韓 國 菌 學 會 誌 第 17 卷 第 3 號
Kor. J. Mycol. Vol. 17, No. 3, 119-123, 1989

Interorder Protoplast Fusion between *Pleurotus ostreatus* and *Ganoderma applanatum*

Young-Bok Yoo, Moon-Tae Song, Seung-Joo Go, Chang-Hyun You, Dong-Yeul Cha, Yong-Hwan Park and Kwon-Yawl Chang*

*Applied Mycology and Mushroom Division, Agricultural Sciences Institute, R.D.A. Suweon 440-707 and *Department of Agronomy, College of Agriculture, Gyeongsang National University, Chinju 660-701, Korea*

느타리버섯과 잔나비걸상버섯과의 異目間 原形質體 融合

劉英福·宋文台·高昇柱·柳昌鉉·車東烈·朴容煥·張權烈*

農村振興庁 農業技術研究所 菌蕈科 *慶尚大学校 農科大学 農学科

ABSTRACT: Interorder heterokaryons were obtained by polyethylene glycol induced fusion of protoplasts from auxotrophic mutants of *Pleurotus ostreatus* in agaricales and *Ganoderma applanaturm* in aphyllophorales. When transferred to MMM plates, all fusion colonies exhibited an extremely slow growth rate. During three times subcultivation on MCM the growth rate of fusants showed faster little by little. Seventy-five % fusion products of potoplasts showed mixed morphologies between those of *P. ostreatus* and *G. applannatum* in the first subcultivation on MCM and MGM. The phenotype of these fusants changed similar those of *P. osteatus* type after three times subcultivation on MCM. However, phenotype of 25% stable strains did not change on subcultivation. Hyphae of all fusion products did not form true clamp connection. All these types did not produce primordia. A comparrison of interorder somatic hybrids was made using isozyme analysis of esterase, malate dehydrogenase and peroxidase. In most cases the enzyme patterns of *G. applanat um* were not distinct, however, fusant showed non-parental bands.

KEYWORDS: Interorder protoplast fusion, *Pleurotus ostreatus* in agaricales, *Ganoderma applanatum* in aphylloporales, Basidiomycotina.

Edible mushrooms including *Pleurotus* and *Ganoderma* have for food and herb medicine from the earliest times. Our knowledge of genetics and breeding mechanisms in higher fungi has been less extensive in comparison with both bacteria and lower fungi due to complex sexuality. The protoplast fusion has been developed to break down the barrier to genetic exchange imposed by conventional mating systems. Protoplast fusion may be used to produce interspecific or even intergeneric hybrids. Intra- (Gold *et al.* 1983; Kiguch and Yanagi, 1985) and interspecific somatic hybrids of protoplasts (Yoo *et al.*, 1984, 1987; Toyomasu *et al.*, 1986; Park *et al.*, 1988; Um *et al.*, 1988) have been obtained in higher fungi. In intergenus or interorder protoplast fusion, however, few works were reported.

This investigation describes interorder somatic hydridization of protoplast between *Pleurotus ostreatus* in agaricales and *Ganoderma applanatum* in aphyllophorales.

Materials and Methods

Strains and Growth Conditions

The isolation of auxotrophic mutants was performed as described by Yoo *et al.*, (1985) and Park *et al.*, (1987). The monokaryon strain of *Pleurotus ostreatus* ASI 2-1 (arg) was induced by irradiation of protoplasts of *P. ostreatus* ASI 2018-106-6 to ultraviolet lights. The strain of *Ganoderma applanatum* ASI 7-8 (cys met) was induced by irradiation of the mycelial fragments of *G. applantaum* ASI 07031 to ultraviolet lights. The strain *P. ostreatus* ASI 2-1 was maintained on the Mushroom Complete Medium (MCM) containing (gl^{-1}) $MgSO_4 \cdot 7H_2O$ 0.05, KH_2PO_4 0.46, K_2HPO_4 1.0, Peptone 2.0, Yeast extract 2.0, glucose 20.0 and agar 20.0. The strain *G. applanatum* ASI 7-18 was maintained on the *Ganoderma* Complete Medium (GCM) containing (gl^{-1} casamino acid 5.0, glusose 30.0, KH_2PO_4 0.46, K_2HPO_4 1.0, $MgSO \cdot 7H_2O$ 0.5, peptone 4.0, sucrose 20.0 yeast extract 10.0 and agar 20.0. Heterokaryon selection after protoplast fusion was carried out on 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, DL-asparagine 2.0, glucose 20.0, Bacto-agar 20.0 and was supplemented with 0.6 M sucrose. Bottom agar was of 2.0% while overlaying soft agar was of 0.75%.

