Bull. Fac. Agr. Shimane Univ., 28 : 83−92, December 20, 1994

Preliminary Study on the Trasfer of Plasmid DNA to Basidiomycetes and its Expression[1)]

Takeshi FURUNO, Kenta SHIMADA,[2)] Tsuyoshi NAKAGAWA,[3)]
Hideyuki MATSUDA and Tohru UEHARA

Abstract To attempt the gene transfer to the basidiomycetes such as wood-decaying fungi, the experimental system of transferring plasmid DNA and seeing the expression was investigated. Using two species of *Tyromyces palustris* and *Coriolus versicolor*, their protoplasts from mycelia cultivated in SMY liquid culture media were prepared by treatments with the cell wall-lysing enzymes and others. As plasmid DNA, pUC4K having a kanamycin-resistant gene was selected because of obstructing the growth of mycelia. The transfer of plasmid DNA was examined by two ways of polyethylene glycol and electroporation.

The transformants of *Tyromyces palustris* obtained by electroporation showed obviously a faster growth than the non-transformed cells in kanamycin-containing liquid media. As a result of agarose gel electrophoresis for chromosomal DNA and plasmid DNA extracted from these transformants, the bands could be detected at the same position of plasmid pUC4K. This verified the transformation to *Tyromyces palustris* by transferring pUC4K.

Keywords: transformation; basidiomycetes; *Tyromyces palustris*; plasmid pUC4K; electroporation.

Introduction

Lignin in the wood is degraded by white-rot fungi belonging to the Class Basidiomycetes, and among them *Phnanerochaete chrysosporium* and *Coriolus versicolor* are widely used for studies of lignin-degrading mechanism. The clarification of lignin-degrading mechanism by microorganisms is very important as fundamental problems involving in the treatment of biological delignification without environmental pollution, and the establishment of techniques for biochemical conversion of lignin, and others. Studies about these subjects have been numerously reported recently.[1−4)]

Even in the field of biodegradation of lignin, genetic studies have increased very lately: for example, papers on the sequencing of the β-etherase gene involved in cleavage of β-arylether,[5)] on the isolation of the gene involved in degradation of biphenyl compounds,[6)] and on the expression of a lignostilbene-α, β-dioxygenase gene and the production in *Escherichia coli*,[7)] and other papers.

Thus, studies at the genetic level is required to clarify the lignin-degrading abilities of the basidiomycetes. In this study, it is our final goal to let the lignin-degrading abilities, as seen in *Coriolus versicolor*, express even in *Tyromyces palustris*.

[1)] Presented partly at the 42nd Annual Meeting of the Japan Wood Research Society in Nagoya, April 1992.

[2)] Kenta SHIMADA: Kishu Paper Co. Ltd., Kishu Mill, Shingu, Wakayama 647

[3)] Tsuyosi NAKAGAWA: Research Institute of Molecular Genetics, Shimane University.

Tyromyces palustris is a brown-rot fungus to degrade mainly cellulose and hemicellulose, and in addition, to degrade a little amount of lignin. If the genes encoding the lignin-degrading abilities can be transferred to *Tyromyces palustris* and the improvement of such abilities becomes practically possible, this technique seems to be greatly useful for pulping, treatment of wastewater with lignin, waste disposal of wood-based materials, and so on.

In this study, aiming to establish the experimental system of gene transfer to the basidiomycetes, antibiotics to obstruct the growth of two fungi, *Coriolus versicolor* and *Tyromyces palustris*, were selected, and the transfer of plasmid DNA having a resistance against the antibiotics and its expression were investigated preliminarily.

Materials and Methods

Fungal strains and cultivation

Two types of fungal strains were used: a brown-rot fungus, *Tyromyces palustris* (Berk. et Curt.) Murr. TYP6137, and a white-rot fungus, *Coriolus versicolor* (L. ex Fr.) Quél. COV1030.

