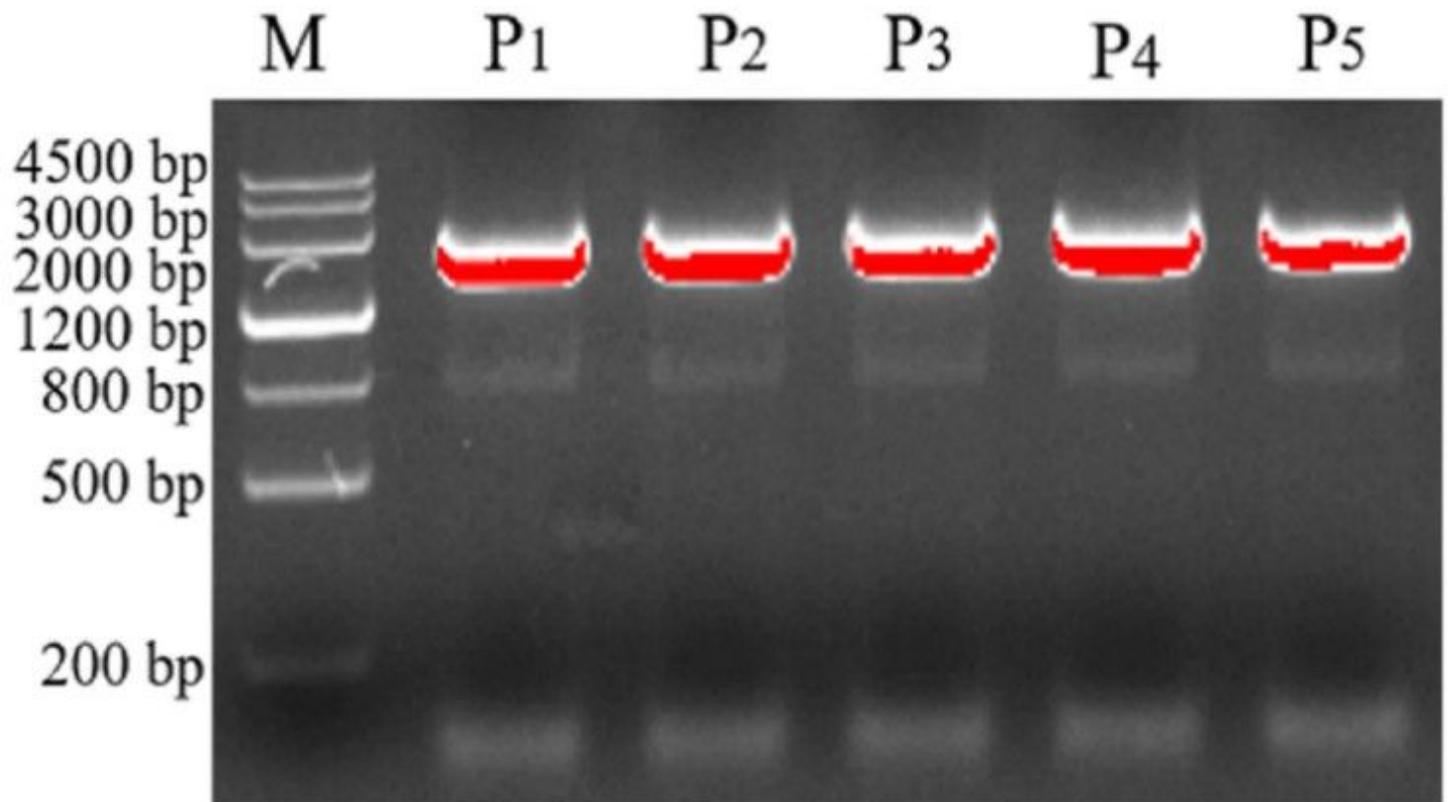


Figure 3

The pyrG deletion mutants was confirmed by PCR M: Marker III; P1-P5: pyrG mutant strains

```
p0.txt ACCGGTCAATATACTACTTCTTCTGTTGACTATGCTCGGA 600
p1.txt ACCGGTCAATATACTACTTCTTCTGTTGACTATGCTCGGA 600
Consensus accggtcaatatactacttcttctgttgactatgctcgga

p0.txt AGTATAA AAGTTTGTGATGGGATTCGTC TCGACACGTC 639
p1.txt AGTATAA AAGTTTGTGATGGGATTCGTC TCGACACGTC 639
Consensus agtataa aagtttgtgatgggattcgtc tcgacacgtc

p0.txt ACCTTGGCGAGGTTTCAGTCTGAAGTTAGCTCGCCTTCGGA 679
p1.txt ACCTTGGCGAGGTTTCAGTCTGAAGTTAGCTCGCCTTCGGA 679
Consensus accttggcgaggtttcagtctgaagttagctcgccttcgga

p0.txt GGAGGAAGATTTTGTCTTCACGACAGGTGTCAACCTC 719
p1.txt GGAGGAAGATTTTGTCTTCACGACAGGTGTCAACCTC 719
Consensus ggaggaagatTTTgtcttCacgacaggtgtcaacctc
```

Figure 4