Protoplast Formation and Fusion

Protoplasts of *P. ostreatus* were prepared using a mixture of Novozym 234 (Novo Biolabs), β-Glucanase (BDH) and β-Glucuronidase (Sigma) basically as described by Yoo *et al.*, (1985). Protoplasts of *G. applanalum* were prepared using a mixture of Novozym 234. Cellulase onozuka R-10 (Yakult) and β-Glucuronidase basically as described by Park *et al.*, (1976). The procedure of protoplast fusion was based on those of Anne and Peberdy (1987) and Ferenczy *et al.*, (1975). 4.15×10^7 protoplasts of each strain were combined in a fusion tube and centrifuged at 500×g for 8 min. The pellet of protoplasts was resuspended in 1 m*l* of a solution of 30% polyethylene glycol 4000 (PEG) containing 100 mM $CaCl_2 \cdot 2H_2O$ and 50 mM glycine, adjusted to pH 8.0 with 10 mM NaOH. After incubation for 15 min at 30°C, the suspension was diluted with 0.6 M sucrose, washed once by centrifugation and resuspended in 5 m*l* 0.6 M sucrose. Serial dilutions of treated protoplasts were plate onto MCM stabilized with 0.6 M sucose for viability and onto MMM to select for fusion products. The fusion frequency was expressed as the number of colonies on MMM to the number of colonies reverted on MCM after 25-30 days incubation at 27°C.

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 pre-cooled mortar for 10-20 min. The mycelial fragments were removed by centrifugation at 13,000g for 30 min.

Gel electrophoresis and enzyme staining procedures

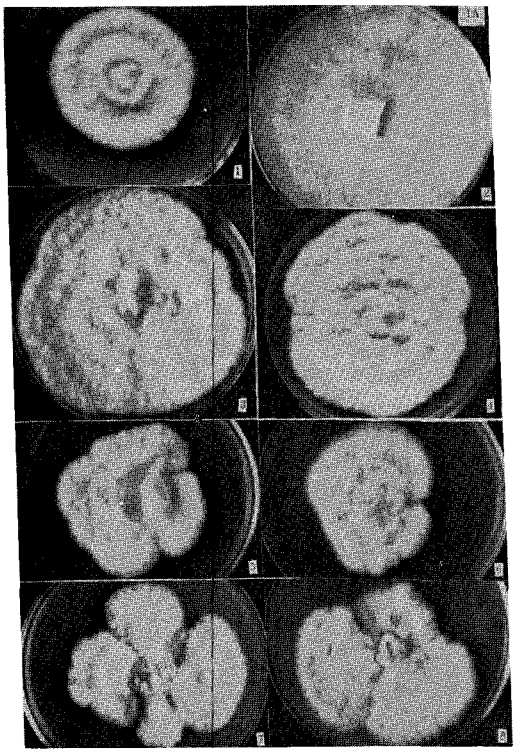
The mycelium homogenates were analysed by the polyacylamide gel and discontinous buffer system as described by Ornstein (1964) and Davis (1964). Electrophoresis was done at 5°C at constant voltage (7-10 mA 1 cm gel).

Esterase were 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 acetate solution (1 capsule α-naphthyl acetate in 2 m*l* ethylene glycol monomethyl eter) were added. The gel in staining solution was incubation of the gels in a solution containing 0.1 M Tris-HCl pH 8.0 100 m*l*, 1 M L-Malate 3 m*l*, NAD 30 mg, MTT 20 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* (sol. B) and shaked the tray to ensure good mixing. The gel was incubated at 37°C until blue band appeared.

