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RESEARCH ARTICLE

Application of Membrane Filtration Method to Isolate Uninuclei Conidium in *Aspergillus oryzae* Transformation System Based on the *pyrG* Marker

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Abstract The purpose of this study was to develop a membrane filtration method to isolate uninuclei conidium of *Aspergillus oryzae*, then the method was adopted to develop a transformation system of *A. oryzae. A. oryzae* 3.951 contained 1–4 nuclei in each conidium. The percentages of uninucleate and binucleate conidia were approximately 16.15 and 74.22%, respectively. Conidia suspension was filtrated with a 5-μm membrane to overcome the bottleneck caused by multinucleate conidia and to remove excess multinucleate conidia before UV mutagenesis. Uninucleate conidia of 5-fluoroorotic acid (5-FOA)-resistant strains were enriched by filtration with a 3-μm membrane. The *pyrG* mutant strain AS11 was obtained and GFP-*pyrG* was successfully transformed into AS11.

Keywords *Aspergillus oryzae*, filtration method, *pyrG* marker, transformation system, uninucleate conidia

Introduction

Aspergillus oryzae is an industrially important filamentous fungus that has been used for over a thousand years in traditional Oriental fermented food. It is also a promising and excellent host for the production of homologous and heterogonous proteins because it is biological safety and can secrete copious amounts of protein (1). Since 2005, when the whole genome sequence of *A. oryzae* has been mapped (2), there has been an increasing amount of research on genetic research in *A. oryzae* (3,4), and molecular genetic manipulation is a promising approach in improving industrial strains and developing strains that produce recombinant proteins, an effective transformation system was required in those studies.

A practical transformation system in *A. oryzae* generally involves vectors that carry suitable selective marker genes, which either complement with an auxotrophic mutation in the recipient strain or introduce antibiotic resistance. However, developing a dominant selection marker for *A. oryzae* is difficult because it is resistant to most of the antimicrobial agents (5). Furthermore, the use of antibiotic resistance markers has been avoided in food applications for security considerations. Thus, the homologous complementation of an auxotrophic marker is preferred for *A. oryzae* transformation systems. However, gene disruption on *A. oryzae* is extremely difficult because it lacks a sexual life cycle and can produce multinucleate conidia, which prevent the characterization of its auxotrophic mutant. Thus, the purification of homokaryotic auxotrophic mutant is time-consuming and labor-intensive. Although it has previously been reported auxotrophic mutation in *A. oryzae* (6,7), single and double auxotrophic mutant strains were previously constructed and used as suitable transformation hosts, *A. oryzae* niaD300 (*niaD*$^-$) (8) and NS4 (*niaD*$^-$, *SC*$^-$) (9) from the model strain RIB40 is currently one of the most frequently used host strains. *A. oryzae* NSR1 (*niaD*$^-$, *SC*$^-$, *adeA*$^-$), NSR13 (*niaD*$^-$, *SC*, *adeB*$^-$), and quadruple auxotrophy in *A. oryzae* were constructed via UV mutagenesis of *A. oryzae* (*niaD*$^-$, *SC*$^-$) (10,11), there is no description about how to solve multinucleate problems. Uninucleate conidia are considered to be useful in mutagenesis for genetic analysis and strain breeding, uninucleate conidia

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has been isolated by fluorescence-activated cell sorting (FACS) (12) and membrane filtration method (13).

In the present study, in order to overcome bottleneck of multinucleate conidia in the genetic manipulation, membrane filtration method was employed and a host-vector system for *A. oryzae* was developed using *pyrG* as a dominant selectable marker. To verify the constracted transformation system, a plasmid (GFP-*pyrG*) in *A. oryzae* carrying *pyrG* and GFP was transformed into AS11 by using the PEG-$CaCl_2$ transformation method.

Materials and Methods

Fungal strains and media *Aspergillus oryzae* wild-type strain 3.951 was provided by the Chinese Academy of Sciences, A Czapek-Dox (CD) medium (3 g/L $NaNO_3$, 2 g/L KCl, 1 g/L KH_2PO_4, 0.5 g/L $MgSO_4$, 0.02 g/L $FeSO_4 \cdot 7H_2O$, 10 g/L glucose, and 18 g/L agar at pH 5.5) was used for fungal growth. The medium used for uridine auxotrophy tests contained 0.5%(v/v) Triton X-100, 10 mmol/L uridine, and 15 g/L 5-fluoroorotic acid (FOA) (Sigma-Aldrich, St. Louis, MO, USA).

