J Wood Sci (2007) 53:339–343
DOI 10.1007/s10086-006-0861-9

ORIGINAL ARTICLE

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Introduction of mitochondrial DNA from *Pleurotus ostreatus* into *Pleurotus pulmonarius* by interspecific protoplast fusion

Received: April 19, 2006 / Accepted: October 5, 2006 / Published online: February 25, 2007

Abstract Interspecific protoplast fusion between two monokaryotic strains (a methionine auxotrophic and chloramphenicol-resistant *Pleurotus ostreatus* strain and a wild-type strain of *Pleurotus pulmonarius*) was carried out to introduce mitochondrial DNA (mtDNA) from *P. ostreatus* into *P. pulmonarius*. Because mycelial colonies regenerated on minimum medium containing chloramphenicol only after the treatment of protoplast electrofusion, the regenerants were regarded as protoplast fusants. The fusants isolated from regenerated colonies were uninucleate, and their resistance of chloramphenicol seemed to be a stable trait. The fusants mated with *P. pulmonarius* but not with *P. ostreatus*, and showed almost identical random amplified polymorphic DNA (RAPD) patterns to that of the parental *P. pulmonarius* monokaryon. The sizes of mitochondrial genomes were estimated to be 81.5 kb for *P. ostreatus* monokaryon, 54.9 kb for *P. pulmonarius* monokaryon, and 84.5 or 86.0 kb for the fusants. *Bam*HI and *Eco*RI digest patterns of the fusant mtDNAs were almost the same as the parental *P. ostreatus* monokaryon. From the above results, the fusants seemed to carry nuclei derived from the monokaryon of *P. pulmonarius* and mtDNA including the chloramphenicol-resistance gene from the *P. ostreatus* monokaryon, suggesting that the *P. ostreatus* mtDNA had been introduced into *P. pulmonarius* cells by interspecific protoplast fusion.

Key words Protoplast fusion · *Pleurotus ostreatus* · *Pleurotus pulmonarius* · Mitochondrial DNA

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Contribution No. 383 of the Tottori Mycological Institute

Introduction

Protoplast fusion has been a potentially useful method for the improvement of desired traits in higher basidiomycetes since Gold et al.[1] first reported protoplast fusants between different auxotrophic strains of *Phanerochaete chrysosporium* Burdsall in 1983. In particular, interspecific protoplast fusion has made possible the development of new strains carrying novel properties that could not have been created by traditional breeding techniques such as artificial mating within intraspecific compatible combinations. Interspecific hybrids constructed by protoplast fusions in several species of edible mushrooms have been reported; the control of their vegetative and generative phenotypes by nuclear genes has been investigated by culturing tests, isozyme characterization, and DNA fingerprinting analyses.[2–6] However, little attention has been paid to extranuclear genes in mitochondrial DNA (mtDNA) because of the scarcity of useful genetic or phenotypic markers such as the cytochrome mutation and chloramphenicol-resistance mutation reported on *Coprinus cinereus* (Schaeff.:Fr.) S.F. Gray.[7,8]

Matsumoto and Fukumasa-Nakai[9] isolated a chloramphenicol-resistant mutant of the edible mushroom *Pleurotus ostreatus* (Jacq.:Fr.) Kummer by manganese treatment. We performed the present study to reveal whether the introduction of *P. ostreatus* mtDNA into *Pleurotus pulmonarius* (Fr.) Quél. cells is possible by interspecific protoplast fusion, using the chloramphenicol-resistant mutant of *P. ostreatus*.

Materials and methods

Organisms

Two monokaryotic strains of *Pleurotus ostreatus* TMIC-400(35) and *Pleurotus pulmonarius* TMIC-30064(2), species that are sexually incompatible, were used in this study. They

