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Protoplast fusion between *Lentinula edodes* and *Coriolus versicolor*

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Protoplast fusion between isoleucine-, arginine- and thymidine-requiring auxotroph (Ile^-, Arg^-, Thy^-) of *Lentinula edodes* and arginine-requiring auxotroph (Arg^-) of *Coriolus versicolor* has been achieved using 30% polyethylene glycol (M.W. 4000) in 10 mM $CaCl_2$-glycine solution (pH 8.0). Fusion hybrids were selected in the 0.6 M sucrose supplemented minimal media on the basis of nutritional complementation with fusion frequency of 7.4×10^{-6}. The hybrids included both parental and non-parental types in colony morphology, growth rate and isozyme patterns. We succeeded inter-order protoplast fusion between the auxotrophs of *Lentinula edodes* and Coriolus versicolor overcoming the natural barriers of incompatibility. We examined the characteristics of the hybrids and clarified the fusion process using electron microscopy.

Key words : *Lentinula edodes, Coriolus versicolor,* Inter-order protoplast fusion, Isozyme pattern

INTRODUCTION

Protoplast fusion has proved to be a suitable tool for overcoming the natural barriers of incompatibility and for establishing heterokaryosis in many basidiomycetes. Inter-generic and even inter-family protoplast fusions have been successfully made in basidiomycetes. Fusions between *Lentinula edodes* and *Ganoderma lucidum, G. lucidum* and *Coriolus versicolor,* and *L. edodes* and *Pleurotus florida* were reported (Liang and Chang, 1989; Bok *et al.*, 1994; Park *et al.*, 1991; Yoo and Shin, 1996).

Lentinula edodes and *Coriolus versicolor* showing anatomical incompatibility were grown on complete media (Fig. 1). The basidiocarps of *L. edodes* are the most popular edible mushroom in Korea. Several pharmacophysical effects of *L. edodes* were reported. The antitumor effect of the extract of *L. edodes* in tumor-transplanted mice and anticarcinogenic effects of an alcohol-insoluble fraction from the culture medium were reported (Maeda and Chihara, 1971). LEM, an extract of the culture medium of *L. edodes* mycelia completely inhibited the HIV-1-induced cytopathic effect *in vitro* at concentrations of > 10 ug/ml and strongly increased the glucose consumption of macrophages and induced the proliferation of murine bone marrow cells *in vitro* (Suzuki *et al.*, 1989).

The polysaccharide fraction of *C. versicolor* inhibits the tumor growth of sarcoma-180 and rat ascites hepatoma AH-13 (Tsukagoshi *et al.*, 1974; Kim *et al.*, 1979). PS-K, an extract of *C. versicolor* inhibited antibody-forming capacities in tumor-bearing mice, human immunodeficiency virus infection and reverse transcriptase (Nomoto *et al.*, 1975; Ohno *et al.*, 1972; Hirose *et al.*, 1987; Tochikuro *et al.*, 1987).

The production of hybrids which have combined or enhanced pharmaco-physical effects of *L. edodes* and *C. versicolor* is very desirable. Investigation of a possibility of inter-order protoplast fusion between two strains showing incompatibility in nature is prerequisite. To investigate a possibility of inter-order protoplast fusion, we performed protoplast fusion of *L. edodes* and *C. versicolor,* and obtained several hybrids by overcoming the natural barriers of incompatibility and determined their characteristics.

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MATERIALS AND METHODS

Strains and culture condition

The isoleucine-, arginine-, and thymidine-requiring auxothroph of *L. edodes,* LE(eb)26, was isolated by ethidium bromide enrichment after ultraviolet irradiation (Kim *et al.*, 1996). The arginine-requiring auxotroph of *C. versicolor,* CV17, was also isolated in our laboratory (Park *et al.*, 1989). Complete media (CM) consisted of glucose 20 g, yeast extract 2.0 g, peptone 2.0 g, K_2HPO_4 1.0 g, KH_2PO_4 0.46 g, $MgSO_4\cdot7H_2O$ 0.5 g, agar 15 g per liter (pH 6.2). Minimal media (MM) consisted of glucose, minerals and agar. In protoplast

