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Studies on Protoplast Fusion between *Lentinula edodes* and *Ganoderma lucidum*

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The potential of protoplast fusion as means of establishing genetic crosses was realized about 20 years ago in *Geotricum candidum* (Frenczy *et al.*, 1974). The list of species of yeasts and filamentous fungi to which the technique of protoplast fusion has been applied increases year by year (Bok *et al.*, 1990; Chadegani and Ahmadjian, 1991).

Nevertheless, very few papers have been reported on inter-order protoplast fusion, especially in basidiomycetes, though intergeneric and even more distant fusion in yeasts have been successfully archieved (Kigichi and Yanagi, 1985). The essence of protoplast fusion is to bring together of nuclear and cytoplasmic genomes of two fungal strains. The main problem in protoplast fusion as breeding tool is that it is difficult to obtain a stable hybrid since the nuclear fusion rarely occurs, especially in fusion between more distant basidiomycetes in lineage. Direct transfer of isolated nuclei may render it possible to hybridize taxonomically more distant species (Briggs and King, 1952; You *et al.*, 1988).

To investigate a possibility of inter-order protoplast fusion and nuclear transfer between two different basidiomycetes, *Lentinula edodes* (LE) and *Ganoderma lucidum* (GL) showing anastomotical incompatibility, the mycelia of both fungi were grown on ten different media for 15 and 10 days at 28℃, respectively. The isolation of protoplast was maximized by the treatment with a combination of Novozym 234 (Novo Industry, Denmark) and cellulase Onozuka (Yakult Honsha, Japan). As an osmotic stabilizer for protoplast formation and regeneration, 0.6 M sucrose pH 5 were the most suitable. The regeneration frequency of the protoplasts of *L. edodes* was 0.50% and that of *G. lucidum* was 0.88%. For isolation of auxotrophic mutants containing specific markers, UV was irradiated and specific mutants were selected on minimal medium. Various amino acid-, nucleic acid-, and vitamin-requiring auxotrophs of *L. edodes* and *G. lucidum* were obtained at survival rates of 0.0001~0.254% and 0.9~27.2%, respectively (Table I). Then these mutants were used in protoplast fusion and nuclear transfer. Back mutation frequency of these auxotrophs used in fusion was 10^{-4}. Polyethylene glycol (M.W. 6,000) in 10 mM $CaCl_2$-glycine solution (pH 8.0) induced protoplast fusion and nuclear transfer. The inter-order fusion frequency between the mycelial protoplasts of the various mutants was 6.7×10^{-3}% (Table II). Viable hybrids were obtained by the transfer of the nuclei of *L. edodes* into the protoplasts of *G. lucidum*. The rate of hybrid formation by nuclear transfer was higher than that of the protoplast fusion. To ascertain formation of hybrids via inter-order protoplast fusion and nuclear

Table I. Isolation of auxotrophic mutants of *L. edodes* (LE) and *G. lucidum* (GL) after UV mutagenesis

Mutant No.	Phenotype*	Exposure time (min)	Mycelial growth on solid media
LE1	Guanine, Uracil	20	Slow
LE2	Met, Thr, Trp	20	Medium
LE3	Asn, Gln	5	Medium
LE4	PABA	10	Fast
GL1	Hypoxanthine	5	Fast
GL2	Asn, Thr, Trp, Glu	5	Medium
GL3	Asp, Glu, His, Tyr, Val	5	Medium
GL4	Thiamine, Pyridoxine	5	Fast

*Met: methionine, Thr: threonine, Trp: tryptophan, Asn: asparagine, Gln: glutamine, PABA: para-aminobenzoic acid, Glu: glutamic acid, His: histidine, Asp: aspartic acid, Tyr: tyrosine, Val: valine

Table II. Inter-order protoplast fusion and nuclear transfer between the two auxotrophs of *L. edodes* and *G. lucidum*

Cross (Genotype)	Symbol	Fusion frequency (%)
Protoplast Protoplast LE4($PABA^-$)+GL1($hypoxanthine^-$)	L4G1P	6.7×10^{-3}
Nuclei Protoplast LE4($PABA^-$)+GL1($hypoxanthine^+$)	L4G1N	–

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Table III. Characterization of the protoplast fusants between *L. edodes* and *G. lucidum*

