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Generation of Nuclear Hybrids Overcoming the Natural Barrier of Incompatibility: Transfer of Nuclei from *Lentinula edodes* into Protoplasts of *Coriolus versicolor*

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Heterokaryotic nuclear hybrids overcoming the natural barriers of incompatibility have been studied in basidiomycetes. To produce these nuclear hybrids between incompatible mushrooms, which have several potent pharmacological effects, nuclear transfer was performed between *Lentinula edodes* and *Coriolus versicolor*. Nuclei from serine auxotrophs of *Lentinula edodes*, LE207 (Ser^-) were transferred into the protoplasts of arginine auxotrophs of *Coriolus versicolor*, CV17 (Arg^-), using 30% polyethylene glycol 4000 in 10 mM $CaCl_2$-glycine solution (pH 8.0). Nuclear transfer progenies were selected by nutritional complementation on minimal media supplemented with 0.6 M sucrose. The progenies were classified based on colony morphology to *L. edodes*-like, *C. versicolor*-like and non-parental type. Most of the progenies grew slower than either parent. The number of nuclei per cell was similar but the DNA content varied between progenies. The isozyme patterns of nuclear hybrids resembled either of the parent profiles or showed a mixed profile.

Key words: *Lentinula edodes*, *Coriolus versicolor*, Nuclear transfer, Nuclear hybrid, Progeny, Isozyme

INTRODUCTION

Lentinula edodes, one of the most popular edible mushrooms, has been reported to possess several pharmacological effects. Polysaccharides from the basidiocarps and mycelia of *L. edodes* (Lentinan, LEM and KS-2) have been shown to inhibit the growth of tumor cells (Maeda and Chihara, 1973; Fujii *et al.*, 1978; Hamuro *et al.*, 1980). The reduction of blood cholesterol level and anti-thrombosis by *L. edodes* were described by Kabir *et al.* (1987) and Hokama and Hokama (1981). Additionally, antiviral activity against the influenza virus, herpes simplex virus, scrapie, orthomyxoviruses and HIV-1 has been reported (Suzuki *et al.*, 1979; Sarkar *et al.*, 1993; Suzuki *et al.* 1989).

Coriolus versicolor, which has generally been used in hot water decoctions, is also claimed to possess several pharmacological effects. For example, PS-K (Krestin), a protein-bound polysaccharide extracted from *C. versicolor*, inhibits tumor growth in mice and humans (Tsukagoshi *et al.*, 1974; Ohno *et al.*, 1975; Dong *et al.*, 1996). Additionally, it prevents the interaction between HIV-1 gp120 and immobilized CD4 receptor and inhibits HIV reverse transcriptase activity (Tochikura *et al.*, 1987; Hirose *et al.*, 1987; Collin and Ng, 1997).

Generation of the chimeric mushroom between *L. edodes* and *C. versicolor* is very interesting for the development of strains having combined or synergistic pharmacological effects. However, generating natural hybrids between *L. edodes* and *C. versicolor* is difficult, because *L. edodes* (*Tricholomataceae*) and *C. versicolor* (*Polyporaceae*) belong to genetically unrelated orders. Previously we generated protoplast fusants between *L. edodes* and *C. versicolor* (Kim *et al.*, 1997b,c). In the current study, we successfully isolated new progenies by transferring nuclei of *L. edodes* into the protoplast of *C. versicolor*. Here we report the morphological, genetic, and biological characteristics of newly isolated progenies.

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MATERIALS AND METHODS

Strains and culture condition

Serine auxotroph (LE207) of *L. edodes* and arginine auxotroph (CV17) of *C. versicolor* were isolated after ultraviolet ray irradiation as described previously (Kim *et al.*, 1996; Park *et al.*, 1991). The auxotrophs were incubated on complete medium (CM) containing 2.0% glucose, 0.2% yeast extract, 2.0% peptone, minerals and agar (pH 6.2). Minimal medium (MM) was composed of only glucose, minerals and agar. In nuclear hybrid regeneration, both media were supplemented with 0.6 M sucrose as an osmotic stabilizer (RCM and RMM). Yeast extract, peptone and agar from Difco (Detroit, MI) were used in media. Other reagents were purchased from Sigma (St. Louis, MO) unless otherwise stated.

