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Interspecific Hybridization between *Pleurotus cornucopiae* and *Pleurotus florida* Following Protoplast Fusion

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原形質體 融合에 의한 노랑느타리버섯과 사철느타리버섯의 種間 交雜

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ABSTRACT: Interspecific fusion products were obtained by protoplast fusion induced by polyethylene glycol, from auxotrophic mutants, *Pleurotus cornucopiae* and *P. florida*. The fusants were classified into allodiploidy, stable heterokaryon, and spontaneously segregated heterokaryons. Fruiting body of the clamped or clampless fusants was produced by light-dark cycle on the sawdust medium in glass bottles. Most of these clampless fusants produced mature basidiocarps. The pilei showed various mixed colors resembling the parents. All fruit bodies presented clamp connections except two fusants. When small tissues of stipe from basidiocarps were cultured on a complete medium, mycelial colonies grew more vigorously than that of the original clampless fusants. Five fusants in three crosses were analysed with the distribution of progenies and segregation of genetic characters by random spore analyses. The genetic markers were shown to segregate and recombine in the first generation of monospores isolated from basidiocarps. The analysis indicated the heter-okaryosis and strong evidence for haploidy of vegetative nuclei, a sexual cycle consisting of nuclear fusion and meiosis. Genotypes of a large number of auxotiophic progenies were not detected. The aberration ratio of segregants indicated the gene interaction resulting from different genome structure between vegetatively incompatible species.

KEYWORDS: Interspecific hybridization, *Pleurotus cornucopiae*, *Pleurotus florida*, Protoplast fusion, Genetic analysis.

The production and consumption of the edible fungi have rapidly increased during the last three decades in Korea. The production of commercially cultivated mushroom in 1989 was estimated to be 71,484 tonnes; The species of *Pleurotus* accounted for about 52.3% of the total production, *Lentinus edodes* for an additional 19.3%, *Agaricus* spp. 16.8 %, *Ganoderma lucidum* 10.1%, *Tricholoma matsutake* 1.3% and the others (contain *Flammalina velutipes, Agrocybe aegerita* and so on) 0.2%. The mushroom *Pleurotus ostreatus, P. florida* and *P. sajor-caju* were cultivated on the rice straw, cotton waste or various sawdust substrates in Korea.

The major species of *Pleurotus* were all bifactorial heterothallic. Single-spore isolates from fruiting body were homokaryotic and self-sterile. Monokarkyotic auxotrophs of *P. cornucopiae* and *P. florida* used in this experiments were self-sterile. However, auxotrophs of *P. cornucopiae* formed primordium initials on poplar sawdust substrates.

Protoplast fusion can be a potentially valuable tool for mushroom genetics and breeding. Intraspecific and interspecific hybridization of protoplast have been obtained in the genus *Pleurotus* (Yoo *et al.*, 1984; 1986; 1987; Toyomasu *et al.*, 1986; Toyomasu and Mori, 1987a; 1987b; 1989; Go *et al.*, 1989). This investigation described somatic hybridization and genetic recombination between vegetatively incompatible species by protoplast fusion.

Table 1. List of strains used

Species	Code no.	Genetic marker	Origin
P. cornucopiae	ASI 2-28-c2011-254	gln arg	Irradiation of basidiospores
	ASI 2-29-c2011- 47	ade	to UV
	ASI 2-39-c2011- 43	cit pan	
P. florida	ASI 2- 3-f 2016-29	rib	Irradiation of protoplasts
	ASI 2-73-f 2016-22	ura	to UV
	ASI 2-74-f 2016-26	pro	Irradition of basidiospores
	ASI 2-75-f 2016- 5	gua	to UV
	ASI 2-76-f 2016-37	leu ura	

1) Mutant symbols : ade (adenine), arg (arginine), gln (glutamine), gua (guanine), leu (leucine), pan (panthothenic acid), pro (proline), ura (uracile).