were deposited in the culture collection of the Tottori Mycological Institute. TMIC-400(35), a methionine auxotrophic and chloramphenicol-resistant mutant[9] derived from a basidiospore formed by the dikaryotic strain TMIC-400, was maintained on potato sucrose agar (PSA; hot-water extract of 200 g fresh potato, 20 g sucrose, and 20 g agar per liter of water) containing 4 mg/ml chloramphenicol (Cap-PSA). The methionine requirement and the chloramphenicol resistance of TMIC-400(35) are caused by nuclear and mitochondrial gene mutations, respectively.[9] On the other hand, TMIC-30064(2) was produced through artificial dedikaryotization by the protoplast regeneration method[10] from the *P. pulmonarius* wild dikaryotic strain TMIC-30064 and also was maintained on PSA. Mycelial growth of TMIC-30064(2) is completely inhibited on Cap-PSA medium. Two single spore isolates of *P. ostreatus* O-11 and *P. pulmonarius* P-8, derived from wild-type fruiting bodies, were used as mating-type testers for TMIC-400(35) and TMIC-30064(2), respectively.

Electrofusion of protoplasts

Protoplast isolation was carried out according to the procedure of Fukumasa-Nakai et al.,[11] and electrofusion of protoplasts of TMIC-400(35) and TMIC-30064(2) was performed as described previously.[12] After electrofusion, the protoplast suspension was plated on minimal medium [20 g glucose, 1.5 g $(NH_4)2HPO_4$, 0.46 g KH_2PO_4, 1 g K_2HPO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 120 μg thiamine-HCl, and 20 g agar per liter of distilled water] containing 4 mg/ml chloramphenicol and 0.5 M sucrose, and was incubated at 25°C. Regenerated mycelial colonies were transferred to Cap-PSA medium.

Staining of nuclei

Nuclei of hyphal cells were stained by the HCl-Gimsa method of Aist[13] and observed by light microscopy.

DNA isolation

To prepare mycelium for DNA isolation, a small MYG (2% malt extract, 0.2% yeast extract, 2% glucose) agar plug (5 mm in diameter) containing the mycelium was inoculated in 15 ml of MYG liquid medium in a 100-ml Erlenmeyer flask, and was incubated stationary in darkness at 25°C for 14 days. Afterward, cultures were fragmented with a Waring blender, and 10 ml was used to inoculate a 500-ml Erlenmeyer flask containing 100 ml MYG liquid medium. The flask cultures were incubated in darkness at 25°C for 14 days, harvested, washed with distilled water, and lyophilized. Extraction of total DNA and isolation of mtDNA from the lyophilized mycelia were done by the method of Fukumasa-Nakai et al.[14] mtDNA was separated from total DNA by CsCl/bisbenzimide density gradient equilibrium centrifugation (1.04 g/ml CsCl and 0.1 mg/ml bisbenzimide) at 20°C and 110 000 *g* for 36 h.

RAPD analysis

Random amplified polymorphic DNA (RAPD) analysis was performed by the procedure of Williams et al.,[15] with minor modifications. Two primers, OPA-02 (5′-TGCC GAGCTG-3′) and OPA-04 (5′-AATCGGGCTG-3′) (Operon, Alameda, CA, USA), were used to detect multiple RAPD bands for the two strains, *P. ostreatus* TMIC-400(35) and *P. pulmonarius* TMIC-30064(2). Polymerase chain reaction (PCR) was done as described previously[16] in a 25-μl reaction mixture containing 25 μg total DNA. Electrophoresis of amplification products was carried out on 1.4% agarose (Type S, Nippon Gene, Tokyo, Japan) slab gels in TAE [40 mM Tris/acetate, 10 mM ethylenediaminetetraacetic acid (EDTA), pH 8.0] at 5 V/cm for 2.5 h, before the gels were stained with ethidium bromide (0.5 μg/ml). RAPD patterns were recorded by photographing the gels on a ultraviolet (UV) transilluminator. RAPD reactions were repeated at least twice to confirm the reproducibility of each RAPD band.

mtDNA restriction analysis

mtDNA was digested separately with two restriction endonucleases, *Bam*HI and *Eco*RI (Nippon Gene), following the supplier's specification. Digests were analyzed using the method described above for RAPD analysis (except that 1% agarose gels were used). Lambda phage DNA digested with *Hin*dIII served as a molecular size standard.

