Transformation of the Edible Basidiomycete *Lentinus edodes* by Restriction Enzyme-Mediated Integration of Plasmid DNA

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We have used the restriction enzyme-mediated DNA integration (REMI) method to establish a transformation system in *Lentinus edodes* using the recombinant plasmid pLC1-hph, which contains the *L. edodes* transcriptional signals and an *Escherichia coli* hygromycin B phosphotransferase gene. Protoplasts of *L. edodes* were treated by the PEG transformation mixture containing 50 units of *Sal*I, which cleaves pLC1-hph at a single site, yielding about 15 transformants per 2.5 μg of DNA. The conventional PEG transformation without *Sal*I, however, yielded only 1.5 transformants per 25 μg of DNA. The optimal amount of *Sal*I for increased transformation was 50 units. In the case of transformation with *SpI*, which cleaves the plasmid at one site, the optimal amount of the enzyme was 2.5 units. Southern blot analysis of the *SpI*-derived transformants suggested that 50% of the plasmid integrations were REMI events.

**Key words:** *Lentinus edodes*; REMI; transformation; hygromycin B

DNA-mediated transformation of edible basidiomycete species have been reported in *Agaricus bisporus*, (*Saccharomyces cerevisiae*, *Agrocybe aegerita*, and *Pleurotus ostreatus*. The transformation of *Lentinus edodes*, which is one of the most important edible mushrooms in Japan, has not been reported so far.

The restriction enzyme-mediated integration (REMI) procedure, first described for *Saccharomyces cerevisiae* and later refined for *Dictyostelium discoideum*, offers the prospect of introducing random tagged mutations into the host genome with a high frequency. Restriction enzymes have been reported to increase transformation efficiencies in the case of *Cochliobolus heterostrophus*, *Magnaporthe grisea*, *Alternaria alternata*, and *Coprinus cinereus*. Therefore, the REMI method has recently received attention as a new technique in fungi for increasing transformation efficiencies and for gene tagging. The principle of REMI is as follows: the enzymes penetrate the cell and the nuclear membranes and cleave the chromosomal DNA *in vivo* at their specific restriction sites. The free chromosomal DNA ends generated can be ligated to restriction enzyme-linearized plasmid DNA by host cell enzymes.

The chromosome-integrating vector pUC19, which is a pUC19-based vector carrying the *L. edodes* ras gene promoter and *priA* gene terminator, has been shown to be very useful for expression of foreign genes in basidiomycetous fungi. The *P. ostreatus* transformants expressing the *Escherichia coli* β-glucuronidase gene (GUS) and the *Streptomyces hygroscopicus* bialaphos-resistance gene (*bar*) and the *C. cinereus* transformant expressing the *P. ostreatus* manganese (II) peroxidase gene (*mpn*) were easily obtained by the conventional PEG transformation method using the vector. In the case of *L. edodes*, however, the conventional PEG method gave only a very limited number of transformants, even though pLC1 was used for the experiment (unpublished data). This led us to attempt to use the REMI method for transformation of *L. edodes*. The *E. coli* hph gene encoding a hygromycin B phosphotransferase was used as a marker. We now report the successful experimental results.

**Materials and Methods**

*L. edodes* strain and culture conditions. A wild type dikaryotic strain of *L. edodes* S-I was used as the recipient host in all transformation experiments. For protoplast preparation, strain S-I was grown on MYPG (0.25% malt extract, 0.1% yeast extract, 0.1% peptone, 0.5% glucose) agar medium for 2 weeks at 25°C, then mycelia were transferred to MYPG liquid medium and growth was continued at 25°C for 1–2 weeks with shaking. The cultivated mycelia were collected, homogenized, and filtered through a 100 μm nylon mesh. Mycelia shorter than 100 μm in length were further grown in MYPG liquid medium at 25°C for 5–6 days without shaking.