Materials and Methods

Strains and Growth Conditions: The isolation of auxotrophic mutants was performed as described by Yoo *et al.* (1988). The strains used in these experiments were listed in Table 1. They were maintained on the mushroom complete medium (CM) containing (per liter) $MgSO_4 \cdot 7H_2O$ 0.5g, KH_2PO_4 0.46g, K_2HPO_4 1.0g, Peptone 2.0g, Yeast extract 2.0g, glucose 20.0g and agar 20.0g. Heterokaryon selection after protoplast fusion was carried out on osmotically stabilized mushroom minimal medium(MM). It consist of (per liter) $MgSO_4 \cdot 7H_2O$ 0.5g,KH_2PO_4 0.46g, K_2HPO_4 1.0g, DL-asparagine 2.0g, Thiamine-HCL 120 μg, glucose 20.0g, Bacto-agar 20.0g and was supplemented with 0.6 M sucrose. The concentration of bottom agar was 2.0% while that of overlaying soft agar was 0.75%.

Protoplast Formation and Fusion: Disks of sterile cellophane sheets were placed on the surface of CM in petridishes. When the colonies of *P. cornucopiae* and *P. florida* on the cellophane sheets grew enough for protoplast isolation, the cellophan sheets were transfered to sterile petridishes. Protoplasts of P. cornucopiae were obtained using a mixture of Novozym 234 (Novo Biolabs), β-Glucanase(BDH), and β-Glucuronidase (Sigma) basically as described by Lee *et al.* (1986). Protoplasts of *P. florida* were prepared using a mixture of Novozym 234 and Cellulase CP (Sturge) with 0.6 M sucrose as described by Yoo *et al.* (1985).

The procedure of protoplast fusion was based on those of Anne and Peberdy (1976) and Yoo *et al.* (1984). Approximately 10^7-10^8 protoplasts of each strain were combined in a fusion tube and centrifused at 500g for 10 min. The pellet of protoplasts was resuspended in 1 m*l* of a solution of 30% polyethylene glycol 8,000(PEG) containing 10 mM $CaCl_2 \cdot 2H_2O$ and 50 mM glycine, adjusted to pH 8.0 with 10 mM NaOH. After incubation for 10min. at 30℃, 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 CM stabilized with 0.6 M sucrose for viability and onto MM for selection of fusion products. The fusion frequency was expressed as the number of colonies on MM to the number of colonies reverted on CM after 10-20 days incubation at 27℃ or

$$F_f = \frac{\text{number of colonies on MM}}{\text{number of colonies on CM}} \times 100$$

Basidiocarp Production: Induction of carpophores was attempted using the 570g sawdust substrates containing poplar tree plus 20% rice bran in 1,000 m*l* glass bottle. For cultivation under sterile conditions, the media was autoclaved at 121℃ for 90 min. On cooling the media was inoculated with spawn. The bottle was plugged with cotton. The cultures were incubated at 27℃ for 25-50 days under low intensity of lights. When mycelia

were grown completely on sawdust media, the bottle was transferred to a light room. In order to get primordia, the mycelia in a bottle with cotton plug were exposed to high intensity of white lights for 20-60 days at 5-15℃. Lighting illuminated for 14 hours per day. When primordia initiation developed primordia the bottle which removed cotton plug was transfered to a light-moisture room.

Basidiospore Germination and Genetic Character Identification: Basidiospore prints obtained from the carpophores of fusion products were stored at 4℃ for the analysis of progeny. The procedure of genetic analysis was based on those of Yoo *et al.* (1986). Spores were spread on mushroom complete agar medium in petridish and incubated for 5-10 days at 27℃. Sporelings were individually transferred from the germination medium to complete medium and incubated for a week at 27℃. All colonies or sectors were transferred to minimal medium 12 colonies per plate. After 7-20 days incubation, prototrophs and auxotrophs could be distinguished, and the latter were identified by testing, again in replicate sets of 12 inocula, on the appropriate screening media.