pyrG sequence comparison RIB40 and mutant strain p0: RIB40; p1: mutant strain

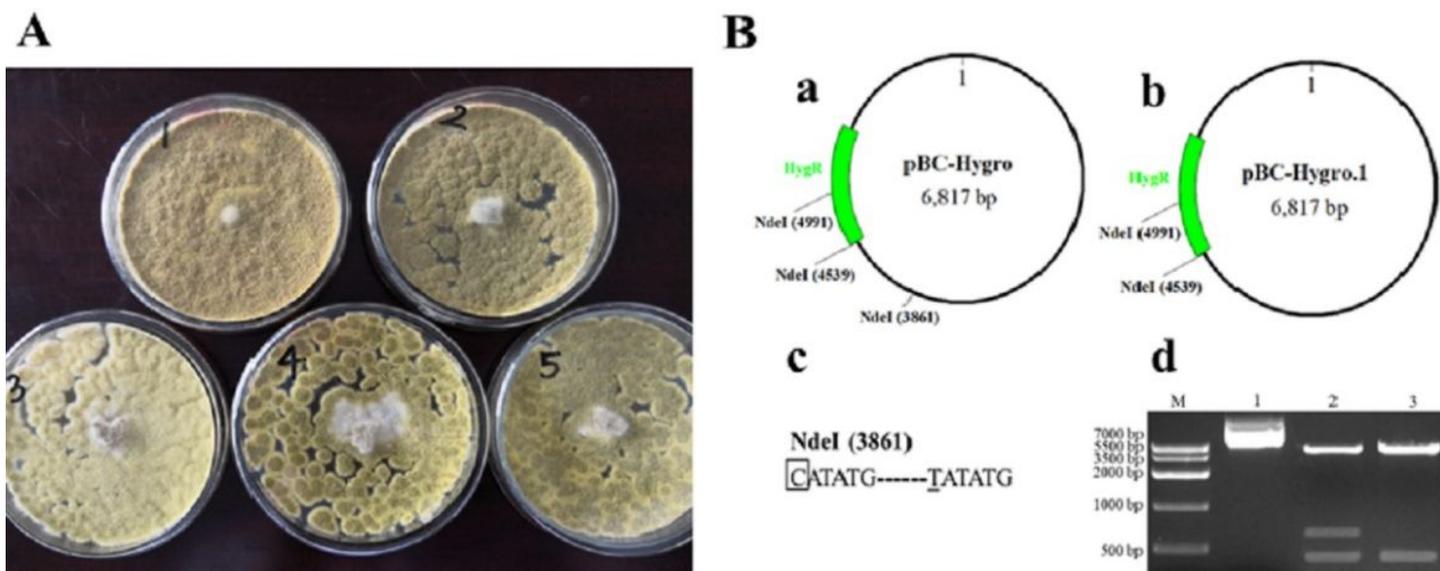


Figure 5

Inhibition hygromycin B on growth of RIB40 Δ pyrG 1-5: The growth of RIB40 Δ pyrG on the MM plate containing hygromycin B containing 0, 0.5 mg/mL, 1.0 mg/mL, 1.5 mg/mL, 2.0 mg/mL respectively. Fig. 5B The scheme for destroying hygromycin sequences of plasmid pBC-Hygro A: Mapping of pBC-Hygro plasmid marking Nde I locus; B: Mapping of pBC-Hygro mutant plasmid marking Nde I locus; C: Mutation 3861 restriction site. The box and underling indicate deleted nucleotides mutation sites; D: Verification of mutant plasmid pBC-Hygro.1 by Nde I. M, DNA Marker IV; 1, plasmid pBC-Hygro; 2, plasmid pBC-Hygro by Nde I digestion; 3, mutant plasmid pBC-Hygro.1 by Nde I digestion.

