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Transformation of *Pleurotus sajor-caju* by Complementation of PABA Requiring Mutant

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여름 느타리버섯에서 PABA 변이주의 Complementation에 의한 형질전환

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ABSTRACT: A PABA auxotroph of *Pleurotus sajor-caju* were transformed to prototrophy by using a plasmid containing pab 1 gene from *Coprinus*. The efficiencies of transformation of *Pleurotus sajor-caju* was five transformants per μg of plasmid DNA. Southern blot analysis of DNA extracted from transformants demonstrated that plasmid DNA was integrated into the chromosomal DNA in multiple tandem copies. Progenies of heterokaryons between transformants of PABA and other auxotropic strains produced pab- progeny, which indicated that integration occurred at a site(s) other than the resident pab biosynthetic gene.

KEYWORDS: Pab 1 complementation, *Pleurotus sajor-caju*, Transformation

Oyster mushroom is one of the most popular mushrooms cultivated in Korea. *Pleurotus sajou-caju* introduced from sub-tropical area such as India and Papua New Guinea is cultivated in summer season due to its requiring relatively high temperature for fruitbody formation. The application of biotechnology to the mushroom breeding could greatly improve crop yield and quality. Transformation of basidiomycetes has been achieved by several groups of workers; mutant strains being transformed to prototrophy by either cloned structural genes of a functionally equivalent gene from the related fungus.

P-aminobenzoic acid (PABA) is an intermediate in the biosythesis of tetrahydrofolic acid; its biosynthesis diverges from the pathway to aromatic amino acid at chorismic acid and involves three genes. Two of these are pab 1 and pab 2 (Nichols, 1989). In this paper, we isolated PABA requiring auxotrophic mutant by UV irradiation and transformation was made by complementation of auxotrophic marker using *Coprinus* pab 1 gene.

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Materials and Method

Strains and media

Pleurotus sajor-caju strains (ASI 2139) were obtained from the wild flora of Papua New Guinea and ASI 2146 obtained from India (Byun *et al.*, 1995). Those strains were

preserved on mushroom complete medium (MCM) for further use.

Preparation of PABA requiring mutant

Spores were collected from the fruitbody of *Pleurotus sajor-caju* ASI 2146. Spores were diluted in sterile distilled water and illuminated by UV light from 30 cm distance for 1 min. Spores were plated on mushroom complete medium (MCM) and incubated at 25°C about 4~8 days. Germinated single colony was transferred to MCM and mushroom minimal medium (MMM). Colonies which can grow well on MCM but can not grow on MMM, were selected and tested fortheir growth on MMM, MMM containing para aminobenzoic acid (PABA) and MCM to identify their requirement of PABA.

Transformation

Transformant was screened by complementation of PABA mutant using pST 1 vector containing *Coprinus* pab gene in pUC 13 vector (Fig. 1). The pST 1 was kindly supplied by Dr. L. Casselton. PEG mediated transformation of *P. sajor-caju* was conducted as previously described by Byun *et al.* (1989).

DNA isolation and Southern hybridization

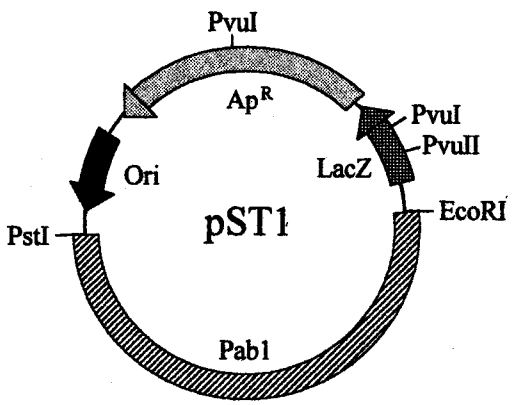

Fig. 1. Restriction map of pST1, Hatched line is *Coprinus* pab1, *Eco*RI-*Pst*1 fragment in pUC13.

P. sajor-caju DNA was isolated from freeze-dried mycelia grown in MCM. Forty mg of lyophylized mycelia ground in a mortar was resuspended by vortexing in buffer A (1% hexadecyl trimethyl ammonium bromide, 0.7 M NaCl, 50 mM Tris-HCl (pH 8.0), 10 mM EDTA and 1% 2-mercaptoethanol) and incubated at 60°C for 30 min. This preparation was extracted with an equal volume of chloroform/isoamyl alcohol (24:1). The nucleic acids were precipitated with the equal volume of isopropanol. The precipitate was resuspended in 300 μl TE. RNAse was treated in 100 μg/ml concentration at 37°C for 30 min. DNA was extracted with an equal volume of chloroform/isoamyl alcohol and precipitated with 2 volume of 95% ethanol in the presence of ammonium acetate.

For Southern hybridization, total genomic DNA (2~3 μg) from transformants was restricted with *Eco*R 1, electrophoresed in 1% agarose gel, followed by transfer to nylon membrane and cross-linking with UV light. The membrane was hybridized with labelled probes encoding the Pab 1 gene, washed, and then subjected to autoradiography.