Results and Discussion

Fusant Isolation and Carpophore Formation: Interspecific fusion products of protoplasts were derived from auxotrophic mutants of *P. cornucopiae* and *P. florida* after PEG treatment. The fusion colonies were produced after 10-20 days of incubation on MM plates. When transferred to MM, almost all fusion colonies exhibited slow growth rate and irregular shape in appearance. Fusion frequency of protoplasts was 0.004-1.24%. Based on the growh rate and cultural characters of each individual fusants grown on CM, they were classified into vigorous growing, stable slowly growing, and segregated slowly growing types (Fig.1).

Strain P554 was more vigorously growing and stable mycelial colony. The colony was abundant mycelium, and showed secretive yellowish brown pigment on CM. When cultured on the CM containing benomyl 100 mg per m*l*, the mycelial colony could be broken. The hyphae did not form

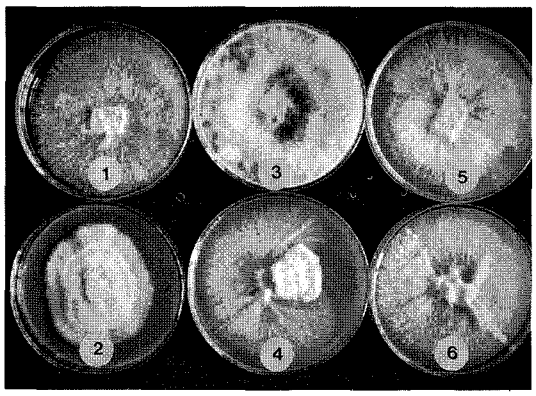

Fig. 1. Morphological variants of fusion products of protopasts between *Pleurotus cornucopiae* and *P. florida*: (1) *P. cornucopiae* ASI 2-29-ade, (2) *P. florida* ASI 2-3-rib, (3) Allodiploid, (4) Non-parental segregant, (5) Segregation colony both parents, and (6) Stable fusant.

clamp connections when observed under microscope. This fusant was assumed to be a nuclear hybrid or allodiploid after nuclear fusion in a cell. Behavior and stability of the hybrid were similar to that of nuclear transfer except pigment production (Yoo *et al.*, 1987). The pigmentation produced by the hybrid was proved to be melanin (Kevei and Peberdy, 1985), and was could be associated with tyrosine metabolism as reported earlier (Trias *et al.*, 1989). Nuclear hybrids derived from filamentous fungi showed basically the same phenotype, but some differences could be seen in pigmentation and fertility (Anne and Peberdy, 1985; Kevei and Peberdy, 1985). The majority of protoplast fusion products were stable slowly growing colonies. There were parental and non-parental types in segregated slowly growing colonies. When each colony derived from segregants of both parents was cultured on MM, it grew, though auxotrophic parents could not survive.

Interspecific hybrids were not obtained from homokaryotic strains of *P. cornucopiae* and *P. florida* by hyphal anastomosis. Out of 59 fusants, however, 2 somatic hybrids of protoplasts formed clamp connections in pairing of ASI 2-28+ASI 2-73. Two clamped and ten clampless heterokaryons among them produced primordia and developed mature carpophores (Table 2). The clamped

Table 2. Characteristics of fusion products of protoplasts between *P. cornucopiae* and *P. florida*

Fusion combination	Genetic background	Clamp[1)]		Fruiting[2)]		No. of isolate	% Isolate
		Anasto mosis	Protoplast fusion	Type	Clamp		
ASI 2-28+ASI 2-3	heterokaryon	−	−	non-fertile		6	100
ASI 2-28+ASI 2-73	heterokaryon	−	+	intermediate	+	2	100
ASI 2-28+ASI 2-74	heterokaryon	−	−	non-fertile		5	100
ASI 2-28+ASI 2-75	heterokaryon	−	−	non-fertile		10	100
ASI 2-29+ASI 2-3	heterokaryon 1	−	−	intermediate	+	8	36.4
	2		−	primordia	−	3	13.6
	3		−	non-fertile		10	45.5
	allodiploid		−	non-fertile		1	4.5
ASI 2-39+ASI 2-3	heterokaryon 1	−	−	intermediate	+	1	7.1
	2		−	intermediate	−	2	14.3
	3		−	primordia	−	6	42.9
	4		−	non-fertile		5	35.7