Hyphae incubated on a SMY agar culture medium (saccharose 1%, malt extract 1%, yeast extract 0.4%, and agar 2%) at 26°C for 4-6 days were taken out, and were inoculated into a conical flask (100 ml) including a SMY liquid culture of 40 ml. After incubating stationarily them at 26°C for 2-4 days, a suspension of hyhae was prepared by stirring from grown mycelia with a magnetic stirrer. A suspension of 2 ml was inoculated into a new liquid culture and incubated again. This inoculation was repeated each inoculating period of 4-6 days.[8]

DNA preparation from mycelia

DNA preparation from mycelia was basically conducted according to the method by U. Raeder and P. Broda.[9] Mycelia cultivated by a shaking culture were freeze-dried and pulverized using a mortar and a pestle. The powdered material was suspended in a DNA extraction buffer solution (200mM Tris-HCl (pH8.5), 250mM NaCl, 25mM EDTA, and 0.5%SDS). Then it was extracted with phenol and chloroform.[10] Finally, the DNA obtained by ethanol precipitation was confirmed by using an agarose gel electrophoresis.

Preparation of protoplasts

Protoplasts were prepared from young mycelia by a modification of the procedure developed by F. Eguchi and others.[8] The cultivated mycelia were collected by filtration with nylon meshes (pore size 10×10 μm). Cell wall-lysing enzymes (2% Novozyme-234 from *Trichoderma harzianum*, cellulase from *Trichoderma viride*, and uskizyme from *Trichoderma* sp.) and a maleic acid buffer containing an osmotic stabilizer (mannitol) were added to the mycelia, and they were incubated at 26°C for 2 h by shaking at 75 rpm. After filtrating the treated solution with miracloth (Calbiochem Corporation), the filtrate was centrifuged for 10 min at $1000\times$g. The precipitate was suspended in an osmotic stabilizing solution to recentrifuge. This

procedure was repeated three times to prepare the purified protoplasts after washing.

Selection of antibiotics

In this study, kanamycin and ampicillin were used as antibiotics obstructing the growth of *Tyromyces palustris* and *Coriolus versicolor*. SMY agar culture media in petri dishes contained each of antibiotics of 200-350 μg/ml. Hyhae were inoculated at the center of dishes, and the growing areas of mycelia were determined regarding as the growth rate to compare them with those in the controls.

Preparation of plasmid DNA

Escherichia coli competent cells being capable to take foreign DNA were prepared. Next, the plasmid DNA provided in advance was transferred to *E. coli*. Plasmid DNA was extracted by the alkali method.[10] Also, plasmid DNA of transformed protoplasts of the basidiomycetes was extracted by the same method.

Transformation of protoplasts

Polyethylene glycol method: Protoplasts happen to fuse naturally followed by the expansion of protoplasmic connection between the cells during the action of cell wall-lysing enzymes.[11] By using the polyethylene glycol (PEG) method for the protoplast fusion, experiments to produce asexually hybrid cells have been considerably put into practical use.[12]

In this experiment, the transformation was done by introducing plasmid DNA into protoplasts of the basidiomycetes owing to the cohesion of PEG solution. Protoplasts prepared in Section Preparation of protoplasts were suspended in an osmotic stabilizing solution. A solution of 60 μl containing plasmid DNA (1 μg/1 μl) was added to a solution of 60 μl containing protoplasts (10^5/1 μl), mixed gently, and incubated on ice for 10 min. The samples were underlaid with 160μl of 40% PEG4000 in 10mM MES (2-(N-morpholino) ethanesulfonic acid), incubated on ice for 10 min, mixed gently, and incubated for an additional 10 min. The transformed protoplasts were inoculated on the culture media containing antibiotics, and then the expression and growth were observed with time.

Electroporation methood: By the electroporation method giving high-voltage pulses to a mixed solution of protoplasts and foreign genes, transferring DNA to the cells was attempted in this study.

