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Transfer of Isolated Nuclei from *Pleurotus florida* into Protoplasts of *Pleurotus ostreatus*

Young-Bok Yoo, Chang-Hyun You, Pyung-Gyun Shin, Yong-Hwan Park and Kwon-Yawl Chang*

*Applied Mycology and Mushroom Division, Agricultural Sciences Institute, R.D.A. Suweon 170, Korea and Department of Agronomy, College of Agriculture, *Gyeongsang National University , Chinju 620, Korea*

느타리버섯 原形質體內에 사철느타리버섯 核의 轉移

劉英福·柳昌鉉·申平均·朴容煥·張權烈*

農村振興庁 農業技術研究所 菌相科 ·*慶尚大学校 農科大学 農学科

ABSTRACT: The transfer of the isolated nuclei from *P. florida* into protoplasts of *P. ostreatus* was induced with polyethylene glycol and $CaCl_2$. Three types of transfer products of nuclei were obtained when transferred to MMM. Type 1 colonies were more vigrously growing mycelium and stable on MCM. One of the type 1 colonies, appeared segregation on MCM plus benomyl. The mycelium did not form clamp connection. These results suggest that type 1 colonies were nuclear hybrids or allodiploids. Type 2 was main products of nuclear transfer. The mycelium formed clamp connection and fertile on sawdust media. Type 3 was very slow growing or non-viable colonies after debris of nuclei or chromosomes transfer into recipient protoplasts.

Isozyme pattern of esterase in type 1 produced a new band. Type 2 and type 3 could be characterized by parental bands.

KEYWORDS: Nuclear transfer, Protoplast, Nuclear hybrid, Esterase, *Pleurotus ostreatus*, *Pleurotus florida*, Basidiomycetes.

Protoplasts are valuable vehicles for gene transfer in filamentous fungi. Nuclei transfer system for *Pleurotus* depend on the availability of protoplasts in large numbers and sexuality in this fungus. Protoplasts provide an opportunity for genetic manipulation by including the uptake of foreign genetic materials(Peberdy, 1979 Ferenczy, 1985).

In relation to the nuclei transfer into protoplasts some investigations have been studied on fungi including *Saccharomyces cerevisiae* (Becher *et al.*, 1982; Ferenczy and Pesti, 1982). In higher fungi, however, few works on nuclear transfer into protoplasts were reported.

In this communication we report function of the donor nuclei in the recipient cells.

Materials and Methods

Strains and Growth Conditions

Pleurotus ostreatus ASI 2-1(Arg: recipient) from ASI 2018 and *Pleurotus florida* ASI 2-3(Ribo-1: donor) from ASI 2016 were obtained from the Agricultural Sciences Institute. They were maintained on the Mushroom Complete Medium(MCM) containing(gl^{-1}) $MgSO_4 \cdot 7H_2O$ 0.5, KH_2PO_4 0.46, K_2HPO_4 1.0, Peptone 2.0, Yeast extract 2.0, glucose 20.0, Agar 20.0. Selection of fusion products after nuclei transfer was carried out on a osmotically stabilized Mushroom Minimal Medium(MMM). It consists of(gl^{-1}) $MgSO_4 \cdot 7H_2O$ 0.5, KH_2PO_4 0.46, K_2HPO_4 1.0, Thiamin-HCl 120 ug, DL-asparagine 2.0, glucose 20.0, agar 20.0, and is supplemented with 0.6 M sucrose. Bottom agar was of 2.0% while overlaying soft agar was of 0.75%.

Protoplast Formation

Disks of sterile cellophan membrane were placed on the surface of MCM in petri dishes. The mycelial disks were ready for protoplast production when mycelia had grown over the disks.

Mycelial disks of *P. ostreatus* and *P. florida* from 4 days culture at 25℃ were removed to clean sterile petri dishes and the lytic enzyme stabilizer solution was added immediately. The lytic mixture consisted of Novozym 234(Novo), β-Glucuronidase (Sigma) and β-D-Glucanase(BDH) in 0.6 M sucrose. The petri dishes incubated on reciprocal shaker(120 strokes min^{-1}) at 28℃ for 4 hours. Protoplasts from lytic mixture were separated from mycelial debris by filtration through sintered glass filter(porosity 1) and protoplasts were washed twice with 0.6 M sucrose.

Preparation of Nuclei

The isolation of nuclei was performed as described by Ohyama *et al.*, (1977) and Lorz and Potrykus(1978). All isolation procedures were carried out on a cooling at 5℃. The nuclear donor strain(ASI 2-3) was converted into protoplasts. The donor protoplasts were resuspended in nuclei isolation medium(NIM) containing 0.3 M Sucrose, 5 mM magnesium chloride, 10 mM calcium chloride, 20% Glycerol, 7% Ficoll 400, and 0.5% Triton X-100. The protoplasts were disrupted by homogening at 1,000 rpm for 10 min. Isolating intact nuclei in NIM were separated from cytoplasmic debris and whole cell by vaccum filtration through millipore(pore size 0.8 um). Filtrates were collected by centrifugation at 1,000g for 15 min. Nuclei pellets were resuspended in 0.6 M sucrose which maintained at 4℃ until used for transfer.