Results and discussion

Protoplasts from the two monokaryotic strains of *Pleurotus ostreatus* TMIC-400(35) and *Pleurotus pulmonarius* TMIC-30064(2) were treated by electrofusion procedures and then plated on minimal medium containing 4 mg/ml chloramphenicol. In this condition, only hybrid cells carrying wild-type nuclear genes conferring methionine autotrophy as well as the mitochondrial chloramphenicol-resistance gene can regenerate. After 2 weeks of incubation, regenerated mycelial colonies were transferred to Cap-PSA medium. These regenerants were regarded as protoplast fusion products, because no colonies developed in control tests in which the electrofusion step had been omitted. All of the 21 fusants isolated randomly from regenerated colonies grew well on Cap-PSA medium after three subcultures at intervals of 30 days, indicating that their resistance of chloramphenicol was a stable trait. As shown in Fig. 1, the morphology of fusant colonies was nearly identical with the parental strain, *P. pulmonarius* TMIC-30064(2). None of the fusants showed any clamp connections, and nuclear staining by the HCl-Gimsa method revealed that they were uninucleate (data not shown).

To examine the nuclear type of the fusants, mating tests and RAPD analysis were carried out. All of the fusants mated exclusively with *P. pulmonarius* P-8, which is compatible with *P. pulmonarius* TMIC-30064(2), but not with

P. ostreatus O-11, which is compatible with *P. ostreatus* TMIC-400(35). Out of the 21 fusants isolated, 5 fusants (F1–F5) were randomly selected for RAPD analysis. The two parental strains, *P. ostreatus* TMIC-400(35) and *P. pulmonarius* TMIC-30064(2), differed in their RAPD patterns (Fig. 2), and the 5 selected fusants showed almost identical RAPD patterns to that of *P. pulmonarius* TMIC-30064(2). Thus, the results from the mating test and RAPD analysis indicated that the fusants had nuclei derived from the monokaryon of *P. pulmonarius* TMIC-30064(2). However, a part of nuclear DNA of the *P. ostreatus* TMIC-400(35) may be inserted to the *P. pulmonarius* TMIC-30064(2) nuclear genome. Hereafter, further studies are required to clarify the nuclear composition of the fusants.

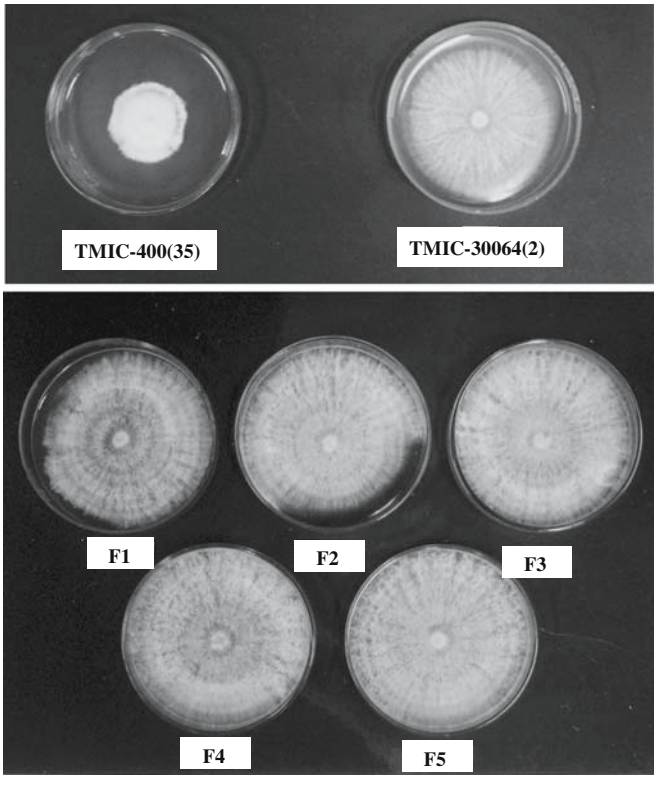

Fig. 1. Colony morphology of cultures of *Pleurotus ostreatus* TMIC-400(35) (*top left*), *Pleurotus pulmonarius* TMIC-30064(2) (*top right*), and the fusants F1–F5 (*bottom*) on potato sucrose agar (PSA)