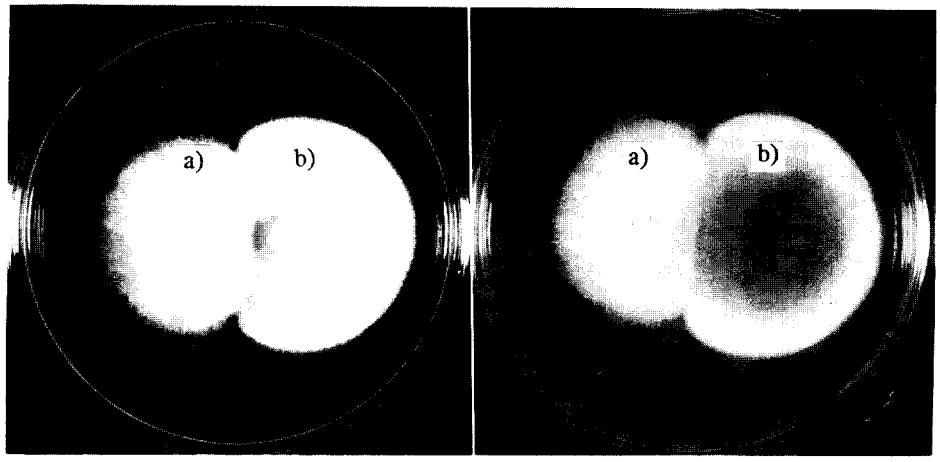

Fig. 1. The plates show anatomical incompatibility between *Lentinus edodes* (a) and *Coriolus versicolor* (b).

regeneration and fusion experiments, both media were supplemented with 0.6 M sucrose as an osmotic stabilizer (RCM and RMM).

Protoplast isolation, fusion and regeneration

Protoplasts were isolated from the liquid-cultured-mycelia of *L. edodes* as described by Kim *et al.* (1997) and from the mycelia of *C. versicolor* which were grown on cellophane sheet as described by Kevei and Peberdy (1977). A combination of 10 mg/ml Novozyme 234 (Novo Industries, Bagsvaerd, Denmark) and 10 mg/ml Cellulase Onozuka R10 (Yakult Co., Japan) was used regularly as lytic enzyme. Protoplast fusion was performed by using 30% polyethylene glycol (PEG) 4000 in the presence of 10 mM $CaCl_2$. The fusion mixture was suspended in 10 ml of 0.6 M sucrose and plated on RMM and RCM. The plates were over-layered with 0.75% top agar and incubated at 25°C. Fusion frequency was calculated as the ratio of colonies growing on RMM and RCM.

Induced segregation

The artificial segregation was induced by the modified method of Kevei and Peberdy (1979). Small discs of the mycelia of the fusants were placed onto CM containing 1.0 ug/ml benomyl (Chinoin, Budapest, Hungary) and segregates were isolated after incubation at 25°C.

Cell lysates and electrophoresis

After 25 days culture, the mycelium was collected and rinsed with ice cold 0.1 M Tris-HCl (pH 7.6). The mycelium was homogenized with dry ice and centrifuged at 15000 rpm for 50 min at 4°C. The enzymes in the supernatant were quantitated by the modified Lowry method with bovine serum albumin as a standard. Enzyme activities were determined after electrophoresis on 10% nondenaturing polyacrylamide gels.

Peroxidase and esterase isozymes

The presences of intracellular peroxidase and esterase were examined by the methods that compare the production of a colored final product.

Peroxidase (EC 1.11.1.7) was detected by a modified method of Tanksley (1979). Briefly, gels were incubated in TMBZ solution (3,3',5,5'-tetramethyl benzidine 25 mg, methanol 25 ml, 1 M sodium acetate 50 ml, and pH 4.7) for 30 min at 30°C in the dark, and reacted with 1 ml of 3% H_2O_2. TMBZ is a substrate of peroxidase activity and is oxidized by the hydrogen peroxide to give a black product.

