韓 國 菌 學 會 誌 第17卷 第4號
Kor. J. Mycol. Vol. 17, No. 4, 209~213, 1989

Transformation of *Pleurotus florida* with *Neurospora pyr* 4 Gene

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Neurospora pyr 4 유전자를 이용한 사철 느타리버섯의 형질전환

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ABSTRACT: Transformation of an auxotrophic requirement for uracil in *Pleurotus florida* P101 has been achieved using chimeric vector containing *Aspergillus nidulans ans* 1, and *Neurospora crassa pyr* 4 DNA. Protoplasts of Ura^-strains of *P. florida* were incubated with plasmid pDJB3 containing the cloned *pyr* 4 gene in the presence of polyethylene glycol and $CaCl_2$. Transformants could grow on MMM showing mitotical stability. Southern hybridization analysis of DNA isolated from transformants showed that the *Neurospora pyr* 4 gene and vector sequence might be intergrated into the *P. florida* chromosomes. As the transformants were monokaryon, each transformant was mated with the other monokaryon. Fruitbody shape of untransformant was eroded type but those of transformants were eroded type, funnel type, plane type and ungrowing cap type.

KEYWORDS: Transformation, *Pleurotus florida* (Ura^-), *Neurospora crassa pyr* 4 gene

Pleurotus florida is one of the very popular mushroom cultivated in Korea. Genetic researches of this species are being started with auxotrophic mutant selection (Yoo *et al.*, 1985), protoplast fusion (Yoo *et al.*, 1984), and transformation (Byun *et al.*, 1989). For transformation of filamentous fungi, selectable marker gene and appropriate host are indispensible. *pyr* 4 gene of *Neurospora crassa* was used for transformation of *Aspergillus nidulans Ura*-mutant

In this report, we describe the transformation of protoplast of *P. florida* via complementation of an uracil auxotrophic gene with the orotidine 5'-phosphate carboxylase of *Neurospora crassa.*

Materials and Methods

Strains

Pleurotus florida P101, (*leu, cyto, ura*) auxotroroph (Byun *et al.*, 1989) was used as transformation recipient. *Escherichia coli* strain HB101 was used for propagation of plasmids. Plasmid pDJB3 (Fig. 2) was used for transformation.

Media

Auxotrophic mutant was maintained on slants of mushroom complete medium (MCM). Complete medium contained (gl^{-1}) yeast extract 2, peptone 2, dextrose 20, KH_2PO_4 0.46, K_2HPO_4 1.0, $MgSO_4{\cdot}7H_2O$ 0.5 and Agar 20. Transformants were selected on mushroom regeneration minimal medium (MMM). Minimal medium was containing (gl^{-1}) Dextrose 20, KH_2PO_4 0.46, K_2HPO_4 1.0, $MgSO_4{\cdot}7H_2O$ 0.5, DL-Asparagine 2 and Bacto-agar 20. Regeneration minimal medium was supplemented with 0.6 M sorbitol as an osmotic stabilizer. Mycelia to analyze transformants were obtained from MCM without agar. *E. coli* cultures for plasmid preparation were grown using Luria Broth medium with ampicillin.

Protoplast formation

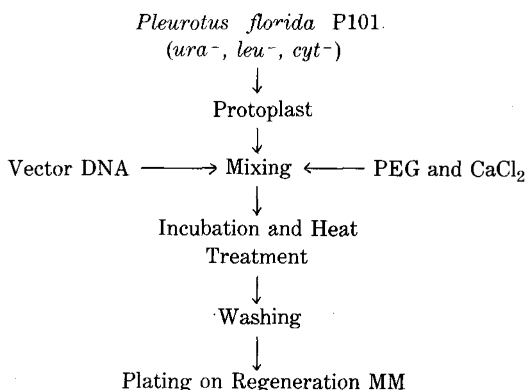


Fig. 1. Transformation procedure.