The prepared protoplasts were put into the buffer in a fusion chamber, plasmid DNA was added with a pasteur pipette and suspended. The mixed solution was placed in a cuvette of an electroporation apparatus (Shimadzu GTE-10). The conditions of electric pulses were as follows: width of parallel electrode chamber, 2 mm; electric-field strength, 0.3 Kv/cm; pulse width, 20μs; and the number of pulse times, 6 times.

Screening

Screening on culture media containing antibiotics: Transformed protoplasts obtained by the gene-transferring method and non-transformed cells were inoculated on SMY agar culture media, and the periods till the generation of colonies were

compared between the two. Furthermore, the generated colonies were inoculated on new culture media and the growing areas of mycelia were measured with time.

Another method was performed as follows: after cultivating the transformants and non-transformed cells on agar culture media, the media were punched out with a cork borer, and the mycelia were incubated in a stationary liquid culture of 40 ml containing antibiotics in a flask (100 ml) for 10-14 days. The fully grown mycelia were filtrated with filter paper and oven-dried at 105°C. The growth rates were compared by measuring the mycelial dry weight.

Agarose gel electrophoresis: Chromosomal DNA and plasmid DNA extracted from transformants as described in Sections Preparation of protoplasts and Preparation of plasmid DNA were provided for an agarose gel electrophoresis to confirm the plasmid DNA transferred.

Results and Discussion

Preparation of protoplasts

As cell wall-lysing enzymes of the basidiomycetes, three species originating from *Trichoderma* belonging to the Imperfect fungi (Deuteromycotina) were provided for this study. Novozyme-234 and cellulase were used by mixing and uskizyme was used independently.

Consequently, the most satisfactory protoplasts were obtained when using 1% uskizyme. Protoplasts prepared from *Tyromyces palustris* and *Coriolus versicolor* were isolated from hyphae in globular forms surrounded with plasma membranes alone under the observation with a light microscope, and their sizes were about 5-10 μm in diameter. It was reported that in protoplasts of the basidiomycetes a cell causing plasmolysis was divided into two parts or more through the weakened region of cell walls,[13, 14] and the expanding-out of protoplasm from hyphae was observed. The uskizyme was said to be capable of lysing the cell walls of the Eumycetes including molds, yeasts, and basidiomycetes, and of forming easily protoplasts.[15] In this study, the uskizyme was found to be excellent for lytic enzymes of basidiomycetes.

Growth of mycelia on culture media containing antibiotics

As a result of examining the growth of *Tyromyces palustris* and *Coriolus versicolor* on agar culture media containing kanamycin and ampicillin, kanamycin proved to obstruct the growth of both strains. Figures 1 and 2 show the growth areas on kanamycin-containing culture media for 7 days whose data were averaged for five strains, and Fig. 3 shows culture media of *Coriolus versicolor* incubated for 4 days. Therefore, we decided to use pUC4K as a plasmid DNA having the drug-resistance marker originating from transposon Tn 903 by coding aminoglycoside 3'-phosphotransferase gene, giving the kanamycin, neomycin, and G-418 resistances.[16,17]

The base pair of pUC4K is 4000 bps and the cleavage sites by restriction enzymes of *Pst* I, *Sal* I, *Bam*H I, and *Eco*R I are determined. As *E. coli* being a host for preparing plasmid, W3110 was provided from the Applied Microbiology Laboratory, Faculty of Agriculture, Shimane University.

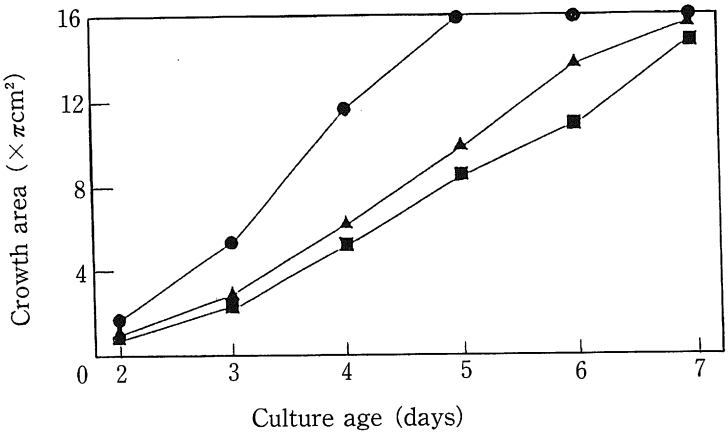

Fig. 1. Mycelial growth of *Tyromyces palustris* on SMY culture media containing kanamycin.
● : Control, ▲ : 200 μg of kanamycin/ml, ■ : 350 μg of kanamycin/ml.

