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Interspecific protoplast Fusion and Sexuality in *Pleurotus*

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느타리의 種間 原形質體 融合 및 有性

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ABSTRACT: Interspecific heterokaryons were obtained by polyethylene glycol induced fusion of protoplasts from auxotrophic mutants of *P. ostreatus*+*P. cornucopiae*, *P. ostreatus*+*P. florida*, *P. ostreatus*+*P. sajor-caju*, *P. florida*+*P. cornucopiae*, *P. florida*+*P. sajor-caju* and *P. sajor-caju*+*P. cornucopiae* protoplasts.

The Fusion products of protoplasts were induced by MCM+benomyl, but segregation of sectors were not identified. Interspecific fusion products of protoplasts between incompatible strains did not form true clamp connections and did not produce fruit bodies. For induction of fertility, interspecific heterokaryons crossed with their parents by hyphal anastomosis.

KEYWORDS: Interspecific protoplast fusion. Clamp connection, Fertility, *P. cornucopiae*, *P. florida*, *P. ostreatus*, *P. sajor-caju*, Basidiomycetes.

Gene transfer mediated by protoplast fusion is a powerful tool of somatic cell genetics and breeding in microorganisms(Anne, 1977; Peberdy 1980; Ferenczy 1984). Interspecific fusion products of protoplasts have been obtained in filamentous fungi(Ferenczy 1976; Anne and Peberdy 1976; Ohnuki *et al.*, 1982; Minuth and Esser, 1983). In basidiomycetes, however, a few work on intraspecies(Santiago, 1981; Gold *et al.*, 1983; Kiguchi and Yangai, 1985) and interspecies protoplast fusion(Yoo *et al.*, 1984; Toyomasu *et al.*, 1986) were reported.

Incompatibility is present in asexual species and in both homothallic and heterothallic sexual species. Most of *Pleurotus* species are all tetrapolar incompatibility(Whitehouse, 1949; Anderson *et al.*, 1973; Roxon and Jong, 1977; Raper, 1978). Four types of single spore isolates are obtained with two different A and two diffent B factors. Bifactorial fungi have two incompatibility factors with together control mating competence.

Recently, we have reported interspecific protoplast fusion and genetic analysis in *Pleurotus*(Yoo *et al.*, 1984, 1986). This paper describes hybridization and induction of fertility using hyphal anastomosis and protoplast fusion with incompatible strains in *Pleurotus*.

Materials and Methods

Stranis and Growth Conditions

All strains were derived from Agricultural Sciences Institute Collection(ASI). Auxotrophs and their wild types used in these experiments, were listed in Table I. They were maintained on the Mushroom Complete Medium(MCM), containing(g/ *l*) $MgSO_4 \cdot 7H_2O$ 0.05, KH_2PO_4 0.46, K_2HPO_4 1.0, peptone 2.0, yeast extract 2.0, glucose 20.0, and Bacto-agar 20.0. Heterokaryon selection after protoplast fusion was carred out on os-

Table I. List of strain used

Species	Strain no.	Marker genetype	Origin	Clamp*	Fruiting**
P. cornucopiae	ASI 2011	none	wild	+	F
	ASI 2-29	Ade	SI 2011	-	S
P. florida	ASI 2016	none	wild	+	F
	ASI 2-3	Rib-1	ASI 2016	-	S
	ASI 2-24	Rib-2	ASI 2016	-	S
P. ostreatus	ASI 2018	none	wild	+	F
	ASI 2-1	Arg	ASI 2018	-	S
	ASI 2-2	Gly Ser	ASI 2018	-	S
P. sajor-caju	ASI 2070	none	wild	+	F
	ASI 2-44	Cys	ASI 2070	-	S
	ASI 2-52	Ane Nia	ASI 2070	-	S
	ASI 2-53	Rib Ane	ASI 2070	-	S
	ASI 2-55	Ane PN Asn	ASI 2070	-	S

*+: Presence clamp connection.
-: Absent clamp connection
**F: Fertile S: Sterile

motically stabilized Mushroom Minimal Medium(MMM). It consists of(g/*l*) $MgSO_4 \cdot 7H_2O$ 0.5, KH_2PO_4 0.46, K_2HPO_4 1.0, Thiamin-HCl 120 ug, DL-asparagine 2.0, glucose 20.0, Bacto-agar 20.0, and was supplemented with 0.6M Potassium chloride or sucrose bottom agar was of 2.0% while overlaying soft agar was of 0.75%.