Formation of protoplasts

Protoplasts were prepared from LE207 grown in liquid culture and CV17 grown on cellophane sheet as described by Kim *et al.* (1997a). The mycelia were washed with distilled water and treated with a mixture of 10 mg/ml Novozyme234 (Novo, Bagsvaerd, Denmark) and 10 mg/ml Cellulase Onozuka (Yakult, Japan) for 2-6 h at 30°C. Hyphal debris was removed by filtration over a sintered glass filter (porosity 1), followed by centrifugation for 5 min at 1000 rpm. The supernatant was centrifuged for 15 min at 1000 rpm to remove enzyme solution. After washing twice with 0.6 M sucrose, the precipitate was suspended in 1 ml of 0.6 M sucrose. Protoplast numbers were counted by hematocytometer.

Nuclear isolation from the protoplast of *L. edodes*

Protoplasts of LE207 were suspended in ice-cold nuclei isolation buffer (10 mM $MgCl_2$, 0.5 M sucrose and 0.4% Triton X-100 in 10 mM Tris-HCl, pH 7.2), and ruptured for 3 min with a micro-blender followed by centrifugation for 15 min at 1000 rpm. The supernatant was filtered through a 0.8 mm filter membrane (Millipore, Bedford, MA), and the filtrate was centrifuged for 30 min at 3000 rpm. The pellet containing the nuclei was suspended in 0.4 ml of 0.6 M sucrose. The suspension was layered over a discontinuous sucrose gradient prepared in 5 ml ultra-clear tubes (Beckman, Palo Alto, CA) and formed by layering 0.5 ml of 1.2 M sucrose over 1 ml of 1.5 M and 1.8 M, and 1.5 ml of 2.0 M. Tubes were centrifuged for 30 min at 49000 rpm and the nuclei at the 1.8 M and 2.0 M sucrose interface were collected and suspended again in 0.6 M sucrose.

Nuclear transfer and regeneration

The nuclei isolated from 1×10^8 protoplasts of LE207 in 1 ml of 0.6 M sucrose were mixed with 1×10^7 protoplasts of CV17 in 1 ml of 0.6 M sucrose. The mixture was centrifuged and the pellet was reconstituted in 1 ml of 30% polyethylene glycol (PEG 4000) in 10 mM $CaCl_2$-glycine solution (pH 8.0). The fusion mixture was incubated for 15 min at 30°C with gentle shaking and suspended in 10 ml of 0.6 M sucrose. After centrifugation for 7 min at 2500 rpm, the pellet was suspended in 0.6 M sucrose and plated on RCM and RMM. After 45-60 days of incubation at 25°C, the regenerated colonies on RMM were isolated and transferred to MM. The colonies were passaged 3-5 times on MM before transfer to CM. After 20 generations on CM, the stable nuclear transfer progenies were characterized based on colony morphology.

Nuclear number and DNA content

The nuclear number of the protoplast was determined by using Giemsa stain or 4,6-diamidino-2-phenylindole (DAPI). Briefly, the protoplasts were fixed by Hellys fixing solution containing 0.6 M sucrose for 15 min and washed with 70% methanol. The fixed protoplasts were incubated in 1% NaCl for 1 h, followed by 1N HCl for 15 min at 60°C. The resulting protoplasts were washed with phosphate buffer (pH 7.0), and stained with Giemsa solution for 45 min. For fluorescence staining, the protoplasts were fixed with 3% glutar-aldehyde containing 0.6 M sucrose for 1 h and stained with 20 mg/ml of DAPI.

The amount of DNA per protoplast was determined by the modified method of Labarca *et al.* (1980). Briefly, protoplast and nuclear membranes were homogenized in phosphate saline buffer (0.05 M Na_2HPO_4, 2.0 M NaCl and 2 mM EDTA, pH 7.4), followed by sonication for 3 min on ice. The sonicated cells were incubated with 1 mg/ml of Hoechst 33258 (Farbwerke Hoechst, Germany). Fluorescence was determined at an excitation wavelength of 330 nm and an emission wavelength of 470 nm (FP-777, Jasco, Japan) using salmon testis DNA Type III as a standard.