Uptake of Nuclei

The procedure of nuclei transfer was based on protoplast fusion technique(Yoo *et al.*, 1984). The suspension of recipient protoplasts(8.0×10^7) and donor nuclei(1.2×10^8 protoplast equivalent) were combined in a fusion tube and centrifuged at 700× g for 15 min. The pellet was resuspended in 1 m*l* of a solution of 30% polyethylen glycol 4000(PEG) containing 10 mM $CaCl_2\cdot2H_2O$ and 50 mM glycine, adjusted to pH 8.0 with 1 mM NaOH. The suspension was diluted with 0.6 M sucrose, washed once by centrifugation, and resuspended in 5 m*l* osmotic stabilizer. Serial dilutions of treated protoplasts were plated onto hypertonic MMM to select complemented colonies.

Preparation of Mycelial Extracts

Mycelia were grown in squat 1 conical flasks containing 300 m*l* MCM solution. The flasks were incubated for 7-10 days at 25℃. The harvested mycelium with liquid nitrogen(-196℃) was ground at 4℃ in a pre-cooled mortar for 10-20 min. The mycelial fragments were removed by centrifugation at 13,000g at 30 min.

Electrophoresis

The mycelium homogenates were analysed by the polyacrylamide gel and the discontinuous buffer system as described by Ornstein(1964) and Davis(1964). Electrophoresis was done at 5℃ at constant voltage(7-10 mA/cm. gel).

Detection of Esterase

Gel was stained for esterases by the method of Peberdy and Turner(1968) and Gabriel(1971). The gel was flooded with a solution of TRIZMAL™ 7.6 buffer(pH 7.6) 50 m*l*; 1 Part TRIZMAL™ 7.6 Buffer concentrate(sigma), 9 parts deionizer water and 1 capsule fast blue RR salt. When salt is completely dissolved in buffer, a-naphthyl acetate solution(1 capsule a-naphthyl acetate in 2 m*l* ethylene glycol monomethyl ether) were added. The gel in staining solution was incubated at 37℃ for 30 min.

Results and Discussion

After PEG solution treatment of a mixture of complementing donor nuclei of *P. florida* and recipient protoplasts of *P. ostreatus*, small prototrophic colonies developed on MMM. Frequency of nuclei transfer was 7.52×10^{-4}%. After 10-20 days culture on hypertonic MMM, complemented colonies produced sectors of growing mycelium. When complemented colonies were transferred to MMM and MCM they were classified into three types; fast growing(Type 1), moderate growing(Type 2), and slow growing(Type 3) colonies(Fig.1).

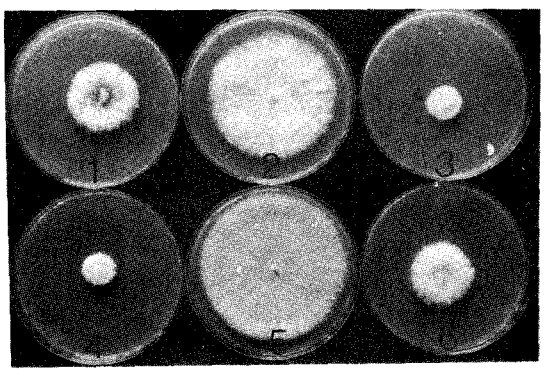

Fig.1. Products of nuclei transfer growing for 7 days on MCM; type 1(2: p426, 5: p427), type 2(6: p417), type 3(3: p431), donor or nuclei(1: Ribo-1) and recipient(4: Arg).

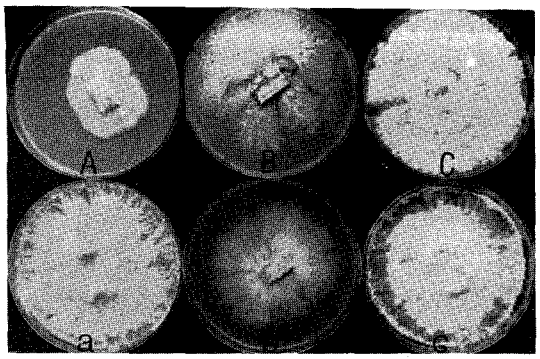

Fig.2. Products of nuclei transfer growing on MCM containing 100 mg^{-1}(A,B,C) and 10 mg^{-1}(a,b, c) benomyl; type 1(B: p427), type 2(C: p417) and type 3(A: p431).