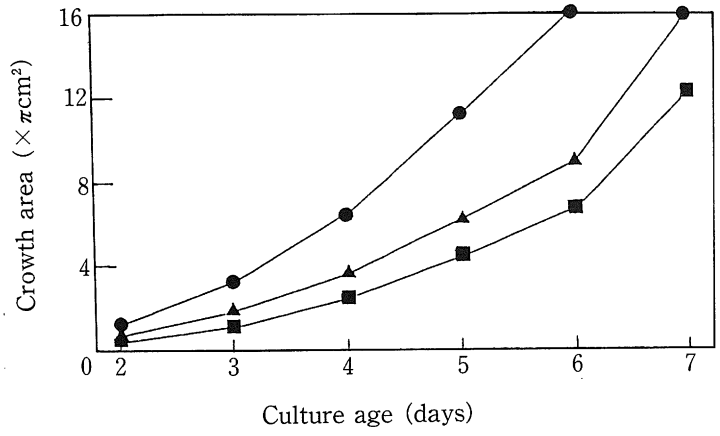

Fig. 2. Mycelial growth of *Coriolus versicolor* on SMY culture media containing kanamycin.
● : Control, ▲ : 200 μg of kanamycin/ml, ■ : 350 μg of kanamycin/ml.

Transformation to basidiomycetes

Polyethylene glycol method: Transformants of *Tyromyces palustris* in which a plasmid DNA had been introduced by the PEG method and non-transformed cells were inoculated on kanamycin-containing culture media. The colonies of the transformants appeared after 4 days, whereas in the non-transformed cells the colonies did not appear yet at this time. The expressed colonies were inoculated on kanamycin-containing culture media. Then, the growth rate was greater in the transformants than in the non-transformed cells.

An agarose gel electrophoresis of DNA extracted from the transformants is shown in Fig. 4. A band of chromosomal DNA could be seen. But any bands of

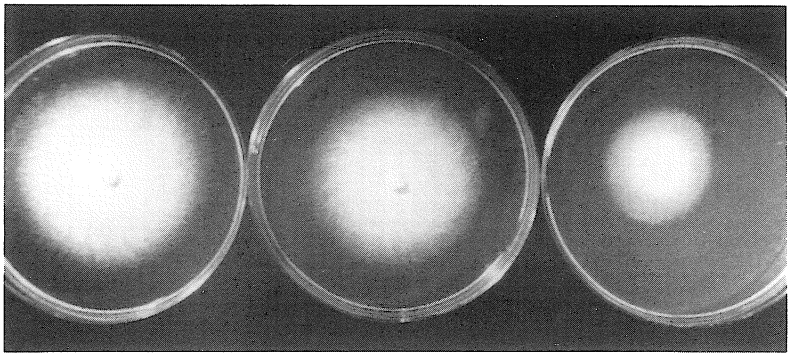

Fig. 3. Mycelial growth of *Coriolus versicolor* on SMY culture media containing antibiotics after the cultivation of 4 days.
A : Control, B : 350 μg of ampicillin/ml, C : 350 μg of kanamycin/ml.