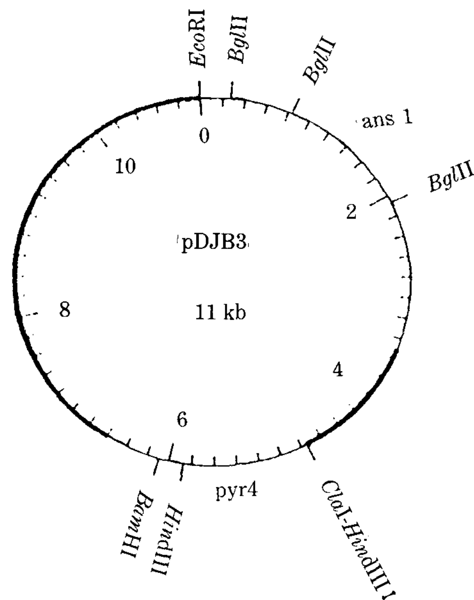


Fig. 2. Restriction enzyme map of pDJB3. Heavy line = pBR325 sequences. The locations of the *N. crassa* segment containing the *pyr*4 gene and the *A. nidulans* sequence *ans*1 (Ballance and Turner, 1985) are indicated.

Protoplasts were obtained from the mycelia which were cultured on cellophane sheet of mushroom complete agar medium for 3-4 days. Mycelia were treated with sterile lytic enzyme Novozyme 234 and Cellulase onozuka R-10 dissolved in 0.6 M sucrose solution. Protoplasts were formed after shaking at 30°C for 3 hrs. Protoplasts were isolated from the mycelial debris through sintered glass filter #1, collected by centrifugation and washed with 0.6 M sorbitol, 10 mM MOPS pH 6.75 twice.

Transformation

Protoplasts were washed with 0.6 M sorbitol, 10 mM MOPS and 10 mM $CaCl_2$. Plasmid DNA was mixed with protoplasts in 0.6M sorbitol, 10 mM MOPS and 10 mM $CaCl_2$. After 20 min at room temperature, the same volume of 50% PEG solution was treated and incubated on ice for 20 min. Heat treatment was done at 37°C for 5 min. For PEG elimination, washing was done with 0.6 M sorbitol and 10 mM MOPS solution twice. Protoplast suspension was then diluted to 10^6 and plated in mushroom regeneration minimal medium overlayered with 0.75% soft agar medium.

DNA preparation from transformants

Vector DNA was prepared (Byun *et al.*, 1989) and total DNA of transformants was isolated from the mycelia by rapid method. Mycelia grown in liquid mushroom complete medium were collected and washed with TE buffer. Lyophillized mycelia were pulverized in the mortar and suspended in 50 mM Tris HCl pH 8.0, 100 mM EDTA, 150 mM NaCl and 2% SDS solution. After incubation for 2 hrs, phenol and chloroform extraction, chloroform extraction and ethanol precipitation were followed.

Southern hybridization

Total DNA was run the agarose gel, and denatured DNA was transfered to gene screen plus paper (Dupont, USA). Neutralized and dried gene screen plus paper was hybridized with the ^{32}P-labelled plasmid vector for 24 hrs at 65°C. Washing of paper was done stringently and autoradiographed at −70°C for 24 hrs.

Crossing

Each transformant was mated with monokaryon strain obtained from *Pleurotus florida* single spore. Transformant and monokaryon strain were inoculated 1 cm separated on mushroom complete medium. After incubation for 2 weeks at 25°C, clamp connection was investigated under light microscope. Hyphae with clamp connections was considered mated dikaryon.

Fruitbody test

Dikaryon was grown on mushroom complete medium. Fruitbody formation was induced on sawdust medium. Sawdust medium was prepared by mixing of poplar sawdust and rice bran by 80:20 ratios. Mixed substrates were adjusted with 65% moisture content by spraying tap water. About 454g sawdust substrates were packed into 1000 m*l* glass bottle using filling equipment. Capping the bottle was done using cotton. After autoclaving culture medium, mated dikaryon of transformants was inoculated into the medium bottle. It takes about 20-30 days to grow fully the culture medium at 28°C incubator. Primordia formation was induced using light, temperature adjustment to 15-18°C and watering.