[1,2)]+: Present clamp connection, −: Absent clamp connection

fusants produced fruit bodies rapidly and abundantly on sawdust medium (Yoo *et al.*, 1984). We have never obtained mature fruit bodies from clampless fusant as described by Yoo *et al.* (1987). However, primordia and fruit bodies from clampless fusants were induced by light-dark cycle in a glass bottle. All hyphae or small tissues from basidiocarps of clampless fusion colonies presented true clamp connections, but fusants P847 and P850 lacked them. Basidiospores of the these two strains bore only a small amounts or zero. A certain part of hyphae of clamped fruiting bodies in sawdust substrates formed also clamp connections. When small tissues of stipe from carpophores cultured on CM agar plates, mycelial colonies grew more vigorously than original clampless fusion products. The somatic hybrid formed intermediate morphology of fruit bodies. However, Most of fruiting character of fusants such as lamellae, stipe, and spore print color were developed similar to those of *P. florida* (Fig. 2). Parental *P. cornucopiae* ASI 2011 and *P. florida* ASI 2016 exhibited yellow and orange white color on pileus in the young mushroom, respectively. Fusion products showed various mixed color of parental species (Table 3). By the way, intraspecific and interspecific hybrids produced almost abnormal morphology of fruit bodies (Toyomasu and Mori, 1989).

Segregation and Recombination of Genetic Markers: Five fusion products in three crosses were analysed with respect to the distribution of progenies and segregation of markers by random spore analysis. The genetic characters were shown to segregate and recombine in the first segregation of monosporus isolates from carpophores of somatic hybrids (Table 4, 5). Basidiospores of strain P564 could yield progeny of four genetypes in the cross ade x rib for protoplasts, auxotrophs of one parental type, auxotrophs of the other parental type and double auxotrophs, respectively. In almost fusants prototrophic recombinants were recovered in large numbers against auxotrophic characters. The allele ratio of could be expected 1 : 1 from one cross *P. cornucopiae* ASI 2-29-ade×*P. florida* ASI 2-3-rib. However, the ratio would be changed to 2 : 1 with increasing proporation of rib. Parental and auxotrophic recombinants were not recovered in the cross *P. cornucopiae* ASI 2-39-cit pan and *P. florida* ASI 2-3-rib. The genotypes of a large number of auxotrophic progenies were not detected in the cross ASI 2-28-gln cit arg×ASI 2-73-ura (Table 6). The modified