For the esterase (EC 3.1) detection, the gels were fixed in citrate-acetone-methanol fixative (38.3 mM citrate 18 ml, acetone 27 ml, methanol 5 ml) for 30 seconds, washed twice with deionized water, and air-dried at least 20 min at room temperature. The dried gels were incubated for 30 min at 37°C in the solution mixture: 10 mg of Fast Garnert GBC salt (Sigma, MO, USA) in 50 ml of 20 mM tris-HCl buffer (pH 7.6), 20 mg of α-naphthyl acetate in 2 ml of ethylene glycol monomethyl ether. The naphthol radical of the carboxylic esters is released by esterase and joins with diazonium salt to form an insoluble black product.

Microscopic observations

Samples were fixed in 3% (v/v) glutaraldehyde in sodium phosphate buffer (pH 7.6) containing 0.6 M sucrose for 5 h. For scanning electron microscopy (SEM), samples were washed with 0.6 M sucrose, dehydrated in ethanol (30, 50, 60, 70, 80, 90, 95, 100%, serially) and acetone. Gold particle coated samples were scanned using SEM (JSM-T200, Jeol, Japan). For transmission electron microscopy (TEM), glutaraldehyde fixed samples were followed by post fixations in 1% osmi-

um tetroxide for 2 h at 4°C. The samples were washed with 0.5 M sucrose, dehydrated in ethanol and propylene oxide, and embedded and polymerized in Epon (nadic methyl anhydride 11.3 ml, dodecenyl succinic anhydride 9.9 ml, EM bed-812 18.8 ml, and 2,4,6-tri (dimethylaminoethyl)phenol 0.6 ml; Electron Microscopy Sciences, PA, USA). Ultrathin sections (50~70 nm) were stained with uranyl acetate and lead citrate, and scanned using TEM (JEM-1200 EXII, Jeol).

RESULTS AND DISCUSSION

Properties of fusion hybrids

The protoplast fusion hybrids appeared on the semi-solid RMM after 45~60 days incubation at 25°C. The reported optimum temperature for the regeneration of *L. edodes* was 22±1°C (Leatham, 1984), 22~26°C (Kim *et al.*, 1987), 25°C (Ishikawa, 1967) and 22~27 °C (Hiroe and Ikuda, 1960), that for *C. versicolor* was 25~30°C (Park *et al.*, 1989). We incubated the hybrids at 22, 25, 28, 30°C and the best regeneration yield was obtained at 25°C (data not shown).

The hybrids were transferred to complete medium after several passages on MM. The hybrids gave rise to parental segregation when transferred to CM from MM. After twenty passages, stable fusants were isolated and their characteristics were analyzed (Table I).

Based on the colony morphology, the fusants were classified into three types: *L. edodes*-like, *C. versicolor*-like and non-parental types. LE(eb)26 was characterized by fast growing, white aerial mycelia and CV17 was characterized by fast growing pigmented mycelia and advancing zone (Fig. 1). Colony morphologies and growth rates of these fusants were variable and aerial mycelia ranged from sparse to dense. Most of the fusants grew slower than either parents and the colony morphology ranged from those that resembled *L. edodes* to those that resembled *C. versicolor*. Most of *C. versicolor*-like fusants except F25 and the non-parental fusants produced a brown pigment. The pigmentation became more intense as incubation was prolonged. Kevei (1985) proved the brown pigment to be melanin. He found that the more soluble fraction was excreted into the medium and that a highly polymerized form was present in the cell wall.

In case of segregation induced by benomyl, some of the protoplast fusants segregated at 1.0 ug/ml. The segregates resembled one of their parents in morphology (Fig. 2). Sectors from a hybrid, F8, gave rise to a

Table I. Characterization of the protoplast fusants between LE(eb)26 and CV17

Strain	Colony morphology	Backcross to parents[a]	Aerial mycelia[b]	Mycelial growth[c]	Pigment/ advancing zone[d]
LE(eb)26	white, long mycelia		4	F	-/-
CV17	heavy growth short mycelia		1	F	+/+
L. edodes type					
F3		Le	4	M	-/-
F5		Le	4	M	-/-
F6			4		
C. versicolor type		Le		M	-/-
F13		CV	2	S	+/+
F14		CV	2	M	+/+
F16		nd*	2	M	+/+
F18		CV	2	S	+/-
F19		CV	2	S	+/+
F21		CV	3	S	+/+
F22		CV	3	S	+/+
F24		CV	2	S	+/+
F25		CV	1	M	-/+
F26		CV	2	M	+/+
non-parental type					
F12		CV	2	M	+/+
F15		CV	2	M	+/+
F23		CV	1	S	+/+
F30		nd	2	S	+/+