Results and Discussions

Pleurotus florida P101 (*ura*$^-$, *leu*$^-$, *cyto*$^-$) was used as a host for transformation of pDJB3 vecter containing *Neurospora crassa pyr* 4 gene and *Aspergillus nidulans ans* 1 (Ballance & Turner, 1986). Plasmid DNA was purified through CsCl-EtBr density gradient centrifugation. 10 μg of plasmid pDJB3 was used to transform *P. florida* P101 protoplasts to uracil prototropy. Transformation frequency was very low. Protoplasts were regenerated at about 0.09% ratio after 6 days, but complemented colonies of ura$^-$ protoplasts was regenerated to 0.8% after 9 days (Table I). Seven transformants were obtained through several transformation. No spontaneous reversion was detected in the control experiment. Transformants exhibited a stable ura$^+$ phenotype even after repeated transfers onto uracil-supplemented medium. Mycelial growth speed of transformants was very different on MCM but all of the transformants can grow compared to no growth of host strain on MMM (Table II). Transformed colonies showed several colony types. One type showed mycelial growth similar to untransformed colony on MCM but second type very slow and compact on MCM but ordinary on MMM. Third type appeared that center part was relatively dense compared to sparse growth of peripheral part of colonies. Another type showed mixed sector colonies which one was sparse and the other was dense (Fig. 3). Different types of colonies compared to protein band pattern on SDS-PAGE were the same species without any contamination (Unpresented data). Total DNA from mycelia of transformants and host strain was isolated and hybridized with ^{32}P labelled vector DNA pDJB3. Transformants showed containing vector DNA fragments (Fig. 4). Transformants were monokaryon and didn't produce any fruitbody. Therefore, each transformant was mated with monokaryon strain (2016-1) of *Pleurotus florida* ASI 2016. Fruitbody of mated strains with clamp connections was investigated. Of the six transformants tested, one strain did not form any primordia, the other strains formed four different types of fruitbody; plane type, funnel type eroded type and ungrowing cap type (Table III). Untransformed strain MP101 produced eroded cap type of fruitbody and formed

Table I. Transformation of *Pleurotus florida* with plasmid pDJB3

pDJB3	Protoplasts	Regenerated colonies			Ura$^+$ colonies from protoplast treated with			
					pDJB3			Buffer
(μg)	$\times 10^5$/m*l*	No.	ratio*	days	No.	ratio**	days	No.
10	3.7	334	0.09	6	3	0.8	9	0

* Regenerated colonies/protoplasts (%)
**Complemented colonies/regenerated colonies (%)

Table II. Mycelial growth of transformants on mushroom complete medium and minimal medium

Transformants	Mycelial growth*	
	MCM	MMM
1	+ +	+ +
3	+ + + +	+
4	+	+ +
5	+ + +	+
6	+ + + +	+ +
7	+ + +	+
P101 (control)	+ + + +	−

* + + + +; Fast, + + +; Medium, + +; Slow, +; Very slow, −; No growth

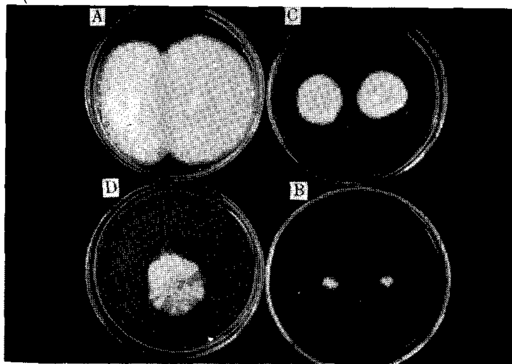
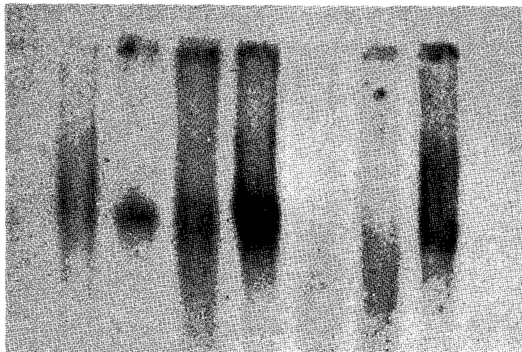


Fig. 3. Mycelial type of transformants on mushroom complete media. A.; Ordinary type, B.; Slow and compact type, C.; Sparse periphery type, D.; Sector type.



P101 1 3 4 5 5 6 7
(Sparse type) (Dense type)

Fig. 4. Southern hybridization of transformants with vector DNA pDJB3.