Cell lysate and non-denaturing gel electrophoresis

After 25 days culture in liquid medium, the mycelium was collected and washed 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 protein concentration of the supernatant was determined by modified Lowry method (Lowry *et al.*, 1951) using bovine serum albumin as a standard. Several enzyme activities were detected after electrophoresis on 5-10% non-denaturing polyacrylamide gel.

Isozyme band patterns

The presence of several intracellular isozymes was

examined by comparing colored end products on the gel. Peroxidase and esterase were detected as previously described (Kim *et al.*, 1997c). Superoxide dismutase (SOD) activity was detected by incubating gels in solution containing 0.01% 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide [MTT] and 0.01% phenazine methosulfate in 0.05 M Tri-HCl, pH 7.5, for 20 min at 30°C in the dark. The gels were then incubated with solution containing 0.02% riboflavin and 0.4% TEMED [N,N,N,N-tetramethylethylenediamine] in 0.05 M Tris-HCl, pH 7.5. After incubation, gels were exposed to light for 15 min. Acid phosphatase activity was detected by incubating gels in acetate buffer for 30 min at 5°C and transferred to the reaction solution (0.5 ml of 1.0 M $MgCl_2$, 50 mg Fast Garnet GBC in 50 ml of 50 mM Na-acetate buffer; 1.5 ml of 1% α-naphtyl acid phosphate dissolved in 50% acetone) for 1-5 h at 30°C.

RESULTS

Properties of nuclear transfer hybrids

The nuclear transfer hybrids appeared on the semi-solid RMM after 45-60 days incubation at 25°C. These hybrids were then transferred to MM and passaged 3-5 times, followed by transfer to CM. After 20 passages on CM, stable nuclear hybrids were isolated and their characteristics were analyzed (Fig. 1 and Table I). Some hybrids spontaneously segregated during incubation on CM, and these segregates were further isolated as individual hybrids.

Based on colony morphology, nuclear hybrids were classified into three types: *L. edodes*-like, *C. versicolor*-like and non-parental type which showed mixed parental morphology. The parental strain LE207 was characterized by fast growing, white aerial mycelia, and CV17 by fast growing pigmented mycelia having advanced zones. Colony morphologies and growth rates of the nuclear hybrids were variable and aerial mycelia ranged from sparse to dense (see Table I for classification). Most of the nuclear hybrids grew slower than their parents, and their colonies resembled either *L. edodes* or *C. versicolor*. The majority of *C. versicolor*-like and non-parental type hybrids produced brown pigment,