Results and Discussion

After PEG solution treatment, from the mixture of complementing *Pleurotus ostreatus* in agaricales and *Ganoderma applanatum* in aphyllophoralles photoplasts, small prototrophic colonies developed on MMM. Fusion frequency for the interorder cross was 0.002%. The fusion

products produced after 25-30 days of incubation on MMM plates. When transferred to MMM plates, all fusion colonies exhibited an extremely slow growth rate. During three times subculture on MCM the growth rate of fusants showed faster little by little. Most of fusants showed segregation or variants in mycelial morphology on GCM or MCM (Fig. 1A). Thirty six heterokaryon fusants were classified into five types; 9 mixed both parents on GCM, 1 mixed both parents on MCM, 17 mixed both parents on GCM or MCM, 3 stable *P. ostreatus* types and 6 stable non parental types (Table I). Seventy five% fusion products of protoplasts showed mixed morphologies or segregants between those of *P. ostreatus* and *G. applanatum* on the first sub-



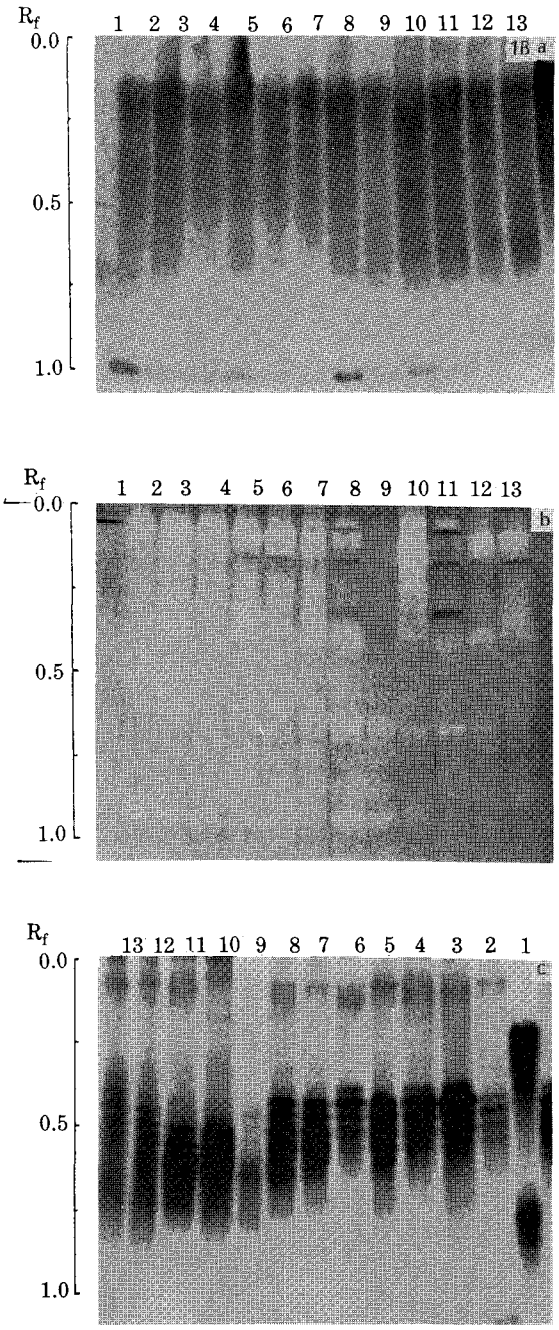


Fig. 1. Morphological variants obtained as fusion products from interorder protoplast fusion between *P. ostreatus* in agaricales and *G. applanatum* in aphyllophorales.