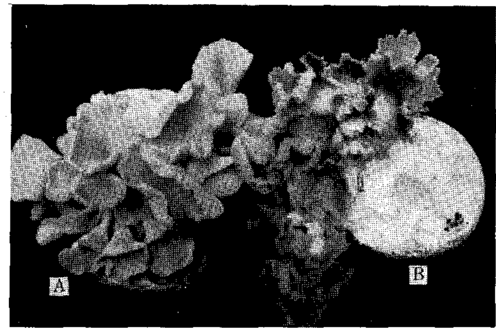


Fig. 5. Funnel type and eroded type of fruit body. A.; Funnel type, B.; Eroded type.

Table III. Characteristics of fruit body of transformants after mating with monokaryon 2016-1 (wild type)

Transformants ×Monokaryon (2016-1)	Fruitbody type	Spore formation*
M1	No primordia	NT
M3	Plane type	+ + +
M4	Funnel shape	–
M5	Funnel shape	–
M6	Funnel shape	–
	Eroded type	+ +
	Ungrowing cap	NT
M7	Funnel shape	–
MP101 (control)	Eroded type	+ +

* + + +; many, + +; a few, –; few, NT; Not tested

basidiospores. M1 transformant did not form any primordia as well as no clamp connection (Table IV). M3 formed plane type fruit body containing lots of spores. M4, M5 and M7 showed funnel type fruit body without spores similar to host strain MP101. However M6 strain produced 3 types of fruit body such as funnel type, eroded type and ungrowing cap type. Eroded type among M6 transformant was similar to untransformed host type in the fruit body morphology and spore formation. Fruit body of ungrowing cap type showed appropriate growth of stem but ungrowth of cap. Therefore, spore test was impossible. Funnel shape didn't disperse any spores on carbon paper. Furthermore, any funnel shape fruit body of several transformants had no basidiospore. It was suggested that the gene related to spore formation linked to the gene of fruit body morphology. As the M6 transformant formed three types of fruitbody such as funnel type, eroded type and ungrowing cap type, mycelia of tissue culture from basidiocarps of three different types were investigated to test whether the mating was complete or not. All of the 3 type fruit bodies had clamp connections and showed different mycelial growing speed. Mycelial growth was tested after collection by tissue culture from

Table IV. Mycelial growth on mushroom complete media of transformants showing different fruitbody type

Transformants ×Monokaryon (2016-1)	Fruitbody type	Clamp connection	Mycelial growth (mm)	
			7 days	14 days
M1	No primordia	–	30	50
M3	Plane type	+	22	90
M6	Funnel type	+	29	53
M6	Eroded type	+	56	90
M6	Un growing Cap	+	29	53

fruitbody, and tested using preserved strain for M1 of no primordia. M1 of no primordia made sparse and slow colonies. Mycelial growth of eroded type and ungrowing cap type was slow compared to the mycelial growth of funnel shape. Plane type is distinguished by slow initial growth followed by a period of adaptation during which growth becomes almost normal (Table IV). Even if the same vector was used for transformation, characteristics of transformant might be diversified by the site of vector DNA intergration into chromosome. Therefore, introduction of DNA into *Pleurotus florida* can lead to sequence instability or DNA rearrangement.

This result shows the introduction of a gene from *Neurospora crassa* into *P. florida* protoplast and subsequent relief of auxotrophy. Gene of basidiomycete *Corprinus cinereus* has also been expressed in *Aspergillus nidulans* (Hynes, 1989). Gene expression of different organism such as *Neurospora* gene in the basidiomycete which is deficient of appropriate genetic marker will be very promising for molecular research of higher fungi.

摘 要

사철 느타리버섯 Uracil 요구성 균주를 *Aspergillus nidulans ans* 1과 *Neurospora crassa pyr* 4 유전자를 지닌 백터를 이용하여 형질전환시켰다. 사철 느타리버섯 *ura* 요구성 균주의 원형질체를 *pyr* 4 유전자를 지닌 백터 pDJB3과 혼합 후 PEG와 $CaCl_2$를 처리하였다. 형질전환 균주는 버섯 최소배지에서 안정되게 생육하였고 southern hybridization 결과도 백터 DNA가 사철 느타리 염색체에 삽입되었다. 형질전환 균주는 단핵성이므로 다른 단핵 균사와 균사 융합 후 자실체 형태, 포자형성 등을 조사하였다. 형질전환이 안된 균주는 톱니형 자실체를 형성한데 비해 형질전환된 균주는 톱니형, 깔대기형, 우산형, 갓 미발육형의 자실체를 형성하였다.

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Accepted for Publication 27 November 1989