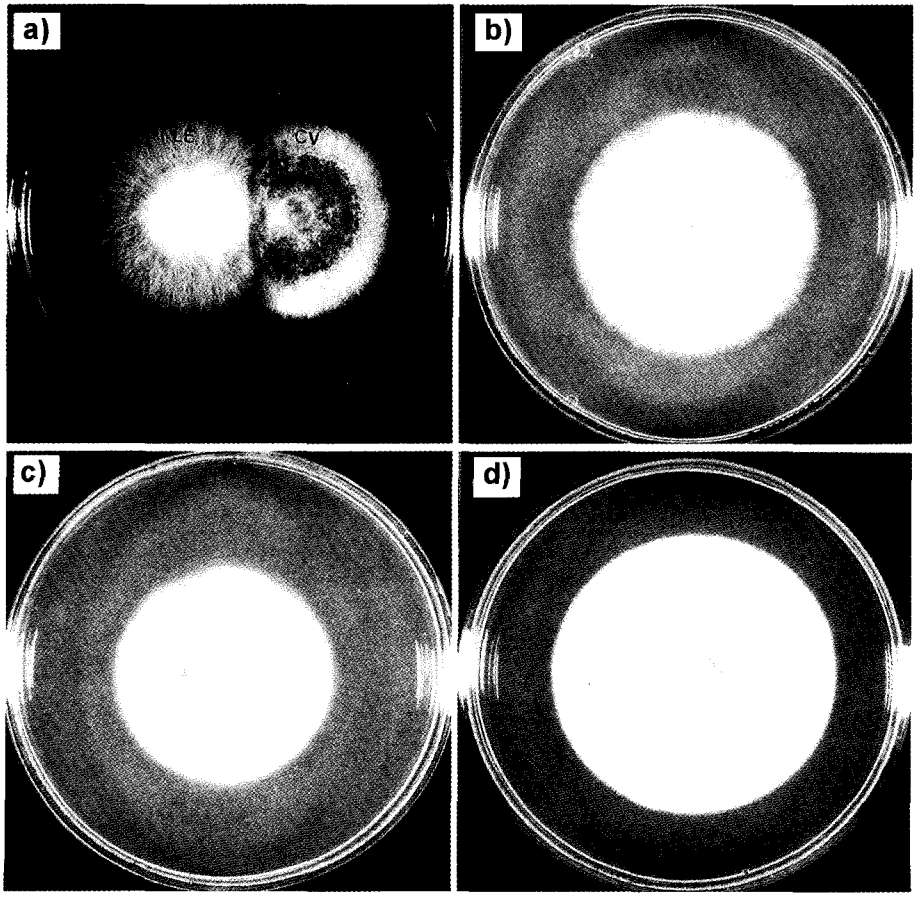

Fig. 1. The nuclear transfer hybrids between *Lentinula edodes* and *Coriolus versicolor*. The parental strains of *L. edodes* and *C. versicolor* show anatomical incompatibility (a), *L. edodes*-like hybrid H11 (b), *C. versicolor*-like hybrid H9 (c), and non-parental type hybrid H22 (d).

Table I. Characteristics of the nuclear transfer progenies between LE207 and CV17[a]

Strains	Morphology			DNA content			Isozyme pattern			
	Aerial mycelia[b]	RGR (10^{-4} m/h)[c]	Pigment/ zone[d]	Clamp connection	Nuclear number/cell	DNA (μg)/10^6cell	POX[e]	EST	ACP	SOD
LE207	4	2.94	-/-	+	1.1	5.7	L	L	L	L
CV17	1	2.80	+/+	-	1.3	3.0	C[f]	C	C	C
L. edodes type										
H10	4	F	-/-	+	*	*	NP	L	L	L
H11	4	S	-/-	+	*	*	NP	NP	L	L
H46	4	F	-/-	+	1.3	16.7	*	*	*	*
C. versicolor type										
H4	3	2.14	+/+	-	1.2	2.3	C	L	C	C
H5	2	*	+/+	*	*	*	NP	L	NP	NP
H6	2	F	+/-	+	*	*	NP	L	NP	NP
H9	2	F	+/+	-	1.3	1.6	C	*	*	*
H17	*	S	+/+	*	*	*	NP	*	C	C
H17-1	2	2.27	+/-	+	1.3	2.3	NP	L	C	C
H23	3	1.85	+/-	+	1.3	4.2	*	L	*	*
H63	2	1.14	+/-	+	1.3	23.3	*	NP	C	*
Non-parental type										
H8	1	S	+/+	-	*	*	C	C	C	*
H12	2	2.34	+/-	*	*	*	C	L	*	C
H22	1	F	+/-	*	*	*	NP	NP	C	*
H89	3	M	+/+	*	1.3	2.3	*	*	*	*

a Data represent 15 of 89 isolates
b 4 indicates best yields
c RGR (radial growth rate), F:faster than 2.0×10^{-4} m/h, M:moderate, between 2.0×10^{-4} and 1.0×10^{-4} m/h, S:slower than 1.0×10^{-4} m/h
d +/+: brown pigment production / zonate growth
e POX: peroxidase, EST: esterase, AP: acid phosphatase, SOD: superoxide dismutase
f L: *L. edodes*-like type, C: *C. versicolor-like type*, NP: non-parental type
* Not determined

which became more intense following the incubation time.