Fruitbody formation

Transformants were mated with compatible ASI 2139-rib monokaryon. Each cross was identified by the presence of clamp connections on the hyphae.

Medium for formation of fruiting body was prepared by mixing poplar sawdust and rice bran at 4:1 ratio. Tapwater was added to the mixed substrate in order to adjust the moisture content to 63~65%. Plastic bottles were filled with moisturized substrates and autoclaved for 60 min. at 121°C (1.2 kg/cm^2). Actively growing mycelia which were grown on potato dextrose agar were inoculated into the substrate bottle and incubated at 27°C for three weeks. Fruiting bodies were induced by removing inoculum and scratching out the

surface of fully colonized substrates.

Relative humidity was maintained 80 to 90% by an automated humidification system. Temperature was maintained at 16±2°C and the carbon dioxide concentration in the air was kept below 600 ppm by regulating the air in the room. The room was illuminated with white fluorescent lights.

Primordia formation occured in 4 to 6 days exposure to the condition for formation of fruiting body. Mushrooms were harvested from the bottle at about 3~5 days after formation of fruiting body.

Spore analysis

Spores were suspended in sterile distilled water and diluted to 0.1, 0.01 and 0.001%. Counts of basidiospore numbers were made using a haemocytometer. 0.25 m*l* of each dilution was plated on MCM agar and incubated at 27°C until the colony was formed. Auxotrophic characteristics of germinated spores were tested on the MMM, MCM, and MMM containing PABA or/and riboflavine.

Results and Discussion

Transformation of pab⁻ *P. sajor-caju* with recombinant plasmid

A PABA requiring mutant was obtained from *P. sajor-caju* by illumination with UV light for 1 minute. The 2146-1-1 mutant could not grow on minimal medium but grow on complete medium or minimal medium containing PABA. Transformants were selected by the complementation of PABA requiring mutant with pab1 gene in pST1 vector.

All transformants were isolated by plating transformed spheroplasts directly on minimal agar medium containing 0.6 M sorbitol to select the transformed pab⁺ phenotype. Colonies appeared after 7~10 days of incubation and were subcultured on minimal medium. Transformation efficiencies with pST1 were

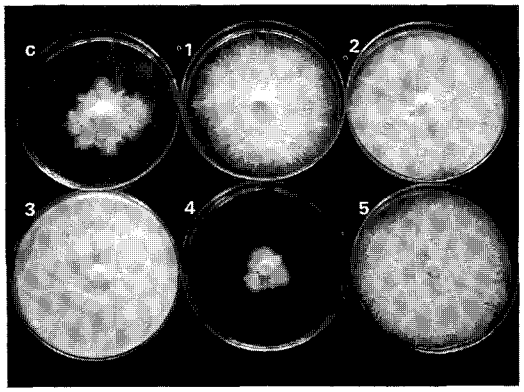

Fig. 2. Colony type of transformants by pab complementation on mushroom complete medium
C: *Pleurotus sajor-caju* 2146-1-1 (pab)1: P1 2: P2, 3: P3, 4: P4, 5: P5

five transformants per μg plasmid DNA and 10^6 viable spheroplasts. Transformants obtained with pST1 were denoted P1 through P 5. The mycelial growth of transformants was so variable that P2 was slow, P3, P4 and P5 were moderate, and P1 was fast, although host strain showed no growth on minimal medium. However, all transformants and host strain 2146-1-1 (pab) showed fast growth on complete medium and minimal medium containing PABA (Fig. 2). Colony types were strandy or fluffy on mushroom complete medium. Host strain showed strandy type but transformants were fluffy types of two and

Table 1. Transformation of *Pleurotus sajor-caju* 2146-1-1 (pab⁻) using pST1 vector containing pab1 gene

Transformant	Mycelial growth[a]			Colony type
	MM	CM	MM +pab	
2146-1-1(pab)	–	+++	++	Strandy
P1	+++	+++	++	Strandy
P2	+	+++	+++	Fluffy
P3	++	+++	++	Fluffy
P4	++	+++	++	Strandy
P5	++	+++	++	Strandy, Primordia

[a]Mycelial growth, –: None, +: Slow, ++: Moderate, +++: Fast growth.

strandy types of three. Especially, transformant P5 produced primordia on MCM agar plate (Table 1).

Southern hybridization analysis of pab$^+$ transformants

Southern blots of DNA extracted from putative transformants were hybridized with labelled pab1 gene fragment of pST1. Non-digested DNA samples of transformants with pab gene gave high molecular weight signals, whereas the nontransformed control did not hybridize to the pab probe, indicating that pST1 sequences had integrated into the *P. sajor-caju* genome. Total DNA was digested with *Eco*R1, run on 1% agarose and blotted to nylon membrane. DNA from the transformant P1, P2, and P3 showed strongly hybridizing band. Different bands appeared in the some pST1 transformants (Fig. 3), indicating that the genomic integration sites differ. Two transformants contained plasmid sized bands (Fig. 3. Lanes 2 and 4), indicating that tandem integrations of the plasmid had occurred, as found in many other fungi. However, the presence of additional hybridizing bands indicates that ectopic integration definitely occurred. Ectopic integration appears to dominate in filamentous fungi (Fincham 1989). In basidiomycetes *Coprinus cinereus*, integration at the homologous locus occurred in only 5% of the transformants examined (Binninger *et al.*, 1987).