Parents and Fusants	Morphology[a]	Aerial mycelia[b]	Mycelial growth[c]	Pigment[d]	Clamp connection[e]
L. edodes	L	++++	M	−	+
G. lucidum	G	+++	F	−	−
L4	L	++++	M	−	+
G1	G	+++	F	−	+
L4G1P2S1	G	++	F	+	−
2S2	G	+	M	+	+
2S3	G	+	F	−	−
3	G	++	F	−	+
5	G	++	F	−	−
6	G	++	F	−	+
15	L	+++	M	−	+
18	L	+++	M	−	−
22	L	+++	F	−	−
30	G	+++	F	−	−
33	N	+	F	−	−
38	G	++	F	−	−
39	L	++++	M	−	+
40	N	++++	S	−	−
42	N	++++	S	−	+
47	L	++++	M	−	+
49	L	++++	M	−	+
55	L	+++	M	−	+
58	L	+++	M	−	+
61S1	G	++	F	−	−
61S2	L	+	F	−	+
66	L	+++	S	−	−
67	L	+++	S	−	N.D.*
68	G	++	F	−	N.D.
69	L	++++	M	−	+
70	L	++++	M	−	+
72	N	+++	S	−	−
80	N	++++	S	−	−
82	L	++++	M	−	+
89	L	++++	M	−	+
92	L	++	S	−	N.D.

[a] L: *L. edodes*, G: *G. lucidum*, N: non L and non G. [b] ++++: indicate best yields. [c] F: fast growth, M: moderate growth, S: slow growth. [d] +: brown pigment production. [e] +: present clamp, −: clampless, *: Not detected

transfer between *L. edodes* and *G. lucidum*, the mycelia of the parents and the hybrids were grown on complete solid and liquid media ($MgSO_4 \cdot 7H_2O$ 0.5 g, KH_2PO_4 0.46 g, K_2HPO_4 1.0 g, Peptone 2.0 g, Yeast extract 2.0 g, glucose 50 g, agar 20 g in 1000 ml distilled water) for 7~15 days at 28℃. Those hybrids were different from their parents in growth rate, mycelial morphology, pigment production, and clamp connection (Tables III and IV), A comparison of the hybrids was made by using isozyme analysis (esterase and acid phosphatase) (Davis, 1964; El-Metainy and Omar, 1981). In these cases, the isozyme patterns of the parents were distinct. The parental isozyme bands as well as the novel ones of the hybrids were observed in different quantities but the hybrids which showed two parental isozymes simultaneously were not observed. Monosaccharides ratio (Tables V and VI) and protein contents of the hot water-extracted protein-bound polysaccharide showed that completely different hybrids from the parents existed in greater numbers than those similar to one parent. When antitumor activities of these protein-bound polysaccharides against sarcoma 180 were examined, some hybrids were shown to have greater antitumor activities than their parents.

Mitochondrial DNA (Avise *et al.*, 1979) was isolated from two fusants, P2S2 and P2S3, and compared with those of the parents. The patterns of mitochondrial DNA digested with restriction enzymes provided the

Table IV. Characterization of the nuclear transferants between *L. edodes* and *G. lucidum*

Parents and Fuscants	Morphology[a]	Aerial mycelia[b]	Mycelial growth[c]	Pigment[d]	Clamp connection[e]
L. edodes	L	++++	M	−	+
G. lucidum	G	+++	F	−	−
L4	L	++++	M	−	+
G1	G	+++	F	−	+
L4G1N2	G	+++	F	−	+
4	N	++++	S	−	−
6	G	+++	F	−	+
10	G	++	F	−	+
13	G	++++	F	−	−
15	L	++++	M	−	+
16	L	++++	M	−	+
24	L	+++++	M	−	+
25S1	G	+++	F	−	−
25S2	L	+++	F	−	−
26	G	+++	F	−	+
27	L	+++++	F	−	+
32	N	++	S	−	N.D.*
34	G	++	F	−	+
37	N	+	F	−	+
39	G	+++	F	−	−
45	N	+	M	+	−
46	G	+++	F	−	+
48	G	++++	F	−	+
50	G	+++++	F	−	+
58	G	+	F	−	+
59	N	+	M	−	+
60	N	+++++	F	−	−
62	G	+++	F	−	−
63	G	+++	F	−	+
68	L	++++	M	−	+
70	N	+	F	−	

[a] L: *L. edodes*, G: *G. lucidum*, N: non L and non G. [b] +++++: indicate best yields. [c] F: fast growth, M: moderate growth, S: slow growth. [d] +: brown pigment production. [e] +: present clamp, −: clampless, *: Not detected