Preparation of protoplasts of *L. edodes*. Protoplasts of *L. edodes* were prepared by the method of Kawasumi *et al.* One gram of mycelia cultivated as described above was collected, washed twice with SM buffer (50 mM succinate (pH 5.6), 0.6 M mannitol), and treated with 10 ml of enzyme solution (2.5% cellulase Onozuka RS (Yakult), 0.1% chitinase (Sigma) in SM buffer) at 28°C for 3–4 h. The protoplasts were filtered through 40 μm nylon mesh, washed with STM buffer (10 mM...
Tris-HCl (pH 7.5), 10 mM CaCl₂, 1.2 M sorbitol), and suspended in STC buffer. About 1 × 10⁶ protoplasts were obtained from 1 g of mycelia of *L. edodes*.

Construction and preparation of pLC1-hph. DNA manipulations and clonings were done by standard procedures using the *E. coli* strain TOP10F'α (Invitrogen). A 1.1-kb fragment containing the *E. coli* hph gene was obtained by BamHI digestion of pCH, and inserted into the BamHI site of pLC1 (6.4 kb) yielding the recombinant plasmid pLC1-hph (7.5 kb) (see Fig. 1). Plasmid DNAs were prepared by the alkaline-lysis method and purified by CsCl density gradient centrifugation and used for transformation before and after digestion with *Sall*.

Transformation procedures
1) Conventional PEG method. Transformation by the conventional PEG method was done as described by Yanai *et al.* Protoplasts of *L. edodes* (0.5–1.0 × 10⁷/100 μl of STC buffer) were transformed with 25 μg of pLC1-hph.

2) REMI method. One hundred μl of this protoplast suspension in STC buffer were added to 150 μl of STC buffer containing 2.5 μg of pLC1-hph and indicated amounts of restriction enzyme, mixed gently, and incubated for 20 min on ice. For next step the following two methods were used, which were differ in PEG treatment. PEG-treatment 1: Two ml of PEG solution (60% PEG4000, 10 mM Tris-HCl (pH 7.5), 10 mM CaCl₂) was added to the mixture of protoplasts, DNA, and restriction enzyme, gently mixed and incubated for 20 min on ice, and then gently mixed with 10 ml of STC buffer. PEG-treatment 2: To the mixture of protoplasts, DNA, and restriction enzyme, 62.5 μl of PEG solution was added, mixed gently, and incubated for 20 min on ice. To this mixture, 3.125 ml of PEG solution was added, mixed and incubated for 20 min at room temperature, and then mixed with 10 ml of STC buffer. The mixtures prepared by the PEG-treatments 1 and 2 were centrifuged at 1,200 × g for 10 min, and the protoplasts were suspended in 4 ml of MS liquid medium and incubated at 25°C for 2–4 days without shaking.

Screening of transformants. After transformation and pre-cultivation in MS liquid medium (2% malt extract, 0.6 M sucrose), the protoplasts were plated onto minimal medium containing 5 μg/ml hygromycin B and incubated at 25°C for 5 days. The plates were then overlaid with MYPG containing 20 μg/ml hygromycin B and 1.5% agar and incubated for 1 week at 25°C. Individual transformants that appeared on the plates were purified twice by subculturing on MYPG agar medium containing 20 μg/ml hygromycin B.

Preparation of genomic DNA and Southern blot analysis. For DNA extraction, mycelia of transformants were transferred to MYPG liquid medium and grown without selection for hygromycin B at 25°C for 3–4 weeks. Genomic DNA was extracted from 3 g of frozen mycelia with liquid nitrogen using ISOPLANT (Nippon Gene). The genomic DNA was further purified using a Nucleon Phytopure DNA extraction resin (Nucleon Phytopure Plant DNA Extraction Kit, Scotlab) according to the supplier's instructions.

About 5 μg of genomic DNA digested with *Sphl* or *Dral* was separated on a 0.7% agarose gel electrophoresis and transferred to a Hybond N+ nylon membrane (Amersham) by the method recommended by the manufacturer. DNA probe labelling, hybridization, and signal detection were done using the ECL direct nucleic acid labelling and detection system (Amersham) according to the manufacturer's instructions.