Clamp connections were observed using hyphae which were grown on sterile cover glass. The hyphae wall was swollen by 4% KOH and stained with 1% phloxine. The presence of clamp connections is generally indicative of a dikaryotic condition and was found in the hyphae of LE207, but not in CV17. In nuclear hybrids, the majority of *C. versicolor*-like hybrids showed no clamp connection, whereas most of the *L. edodes*-like hybrids showed clamp connections (Fig. 2). However, some *C. versicolor*-like hybrids including H6, H17-1, H23 and H63 produced clamp connections (Table I). A possible explanation why the *C. versicolor*-like hybrids produce clamp connections is that two *L. edodes* nuclei entered into one protoplast of *C. versicolor* generating reconstituted products.

Genetic traits and DNA contents

The stable heterokaryosis and genetic recombination of nuclear hybrids were confirmed by nutritional complementation. The protoplasts from nuclear hybrid, H4, were cultured separately on RMM, RMM+arginine, and RCM. From these cultures, 368 colonies from RMM, 688 colonies from RMM+arginine, and 528 colonies from RCM were isolated and cultured again to confirm their regeneration efficiency on MM, MM+arginine and CM. All colonies were regenerated on MM as well as MM+arginine and CM. This confirmed that nutritional complementation occurred in nuclear hybrids, H4. The nuclear number per protoplast and amount of DNA per nucleus were studied. The hybrids differed in their DNA content, whereas the nuclear number per cell was consistent (Table I). Compared with the parents, some hybrids contained more than three times the DNA content, demonstrating nuclear hybridization. Other hybrids possessed less DNA than the parents. This suggests the entire chromosome did not integrate into the recipient nucleus or some parts of the genome were deleted during selection. In addition, 70-90% of protoplasts contained only one nucleus, whereas other