[a]Le: LE(eb)26, CV: CV17
[b]4: indicate best yields
[c]F: fast growth, represent radial growth rate > 2.0×10^{-4} m/h, M: moderate growth, represent 2.0×10^{-4} m/h > radial growth rate > 1.0×10^{-4} m/h, S: slow growth, represent 1.0x10-4 m/h > radial growth rate
[d]+/+: brown pigment production and zonate
nd*: not determined

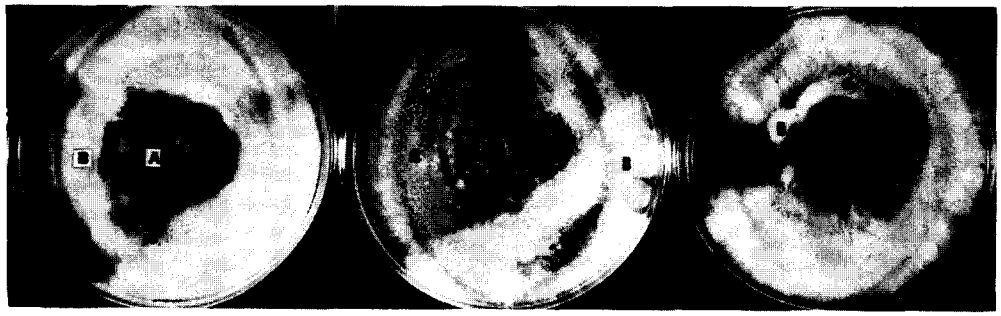

Fig. 2. Benomyl-induced segregation of the fusant F8 at 1.0 ug/ml. The segregates resembled one of their parents in morphology.

strain (A) with sparse aerial, wrinkled and brown pigmented mycelia and a more rapidly growing white strain (B).

Isozyme band patterns

The isozyme components of the mycelial extracts were a potentially useful tool for assessing the interaction of different genomes in a fusant. An intracellular buffer-soluble protein from the mycelia was used for peroxidase and esterase assay on nondenaturing polyacrylamide gels. The isozyme patterns of LE(eb)26 and CV17 were distinctly different and their fusants dis-

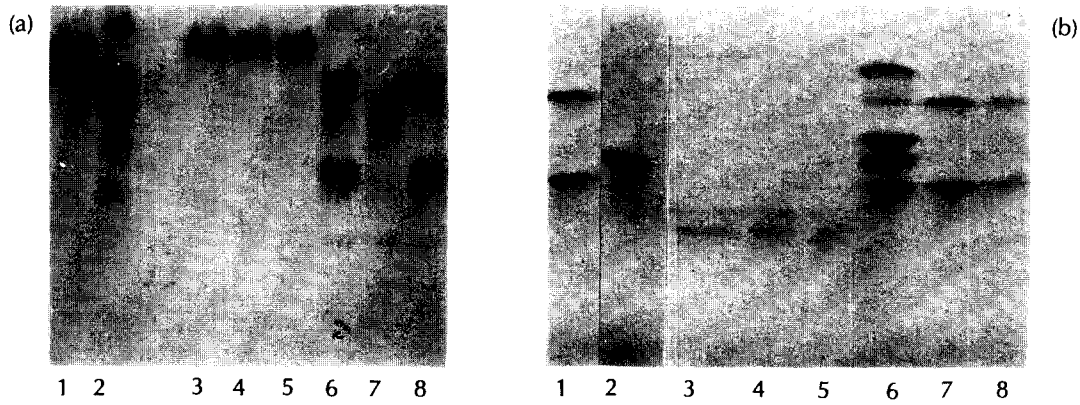

Fig. 3. Electrophoretic isozyme patterns of peroxidase (a) and esterase (b) of six protoplast fusants (3-8) and their parents, LE (eb)26 and CV17. In both panels, 1: LE(eb)26; 2: CV17; 3: F3; 4: F5; 5: F6; 6: F10; 7: F11; 8: F12.