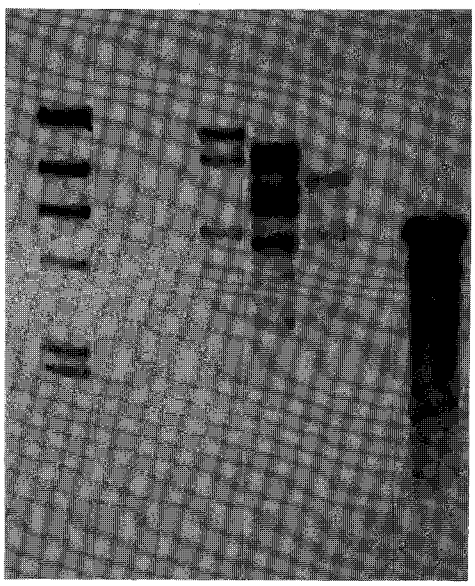

Fig. 3. Southern hybridization of genomic DNA from transformants with probe DNA
M: Lambda Hind III
C: *Pleurotus sajor-caju* 2146-1-1 (pab$^-$)
1-5: Transformants P1-P5
P: Positive control fragment encoding PABA gene in pST 1.

Genetic Analysis

Dikaryon was constructed by mating the transformants and a compatible monokaryon 2139-rib. Fruitbody of dikaryon produced on sawdust medium showed similar grayish brown funnel fruitbody type in all transformants and 2146-1-1 (pab$^-$) as shown in Table 2. Germination frequencies were generally low, ranging from 6 to 32%. Single spore germlings were collected and scored for the segregation of prototrophy and auxotrophy for which a 1:1 ratio is predicted. A chi-square test for goodness of fit to the predicted ratio was performed in 2146-1-1 (pab), P1, P2 and P3. In progeny test of pab transformants crossed with rib auxotrophy, pab auxotrophs

Table 2. Formation of Fruiting body of transformants mated with monokaryon

Hybrid	Fruitbody type	Period for fruitbody formation[a]
2146-1-1(pab) X 2139-rib	Greish brown funnel type	++
P1 X 2139-rib	"	+
P2 X 2139-rib	"	++
P3 X 2139-rib	"	++
P4 X 2139-rib	"	+
P5 X 2139-rib	"	+++

[a]Period to pinheading after induction by scratching the surface: +: Short, ++: Average, +++: Long.

Table 3. Recombination analysis of hybrids between transformant and auxotroph (2139-rib) of *Pleurotus sajor-caju*

Strain	Germination ratio (%)	Parentals		Recombinant				Recombinant. (%)
		Pab⁻	Rib⁻	Prototroph	Prototroph	Pab⁻	Rib⁻	
2146-1-1 (pab⁻) ×2139-rib	31.5 ±6.01	5	14	42	59.2	10		73.2
P1×2139-rib	22.0 ±0.71	0	9	26	66.7	4		76.9
P2×2139-rib	6.03±0.16	0	4	25	65.8	9		91.9
P3×2139-rib	10.01±0.96	1	11	50	71.4	8		82.9
P4×2139-rib	19.33±2.72	0	4	67	83.8	9		95.0
P5×2139-rib	31.34±2.36	2	13	77	84.6	9		95.6

were not obtained in P1, P2 and P4 but recombinants requiring both pab and rib were produced in all transformant (Table 3). Thus, all such crosses yielded some pab auxotrophs, which suggested that integration of the transforming gene into recipient genome did not occur homologously with the resultant replacement of the defective PABA biosynthetic gene. Such heterologous integration of tandem multiple copies of transformed DNA has been also reported in *Phanerochaete chrysosporium* (Alic *et al.*, 1989).

In *P. sajor-caju, pab* gene of *Coprinus* was a useful selection marker for complementation of pab requiring host strain. This transformation system will be effective for genetic research of higher fungi and the breeding of industrially important mushroom.

적 요

여름느타리버섯의 단포자에 UV를 처리하여 pab 요구성 영양요구주를 작성하였으며 이 균의 균사에서 원형질체를 분리한 후 *Coprinus* pab 1 유전자를 함유하는 plasmid를 이용하여 prototrophy로 형질전환 하였다. 형질전환율은 μg의 DNA 당 5개의 형질전환주를 얻을 수 있었다. 형질전환주는 Southern 분석결과 염색체 DNA 속으로 integration된 것으로 확인되었으며 영양생장과 생식생장시 모두 안정하게 유지되었다. 형질전환주와 화합성인 다른 영양요구주와 교배후 자실체의 포자를 분리하여 유전분석 결과 pab 생합성 유전자 보다는 그 유전자 주위에 integration이 일어난 것으로 추정되었다.

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