Staining of nuclei in conidia and microscopy Nuclei staining in conidia was carried out as described by Prigione with modification (14). Briefly, 5%(v/v) sodium hypochlorite solution was added to conidia suspensions (5×10^7 conidia/mL), which were incubated for 5 min at room temperature. The suspensions were washed by 2 consecutive centrifugations at 3,800×*g* for 10 min. Conidia was then resuspended in a phosphate buffer saline solution (pH 7.4) with a final concentration of 5×10^6 conidia/mL. The suspensions were incubated with 8 μg/mL 4,6-diamidino-2-phenylindole (DAPI) and centrifuged at 35×*g* for 2 h in the dark at 25°C. Stained conidia were observed using a Nikon Eclipse E-600 epifluorescence microscope (Nikon, Tokyo, Japan) equipped with a 100-W mercury lamp and filter sets for fluorescent dye. A total of 2,000 conidia were analyzed on nonsuperpose fields.

Isolation of *A. oryzae* uridine auxotrophic mutant via membrane filtration method Conidia suspension (1×10^8 conidia/mL) was filtered using a 5-μm membrane. The filtrate was collected at 3,800×*g* for 10 min. Conidia was then resuspended in physiological saline solution with 0.05%(v/v) Tween-80 to obtain a final concentration of 1×10^7 conidia/mL. Conidia were irradiated with UV light for 3 min at about 40 μW/cm in a safety cabinet equipped with 13 W UV lamp. Conidia was then collected and plated onto 5-FOA agar plates, 5-FOA-resistant colonies were obtained after incubation at 30°C for 5 day in the dark, and the conidia from these strains were filtered with a 3-μm membrane and placed on 5-FOA plates for further identification. Finally, the uridine auxotrophic mutant strain was successfully screened out and designated as AS11.

Confirmation and analysis of *A. oryzae pyrG* mutation DNA was isolated from *A. oryzae* as described by Yua *et al.* (15). The genomic DNA of AS11 was used as a template to amplify the *pyrG* gene for sequencing. Sequence assembly was performed using DNASTAR. The sequence alignments of DNA and protein were carried out using BLASTn and BLASTp programs, respectively (http://www.ncbi.nlm.nih.gov/BLAST/). The pAO-*pyrG* plasmid (16), which contained the full-length *A. oryzae pyrG* gene, was transformed into *A. oryzae* AS11. Every generation of conidia was collected and plated on CD plates with 0.05%(v/v) Triton X-100 for 10 continuous generations to obtain stable uridine auxotrophic strains. The rate of reversion was calculated after 72 h.

***Koji* fermentation and proteinase activity assay** The *koji* medium was composed of 10 g wheat bran and soybean meal (6:4, w/w) moistened with 7.8 mL water. The medium was placed in 250-mL triangular flasks and autoclaved at 121°C for 30 min. About 1×10^8 conidia was inoculated in the *koji* medium and mixed well. Cultivation was carried out at 30°C for 3 days. The enzyme assay was summarized as follows: 5 g *koji* was added into 50 mL water, and were kept at 40°C for 1 h. The supernatant was obtained by filtration and proteinase activity was determined with the Folin phenol reagent (17). One unit of protease was defined as the amount of enzyme required to release l g of tyrosine/min from casein. Using the same medium composition and under the same culture conditions, the number of conidia was compared between the AS11 strain and the wild-type strain.

Transformation of *A. oryzae* AS11 with the *pyrG* as a selectable marker Complementation of the *A. oryzae pyrG* mutant strain was performed by transforming pGFP-*pyrG* (15) into AS11. Protoplasts were prepared by treating fresh mycelia of *A. oryzae* AS11, which were cultured for 16 h, with 1%(w/v) lyase (Sigma-Aldrich), 1%(w/v) cellulose, and 0.1%(w/v) snailase at 30°C for 3 h in a hypertonic solution (0.8 M NaCl). The plasmids were introduced into strain AS11 using the protoplast-PEG method (18). Conidia of transformants were cultured on a CD medium at 30°C for 36-40 h. The hyphae were observed using a fluorescence microscope.

