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Transformation of the β-Isopropylmalate Dehydrogenase Gene of *Flammulina velutipes* into *Pleurotus florida*

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팽나무버섯 Leu 2 gene(β-isopropylmalate dehydrogenase)의 사철 느타리버섯 형질전환

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ABSTRACT: ***F. velutipes*** **Leu 2 gene (β-isopropylmalate dehydrogenase gene) was used for transformation of** ***P. florida*** **leucine requiring auxotrophic mutant P101. Transformation frequency was very low but the transformed colony can grow on minimal medium very slowly. Transformation was identified by Southern hybridization and reverse transformation into** ***E. coli*** **using chromosome DNA isolated from transformed** ***P. florida.***

KEYWORDS: ***Flammulina velutipes,*** **β-isopropyl malate dehydrogenase,** ***Pleurotus florida,*** **Transformation**

Flammulina velutipes and *Pleurotus florida* belong to the family Tricholomataceae in Basidiomycetes. Cultivation of *Pleurotus* spp. has been increasing for the ast decade and become the most inportant mushroom produced in Korea.

Development of cloning vector and transformation system is a prerequisite for molecular genetics research. Dominant selectible markers are required to screen transformants. These marker genes obtained by complementation of auxotrophic mutants by biosynthetic genes cloned in a bacterial plasmid have been described for some fungi (Hinnen, 1978; Case, 1979; Ballance, 1985; Munoz-Rivas, 1986). Transformation in basidiomycetes auxotrophic mutants has been reported on three occasions. Munoz-Rivas *et al.*(1986) described the use of wild type trp 1 sequence to transform trp auxotroph of *Schizophyllum commune* Mellon *et al.* used the *Coprinus cinereus* isocitrate gene for cotransformation of *C. cinereus* double mutant, and Binninger *et al.* transformed *Coprinus cinereus by* Coprinus tryptophan synthetase gene. We report the transformation of *P. florida* mutant, using *F. velutipes* β-isopropyl malate dehydrogenase (E.C.1.1.1.85) isolated by complementation of *E. coli* Leu B mutant.

Materials and Methods

Strains

P. florida P101, Leu, Cyto, Ura auxotroph selected by U. V. irradiation form the spores of *P. florida* ASI 2016, was used for transformation.

Media

Mushroom complete medium (MCM) was used for preparation of protoplast and DNA isolation from transformants. Mushroom minimal medium (MMM) was used for the selection of transformants.

Bacterial transformants were selected on Luria Broth agar plates containing ampicillin (100 mg m*l*/*l*.

Isolation and manipulation of DNA

Total *P. florida* DNA isolation and southern hybridization was as described previouly (Byun, 1989).

Preparation of transforming DNA

Plasmid DNA pM 301 (Byun, 1989) was isolated from strain of *E. coli* using alkaline lysis proce-

dure of Birnboim and Doly (1979) and purified on cesium chloride/ethidium bromide gradients as described by Maniatis *et al.*(1982).

Preparation of protoplast

Protoplasts were prepared from cellophane cultures of *P. florida* P101 as previously described by Yoo *et al.* (1984). After filtration through sintered glass filter (porosity 1), they were washed twice with 0.5 M sorbitol and 10 mM MOPS pH 6.75 and once with 0.5 M sorbitol, 10 mM MOPS and 10 mM $CaCl_2$. The protoplasts were resuspended in 0.5 m*l* of 0.5 M sorbitol, 10 mM MOPS and 10 mM $CaCl_2$ and dispensed into 0.25 m*l* aliquots. Plasmid DNA was then added and mixed well. Five-tenth m*l* of 30% PEG 4000 was added and incubated at about 30°C for 20 min. The protoplasts were washed twice with 0.5 M sorbitol and 10 mM MOPS. The washed protoplasts were plated on MMM and overlayered with 0.75% molten agar containing 0.6 M KCl.

Results and Discussion

Isolation of *P. florida* auxotrophic mutant

Spores from *P. florida* were collected and suspended in sterile distilled water. The spore suspension was irradiated by U. V. ray until 5% survival ratio. Leucine auxotrophic mutant was characterized by its ability to grow on mushroom minimal medium (MMM) supplemented with leucine, but not to grow on MMM without leucine. This mutant was designated as P101. As the auxotrophic mutant was complemented by the β-isopropylmalate dehydrogenase of *F. velutipes* cloned into PBR 322 it is assumed that mutant may be Leu 2 mutant.

Transformation of *P. florida* mutant P101

Plasmids containing Leu 2 gene (β-isopropylmalate dehydrogenase) isolated from the *F. velutipes* genomic library was selected by the complementation of Leu B auxotrophic mutant in *E. coli*.

This plasmid was used for transformation of *P. florida* leucine auxotrophic mutant by complementation. Protoplasts from 4-day-old mycelia of *P. florida* P101 were released and washed with buffer with an osmotic stabilizer. Protoplasts and vector DNA were mixed and treated with polyethylene glycol. Protoplasts with DNA were plated for selection on minimal regeneration media. Following incubation for 8-20 days at 25°C some colonies of *P. florida* were observed.

Table I. Transformation of *P. florida* P101 using pM 301 vector.