Protoplast Formation and Fusion

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 *Pleurotus* species from 4 day culture at 27℃ were removed to clean sterile petri dishes and the lytic enzyme stabilizer solution was added immediately. The lytic mixture contained Novozym 234(Novo), β-Glucuronidase(Sigma) and β-D-Glucanase(BDH) 5 mg ml^{-1} each in 0.6M sucrose.

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 500×g for 10 min. The pellet of protoplasts was resuspended in 1 m*l* of a solution of 30% polyethylene glycol 4000(PEG) containing 0.1M $CaCl_2 \cdot 2H_2O$ and 0.05M glycine, adjusted to pH 8.0 with 0.01M NaOH. After incubation for 10 min. at 30℃, the suspension was diluted with 0.6M sucrose, washed once by centrifugation, and respended in 5 m*l* osmotic stabilizer, Serial dilutions of treated protoplasts were plated onto MCM stabilized with 0.6M potassium chloride or sucrose 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 10-20 days incubation at 27℃.

Basidiocarp production

Fruit body production was attempted using the sawdust of a broadleaf tree plus 20% rice bran in 450g bottle. For cultivation under sterile conditions the media autoclaved at 121℃ for 90 min. On cooling, the media were inoculated with spawn. The bottle was plugged with cotton. The cultures were incubated at 25℃ for 30 days and then in the region of 10-20℃ in the light room.

Results and Discussion

Isolation of fusion products

After PEG solution treatment of a mixture of complementing *P. ostreatus*+*P. cornucopiae*, *P.*

Table II. Percentage of protoplast fusion in the various combinations of auxotrophs of *Pleurotus* species

Fusion mixtures	Fusion frequency(%)
P. ostretus P. cornucopiae	
ASI 2-1+ASI 2-29	3.25
P. ostreatus P. florida	
ASI 2-1+ASI 2-3	0.50
ASI 2-2+ASI 2-4	2.00
P. ostreatus P. sajor-caju	
ASI 2-1+ASI 2-44	0.04
ASI 2-1+ASI 2-53	0.35
ASI 2-2+ASI 2-55	0.80
P. florida P. sajor-caju	
ASI 2-3+ASI 2-44	0.002
ASI 2-3+ASI 2-53	0.02
ASI 2-3+ASI 2-55	0.24
P. florida P. cornucopiae	
ASI 2-3+ASI 2-29	2.62
P. sajor-caju P. cornucopiae	
ASI 2-55+ASI 2-29	0.24

ostreatus+*P. florida*, *P. ostreatus*+*P. sajor-caju*, *P. florida*+*P. cornucopiae*, *P. florida*+*P.sajor-caju* and*P. sajor-caju*+*P. cornucopiae* protoplasts, small prototrophic colonies developed on MMM. Fusion frequencies for the various interspecific crosses were ranged between 0.002 and 3.25%(Table II). The fusion products produced after 10-20 days of incubation on MMM plates. When transferred to MMM plates, the sectors gave normal with a compact and regular colony morphology. In the all crosses fusion products were induced by MCM and MCM+benomyl, but segregation of sectors was not identified. These fusion products were probably due to stable heterokaryon.

The growth rate and cultural characters of each individual fusion product of protoplasts grown on MMM they could be classified into fast growing, moderate growing and slow growing colonies(Yoo *et al.* 1984).

Interspecific allodiploid(hybrid) was identified by various criteria including prototrophy, outgrowth, pigmentation, conidia, DNA content and segregation(Kevei and Peberdy, 1977, 1979; Bradshaw, 1983).