Results and Discussion

Observation of nuclei in *A. oryzae* conidia DAPI is one of the most frequently used nucleic acid stains. It binds to the minor groove of DNA at AT-rich sequences. However, conidia of *A. oryzae* have very thick polysaccharide matrix and resistant cell walls, which severely impede staining using fluorescent dyes. Therefore, the addition of 5%(v/v) sodium hypochlorite significantly enhanced permeability of cell walls, along with oxidative and bleaching effects. The micrographs are shown in Fig. 1, conidia of *A. oryzae* 3.951 have 1 to 4 nuclei (A, B, C, and D). When the diameter of the conidia increased, its nuclear number (fluorescent spot) also increased, and a close relationship between nuclear number and conidial size was observed. The percentages of uninucleate and binucleate conidia were approximately 16.15 and 74.22%, respectively. Most of the uninucleate conidia were in the range of 2 to 5 μm in diameter, with maximum frequency of about 3 μm. Uninucleate conidia were enriched for *A. oryzae* RIB40 via filtration using 5-μm membrane as reported by Hara *et al.* (13).

Multinucleate conidia in *A. oryzae* are important in conferring genetic stability, such as resistance against UV-irradiation and freeze-thaw treatments. Moreover multinucleate conidia have higher germination rate and germinate earlier than uninucleate conidia during fermentation (19). However, the use of multinucleate conidia has been an obstacle in classical genetic analysis and industrial strain breeding. Multinucleate conidia can attenuate the phenotypic effect of recessive mutations. Uninucleate conidia are considered useful in mutagenesis during genetic analysis and strain breeding.

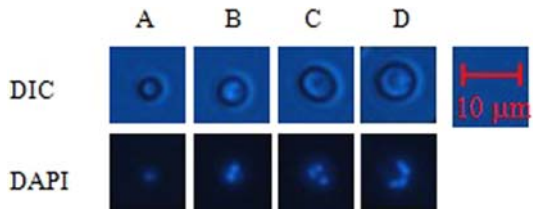

Fig. 1. Conidia size and nuclear number. Conidia of *A. oryzae* 3.951 were collected from MES medium, stained with DAPI, and observed with fluorescence and DIC microscopy. One nuclear of conidia (A), 2 nuclear of conidia (B), 3 nuclear of conidia (C), and 4 nuclear of conidia (D)

Isolation of *A. oryzae* uridine auxotrophic mutant by membrane filtration method As shown in Fig. 2, the percentage of uninucleate conidia in *A. oryzae* 3.951 was approximately 16.15% (Fig. 2), which was difficult for screening auxotrophic mutants. In the present work, uninucleate conidia of *A. oryzae* were enriched by filtration using a membrane. The percentages of uninucleate conidia that passed through 5 and 3-μm membranes were increased to 51.24 and 80.41%, respectively. However, when a 3-μm membrane was used to filter the conidia, the recovery was only 0.04%, which is quite a low value and is insufficient to allow for mutation. When a 5-μm membrane was applied filter the conidia, the recovery was increased to 0.8%. Therefore, a 5-μm membrane was chosen to remove multinucleate conidia before UV mutagenesis. The uninucleate conidia from 5-FOA-resistant colonies were enriched by filtration using a 3-μm membrane to increase the efficiency of screening. At the mean time, heterokaryon clones have been excluded by filtration after mutagenesis. The uridine auxotrophic mutant strain was successfully screened out with the 2-step filtration before and after