Results and Discussion

Effects of PEG treatment and plasmid DNA forms on transformation efficiency

The conventional PEG method with circular pLC1-hph DNA yielded 1.5 transformants per 25 μg of DNA (experiment 1 of Fig. 2). Almost the same number of transformants was obtained with 2.5 μg, 12.5 μg, or 25 μg of circular pLC1-hph DNA (data not shown). The REMI transformations were done using 2.5 μg of pLC1-hph by the two methods different in PEG treatment: PEG-treatment 1 and PEG-treatment 2 (refer to Materials and Methods).

As shown in Fig. 2, PEG-treatment 1 or 2 of REMI transformation with 50 units of *Sall* gave 5.5 or 15 transformants per 2.5 μg of *Sall*-linearized pLC1-hph DNA, respectively (experiments 3 and 5), demonstrating that the PEG-treatment 2 increases transformation frequency by about 10 times that of the conventional PEG method and about 3 times that of the PEG-treatment 1. It was reported that a high transformation frequency has been obtained by the PEG-treatment 1 method of REMI in *M. grisea* and *Ustilago maydis* or by the PEG-treatment 2 method of REMI in *C. cinereus* and *A. alternata*. Thus, optimal condition of PEG treatment on transformation may differ with the host fungi. Our results showed that the PEG-treatment 2 method

![Fig. 1. Restriction Map of the Transforming Plasmid pLC1-hph.](image-url)

*Pras* is the promoter sequence (2.5 kb) of the *L. edodes* ras gene. *hph* is a hygromycin B phosphotransferase gene from *E. coli*. *TpriA* is a terminator sequence (1.2 kb) of the *L. edodes priA* gene. Thin line is pUC19 sequence. *amp* is an ampicillin resistance gene.
was better than the PEG-treatment 1 method in REMI transformation of *L. edodes*.

Then, we examined the effect of plasmid DNA forms on transformation frequency (Fig. 2). In the case of the conventional PEG method, the linearized DNA increased the transformation frequency by about 2 times compared with that of circular plasmid DNA (data not shown). Similar result was obtained in *M. grisea*. This result suggests that linear DNA integrates more readily into the fungal genome than circular DNA. In the REMI method, although addition of 50 units of *SalI* does not linearize circular pLC1-hph in the transformation buffer under the transformation conditions (data not shown), the number of transformants obtained with the circular DNA (experiments 2 and 4) was almost the same as that obtained with the linearized DNA (experiments 3 and 5). These results suggest that the restriction enzyme was active in vivo in cleaving both the plasmid and chromosomal DNAs, and the form of the plasmid in solution is not a critical factor in determining the efficiency of transformation by the REMI method.

**Effects of restriction enzymes on transformation**

Plasmid pLC1-hph (7.5 kb) has unique restriction sites for *SalI* and *SphI* in the pUC19 sequence flanking the ras promoter sequence (Fig. 1). The effects of enzyme amounts on transformation efficiency were measured for these restriction enzymes with PEG-treatment 2 with 2.5 μg of circular plasmid DNA (Fig. 3). The maximum number of transformants was obtained with 50 units of *SalI* or 2.5 units of *SphI*. These results indicate that the optimal amount of restriction enzymes for increased transformation varies between enzymes. This may be owing to differences in the optimal reaction conditions of each enzyme and/or in the ease with which each enzyme enters a cell and a nucleus, and the stability of the enzyme and/or the number and accessibility of sites present in the *L. edodes* genome for the enzyme.

A decrease in transformation frequency was found in the presence of higher amounts of *SalI* or *SphI* (Fig. 3), but no change in viability of protoplasts was observed as a result of the addition of high concentrations of the enzymes (data not shown). This phenomenon was observed similarly in *M. grisea*, *A. alternata*, and *C. cinereus*. These findings suggest that the digestion of chromosomal DNA using *SalI* or *SphI* may have led to no cytotoxic effect on protoplasts.

pLC1-hph has three *DraI* sites located in the pUC19 sequence that are not essential for vector function, a single *KpnI* site in the ras promoter sequence located about 1 kb apart from the *hph* gene start codon, and two *BamHI* sites at the ends of the *hph* gene (Fig. 1). Addition of 50 units each of *DraI* and *KpnI* yielded transformants to about 75% of those obtained with *SalI* done by PEG-treatment 2 with 2.5 μg of circular plasmid DNA. As was expected, no transformants were obtained with *BamHI*.