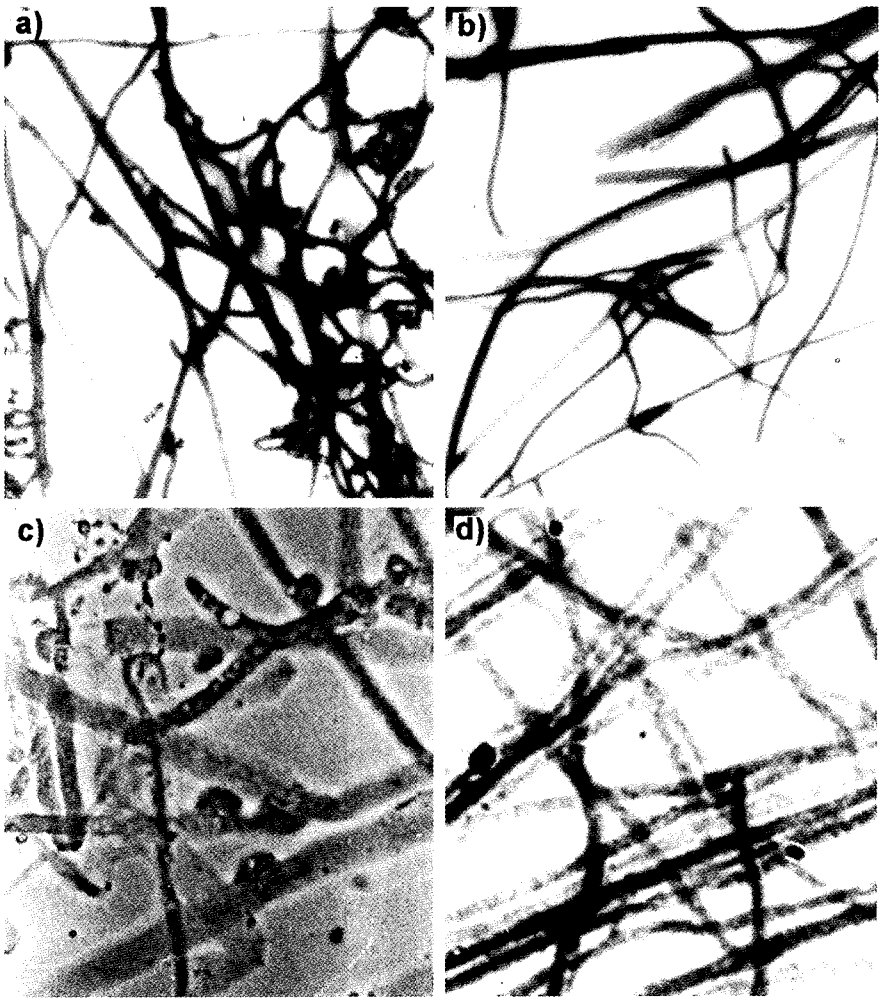

Fig. 2. Clamp connection formation of the mycelia of *Lentinula edodes* and *Coriolus versicolor* and their nuclear transfer hybrids. LE207 with clamp connections (a), CV17 without clamp connection (b), hybrids H45 with clamp connections (c) and hybrid H9 without clamp connection (d).

Table II. Distribution of nucleus in nuclear transfer progenies between LE207 and CV17

Strains	Nuclear number/Cell					Average nuclear No.
	0	1	2	3	4	/Cell
LE207	0.5*	88.5	10.3	0.7	0.0	1.1
CV17	2.5	78.0	16.5	1.5	2.0	1.3
H4	1.5	83.5	12.0	1.0	2.0	1.2
H17-1	1.0	77.5	17.0	2.0	2.5	1.3
H23	2.0	74.5	18.5	3.0	2.0	1.3
H37	0.5	71.5	22.0	3.5	2.5	1.2
H46	3.0	76.5	15.5	1.5	3.5	1.3
H51	0.0	76.0	20.0	3.5	0.5	1.3
H63	1.5	73.0	21.0	3.5	1.0	1.3
H83	2.0	76.5	19.0	2.0	0.5	1.2
H89	5.0	72.5	17.0	2.5	3.0	1.3

* Value represents the percentage of protoplasts and 200 protoplasts were counted for each sample.

protoplasts contained either no nucleus or 2-4 nuclei (Table II).

Comparison of isozyme patterns

An intracellular soluble protein from the mycelia was used to identify and discriminate between hybrids based on isozyme patterns: including peroxidase, esterase, superoxide dismutase and acid phosphatase on non-denaturing polyacrylamide gel. The isozyme patterns of LE207 and CV17 were distinctly different, and their nuclear hybrids displayed either parental or mixed phenotypes (Table I and Fig. 3). The isozymes were also consistently stable during the study except the esterase of *L. edodes*. LE207 showed two peroxidase bands, whereas CV17 had four bands. The peroxidase pattern of *L. edodes*-like hybrids H10 and H11 showed non-parental type, whereas those of *C. versicolor*-like hybrids, H4 and H9, and non-parental hybrids, H8 and H12,