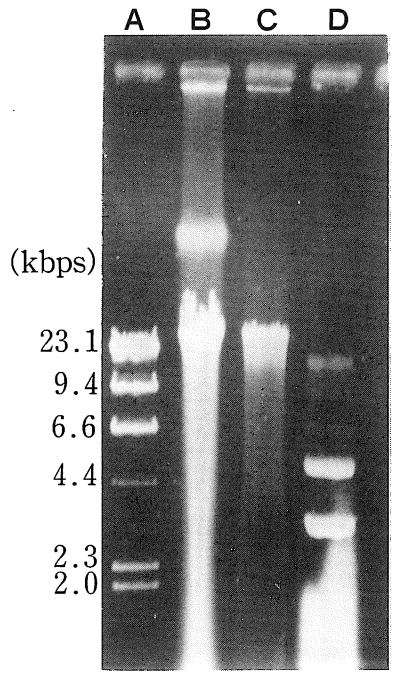

Fig. 4. Agarose gel electrophoresis for DNA extracted from the transformant of *Tyromyces palustris* obtained by the polyethylene glycol method.
Lane A : DNA marker (λ/*Hind* III digest), Lane B : DNA from the transformants, Lane C : Chromosomal DNA from the non-trans formed cells, Lane D : Plasmid pUC4K.

plasmid DNA from the transformants were not shown up even if the extraction of plasmid DNA was done. Although the bands of plasmid DNA could not be clearly recognized, it may be considered that there is a possibility of the integrading of plasmid DNA into chromosome.[12] However, the transfer of plasmid DNA by the PEG

method cannot be judged from this data. Because the confirmation of transferring in *Coriolus versicolor* was reported,[18] for the transformation by PEG, the technique should be required to improve much.

Electroporation method: When cultivating the transformants of *Tyromyces palustris* obtained by the electroporation method in liquid culture media containing kanamycin, the mycelia grew up considerably in 10-14 days as shown in Fig. 5, and there was obviously a large difference compared with the non-transformed cells of which the growth was inhibited. The yields of the mycelial dry weight were 0.11 g for the transformants and 0.06 g for the non-transformed cells. Such a growth of the transformant in antibiotic-containing culture media could be considered to be due to the expression of transferred plasmid pUC4K being kanamycin-resistant.

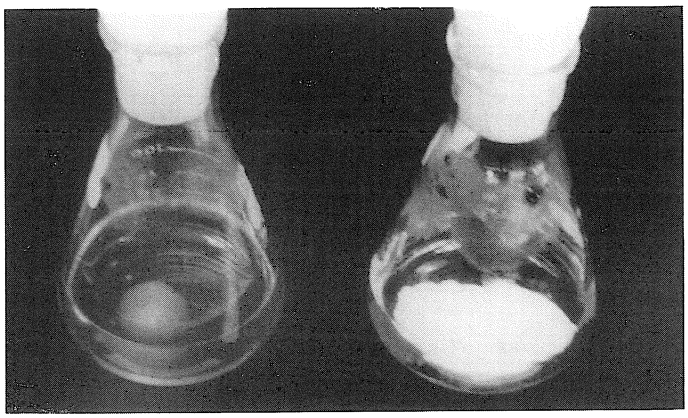

Fig. 5. Mycelial growth of the transformant of *Tyromyces palustris* obtained by the electroporation method in SMY liquid media containing kanamycin. A : non-transformed cells, B : Transformants.

As a result of the agarose gel electrophoresis for chromosomal DNA and plasmid DNA extracted from the transformants, the bands could be seen at the same position of plasmid pUC4K, as is evident from Fig. 6. This substantiated certainly the transfer of pUC4K to *Tyromyces palustris*. Thus, it is very significant that the plasmids, replicated in *E. coli*, expressed even in the basidiomycetes. Because any studies on the transfer of pUC4K and its expression applied to the basidiomycetes have scarcely been reported so far.

