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Transfer of Isolated Nuclei from *Agrocybe aegerita* Mycelia into *Pleurotus florida* Protoplasts

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사철느타리버섯 原形質體內 버들볏짚버섯 核의 轉移

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ABSTRACT: The transfer of isolated nuclei from *Agrocybe aegerita* mycelia of wild type into *Pleurotus florida* protoplasts of auxotroph was induced with polyethylene glycol. The type 1 of nuclear transfer products was spontaneous segregants of both parental morphology of colony. Hyphae of *A. aegerita* type had clamp connections while that of *P. florida* type lacked. Type 2 was main products of nuclear transfer which formed true clamp connections. Type 3 was clampless products. They all produced primordia and developed basidiocarps similar to *Agrocybe aegerita*.

A comparison of nuclei transfer products was made using isozyme analyses of alcohol dehydrogenase, esterase, lactate dehydrogenase and peroxidase. Isozyme band patterns of some type 2 strains produced a new band. Band patterns from mycelial extracts of the other strains could be characterized by parental bands.

KEYWORDS: Nuclear transfer, *Agrocybe aegerita* nuclei from mycelia, *Pleurotus florida* protoplasts, Isozyme analysis, Basidiomycotina.

The uptake of isolated cell organelles by protoplasts is a powerful tool of cell genetics and breeding. The absence of cell walls around protoplasts provides an opportunity for genetic manipulation by inducing the uptake of foreign cell organelles in the form of mitochondria and nuclei. In relation to the nuclei transfer into protoplasts some investigations have been studied on yeast (Becheer *et al.*, 1982; Ferenczy and Pesti, 1982) and higher fungi (Yoo *et al.*, 1987; You *et al.*, 1988). In this investigation we report function of the donor nuclei in the recipient cells.

Materials and Methods

Strains and Growth Conditions

Agrocybe aegerita ASI 19003 (wild: donor) and *Pleurotus florida* ASI 2-3-f2016-29 (rib: recipient) were obtained from the Agricultural Sciences Institute (Korea). *A. aegerita* ASI 19003 was maintained on the yeast glucose (YG; Conney and Emerson, 1964), containing (g/*l*) yeast extract 5.0, glucose 10.0 and agar 20.0. *P. florida* ASI 2-3 was maintained on the mushroom complete medium (MCM; Raper *et al.*, 1972), containing (g/*l*) $MgSO_4 \cdot 7H_2O$ 0.5, KH_2PO_4 0.46, K_2HPO_4 1.0, peptone 2.0, yeast extract 2.0, glucose 20.0 and agar 20.0. Selection of nuclei uptake products after uptake of isolated nuclei by protoplasts was carried out on an osmotically stabilized mushroom minimal medium (MMM; Raper *et al.*, 1972). It consists

of (g/*l*) $MgSO_4 \cdot 7H_2O$ 0.5, KH_2 PO_4 0.46, K_2 HPO_4 1.0, DL-asparagine 2.0, glucose 20.0 and bacto-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

Protoplasts of *P. florida* were prepared using a mixture of Novozyme234 (Novo Biolabs), Cellulase onozuka R-10 (Yakult) and β-Glucuronidase (Sigma) basically as described by Yoo *et al.* (1985).

Preparation of Nuclei

Nuclei were isolated by a modified method of Ohyama *et al.* (1977), Lorz and Potrykus (1978). All isolation procedures were carried out on a cooling at 5°C. The nuclear donor mycelia were harvested by filtration through sintered glass filter (Porosity 1). The mycelia diluted with 100 m*l* of nuclei isolation medium containing 0.3 M sucrose, 5 mM magnesium chloride, 10 mM calcium chloride, 20% glycerol, 7% ficoll 400 and triton x-100 and homogenized for 10 min. at 1 strokes. Isolating intact nuclei in NIM were separated from mycelial fragment and whole cell by vacuum filtration through filterpaper (Whatman 1) and millipore (Pore size 8.0μm).Filtrates were collected by centrifugation at 1000g for 15 min. Nuclei pellets were resuspended in 0.6 M sucrose which maintained at 4°C until used for transfer.

Uptake of Nuclei

The procedure of nuclei transfer was based on the method of Yoo *et al.* (1987). The suspension of recipient protoplasts and donor nuclei were combined in a fusion tube and centrifuged at 1000g for 15 min. The pellet was resuspended in 1 m*l* of a solution of 30% polyethylene glycol 4000 (PEG) containing 10 mM $CaCl_2 \cdot 2H_2O$ and 50 mM glycine, adjusted to pH 8.0 with 1 mM NaOH. After incubation for 15 min. at 30°C, the suspension was washed with 0.6 M sucrose by centrifugation and resuspended in 5 m*l* osmotic stabilizer. Serial dilutions of treated protoplasts were plated onto 0.6 M sucrose stabilized 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 15 days at 25°C. The harvested mycelium with liquid nitrogen (−196°C) was ground at 4°C in a precooled mortar for 10-20 min. The mycelial fragments were removed by centrifugation at 13000g for 30 min.