pBC-Hygro.txt	GGGGCTGACTGCCTCAGGTGGGGCAGTGCTAGTGTGTGTA	160
pBC-Hygro.1.txt	GGGGCTGACTGCCTCAGGTGGGGCAGTGCTAGTGTGTGTA	160
Consensus	ggggctgactgcctcaggtggggcagtgctagtgtgtgta	
pBC-Hygro.txt	CCGACCCGCAGGATTGGTGCTTTGCCAGAGCTCTACAGA	200
pBC-Hygro.1.txt	CCGACCCGCAGGATTGGTGCTTTGCCAGAGCTCTACAGA	200
Consensus	ccgaccgcgaggattggtgctttgccagagctctacaga	
pBC-Hygro.txt	ATAGCGCGCGCATCCATATGTTAGTTCTGCAATTTTCTTG	240
pBC-Hygro.1.txt	ATAGCGCGCGCATCCATATGTTAGTTCTGCAATTTTCTTG	240
Consensus	atagcgcgcgcatc atatgttagttctgcaattttcttg	
pBC-Hygro.txt	TATCGGTGCTGTGACTCATACTTCCCCCTTGGCTGGCCT	280
pBC-Hygro.1.txt	TATCGGTGCTGTGACTCATACTTCCCCCTTGGCTGGCCT	280
Consensus	tatcggtgctgtgactcatacttcccccttggctggcct	
pBC-Hygro.txt	TGCGGCAACCAATAAGAACGCACAGTGAAATCTTGCGGGT	320
pBC-Hygro.1.txt	TGCGGCAACCAATAAGAACGCACAGTGAAATCTTGCGGGT	320
Consensus	tgcggcaaccaataagaacgcacagtgaaatcttgcgggt	