Table V. Monosaccharide contents of the polysaccharide moiety of the hot water extracts of protoplast fusants between *L. edodes* and *G. lucidum*

Parents and Fusants	Gluose	Galactose	Mannose	Fucose	Xylose	Arabinose
L. edodes	53.3	5.6	25.3	6.0	4.7	5.1*
G. lucidum	48.2	7.7	35.7	5.2	1.4	1.8
L4G1P2S1	48.2	9.1	33.3	6.4	1.2	1.8
2S2	34.7	6.0	47.0	11.4	0.9	−
2S3	49.6	12.1	29.6	7.0	0.7	1.0
3	30.0	6.0	46.4	13.9	2.1	1.6
5	41.1	4.8	43.9	8.7	0.9	1.6
6	29.2	6.7	51.3	9.8	1.4	1.6
15	59.3	3.2	29.0	4.8	2.8	0.9
18	69.5	2.9	20.1	3.2	1.2	3.1
22	38.5	7.0	46.1	3.6	3.4	1.4
33	40.8	8.4	29.0	13.9	2.5	5.4
38	26.3	10.8	49.7	11.1	0.5	1.6
39	51.4	8.3	26.0	8.1	6.2	−
47	58.8	8.7	14.3	15.5	2.7	−

Table V. Continued

Parents and Fusants	Gluose	Galactose	Mannose	Fucose	Xylose	Arabinose
49	64.4	24.6	4.3	4.6	1.2	0.9
55	56.6	4.2	25.2	6.7	5.8	1.5
61S1	54.4	8.9	24.0	10.3	1.0	1.4
61S2	37.3	16.4	36.0	8.7	0.7	0.5
70	46.0	4.1	41.2	5.5	0.9	2.3
82	60.9	7.7	21.0	6.7	3.7	–
89	40.3	5.6	37.3	7.5	3.0	6.3
92	56.1	5.2	33.5	5.2	–	–

*: Mole percentage

Table VI. Monosaccharide contents of the polysaccharide moiety of the hot water extracts of nuclear transferants between *L. edodes* and *G. lucidum*

Parents and Fusants	Gluose	Galactose	Mannose	Fucose	Xylose	Arabinose
L. edodes	53.3	5.6	25.3	6.0	4.7	5.1*
G. lucidum	48.2	7.7	35.7	5.2	1.4	1.8
L4G1N10	60.1	3.4	21.1	11.0	4.4	–
12	34.6	6.4	45.7	11.2	1.6	0.5
15	63.9	5.0	15.7	11.2	1.6	0.5
19	30.5	8.0	50.0	9.7	1.0	0.9
24	51.1	4.6	35.5	5.3	2.2	1.3
25	52.3	7.5	31.6	6.1	1.8	0.7
26	29.2	19.5	42.3	8.6	0.4	–
37	50.4	6.4	26.0	14.2	2.3	0.7
48	43.1	7.8	39.5	6.7	1.3	1.6
50	36.1	12.1	40.9	9.3	0.5	1.1
57	37.5	4.1	45.9	11.6	0.9	–
58	28.8	8.3	51.8	10.0	1.1	–
59	23.3	16.5	47.0	13.2	–	–
62	26.5	12.9	43.1	16.6	0.9	–
63	55.0	5.1	26.8	8.7	1.2	3.2
70	34.7	8.3	40.5	14.2	2.3	–

*: Mole percentage

evidence that P2S3 had the same mitochondrial DNA with that of *G. lucidum* but P2S2 had a new/y, recombined DNA. Electrophoretic karyotypes (Brody and Carbon, 1989; Hayes *et al.*, 1993) of the parents and the two nuclear hybrids, L4G1N 45 and 70, were obtained by using field-inversion gel electrophoresis. By using the chromosomes of *Saccharomyces cerevisiae* as size marker, the band patterns of the chromosomes of the hybrids were examined. This technique permitted the separation and visualization of intact chromosomes from viable protoplasts. Five chromosome bands were visualized with *L. edodes* and four chromosome bands with *G. lucidum*. But six and four bands were visualized respectively with two nuclear hybrids, L4G1N 45 and 70. The smallest chromosome of all four strains seems to be similar in size but all the remaining chromosomes observed were dissimilar in size. In these studies, these hybrids showed that an interaction occurred between the two parental genomes.

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