The electroporation method is widely used for the transformation,[19, 20] and the application to *Pseudomonas* species having lignin-degrading activity was reported,[21] so it is effective to the experiment of gene-transferring system. This time we could confirmed the transformation to *Tyromyces palustris*, but not applying yet to *Coriolus versicolor*. Examining thoroughly the pulse conditions such as electric-field strength and pulse time, and the other experimental conditions, it is desirable to establish the system capable of transferring the genes to the basidiomycetes. Also, the re-

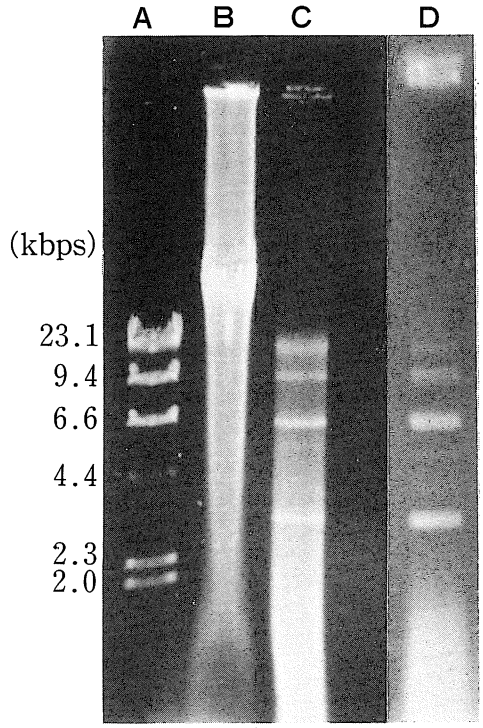

Fig. 6. Agarose gel electrophoresis for plasmid DNA extracted from *Tyromyces palustris* transformed by electroporation.
Lane A : DNA marker (λ/*Hind* III digest), Lane B : Chromosomal DNA from the transformants, Lane C : Plasmid pUC4K from the transformants, Lane D : Plasmid pUC4K.

producibility and efficiency of the transformation by electroporation in the present study, the analysis at the DNA level such as Southern hybridization, and stability of foreigh DNA in the transformants and of its drug-resistance are required to be investigated detailedly in the following experiments.

Conclusion

The transformants of *Tyromyces palustris*, in which plasmid pUC4K having a kanamycin-resistant gene was introduced by the electroporation method, found to grow faster in liquid culture media containing kanamycin than the non-transformed cells.

As a result of agarose gel electrophoresis for plasmid DNA extracted from the transformants, the bands regarded as pUC4K could be confirmed. Therefore, the transfer of plasmid DNA to *Tyromyces palustris* by using electroporation was verified to be possible in this study.

Considering fully the pulse conditions, solutions, temperatures, and others, it is

required to establish the more reliable system of gene-transferring experiment based on the detailed data. There seems to be some room for improving the preparation of protoplasts including cell wall-lysing enzymes. Furthermore, as the final goal, it would be hoped to progress the study so as to express the genes associated with lignin -degrading enzymes in *Tyromyces palustris*.