Protoplast Fusion and Sexuality

Seven strains of parents for hyphal- and protoplast fusion showed their compatibility and incompatibility each other(Table III). All of the colonies from hyphal- and protoplast fusion produced true clamp connections, but combination of ASI 2-1+ASI 2-44, ASI 2-1+2-53, ASI 2-3+ASI 2-44, ASI 2-3+ASI 2-53 and ASI 2-29+ASI 2-55. The colonies with clamp connections from hyphal and protoplast fusion induced primodia and basidiocarps on sawdust media in bottles under certain conditions. Interspecific fusion products of protoplasts with incompatible strains by hyphal fusion did not form true clamp connections and did not produce furit bodies(Table IV-1, Table V-1, Table VI-1, Table VII). For induction of basidiocarps, strains of P185, P188, P150 and P148 crossed with their parents of ASI 2016, ASI 2018 and ASI 2070 by hyphal

Table III. Pairings among the seven monokaryotic mycelia

Strain	ASI 2-1	ASI 2-2	ASI 2-3	ASI 2-44	ASI 2-52	ASI 2-53	ASI 2-55
ASI 2-1		−	+	−		−	−
ASI 2-2			+	−			+
ASI 2-3				−		−	+
ASI 2-44					+	−	−
ASI 2-52						−	+
ASI 2-53							−
ASI 2-55							

+: indicate formation of clamp connection
-: indicate no dikaryon formation

Table IV-1. Protoplast fusion between *P. ostreatus* and *P. florida*

Fusion mixture	No. of	Clamp connection		Fruiting
P. ostreatus *P. florida*	fusion products	Anastomosis	protoplast fusion	
ASI 2-1+ASI 2-3	P5	+	+	F
ASI 2-2+ASI 2-24	P15	+	+	F

Table IV-2. Hyphal anastomosis with fusion products of protoplasts and monokaryon mycelia

Strain	Clamp connection	Fruiting
P5 × ASI 2-1	+	F
× ASI 2-2	+	F
× ASI 2-3	+	F
× ASI 2-4	+	F
P15 × ASI 2-1	+	F
× ASI 2-2	+	F
× ASI 2-3	+	F
× ASI 2-4	+	F

fusion. After 10-20 days hyphal anastomosis between fusion products of protoplasts and their parents, fusion products of P185, P188, P150 and P148 formed true clamp connections. Strain P148 was fusion products of protoplasts between *P. florida* ASI 2-3 and *P. sajor-caju* ASI 2-53. Strain ASI 2-3 was compatible with ASI 2-1, but strain P148 was incompatible with ASI 2-1. Strain P150 was the same case of P148.

Strain P185 is fusion products of protoplasts between *P. ostreatus* ASI 2-1 and *P. sajor-caju* ASI 2-44. Strain ASI 2-1 was compatible with ASI 2-3 and strain P185 was compatible with ASI 2-3. Strain P188 was the same case as P185(Table VI-2, Table V-2, Table VI-2).

Protoplast fusion is efficient at overcoming the

Table V-2. Hyphal anastomosis with fusion products of protoplasts and mono- and dikaryon mycelia

Strain	Clamp connection	Fruiting
P185 × ASI 2-1	−	S
× ASI 2-2	−	S
× ASI 2-3	+	F
× ASI 2-44		
× ASI 2-52	+	F
× ASI 201 1	+	F
× ASI 2016	+	F
× ASI 2018	+	F
× ASI 2070	+	F
P188 × ASI 2-1	−	S
× ASI 2-2	−	S
× ASI 2-3	+	F
× ASI 2-53	−	S
× ASI 2011	+	F
× ASI 2016	+	F
× ASI 2018	+	F
× ASI 2070	+	F
P156 × ASI 2-1	+	F
× ASI 2-2	+	F
× ASI 2-3	+	F
× ASI 2-52	−	S
× ASI 2-55	−	S

Table V-1. Protoplast fusion between *P. ostreatus* and *P. sajor-caju*

Fusion mixture	No. of	Clamp connection		Fruiting
P. ostreatus *P. sajor-caju*	fusion products	Anastomosis	protoplast fusion	
ASI 2-1+ASI 2-44	P185	−	−	S
ASI 2-1+ASI 2-53	P188	−	−	S
ASI 2-2+ASI 2-55	P156	+	+	F