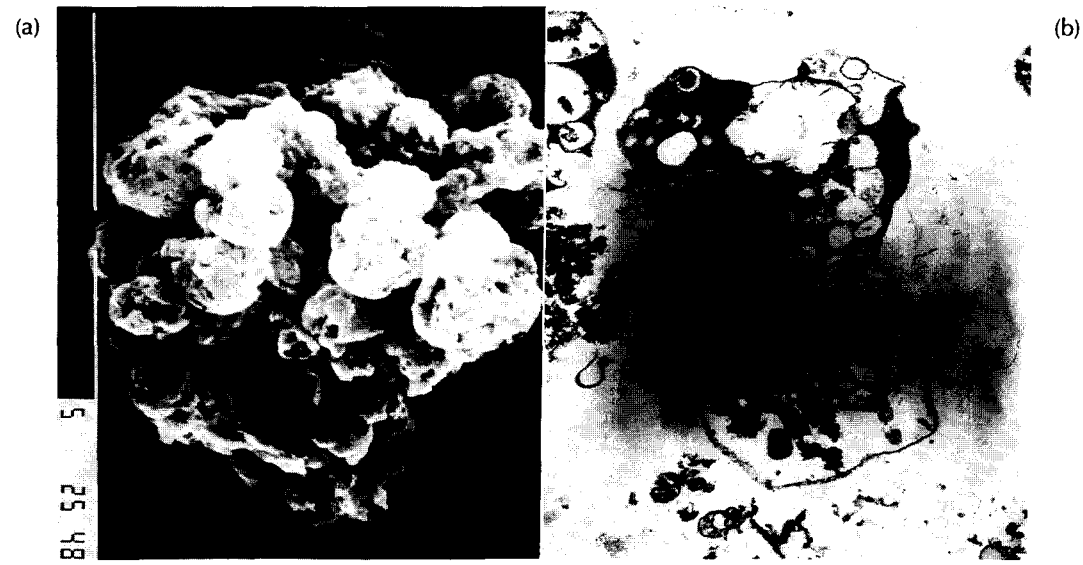

Fig. 4. Electron micrographs of the fusion process between *Lentinus edodes* and *Coriolus versicolor* after PEG 4,000 treatment. (a) Scanning electron micrograph shows aggregated protoplasts (×6,000). (b) Transmission electron micrograph shows fused protoplasts (×12,000).

played mixed profiles or totally different profiles (Fig. 3). LE(eb)26 revealed one broad peroxidase band, whereas CV17 revealed three bands. Peroxidase patterns of the fusants, F1, F3 and F5, were similar to that of LE (eb)26 and those of F10 and F12 were similar to that of CV17. Esterase isozyme patterns of the fusants were different from those of their parents. Esterase patterns of the fusants, F11 and F12 were similar to that of LE (eb)26 and those of F3, F5, F6 and F10 were novel.

Lentinula edodes possessed a high variability in isozyme activities. A total of 37 isozymes of its esterase was studied by Ohmasa and Furukawa (1986). And a total of 16 esterase isozyme bands for 24 strains among 45 isolates was reported by Toyomasu and Zennyozi (1981). Peroxidase activity and multi peroxidase bands of *C. versicolor* were reported (Harkin and Obst, 1973; Park *et al.*, 1989).

The bands of esterase and peroxidase were consistently stable for the study and these isozymes can be used to identify and discriminate fusion products. In addition, determination of the molecular weights of these bands has to be further studied.

Microscopic observation

Protoplast fusion was usually observed between two cells or among more than three cells. Big aggregates of the dehydrated protoplasts were observed after PEG 4000 treatment. Membrane contact and reorganization of membrane components were required for the membrane fusion. Transmission electron micrograph shows fused protoplasts (Fig. 4). Flattening of the cells in the area of the membrane contact was achieved.

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