Next, we isolated mtDNAs of the two parental strains and the five fusants, and digested them with *Bam*HI or *Eco*RI. The mtDNA digests from the two parental strains produced distinct restriction patterns (Fig. 3). Summing the restriction fragment sizes, the sizes of the mitochondrial genomes were estimated to be 81.5 kb for *P. ostreatus* TMIC-400(35) and 54.9 kb for *P. pulmonarius* TMIC-30064(2). *Bam*HI and *Eco*RI digests of mtDNAs from the five fusants produced two distinct restriction fragment length polymorphism (RFLP) patterns, and their mtDNAs could be grouped into two genotypes, F1-mt (F1, F3, F4, and F5) and F2-mt (F2). The estimated fusant mitochondrial genome sizes were 86.0 kb for the F1-mt type and 84.5 kb for the F2-mt type. Fusant mtDNAs seem to consist mostly of portions originating from *P. ostreatus* TMIC-400(35), judging from the molecular sizes and common restriction fragments (Fig. 3). Thus, the RFLP patterns of both fusant genotypes resembled that of *P. ostreatus* TMIC-400(35) to some degree, but the fusant mtDNAs were distinguishable from those of both parental monokaryons. These findings from the mtDNA RFLP analysis did not necessarily imply that complete *P. ostreatus* TMIC-400(35) mitochondrial genome had been introduced into *P. pulmonarius* TMIC-30064(2) cells. In any case, the fusants seemed to carry nuclei derived from *P. pulmonarius* TMIC-30064(2) and mtDNA from *P. ostreatus* TMIC-400(35) including the

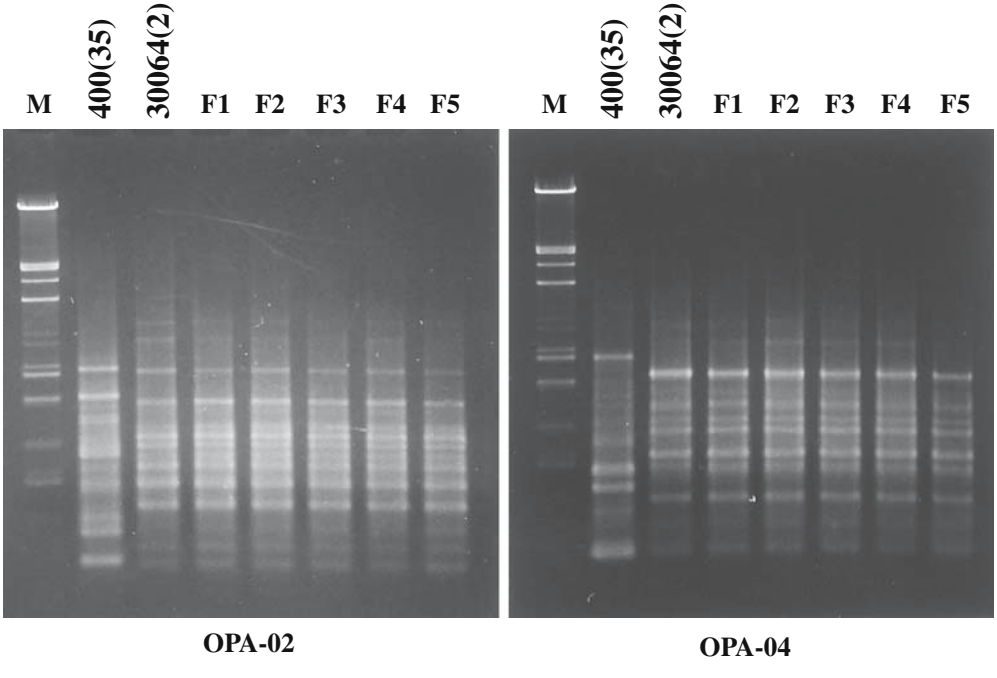

Fig. 2. Random amplified polymorphic DNA (RAPD) patterns produced from *P. ostreatus* TMIC-400(35), *P. pulmonarius* TMIC-30064(2), and the fusants F1–F5 using primers OPA-02 (*left*) and OPA-04 (*right*). Lane *M*, *Hin*dIII and *Eco*RI-digested lambda phage DNA