Table VI-1. Protoplast fusion between *P. florida* and *P. sajor-caju*

Fusion mixture	No. of	Clamp connection		Fruiting
P. florida *P. sajor-caju*	fusion products	Anastomosis	Protoplast fusion	
ASI 2-3+ASI 2-44	P150	−	−	S
ASI 2-3+ASI 2-53	P148	−	−	S
ASI 2-1+ASI 2-55	P110	+	+	F

Table VI-2. Hyphal anastomosis with fusion products of protoplasts and mono- and dikaryon mycelia

Strain	Clamp connection	Fruiting
P150 ×ASI 2-1	−	S
×ASI 2-	−	S
×ASI 2-3	−	S
×ASI 2-44	−	S
×ASI 2-52	+	F
×ASI 2011	+	F
×ASI 2016	+	F
×ASI 2018	+	F
×ASI 2070	+	F
P148 ×ASI 2-1	−	S
×ASI 2-2	−	S
×ASI 2-3	−	S
×ASI 2-53	−	S
×ASI 2011	+	F
×ASI 2016	+	F
×ASI 2018	+	F
×ASI 2070	+	F
P110 ×ASI 2-1	+	F
×ASI 2-2	+	F
×ASI 2-3	+	F
×ASI 2-52	+	F
×ASI 2-55	+	F

effects of vegetative incompatibility(Cropt, 1985). Intraspecific fusion products of protoplasts between antagonistic morphological variants in *Volvariella volvacea* induced fruit bodies(Santiago, 1981). However, kigachi and Yanagi(1985) obtained intraspecific heterokaryons of protoplasts between incompatible strains in *Coprinus macrorhizus*. The heterokaryons did not developed fruit bodies on MYPG medium. These discrepancies of the fertility were probably due to pattern of sexuality.

摘 要

원형질체 융합에 의한 식용버섯의 유전육종연구는 종래의 전통적 육종방법인 균사융합으로는 해결할 수 없는 균주간의 불화합성 장벽을 능가하여 새로운 품종육성에 그 기대가 크다. 한국의 주요재배 버섯이며, 날로 세계의 재배면적이 증가하는 느타리버섯류의 유전육종을 위하여 몇가지 종간의 체세포 잡종의 선발과 이들의 유성에 대하여 조사한 결과는 다음과 같다.

1. 이 종간 원형질체 융합으로 체세포 잡종을 선발하였는데, 느타리+노랑느타리, 느타리+사철느타리, 느타리+여름느타리, 사철느타리+노랑느타리, 사철느타리+여름느타리 그리고 여름느타리+노랑느타리의 원형질체 융합율은 0.002-3.25%였으며, MCM과 MCM+benomyl에서 융합주의 균총분리가 일어나지 않았다.

2. 화합성 균주간에는 균사융합 및 원형질체 융합에 의하여 모두 균사체에 clamp connection을 형성

Table VII. Protoplast fusion of *P. cornucopiae* with other speices

Fusion mixture	Clamp connection		Fruiting
	Anastomosis	Protoplast fusion	
P. cornucopiae(ASI 2-29)+*P. florida*(ASI 2-1)	+	+	F
+*P. ostreatus*(ASI 2-3)	+	+	F
+*P. sajor-caju*(ASI 2-55)	−	−	S

하였으며, 자실체도 형성하였다. 그러나 불화합성 균주간에는 균사 및 원형질체 융합에 의하여 clamp connection을 확인할 수 없었으며, 자실체도 형성하지 않았다.

3. clamp connection이 없고 자실체도 형성하지 않는 원형질체 융합주의 임성을 유도하기 위하여, 이들 모균주와 균사융합한 결과 단핵균주와는 드물게 clamp connection을 형성하였으며, 이 핵균주와는 모두 clamp connetion을 형성하였다.

4. 불화합성 균주간의 원형질체 융합주는 새로운 불화합성을 형성하였는데, ASI 2-3+ASI 2-53의 P 148은 ASI 2-3과 화합성인 ASI 2-1, ASI 2-2와 불화합성을 나타냈다. 또한 ASI 2-3+ASI 2-44의 P 150은 ASI 2-3과 화합성인 ASI 2-1, ASI 2-2와 불화합성이었다.

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