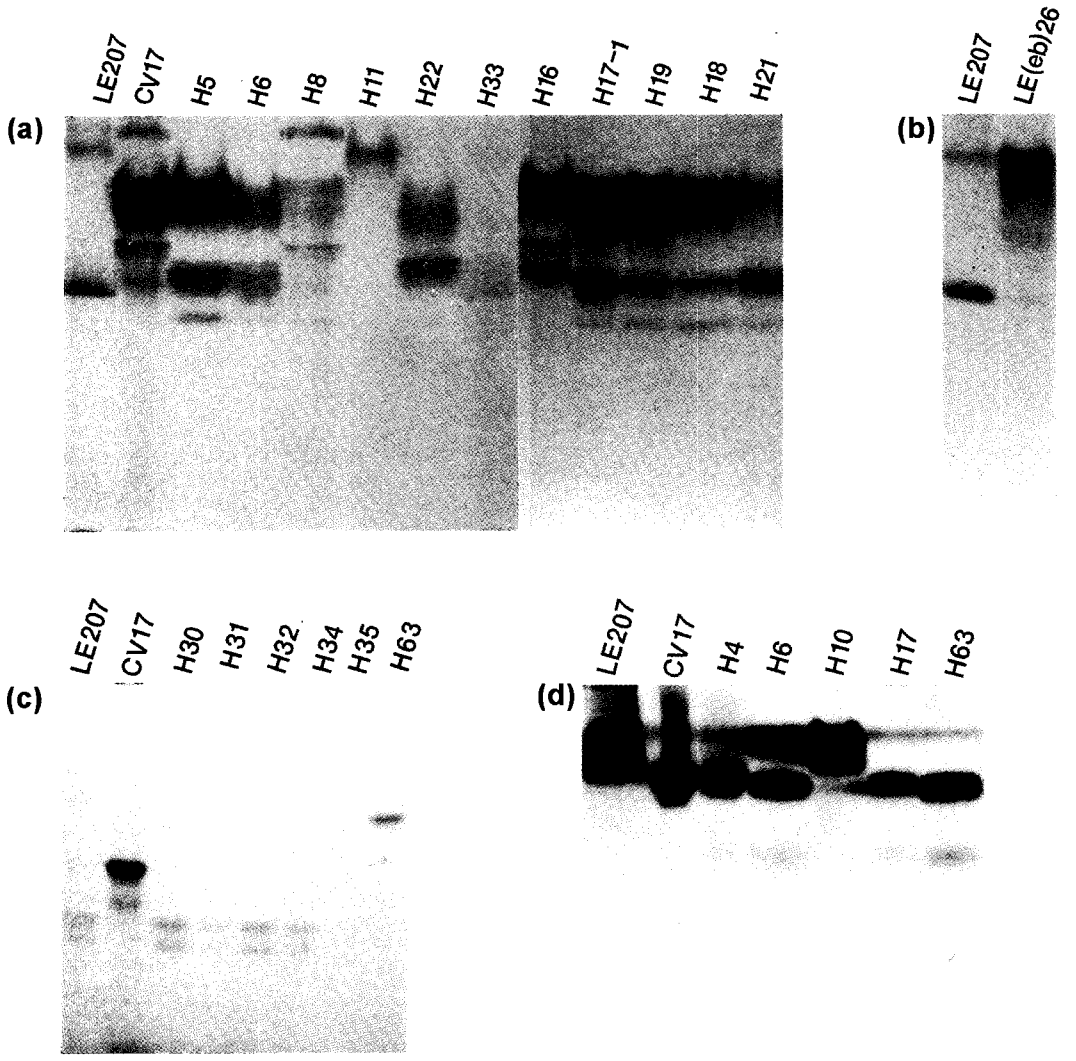

Fig. 3. Peroxidae isozyme patterns on 10% non-denaturing polyacrylamide gel of nuclear transfer hybrids between LE207 and CV17 (a), and two auxotrophs of *Lentinula edodes*, LE207 and LE(eb)26 showed distinctive patterns (b). Esterase isozyme patterns on 10% gel (c), and acid phosphatase on 7.5% gel (d) were identified by comparing colored bands.

were similar to that of CV17 (Fig. 3a). The esterase isozyme patterns for most of the nuclear hybrids were similar to that of *L. edodes* (Fig. 3c). Two transluminant bands corresponding to SOD were detected in *L. edodes*, but no similar band was detected in *C. versicolor* (data not shown). One acid phosphatase band was observed for *C. versicolor* and two bands were detected in *L. edodes* (Fig. 3d). The SOD and acid phosphatase patterns of the nuclear hybrids resembled the parental profile.

DISCUSSION

Nuclear transfer in fungi is a useful technique for establishing fungal hybrids to overcome the natural barriers to gene exchange by conventional breeding systems, and was first reported in *Saccaromyces* (Ferenczy and Pesti, 1982; Becher *et al.*, 1982). Recently hybrid production using *L. edodes* or *C. versicolor* have been studied by several groups. The isolation of protoplast fusants between *L. edodes* and *C. versicolor*, *C. versicolor* and *Ganoderma lucidum*, *L. edodes* and *G. lucidum*, *L. edodes* and *Pleurotus cornucopiae*, and *L. edodes* and *Pleurotus sapidus* has been reported (Kim *et al.*, 1997b,c; Park *et al.*, 1991; Bok *et al.*, 1994; Ogawa, 1993). The nuclear hybrids established between *C. versicolor* and *G. lucidum*, *L. edodes* and *C. versicolor*, and *L. edodes* and *Pleurotus florida* have also been reported (Park *et al.*, 1991; Kim *et al.*, 1997b; Yoo *et al.*, 1996).