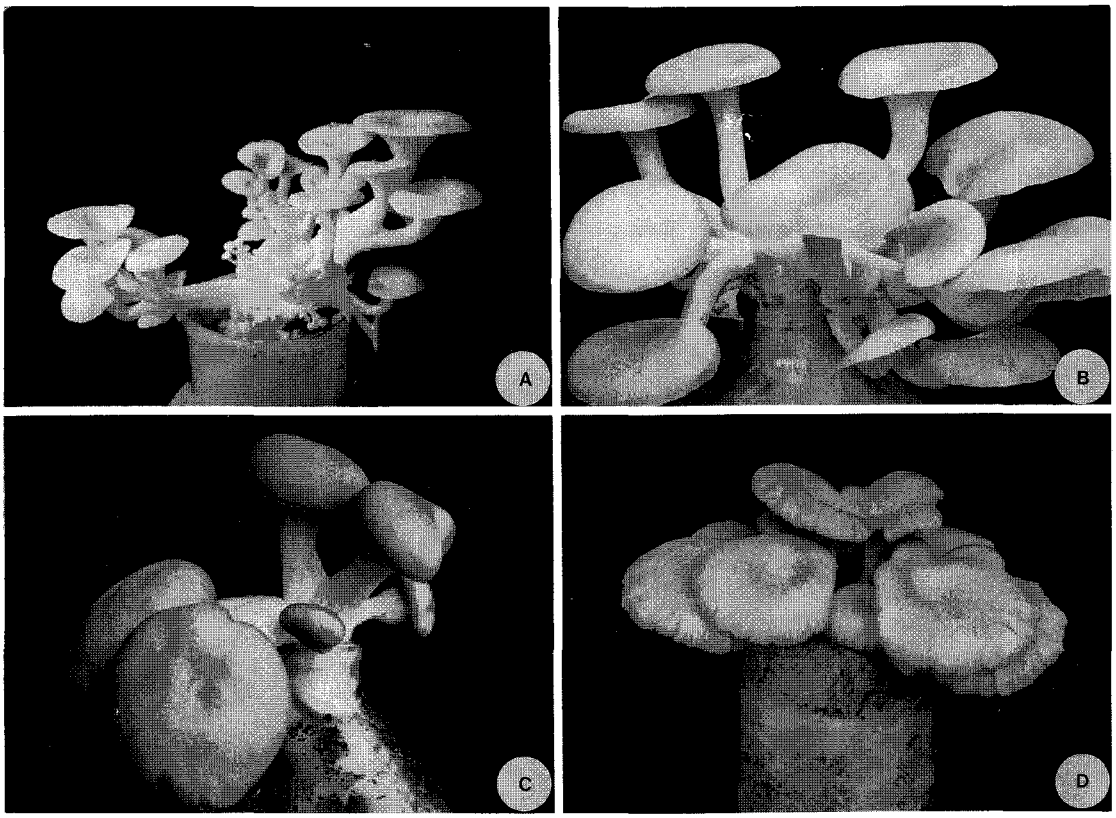

Fig. 2. Carpophores of somatic hybrids of protoplasts between *Pleurotus cornucopiae* and *P. florida*: (A) *P. cornucopiae* ASI 2011, (B) *P. florida* ASI 2016, (C) Fusant P564 of *P. cornucopiae* ASI 2-29-ade+*P. florida* ASI 2-3-rib, and (D) Fusant P863 of *P. cornucopiae* ASI 2-28-gln arg+*P. florida* ASI 2-73-ura.

Holliday method was used for identification of abnormal progenies. These progenies showed adenine, guanine and hypoxanthine requiring mutants. When parental auxotrophs of ASI 2-28-gln arg and ASI 2-73-ura cultured on MM plus adenine, MM plus guanine, MM plus hypoxanthine, and MM plus adenine, guanine and hypoxanthine, respectively, they were non-viable. However, no abnormal phenomena of distribution and segregation were found in intraspecific (Yanagi *et al.*, 1988) and closely related interspecific hybrid (Yoo *et al.*, 1986).

The aberration ratio indicated the gene interaction resulting from the difference of genome structures of distantly related species.

摘 要

노랑느타리 *Pleurotus cornucopiae*와 사철느타리 *Pleurotus florida*의 營養要求株로부터 裸出한 原形質體를 polyethylene glycol로 融合하여 種間 體細胞雜種을 얻었다. 이들은 核融合株 nuclear hybrid 또는 異質二倍體 allodiploid, 異核體 heterokaryon, 自然的 分離性異核體 spontaneous segregated heterokaryon로 나누어졌으며, 6融合組合중에서 1組合에서만 꺽쇠연결체 clamp connection를 가진 것이 나타났다. 총 59융합주 중에서 꺽쇠연결체를 가진 2균주와 가지지 않은 10균주가 완전한 子實體를 형성하였는데 거의 사철느타리 形態와 有似하였으나 버섯의 갓 色澤이 兩親인 노랑느타리가 어릴때 yellow, 사철느타리가 orange white 인데비해 체세포 잡종은 light blond-greyish brown으로 다양한 混合色으로 나타났다. 꺽쇠연결체가 없는 융합주로부터 發芽된 자실체는 두균주를 제외하고 모두 꺽쇠연결체를 가졌으며, 이 子實體 組織을 完全培地에 培養한 결과 菌絲生長이 빠르고 균사도 꺽쇠연결체를 가져 本來의 융합주와는 다른 형태로 변하였다.

