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Transformation of *Lyophyllum decastes* by particle bombardment

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Abstract The basidiomycete *Lyophyllum decastes* was transformed by means of particle bombardment. We isolated five transformants under twelve conditions differing in the two parameters of target distance and helium pressure. The transformation frequency was one transformant/μg DNA. In the transformants, plasmid DNAs were integrated into the genomic DNA and stably maintained. This is the first report on transformation of *L. decastes* by particle bombardment.

Key words *Lyophyllum decastes* · Particle bombardment · Transformation

Lyophyllum decastes (Fr.) Sing. is an important edible mushroom that has been recently cultivated in Japan and used in traditional medicine (Miura et al. 2002). Particle bombardment, or the biolistic process, was originally developed for use with plant cells and tissues by Sanford et al. (1987). This technique relies on high-velocity delivery of DNA-coated microcarriers. Particle bombardment is technically simpler than polyethylene glycol (PEG), electroporation, and restriction enzyme-mediated integration (REMI) because isolation of protoplasts is not needed. Among the fungi, particle bombardment has been used for the transformation of the rust fungus *Puccinia graminis* Pers. f.sp. *tritici* Erikss. & Henning (Schillberg et al. 2000), the ectomycorrhizal fungus *Paxillus involutus* (Batsch) Fr. (Bills et al. 1995), and *Aspergillus nidulans* (Eidam) G. Winter (Fungaro et al. 1995). In this study, we report the transformation of *L. decastes* by particle bombardment.

Lyophyllum decastes, strain N1 was used. This strain is maintained in our laboratory on MMN medium (Murata et al. 1999).

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Plasmid vector pHHM 192 is based on pUC18 and contains the hygromycin B phosphotransferase (*hph*) expression casette (i.e., P*ras-hph*-T*priA*), and the *dsred2* expression casette (i.e., P*gpd*-5′008*gpd-dsred2*-T*nos*) (Murata et al. 2006).

A BiolisticPDS-1000/He Particle Delivery System (Bio-Rad, Hercules, CA, USA) was used for the transformation. The transformation procedure of the plasmid DNA (pHHM 192) was performed as described in a previous report (Sunagawa and Magae 2002). A square mycelial mat (3 × 3 cm) was cut out from the N1 colony grown for 7 days on the potato dextrose agar (PDA) medium and placed in the center of a Petri dish (9 cm in diameter). The distance between the mycelial mat on the Petri dish and the plasmid coated with gold particles on the macrocarrier was 5.0–14.5 cm. Bombardment was performed under a helium pressure of 650–1300 psi (pounds per square inch). After bombardment, the mycelial mat was subdivided into square pieces of about 0.7 × 0.7 cm each. To select transformants, each mycelial mat was transferred to modified Melin–Norkrans (MMN) medium containing 100 μg/ml hygromycin B (Hyg; Wako, Osaka, Japan) and incubated at 26°C.

To confirm whether the mycelia grown on MMN media containing 100 μg/ml hygromycin B were transformants, polymerase chain reaction (PCR) and Southern hybridization were performed. Genomic DNA of *L. decastes* was prepared by the modified cetyltrimethylammonium bromide (CTAB) procedure of Murray and Thompson (1980). Insert Check PCR Mix (Takara Shuzo, Kyoto, Japan), which contained universal primers for pUC vectors, was used for PCR. The primers were M13-M4, 5′-GTTTTCCCAGTCACGAC-3′ and M13-RV, 5′-CAGG AAACAGCTATGAC-3′. PCR was performed with 30 cycles of the following: 5 s at 95°C, 5 s at 55°C, and 1 s at 72°C. Using this mix, inserts flanked by a pUC8 vector were amplified. Amplified DNAs were analyzed by ethidium bromide staining after 0.8% agarose gel electrophoresis. Southern hybridization using the digoxigenin (DIG) system was performed according to the manufacturer's instructions (Roche Diagnostics, Tokyo, Japan). The genomic DNA (10 μg) of *L. decastes* and transformants was digested with

Table 1. Effects of target distance and helium pressure on the number of putative transformants

Helium pressure (psi)	Target distance (cm)			
	5.0	8.0	11.0	14.5
650	0	0	0	0
1100	2	1	0	0
1300	1	1	0	0

*Bam*HI, fractionated on a 0.8% agarose gel, and transferred to a nylon membrane (Hybond-N; Amersham Pharmacia Biotech, Tokyo, Japan). A hybridization probe was labeled using a DIG-labeling kit (Roche Diagnostics) according to the manufacturer's instructions. Detection of the DIG-labeled *hph* gene was undertaken using a DIG detection kit for DNA on the nylon membrane according to the manufacturer's protocol (Roche Diagnostics).

We performed bombardment under 12 sets of conditions. Two parameters, the target distance and the pressure of the bombardment, were varied at 5.0, 8.0, 11.0, and 14.5 cm and 650, 1100, and 1300 psi, respectively (Table 1). Experiments were repeated several times, and on one occasion the transformants were obtained on the same day. Under 12 sets of conditions, we obtained five putative transformants from *L. decastes* (see Table 1). At a pressure of 650 psi, none of the transformants were isolated at any target distance. Furthermore, transformants were not isolated under combinations of 11.0 and 14.5 cm with any pressure. Most transformants were isolated under combinations of target distance 5.0 or 8.0 cm and pressure 1100 or 1300 psi. Three putative transformants were obtained at a pressure of 1100 psi, which was consistent with the results of Takeno et al. (2004). In contrast, Li et al. (1993), using *Uromyces appendiculatus* F. Strauss var. *appendiculatus*, reported a higher pressure of 1550 psi and a shorter distance of 2.5 cm compared with our conditions. This difference may depend on the nature of the target cells (conidia, spores, and mycelia).