In a previous study, we generated protoplast fusants between *L. edodes* and *C. versicolor* based on the report

that elimination of mitochondrial elements improved viability of hybrids (Ziegler and Davidson, 1983). However, protoplast fusion between CV17 and our previously generated mitochondria-deficient auxotroph LE(eb)26 (Ile^-, Arg^-, Thy^-) showed a low level of complementation (Kim *et al.*, 1997c). At the same time, protoplast fusion between auxotrophs LE207 (Ser^-) and CV17, and LE93 (amino acid and vitamin-requiring mutant) and CV17 were attempted. However, the yield from protoplast fusion was insufficient for further analysis. An alternative approach based on the assumption that nuclear transfer using isolated nuclei may allow hybridization between distantly related species, thus facilitating strain improvement and answering basic questions concerning regulatory processes, were applied to LE207 and CV17. As hypothesized, the generation efficiency of nuclear hybrids increased approximately 4 fold over protoplast fusion between LE(eb)26 and CV17 from 7.4×10^{6} to 2.8×10^{-5} (Kim *et al.*, 1997c).

Most of the nuclear transfer hybrids grew slowly compared to the parents, perhaps due to heterogeneity between fused nuclei and cell components. LE207 has clamp connections but CV17 does not. It would be expected that some hybrids of LE207 and CV17 would be monokaryon which do not produce clamp connections. This status can be referred to as a syncaryon where two nuclei cannot exist together. When protoplast fusion or nuclear transfer occurred, the cell initially produced heterokaryon containing both nuclei. In most cases the two nuclei fused into a mononuclear hybrid (personal communication from Y.B. Yoo). It is generally assumed some of the genetic material from the donor can be inserted into the recipient nucleus following nuclear fusion.

Isozyme activities are frequently used in fungal studies since enzyme expression occurs codominantly, and so a recessive gene does not express. Peroxidase and esterase isozymes are the most frequently used isozymes in characterizing fungi, and have been used for *L. edodes* and *C. versicolor* (Ohmasa and Furukawa, 1986; Toyo-masu and Zennyozi, 1981; Park *et al.*, 1991; Kim *et al.*, 1997c). For the isozyme assay, hybrids were cultured for 25 days, this provided the most stable and prominent peroxidase activity. In most cases, the isozyme patterns of the mutants were distinctive from the wild-type, as observed for the peroxidase band patterns of LE207 and LE(eb)26 (Fig. 3b). LE207 and LE(eb)26 are the mutants from the wild-type *L. edodes* (Kim *et al.*, 1996). In comparing hybrids with their parental strains, the hybrids showed differing intensities of the parental bands and the appearance of new bands. This indicates that crossing-over has occurred between the two parent genomes in generating hybrids. Alkaline phosphatase, leucine aminopeptidase, alcohol dehydrogenase and carboxy-peptidase are the enzymes which have previously been studied in fungi and can be detected by colored end products on non-denaturing gel (Kerrigan and Ross, 1989; Roux and Labarere, 1990). We tested the presence of alkaline phosphatase, alcohol dehydrogenase and α-amylase, but none of the enzymes were detected in LE207, CV17 and their nuclear hybrids.

In conclusion, nuclear hybrids between *L. edodes* and *C. versicolor* were selected by nutritional complementation. The hybrids showed either of the parents or mixed patterns in morphology and isozyme patterns. The development of fusion techniques using protoplasts has opened up many possibilities for generating hybrids and understanding the genetic mechanisms of fungi. The successful isolation of nuclear transfer hybrids, between *L. edodes* and *C. versicolor*, provides a method for generating hybrids between genetically unrelated strains. Future work will compare the pharmacological effects of the hybrids with the parental strains.

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