Electrophoresis

The mycelium homogenates were analysed by the polyacrylamide gel and the discontinuous buffer system as described by Ornstein (1964) and Davies (1964). Electrophoresis was done at 5°C at constant voltage (7-10 mA/1 cm gel).

Detection of Enzyme

Esterase was detected in the gels by immersion in a solution of TRIZMAL 7.6 buffer 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 , α-naphyl acetate solution (1 capsule α-naphyl acetate in 2 m*l* ethylene glycol monomethyl ether) was added. The gel in staining solution was incubated at 37°C for 30 min. Alcohol dehydrogenase was detected in the gels by immersion in a solution containing 0.1 M Tris-HCl pH 7.5 100 m*l*, NAD 30 mg, MTT 20 mg, PMS 4 mg and ethanol 6 m*l*. The gel in staining solution was incubated at 37°C for 30 min. Lactate dehydrogenase bands could be observed after incubation of the gels in solution of 0.1 M Tris-HCl pH 7.5 100 m*l*, 1 M $MgCl_2 \cdot 6H_2O$ 1 m*l*, 85% DL-Lactate 10 m*l*, NAD 30 mg and PMS 4 mg. The gel in staining solution was incubated at 37°C for 30 min. Peroxidase bands were detected by immersing the gels in a solution A containing 1 M Na-acetate pH 4.7 50 m*l*, methanol 50 m*l* and TMBZ 50 mg. The gel in staining solution A was incubated at 37°C for 30 min, added 30% H_2O_2 2 m*l* and shaked the tray to ensure good mixing. The gel was incubated at 37°C until blue band appeared.

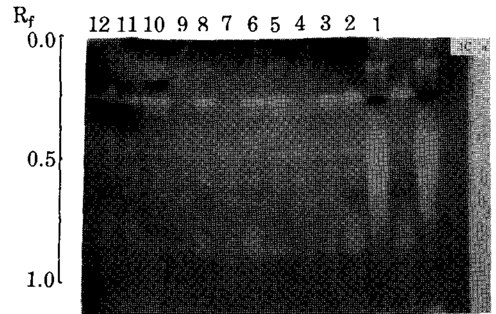
Results and Discussion

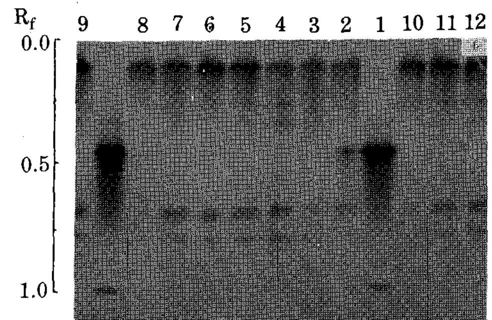
To check for viability of the isolated nuclei of *Agrocybe aegerita* control plates were inoculated on 0.6 M sucrose stabilized MMM and MCM. No reversion colony was seen in such cultures. After PEG + Ca^{++} + glycine solution treatment of mixture of complementing donor

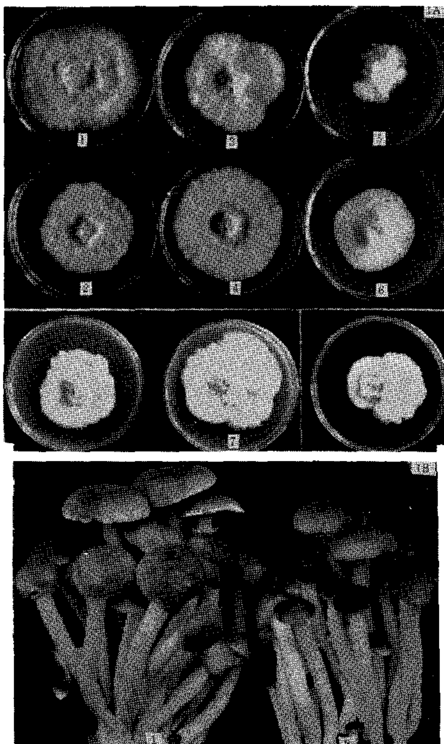
Table 1. Characteristics of transfer products of nuclei from *A. aegerita* mycelia into *P. florida* protoplasts

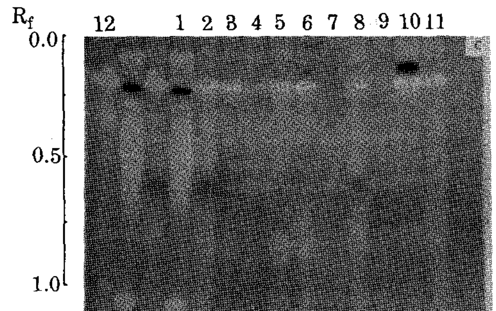
Strain	Clamp*	No. of isolates	Fruiting**
Transfer progeny			
Type 1	±	3	F
Type 2	+	55	F
Type 3	−	42	F
Donor of *A. aegerita* ASI 19003	+	−	F
Recipient of *P. florida* ASI 2-3	−	−	S

* + : Presence of clamp connection, − : Absence of clamp connection, ± : Spontaneous segregation colony
** F : Fertile, S : Sterile