References

1) HIGUCHI, T., H.-m CHANG and T. K. KIRK, Recent Advances in Lignin Biodegradation Research. Uni Publishers, Tokyo, 279 pp., 1983.
2) CRAWFORD, R. L., Lignin Biodegradation and Transformation. John Wiley & Sons, New York *et al.*, 154 pp., 1981.
3) JANSHEKAR, H. and A. FIECHTER, Pentoses and Lignin. *In* FIECHTER, A, (ed.), *Advances in Biochemical Engineering/Biotechnology. Vol. 27*. Springer-Verlag, Berlin *et al.*, pp. 119-178, 1983.
4) KIRK, T. K., T. HIGUCHI and H.-m CHANG (ed.), Lignin Biodegradation: Microbiology, Chemistry, and Potential Applications. Vol. I & II. CRC Press, Boca Raton, Florida, 1980.
5) MASAI, E. *et al.*, Cloning of the genes involved in degradation of lignin model compounds (XVIII). Proc. 36th Lignin Symp. (Sapporo), pp. 69-72, 1991.
6) HANASHIRO, K. *et al.*, Cloning of the genes involved in degradation of lignin model compounds (XIX). Ibid., pp. 73-76, 1991.
7) KAMODA, S. and Y. SABURI, Cloning, expression and identification of a lignostilbene-α, β-dioxygenase gene from *Pseudomonas paucimobilis* TMY1009. Ibid., pp. 77-80, 1991.
8) EGUCHI, F. *et al.*, Preparation and regeneration of protoplasts of edible mushrooms. Mokuzai Gakkaishi, **36**: 232-240, 1990.
9) RAEDER, U. and P. BRODA, Preparation and characterization of DNA from lignin-degrading fungi. Methods In Enzymology, **161**: 211-237, 1988.
10) MURAMATSU, M. (ed.), Laboratory Manual for Genetic Engeneering. Maruzen, Tokyo, 203 pp., 1990. (In Japanese.)
11) YAMAGUCHI, H. (ed.), Operational Techniques for Plant Genes. Genetic Recombination and Cell Fusion. CMC, Tokyo, p. 15, 1986. (In Japanese.)
12) ALIC, M. *et al.*, Transformation by complementation of an adenine auxotroph of the lignin-degrading basidiomycete *Phanerochaete chrysosporium*. Appl. Environ. Microbiol., **55**: 406-411, 1989.
13) DE VRIES, O. M. H. and J. G. H, WESSELS, Release of protoplasts from *Schizophyllum commune* by a lytic enzyme preparation from *Trichoderma viride*. J. Gen. Microbiol., **73**: 13-22, 1972.
14) NISHIGUCHI. Y. *et al.*, Ultrastructure of mycelia and protoplasts of edible basidiomycetes. Res. Bull. Exp. For. Hokkaido Univ., **44**: 1435-1473, 1987. (In Japanese.)
15) OGAWA, K., Breeding of koji fungi by protoplast fusion. Bio Industry, **3**: 812-820, 1986.
16) OKA, A., H. SUGISAKI and M. TAKANAMI, Nucleotide sequence of the kanamycin resistance transposon Tn903. J. Mol. Biol., **147**: 217-226, 1981.
17) VIEIRA, J. and J. MESSING, The pUC plasmids, M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene, **19**: 259-268, 1982.
18) TAKENOUCHI, K. *et al.*, Establishment of transfer system of gene to basidiomycete

Coriolus versicolor. Abst. 41st Ann. Meet. Japan Wood Res. Soc. (Matsue), p. 117, 1991. (In Japanese.)
19) EGUCHI, F. and M. HIGAKI, Production of new species of edible mushrooms by protoplast fusion method I. Mokuzai Gakkaishi, **38**: 403-410, 1992. (In Japanese.)
20) KUSAOKE, H. *et al*., Optimum conditions for electric pulse-mediated gene transfer to *Bacillus subtilis* cells. Agric. Biol. Chem., **53**: 2441-2446, 1989.
21) SAMEJIMA, M. and T. YOSHIMOTO, Transformation of *Pseudomonas* species with plasmid by electroporation. Mokuzai Gakkaishi, **35**: 948-950, 1989.

担子菌へのプラスミドＤＮＡ導入とその発現についての予備的研究

古野　毅・島田健太・中川　強・松田英幸・上原　徹

摘　　要

木材腐朽菌などの担子菌への遺伝子導入をはかるために，まずプラスミドDNAを導入し，その発現を調べる実験系を検討した．オオウズラタケとカワラタケの２種の担子菌を用い，SMY液体培地で培養した菌子体を細胞壁溶解酵素などの処理によって，プロトプラストを調製した．抗生物質としてカナマイシンが菌子体の成長を阻害したので，導入するプラスミドDNAとしてそれに耐性の遺伝子をもつpUC4Kを選んだ．プラスミドDNAの導入は，ポリエチレングリコール法とエレクトロポーレション法の２法で検討した．後者で得られたオオウズラタケの形質転換体はカナマイシン含有培地で野生株よりも明らかに成長量が多かった．この形質転換体から染色体DNA抽出とプラスミドDNA抽出を行い，アガロースゲル電気泳動を行った結果，プラスミドpUC4Kと同位置にバンドが検出できた．これはオオウズラタケにpUC4Kが導入され，形質転換が行われたことを示す．