Experiment	Protoplasts $\times 10^5$	Transformants	Incubation periods for Transformation (days)	Presence of clamp connection
1.	2.25	2	17	–
2.	36.75	6	12	–
3.	5.25	9	14-22	–

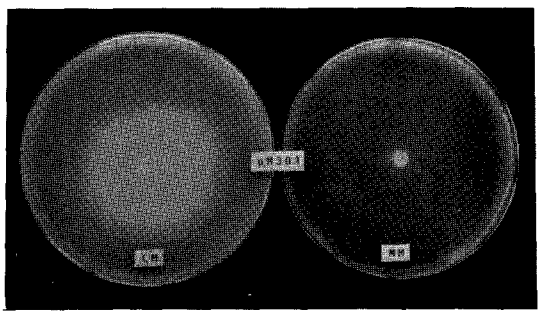

Fig.1. Transformed colony on mushroom complete medium (CM) and mushroom minimal medium (MM).

Putative transformed colonies and untransformed colonies were tested on mushroom minimal media (MMM) and mushroom complete media (MCM). The untransformed colony grew very well on MCM but could not grow on MMM. The Putative transformed colonies grew on MMM as well as MCM but mycelial growth speed on MMM was slow compared to that of MCM. The mycelia of putative transformants have no clamp connection in the same way as untransformed mycelia.

Southern hybridization

Total DNA from transformed colony and untransformed colony was isolated and purified by CsCl gradient ultra centrifuge. Total DNA of transformant was digested with EcoRl and ran on 0.7% agarose gel stained with ethidium bromide. The DNA in the gel was transferred to nitrocellulose, probed with nick-translated pM 301 vector, washed stringently 2XSSPE at 65°C and autoradiographed. DNA from transformed colonies was hybridized with vector pM 301. Hybridized band showed that the plasmid may be integrated into the chromosome.

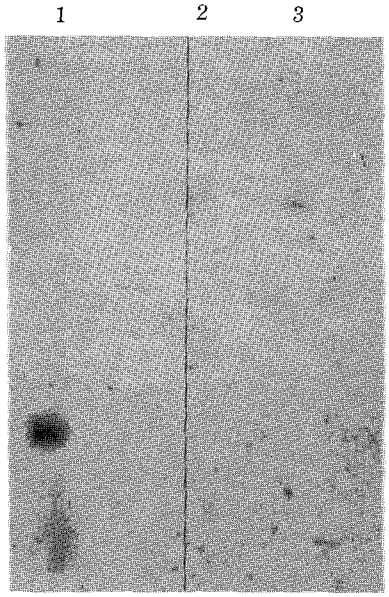

Fig.2. Southern hybridization of pM 301 to the DNA of pM 301 transformants Lane 1, Leu 2 gene fragment of *F. velutipes* DNA; Lane 2, transformant's total DNA; Lane 3, transformant's total DNA.

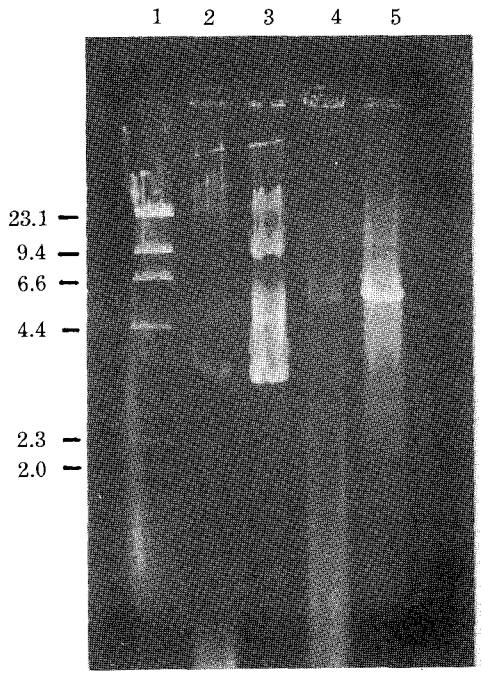

Fig.3. Plasmid pM 301 and recovered plasmid by transformation of *E. coli* HB 101 using transformant's total DNA religated ater Eco R1 digestion, Lane 1, m Hind 3 digested; Lane 2, recovered plasmid; Lane 3, plasmid pM 301; Lane 4, recovered plasmid, Eco R1 digested; Lane 5, plasmid pM 301, Eco R1 digested.

Transformation of *E. coli* using total DNA of *P. florida* transformant

Total genomic DNA of transformants was used for transformation of *E. coli* HB101. Any colonies wasn't recovered by transfer of the cellular DNA into an *E. coli* host. On the other hand, total genomic DNA of transformants was EcoRl digested and ligated. Ligated DNA was used to transform *E. coli* HB101 by ampicillin resistancy. One colony showed ampicillin resistancy. Plasmid DNA was prepared from the colony of ampicillin resistant *E. coli* cells using the modification of the Birnboim and Doly procedure described by Silhavy *et al.* Whether the plasmid was Eco Rl digested or not, plasmid band was the same to the original pM 301 vector on the agarose gel electrophoresis (Fig.3).

Together with the hybridization data these results showed that in *P. florida* this vector may be integrated into chromosome without the capacity of autonomous replication.

These results show that the pM 301 plasmid contains a sequence which can transform to prototrophy the β-isopropylmalate dehydrogenase-deficient strains of *P. florida.* The transformation frequencies observed with this plasmid are still markedly lower than those observed in yeast with self-replicating hybrid plasmid.

摘 要

팽나무버섯 β-isopropylmalate dehydrogenase의 유전자가 PBR322에 크로닝된 백터를 이용하여 사철느타리버섯 Leucine 영양요구성 균주를 형질전환 시켰다. 형질전환율은 저조하였으나 형질전환된 균총은 버섯 최소배지에서 다소 느리지만 생육할 수 있었다. 형질전환은 Southern hybridization과 형질전환된 사철 느타리버섯 염색체 DNA를 다시 대장균에 형질전환시켜 확인하였다.

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