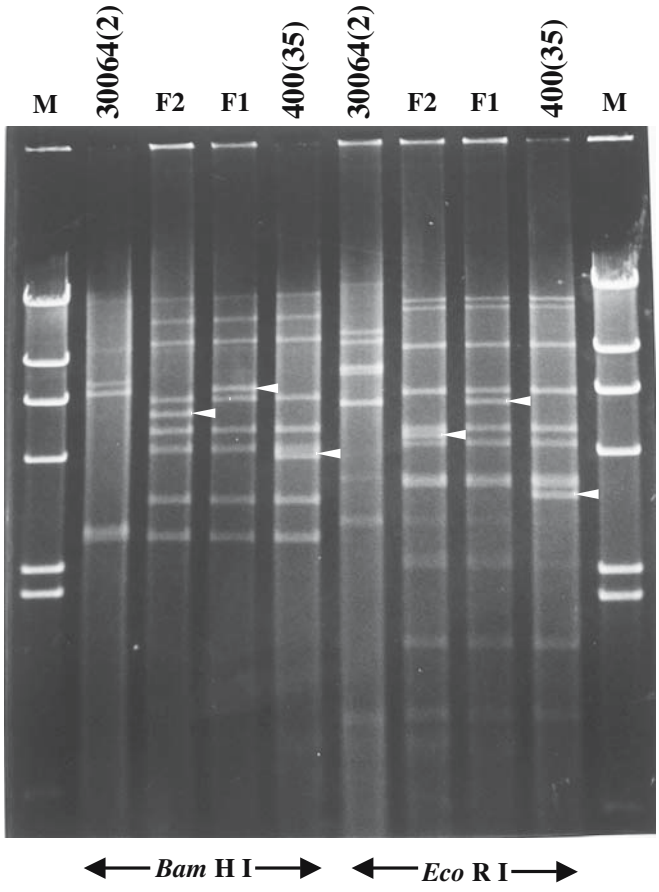

Fig. 3. Restriction fragment length polymorphism (RFLP) patterns of mtDNA produced from *P. ostreatus* TMIC-400(35), *P. pulmonarius* TMIC-30064(2), and representative fusants F1 and F2. The *arrowheads* indicate distinct restriction fragments among the mtDNAs from the fusants and *P. ostreatus* TMIC-400(35). Lane *M*, *Hin*dIII-digested lambda phage DNA

chloramphenicol-resistance gene, suggesting that the *P. ostreatus* mtDNA had been introduced into *P. pulmonarius* cells by interspecific protoplast fusion. Considering the experimental conditions, deficiency of the *P. ostreatus* nucleus and/or *P. pulmonarius* mtDNA possibly occurs during the cell division after the fusion of protoplasts. In addition, there is a possibility that the fusants resulted from the fusion between *P. pulmonarius* TMIC-30064(2) protoplasts carrying nuclei and enucleate protoplasts of *P. ostreatus* TMIC-400(35).

In higher plants, recombination of mtDNA in heteroplasmic cells obtained by protoplast fusion has been reported frequently.[17–20] Similarly, mtDNA recombination was observed in sexual crosses of a few higher basidiomycetes including *C. cinereus*[7,21] and *P. ostreatus*.[22] In addition, possibly recombinant mtDNA has been described in intraspecific protoplast fusants of *Lentinula edodes* (Berk.) Pegler.[12] The present results suggest that the fusant mtDNAs may be recombinant mtDNAs derived from *P. ostreatus* TMIC-400(35) and *P. pulmonarius* TMIC-30064(2). In addition, the recombination of mtDNAs from *P. ostreatus* TMIC-400(35) and *P. pulmonarius* TMIC-30064(2) may occur in the limited homologous regions of the two parental genomes, which would explain why four of the five fusants had identical mtDNA genotypes (F1-mt).

This is the first report of mtDNA transmission in interspecific fusion protoplasts of edible mushrooms. It now appears possible to transfer mtDNA between species, thus increasing the genetic variability of economically important mushrooms. This possibility will be useful in mushroom breeding, because the mitochondrial genome may affect phenotypical properties of edible mushrooms, as demonstrated for mycelial growth in *Agaricus bitorquis* (Quél.) Sacc.[23]

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