0273-2289/03/105-108/0821/$20.00

Active Nuclear Shuffling System Using a Swollen Conidium of *Trichoderma reesei*

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Abstract

Cellulase hyperproducers of *Trichoderma reesei* can be constructed using autopolyploidization and haploidization techniques. To increase the efficiency of this method, the active nuclear shuffling system in a swollen conidium was effective. A dried mature green conidium of a model strain, *T. reesei* QM6a (IFO 31326), was swollen to make room for a larger autopolyploid nucleus. After colchicine treatment, a larger autopolyploid nucleus was produced in such a swollen conidium. Benomyl treatment of swollen conidia generated multiple smaller nuclei from one larger autopolyploid nucleus. Those smaller nuclei were transported through conidia to mycelia after germination. This system could contribute to increasing the efficiency of genetic shuffling.

Index Entries: *Trichoderma reesei*; cellulase; colchicine; cellulose; benomyl.

Introduction

Trichoderma is a well-known cellulolytic fungus *(1)*. For the purpose of breeding this fungus, chemical mutation and genetic engineering techniques have been mainly applied *(2,3)*. Therefore, we attempted to create a new breeding technique using autopolyploid nucleus. Previously we demonstrated that autopolyploidization and genetic recombination can be carried out on this fungus using a mitotic arrester, colchicine, and a haploidizing reagent, benomyl *(4)*. Moreover, we reported the selection system of cellulase hyperproducers using a double-layer selection medium from conidia as genetic recombinants *(5)*. In this article, we present our

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attempt to increase the frequency of genetic recombination using swollen conidia of this fungus.

Materials and Methods

T. reesei QM6a (IFO 31326) was used as a model strain. This fungus was cultivated on potato dextrose agar (PDA) (BBL, Cockeysville, MD) medium at 28°C and preserved at 4°C. For preparation of dried green mature conidia, a mycelial mat (2 × 2 mm) was put on a PDA medium and incubated for 2 wk at 28°C in order to generate a large amount of green mature conidia on the colony. Those conidia were suspended in sterilized water followed by filtration with a glass filter 3G-2 for the purpose of removing mycelia. After filtration, those conidia were collected by centrifugation (Sigma 2-15) at 5510*g* followed by drying in a desiccator. Those treated conidia were used as dried mature green conidia.

At first, swollen conidia were prepared. As the medium for preparation of swollen conidia, Mandel's medium containing 1.0% (w/v) glucose (Wako, Osaka, Japan) and 0.5% (w/v) peptone (Difco, Detroit, MI) (pH 6.0) was used. The conidia of *T. reesei* QM6a are oval and mononucleate *(6)*. After Giemsa staining, one portion was stained in a conidium. The Giemsa-stained portion was also stained with 4.6'-diamidino-2-phenylindole (DAPI) solution, such that the Giemsa-stained portion was regarded as a nucleus *(7)*. The inner volume of the original conidium was increased in order to produce a larger autopolyploid nucleus by swelling the conidium. Five loopfuls of dried green mature conidia were added to 50 mL of the medium for preparation of swollen conidia in a 100-mL Erlenmeyer flask and incubated for 10 h at 28°C using a rotary shaker (Taitec R-30 mini) (160 rpm). After incubation, swollen conidia were observed by microscope in the medium. Those swollen conidia were collected by centrifuging at 5510*g* (Sigma 2-15; Sigma, St. Louis, MO). Conidia were pretreated with 5 *N* HCl (Wako) for 40 min at 60°C. After washing with distilled water, nuclear staining was carried out using Giemsa solution (Merck, Darmstadt, Germany) and DAPI solution (Sigma) followed by photomicrography. Swollen conidia were pretreated with 0.1 *N* HCl for 30 min at room temperature followed by washing with distilled water and nuclear staining. When these swollen conidia were stained with Giemsa solution, one small nucleus was observed. The diameter of the nucleus was the same as that of the original conidium.

Next, colchicine treatment of the swollen conidia was carried out. Swollen conidia were added to 20 mL of the medium in a 50-mL Erlenmeyer flask and incubated at 28°C under stationary conditions. Mandel's medium containing 1.0% (w/v) glucose, 0.5% (w/v) peptone, and 0.1% (w/v) colchicine (Wako) (pH 6.0) was used. Swollen conidia were incubated in the medium for 2 wk at 28°C under stationary conditions. After the colchicine treatment, the swollen conidia were collected by centrifuging at 5510*g* (Sigma 2-15). When the colchicine-treated swollen conidia were stained with Giemsa solution, one larger portion was stained in a swollen conidium.

Table 1
Comparison of Number of White Colonies

	No. of white colonies	No. of green colonies
Experiment A[a]		
1	1	904
2	0	885
3	1	923
4	3	954
Experiment B[b]		
1	7	948
2	11	908
3	9	865
4	15	981

[a]Benomyl treatment of treated swollen conidia in the solid medium.
[b]Benomyl treatment of treated swollen conidia in the liquid medium.

Since it was also stained with DAPI solution, this Giemsa-stained portion was regarded as an autopolyploid nucleus.

The swollen conidia containing autopolyploid nuclei were spread on the solid medium for benomyl treatment for 10 d at 28°C (experiment A). PDA medium containing 0.1% (v/v) Triton X-100 (polyoxyethylene-octylphenylether) (Wako) and 0.6 µg/mL of benomyl(1-[butylcarbamoyl]-2-benzimidazolecarbamate) (Sigma) was used.

Benomyl is a fungicide that deletes chromosomes from the polyploid nucleus with or without chromosomal recombination *(8)*. Therefore, benomyl treatment was carried out in this experiment. Triton X-100 was used for restraining mycelial elongation for ease of colony counting. After incubation, the number of colonies that generated white conidia (white colony) was counted. PDA medium containing 0.1% Triton X-100 (pH 6.0) was used. The number of white colonies generated after incubation is given in Table 1. It was suspected that the white colonies were produced through genetic recombination. Thus, they were used for estimation of genetic shuffling frequency.

Finally, benomyl treatment of swollen conidia in the liquid medium was carried out (experiment B). Mandel's medium containing 1.0% (w/v) glucose, 0.5% (w/v) peptone, and 0.4 µg/mL of benomyl (pH 6.0) was used. Five loopfuls of swollen conidia containing autopolyploid nuclei were added to 20 mL of the liquid medium in a 50-mL Erlenmeyer flask for haploidization followed by incubation for 2 wk at 28°C under stationary conditions. When the benomyl-treated swollen conidia were stained with Giemsa solution, various types of smaller nuclei were observed in a swollen conidium. When such swollen conidia containing smaller nuclei were incubated in Mandel's medium containing 1.0% glucose and 0.5% peptone at 28°C, germination occurred and such smaller nuclei were transported

Table 2
Comparison of Frequency of White Colonies

	White colonies/ Total colonies	Frequency of white colonies (%)
Experiment A	5/3671	0.14
Experiment B	42/3744	1.12

through mycelia. It was suspected that those smaller nuclei are transported to conidia like the original nuclei.

The benomyl-treated swollen conidia were washed with sterilized water and spread on the medium for counting the number of colonies and incubated for 10 d at 28°C followed by the white colony count. As a result, it appeared that the occurrence of white colonies was almost 10 times higher than that in the solid medium containing benomyl (experiment A), as shown in Tables 1 and 2.

Conclusion

From these results, we considered that a higher frequency of genetic shuffling could be obtained by benomyl treatment in the liquid medium. Consequently, such a technique was named "active nuclear shuffling," which we concluded can contribute to obtaining a higher frequency of genetic recombination in *T. reesei*.

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