Table 3. Characteristics of basidiocarps of somatic hybrid of protoplasts between *P. cornucopiae* and *P. florida*.

Strain	Clamp[1)]	Color of pileus	
		Young	Mature
ASI 2-28+ASI 2-73 P863	+	brownish orange	orange grey
P864	+	light blond	brownish orange
ASI 2-29+ASI 2- 3 P547	+	dark brown	greyish brown
P549	+	dark brown	greyish brown
P558	+	dark brown	greyish brown
P564	+	greyish brown	brownish grey
P567	+	greyish brown	brownish grey
P560	+	greyish brown	greyish orange
P548	+	brown	brownish orange
P562	+	brown	brownish orange
ASI 2-39+ASI 2- 3 P842	+	greyish orange	greyish yellow
P847	−	brown	light brown
P850	−	reddish grey	brown
P. cornucopiae ASI 2011	+	yellow	light yellow
P. florida ASI 2016	+	orange white	yellowish white

[1)]+: Preset clamp connection, −: Absent clamp connection

Table 4. Frequency distribution of progenies of somatic hybrid of protoplasts between *P. cornucopiae* ASI 2-29-ade and *P. florida* ASI 2-3-rib (P549).

Genotype[2]		No. of individual
+	+	51
ade	+	0
+	rib	22
ade	rib	29

Allele ratio

Locus	Matant	Wild	χ^2	P
ade	29	73	18.98	<0.005
rib	51	51	0	1

Genetic analysis of paired markers

Parental	Recomb	χ^2	P
22	80	32.98	<0.005

*germination: 2.6%

Table 5. Frequency distribution of progenies of somatic hybrid of protoplasts between *P. cornucopiae* ASI 2-29-ade and *P. florida* ASI 2-3-rib (P654).

Genotype		No. of individual
+	+	70
ade	+	3
+	rib	34
ade	rib	11

Allele ratio

Locus	Matant	Wild	χ^2	P
ade	14	104	68.64	<0.005
rib	45	73	6.64	<0.010

Genetic analysis of paired markers

Parental	Recomb	χ^2	P
37	81	16.40	<0.005

*germination: 0.8%

Table 6. Frequency distribution of progenies of somatic hybrids between *Pleurotus cornucopiae* ASI 2-28-gln arg and *P. florida* ASI 2-73-ura (P863.P864) following protoplast fusion

Phenotype	No. of individual	
	P863	P864
prototroph	191	229
ura	8	6
gln	5	6
arg	6	12
gln arg	9	7
ura gln	2	5
ura arg	2	2
ura gln arg	5	7
ade hyp	9	0
ade gua hyp	36	83

*germination: P863 15.09%, and P864 23.63%.

Table 7. Frequency distribution of progenies of somatic hybrids of protoplasts between *Pleurotus cornucopiae* ASI 2-39-cit pan and *P. florida* ASI 2-3-rib (P 842)

Phenotype	No. of individual
prototroph	320
auxotroph	0

*germination: 0.03%

3組合 5融合株를 遺傳分析한 결과 노랑느타리 ASI 2-29+사철느타리 ASI 2-3 융합주는 사철느타리 遺傳子座의 分離比가 노랑느타리보다 2倍로 많이 나타났고, 노랑느타리 ASI 2-28-gln arg+사철느타리 ASI 2-73-ura에서는 많은 수의 영양요구성 遺傳子再組合體가 양친의 遺傳標識와는 다는 ade hyp, ade gua hyp의 形質로 나타났다. 또한 노랑느타리 ASI 2-39+사철느타리 ASI 2-3은 모두 原營養體 prototroph만 나타내어 非正常的 遺傳現象을 보였다.

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