On MMM and MCM type 1 colonies were more vigrously growing mycelium and stable. Strain p427 of the type 1 colonies, appeared to be in segregation on MCM containing 100 mg^{-1} benomyl(Fig.2). The sexuality of transfer products of nuclei is given Table I. The mycelium did not form clamp connection when observed under microscopy and sterile on sawdust medium. These results suggest that type 1 colonies are homokaryon or uninucleate in a cell.

Table I. Characteristics of the products of nuclei transfer

Strain	Mycelial growth* on MMM	Clamp**	Fruiting***
Transfer progeny			
Type 1 P426	FG	−	S
P427	FG	−	S
Type 2 P417	MG	+	F
P421	MG	+	F
Type 3 P430	SG or N	−	S
P431	SG or N	−	S
Donor(Ribo-1)	MG	−	S
Recipient(Arg)	MG	−	S

*FG: Fast growing
MG: Moderate growing
SG: Slow growing
N : Non- viable
**+: Presence clamp connection
−: Absent clamp connection
***F: Fertile
S: Sterile

Although conclusive evidence in lacking, these observations indicated that type 1 colonies were nuclear hybrid or allodiploid after nuclear fusion in a cell. On MCM and MCM+benomyl, type 2 colonies were main products of nuclear transfer and stable with no visible evidence of sectoring. The mycelium did form clamp connection and fertile on sawdust medium which did exhibit interspecific heterokaryon of protoplasts(Yoo *et al.*, 1984, 1987). On MMM type 3 colonies were very slow growing or non-viable mycelium. When transferred to MCM the sectors gave normal and stable. These colonies did not induce segregation on MCM plus benomyl. The mycelium did not form clamp connection and sterile on sawdust medium. These findings suggests that type 3 colonies are homokaryon or uninucleate in a cell after debris of nuclei or chromosomes transfer into recipient protoplast.

Nuclear hybrids were obtained by transfer of nuclei into protoplasts in *Saccharomyces*(Ferenczy and Pesti, 1982). We previously reported interspecies protoplast fusion in *Pleurotus*(Yoo *et al.*, 1984, 1987). However we never found hybrid following protoplast fusion. Interspecific hybrids in *Aspergillus* were identified by various criteria including vigrous growing sectors, secretive brown pigment, doubled DNA content, few conidia and segregation on CM or CM plus haploidizing agents(Ferenczy *et al.*, 1977; Kevei and Pberdy, 1977, 1979). No pigmentation occurred and the hybrid colonies was white on MM and CM in

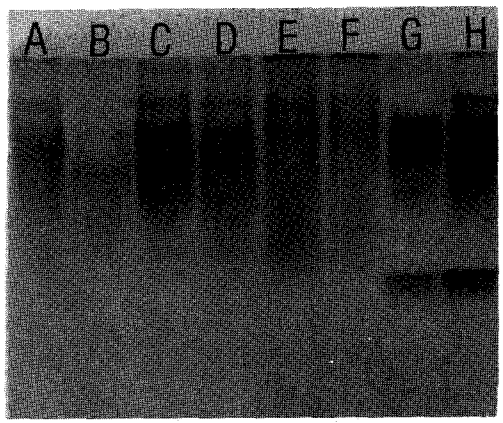

Fig.3. Isozyme pattern of esterase in products of nuclei transfer on polyacrylamide gel; type 1(G: p426, H: p427), type 2(E: p417, F: p421), type 3(C: p430, D: p431), donor of nuclei(B: Ribo-1) and recipient(A: Arg).

Penicillium(Anne *et al.*, 1976; Peberdy *et al.*, 1977).

Isozyme patterns of transfer products of nuclear are given in Fig.3. Type 2 and type 3 could be characterized by parental bands. Type 1 of interspecific hybrids produced a new highly active bands.

摘 要

원형질체내에 외래유전물질 도입에 의한 균주 개발의 가능성 검토의 일환으로 사철느타리의 원형질체로부터 핵을 분리하여 느타리의 원형질체내로 전이하여 얻은 결과는 다음과 같았다.

1. 핵전이주는 크게 3가지 type으로 구분되었는데 type 1은 아주 빠른 균사생장과 clamp connection를 형성하지 않으며 CM+benomyl에서 균총분리가 나타남으로서 이질이배체로 사료되었다. type 2는 주된 핵전이주로서 모균주와 비슷하거나 다소 빠른 균사생장과 clamp connection을 지녀 자실체를 형성하였으며 heterokaryon으로서 원형질체 융합주와 유사하였다. 그리고 type 3은 핵 또는 염색체 단편의 전이로 clamp connection를 지니지 않고 아주 느리거나 MM에서 생육이 거의 불가능한 균주였다.

2. 핵전이주 및 모균주를 전기영동법으로 esterase 동위효소 pattern을 조사한 결과 핵전이주는 모균주와 구별되었는데 type 1인 이질이배체는 새로운 band를 나타내었으며, type 2와 type 3은 모균주들을 합한 것으로 보였다.

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