Figure 6

Partial sequence alignment of plasmids pBC-Hygro and pBC-Hygro.1

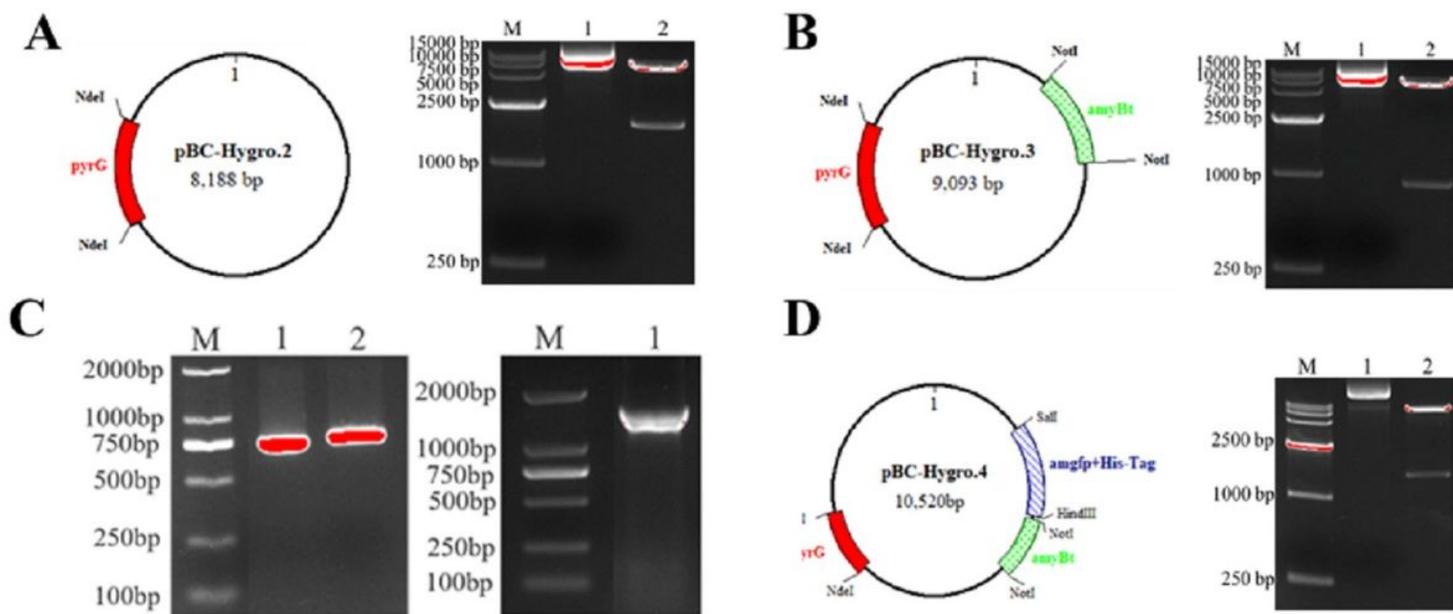


Figure 7

Schematic illustration of pBC-hygro, and binary expression vectors pBC-hygro.2, pBC-hygro.3, and pBC-hygro.4 A: pBC-hygro.2 was constructed using a pBC-hygro backbone, containing a pyrG gene expression

cassette; B: pBC-hygro.3 was generated by insertion of amyB (*A. oryzae* amyB terminator); C: Fusion of target genes amyB(*A. oryzae* amyB promoter) and GFP by fusion PCR(M: DNA Maker DL2000; 1, amyB; 2, GFP; M: DNA Maker DL2000; 1, fusion gene amgfp)Lanes from left to right; D: pBC-hygro.4 was generated by insertion of the fusion gene amgfp.

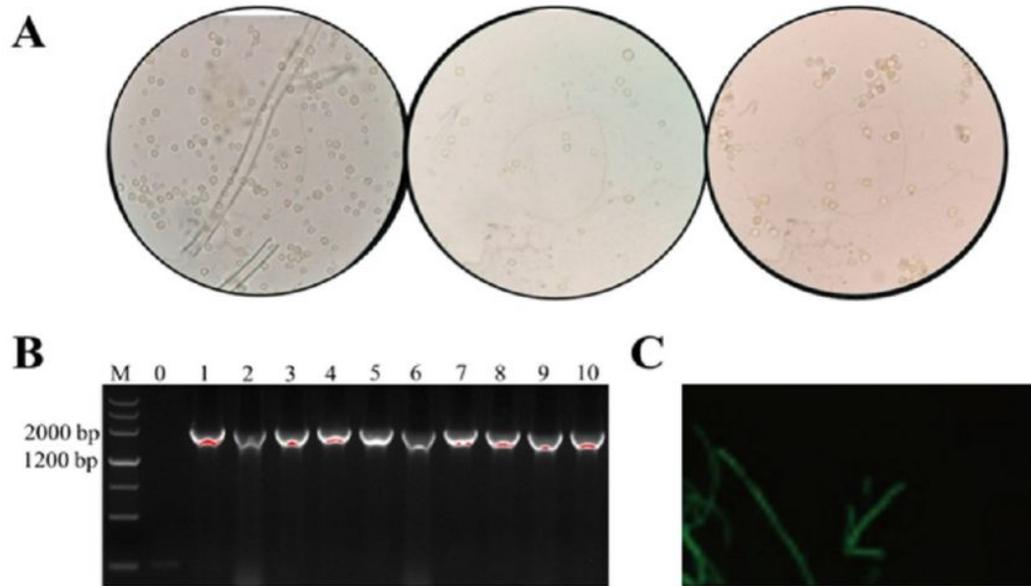


Figure 8

Verification of *Aspergillus oryzae* expression vector A: From left to right, protoplast enzymatic hydrolysis for 2 h, 3 h, and 4 h; B: Verification of transformant colony by PCR; M: DL2000, 0: negative control, 1-10: transformant colonies; C: Observation of GFP expression by fluorescence microscopy