**Southern analysis of DNA isolated from transformants**

To discover the patterns of plasmid integration, genomic DNAs prepared from the transformants obtained by the PEG-treatment 2 using *SalI* and *SphI* were digested with the respective enzymes used to generate the transformants, and analyzed by Southern blotting
using the 1.1-kb BamHI fragment containing the hph gene as a hybridization probe. If integration is mediated by compatible ends generated by the enzyme used in the REMI experiment, the two restriction recognition sites at the ends of the inserted plasmid are restored after integration. Hybridization of a plasmid-size fragment in transformant DNA digested with the same enzyme used in the REMI experiment provides evidence of a REMI event.

The SalI-digests of SalI-derived transformants almost gave specific hybridization signals in the high-molecular-mass DNA region (data not shown), suggesting that SalI could hardly cut the integrated foreign DNAs. We could not analyze the frequency of REMI events in SalI-derived transformants.

Then the SphI-digests of SphI-digested transformants were analyzed. The SphI-digests of 5 transformants (lanes 1, 2, 4, 5, and 10) out of 10 gave the hybridization signal at the position corresponding to the plasmid size (7.5 kb) (Fig. 4A), suggesting that 50% of plasmid integrations occurred through a REMI event. This value was lower than those reported for C. albicans (100%), S. cerevisiae (80–90%), D. discoideum (>70%), and M. grisea (72%), similar to those reported for U. maydis (50%) and C. cinereus (8–56%), and higher than that reported for A. alternata (5.88%).

As shown in Fig. 4A, bands were also observed at the positions larger and smaller than 7.5 kb, indicating other types of integrations. Lanes 3 and 9 showed bands smaller than 7.5 kb, indicating that pLC1-hph might integrate with a deletion. Lanes 1, 4, 6, 7, 8, and 10 showed bands larger than 7.5 kb, indicating that pLC1-hph might integrate at an SphI site without site regeneration or not at an SphI site. Lanes 1, 4, 7, and 10 showed multiple bands, indicating combinations of these types of integrations. Lanes 2 and 5 showed one band with plasmid size indicating pLC1-hph might integrate at 1 site by a REMI event only.

Next, we analyzed the DraI digests of these transformants to confirm the copy number of integrated pLC1-hph by detecting the hybridization signal with the hph gene sequence (Fig. 4B). Lanes 3, 5, and 9 showed 1 band without the plasmid size (6.8 kb), indicating 1 site and 1 copy integration of the plasmid. Lane 2 showed the plasmid-size band (6.8 kb) and another band larger than 5.73 kb SphI-DraI of pLC1-hph containing hph, suggesting that the plasmids might integrate at 1 site with more than 1 copy of the plasmid in tandem. Each of lanes 7 and 10 showed the plasmid-size band and other bands larger than 5.73 kb, suggesting that more than 1 copy of the plasmid with tandem integration and combination of integration events described above had occurred. Lanes 6 and 8 showed 2 bands without the plasmid size (6.8 kb). In the case of the SphI digests (Fig. 4A), these transformants each showed 1 band with non-REMI. These results suggest that tandem integration of the plasmid might occurred at 1 site in these transformants with destruction of the internal SphI site or deletion. Lanes 1 and 4 showed multiple bands. The SphI digests (Fig. 4A) of these transformants each showed fewer bands with the plasmid size compared with the DraI digests (Fig. 4B) suggesting that multiple integration containing tandem integration might occurred with REMI and non-REMI in these transfor-

The REMI procedure offers the prospect of introducing random tagged mutations into the fungal genome at a relatively high rate as reported in C. cinereus, A. alternata, M. grisea, and U. maydis. One copy insertion of the marker gene into the genome at a single site is effective to isolate the tagged gene. Thus, this type of integration in L. edodes was 30%, suggesting that the REMI procedure is a useful tool for isolation of the tagged gene. The REMI transformation system for L. edodes developed in this paper will be useful for basic research of the molecular biology of L. edodes.

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References