A. Somatic hybrids growing on MCM (1) *P. ostreatus* ASI 2-1 (2) *G. applanatum* ASI 7-18 (3) P385 (4) P401 (5) P414 (6) P411 (7) P413 (8) P412

B. Isozyme pattern of somatic hybrids on polyacrylamide gel. (a) Esterase (b) Malate dehydogenase (c) Peroxidase

(1) *G. applanatum* ASI 7-18 (2) *P. ostreatus* ASI 2-1 (3) P413 (4) P412 (5) P403 (6) P400 (7) P396 (8) P 391 (9) P390 (10) P389 (11) P388 (12) P386 (13) P380

Table I. Characteristics of fusion products of protoplasts between *Pleurotus ostreatus* and *Ganoderma applanatum*

Strain	Morphology of mycelial colony on CM		No. of isolates	%	Clamp*	fruiting**
	1st subcultivation	3rd subcultivation				
Fusion Products						
Type 1	Mixed both parents on GCM		9	25.0	–	s
Type 2	Mixed both parents on MCM		1	2.8	–	s
Type 3	Mixed both parents on GCM or MCM	*P. ostreatus* type	17	47.2	–	s
Type 4	*P. ostreatus* type		3	8.3	–	s
Type 5	Non-parental type	Non-parental type	6	16.7	–	
P. osteatus ASI 2-1 (arg)					–	s
G. applanatum ASI 7-18 (Cys Met)					–	s

* – : clampless
** s : sterile

cultivation on MCM and GCM. The phenotype of these fusants changed similar to those of *P. ostreatus* type after three times subcultivation on MCM. Phenotype of 25% stable strain did not change after subcultivation on MCM. *P. ostreasus*-like fusants did not have the *G. applanatum* morphologies, probably indicating that *G. applanatum* chromosome bearing phenotype gene was lost. Hyphae of all fusion products did not form true clamp connection. These all types did not produce primordia.

A comparison of fusion products of protoplasts was made using isozyme analysis of esterase, malate dehydrogenase and peroxidase (Fig. 1B). The esterase banding patterns of fusants were similar to that of the *P. ostreatus* parental strain. The malate dehydrogenase enzyme patterns of *G. applanatum* extract contained one band at 0.07 Rf. However, this band was not observed in *P. ostreatus*. Strains P403, P400, P391, P390, P389, P388 and P386 showed active non-parental bands. The peroxidase banding pattern was characteristic for each parental strains. The *P. ostreatus* extract contained seven bands, the most active band being at 0.07 R_f, 0.42 R_f and 1.0 R_f. In *G. applanatum*, which showed two bands both at 0.25 R_f and 0.75 R_f. All fusants extract contained non-parental bands both at 0.38 R_f and 0.64 R_f. At these R_f values *G. applanatum* had a weak activity. Comparison of the five type fusants and their parents showed that an interaction exists between the two genomes in the interorder somatic hybrids.

We previously reported interspecific protoplasts fusion in *Pleurotus* and *Ganoderma* respectively. However, we never obtained allodiploid strains following protoplast fusion as like this case of interorder protoplast fusion. Most of potoplast fusion products between incompatible dikaryon strains showed spontaneous segregation of mycelial colony, but no segregants were found in fusion combination between compatible monokaryon strains (Yoo *et al.*, 1984, 1987; Park *et al.*, 1988; Um *et al.*, 1988). From these results, spontaneous segregation of heterokaryon fusants probably took place due to unbalance in the cell contents of the mycelium, particularly in the types and number of nuclei.

摘 要

주름버섯目 느타리버섯과 민주름버섯目 잔나비걸상버섯과의 異目間 原形質體를 polyethylene glycol로 유도하여 融合株 heterokaryon을 선발하였다. 버섯최소배지에서 극히 균사생장이 느렸으며 버섯완전배지에서 3번 계대배양되면서 다소 생장이 빨라졌다. 융합주 36균주의 75%는 양친의 균사가 혼합된 균총형태였으며 16.7%는 새로운 형태, 8.3%는 느타리버섯 형태이었다. 이들 중 양친의 菌叢이 혼합된 형태는 3번 계대배양 후 모두 느타리버섯 형태로 변하였다. 菌絲에는 클램프 연결체가 없었고 原基도 형성하지 않았다.

融合株를 전기영동법으로 esterase, malate dehydrogenase, peroxidase의 同位酵素 分析으로 비교하였는데 잔나비걸상버섯 효소는 뚜렷하지 않았으나 새로운 밴드의 형성으로 보아 두 양친 genome 간의 상호작용이 존재하였다.

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Accepted for Publication 12 June 1989