The transformation frequency was 1 transformant/μg of DNA. The transformation frequency (1–2 transformants/μg of DNA) of a monokaryotic strain of *Pleurotus ostreatus* (Jacq.) P. Kumm. using electroporation (Honda et al. 2000) was similar to our results. The optimal conditions of bombardment remain to be optimized to increase the transformation frequency, as noted by Sanford et al. (1993). Transformation frequency is affected by the nature (gold and tungsten) and size of particles, the distance from the mycelial mat to the particles, and the bombardment pressure. Herzog et al. (1996) compared particle bombardment and PEG/$CaCl_2$ treatment of *A. nidulans*. They reported that the transformation frequency with the former was somewhat lower than that with the latter; however, the rate of stable transformation was considerably higher with particle bombardment. Similarly, Lorito et al. (1993) reported, with *Trichoderma harzianum* Rifai and *Gliocladium virens* J.H. Miller, Giddens & A.A. Foster, that the biolistic procedure produced more genetically stable progeny than protoplast-mediated transformation.

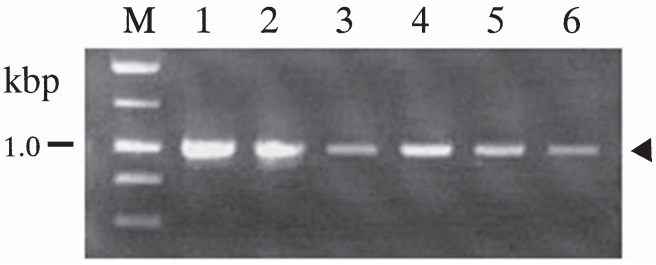

Fig. 1. PCR of plasmid pHHM 192 vector and transformants. *Lanes 1* and *2–6* show plasmid pHHM 192 vector and transformants, respectively; *lane M* is a size marker of the 1-kb DNA ladder. *Arrowhead* shows 1.1-kbp fragment of the *hph* gene

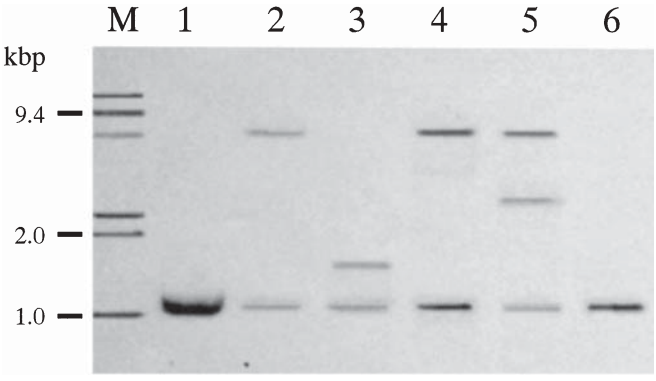

Fig. 2. Southern hybridization of plasmid pHHM 192 vector and transformants. DNA was digested with *Bam*HI. *Lanes 1* and *2–6* show plasmid pHHM 192 vector and transformants, respectively; *lane M* is a size marker of *Hin*dIII-digested λ-DNA

All putative transformants that grew after transfer to MMN medium containing 100 μg/ml hygromycin B were analyzed by PCR. PCR of the putative transformants obtained from *L. decastes* was performed using Insert Check PCR Mix. The amplified *hph* gene of the pHHM 192 vector should appear as a DNA band of 1.1 kb in size. The results are shown in Fig. 1. The DNA band appeared in all the transformants, showing that an intact sequence of the *hph* gene was present in the genome. The PCR bands represent evidence for genomic integration of the transforming plasmid. However, only 73% (11 of 15) of phleomycin-resistant clones could be verified by PCR using a phleomycin marker for the mycorrhizal fungus *Suillus bovinus* (Pers.) Roussel (Pardo et al. 2002). Our result using a hygromycin marker indicated that the DNA transferred by particle bombardment was stable in all the hygromycin-resistant transformants. The fragment of the *hph* gene was not amplified in the N1 strain (data not shown).

We also analyzed the transformants obtained from *L. decastes* by Southern hybridization. The hybridization signal of pHHM 192 appeared as a band of 1.1 kbp (Fig. 2). No hybridization was found with the DNA of N1 strain (data not shown). One of the five transformants (Fig. 2, lane 6) had one band of pHHM 192, and the remainder had several hybridizing signals, as multiple copies (Fig. 2, lanes 2–5). Hanif et al. (2002) detected a multiple integration event (3–8 copies) using *hph* as a probe for *S. bovinus*. In

addition, Saito et al. (2001) indicated that multiple copies of introduced DNA were integrated at various positions in the chromosomal DNA of transformants of the mycorrhizal fungus *Lyophyllum shimeji* (Kawam.) Hongo.

From the results of PCR and Southern hybridization, we concluded that the plasmid pHHM 192 DNA was successfully integrated into the genomic DNA of *L. decaste* by particle bombardment.

All transformants were subcultured twice on MMN media containing 100 μg/ml hygromycin B for 6 months after bombardment. The presence of the pHHM 192 sequence in the genomic DNA of the transformants was confirmed again by PCR (data not shown). This result indicated that the DNA transferred by particle bombardment was stably maintained in the mycelium. The 100% mitotic stability of the five transformants was higher than the 60%–70% of *A. nidulans* (Herzog et al. 1996) achieved by particle bombardment. In addition, Hazell et al. (2000) and Te'o et al. (2002) obtained results similar to ours.

In conclusion, the present results demonstrate that particle bombardment can be used to stably transform *L. decastes*, and that this could be a useful tool for transforming other fungi in which protoplasts are difficult to generate.

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