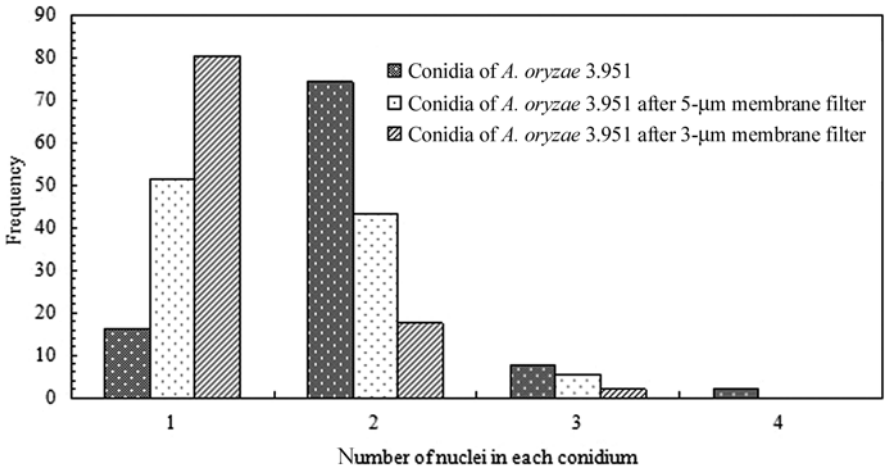

Fig. 2. Number of nuclei in each conidium of *A. oryzae* 3.951. Percentages of the number of nuclei in each conidium. A total of 2,000 conidia, both unfiltered and filtered using 3 and 5-μm membranes, were analyzed on nonsuperpose fields. The number of nuclei in each conidia of *A. oryzae* 3.951 upon DAPI staining was also presented.

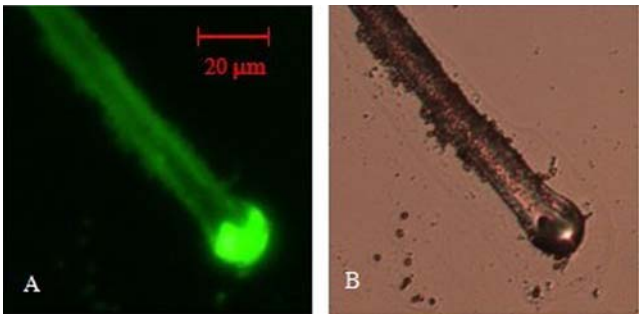

Fig. 3. Fluorescent microscopy of transformant with GFP. A and B are photographs of the transformant with blue and ordinary lights, respectively.

mutagenesis. There are only a few reports on the isolation of mutant strain using the membrane filtration method (9). Although studies have reported that uninucleate conidia were enriched using a flow cytometer equipped with a cell sorter, this method is not perfect because staining the nuclei using a fluorescent dye can kill conidia and affect subsequent tests (9).

Selection and confirmation of a *pyrG* mutant strain The *pyrG* mutant was successfully obtained through mutagenesis based on membrane filtraction and were characterized with phenotype identification, sequence analysis, and genetic complementation. The AS11 strain did not grow on the medium without uridine in the 10^{th} generation (data not shown). The results from sequence analysis revealed that there were 6 point mutations in *pyrG* gene compared with wild type strain. One of these mutations was due to the frame shift for base insertion, and caused the inactivation of orotidine-5'-monophosphate decarboxylase (OMP decase) function. In the *pyrG* transformation system, auxotroph protoplasts will not be able to grow in the medium without uridine until they are transformed using the *pyrG* gene. From the complementation of *A. oryzae* AS11, transformants of *A. oryzae* AS11 were restored using the $pyrG^{+}$ phenotype. The suitability of introducing selectable markers into *A. oryzae* was assessed using the *A. oryzae pyrG* gene to repair uridine auxotrophy. Further analysis on conidia-producing and neutral protease in secretion ability showed that the number of conidia-producing *A. oryzae* 3.951 and AS11 strains were 1.94×10^{8} and 1.47×10^{8}, respectively, and the neutral protease in enzyme activity were 2,650.27 and 2,646.36 U/mL, respectively. Upon continuous passage for 10 generations, the reverse mutation of AS11 was significantly less than 1×10^{-8} conidia/mL for each generation. The hereditary stability of AS11 was confirmed in the reversion rate of the next generation.

Transformation of *A. oryzae* AS11 with the *pyrG* as a selectable marker The expression of the transformed gene was evaluated using the GFP gene. *pyrG* was used as a screening marker to transfer the AS11 strain on the CD medium, and the hyphae of GFP-harboring transformants showed bright green signals (Fig. 3), which suggested the successful transformation and expression of GFP gene.

In conclusion, the membrane filtration method was adopted to isolate uninuclei conidium of *A. oryzae* 3.951, and then a transformation system for *A. oryzae* 3.951 was successfully established using *pyrG* as a selective marker. Uninucleate conidia are considered to be useful in mutagenesis for genetic analysis and strain breeding. We expanded the set of available food-grade markers for the expression of heterologous proteins using our selection system.

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