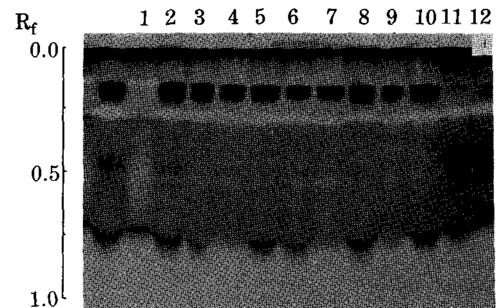


Fig. 1. Transfer of isolated nuclei from *A. aegerita* mycelia into *P. florida* protoplasts.
A. Transfer products of nuclei growing for 10 days on MCM (1) Recipient of *P. florida* ASI 2-3 (2) Donor of *A. aegerita* ASI 19003 (3) P604 (4) P603 (5) P608 (6) P602 (7) P578 of segregation colony.
B. Basidiocarp of transfer products of nuclei on sawdust substrates
(1) Transfer products of nuclei (2) *A. aegerita* ASI 19003
C. Isozyme pattern of nuclei transfer products on polyacrylamide gel (a) Alchol dehydrogenase (b) Esterase (c) Lactate dehydrogenase (d) Peroxidase (1) Recipient (2) Donor (3) P599 (4) P600 (5) P601 (6) P602 (7) P603 (8) P604 (9) P605 (10) P606 (11) P607 (12) P608

nuclei and recipient protoplasts of *Pleurotus florida*, small prototrophic colonies developed on MMM. Frequency of nuclei transfer was 0.82×10^{-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(Table. I). Type 1 produced 3% spontaneous segregants of both parental types. Hyphae of *A. aegerita* type was present true clamp connections while mycelia of *P. florida* type lacked them. Type 2 showed 55% stable colonies of nuclei transfer products which formed clamp connections. In this case the hyphae may contain binucleate from donor and recipient cells. Type 3 was 42% stable transfer products of nuclei which did not form clamp connections. These hyphae contained uninucleate from donor or binucleate from donor and recipient cells. Ninety five percents of nuclei transfer products in mycleial colony morphology were similar to those of *A. aegerita* (Fig. 1A). These all types produced primordia and developed basidiocarps similar to those of *A. aegerita* (Fig. 1B). The uninucleate colony of type 3 was reconstitute cells between donor karyoplast and recipient cytoplast because donor of *A. aegerita* was wild type. The proportion of reconstituted cell could be quite high because the proportion of enucleate protoplasts was 20-50% after 1-3 h digestion (Peberdy, 1979). The clamp connection in type 2 was derived from donor nuclei of *A. aegerita* because the hyphae of fusion products between incompatible strains did not form true clamp connections and could not develop fruit bodies (Yoo *et al.*, 1987; You *et al.*, 1988).

A comparison of nuclei transfer products was made using isozyme analysis of alcohol dehydrogenase, esterase, lactate dehydrogenase and peroxidase (Fig. 1C). The enzyme pattern of alcohol dehydrogenase was similar to that of lactate dehydrogenase. The *P. florida* extract contained two bands at both 0.16 R_f and 0.20 R_f. In *A. aegerita* which showed one band at 0.53 R_f. Mycelial extracts of nuclear transfer products P602, P604, P606 and P607 contained some *A. aegerita* like bands at 0.16 R_f. Mycelial extracts of the other strains did not contain any bands under the conditions tested. The esterase banding patterns of nuclear transfer products were similar to those of the *A. aegerita* parental strains. The *P. florida* extract contained eight bands, the most active band being both 0.45 R_f and 0.97 R_f. At these R_f value fusants were observed having a very weak activity, probably indicating that the *P. florida* chromosome bearing the esterase was lost. The peroxidase enzyme patterns of nuclei transfer products were similar to those of the *A. aegerita* except two strains. Strains P607 and P608 showed very active non-parental bands at 0.4 R_f. These strains were of type 2.

摘　　要

野生形이며 self-fertile인 버들볏짚버섯(버들송이) 菌絲體로부터 分離한 核을 營養要求株이며 self-sterile인 사철느타리버섯 原形質體內에 polyethylene glycol+$CaCl_2$+glycine 용액으로 攝入하였다. 100菌株의 核轉移株 檢定으로 兩親이 菌叢分離되어 버들볏짚버섯親 形態에는 클램프 連結體가 菌絲에 있고, 사철느타리버섯親 形態에는 클램프 連結體가 없는 것이 3菌株, 菌叢形態가 安定性이며 클램프 連結體가 菌絲에 形成된 것이 55菌株, 菌叢形態가 安定性이며 클램프 連結體가 菌絲에 存在하지 않는 것이 42菌株였다.

이들 核轉移株는 모두 原基 形成되어 子實體로 성숙되었으며 그 形態는 버들볏짚버섯과 거의 類似하였다.

核轉移株를 전기영동법으로 alcohol dehydrogenase, esterase, lactate dehydrogenase, peroxidase의 同位酵素 分析으로 비교하였는데 클램프 連結體를 가지는 形態의 몇 菌株가 새로운 band를 形成하였으며 다른 菌株들은 兩親株에서 유래된 것이었다.

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