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The Efficient Transformation of *Pleurotus ostreatus* using REMI Method

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Restriction enzyme-mediated integration (REMI) was used to transform uracil auxotrophs of *Pleurotus ostreatus* to prototrophy. When protoplasts of *Pleurotus ostreatus* were treated by the reaction mixture containing 10 units of *Bam*HI, the frequency of REMI was about 64 transformants per 1 μg of DNA. This efficiency was increased by 14.2 times compared with that of the conventional PEG transformation. The optimal condition for REMI of *P. ostreatus* was achieved when 1 μg of linearized pTRura3-2 DNA was added into 1×10^7 protoplasts along with 10 units *Bam*HI. Southern blot analysis revealed that about 50% of transformants examined were caused by REMI event and 30% carried single copy insertion at the genome. This suggested that the REMI method might be a useful tool for efficient transformation and tagging mutagenesis of *P. ostreatus*.

KEYWORDS: *Pleurotus ostreatus*, Restriction enzyme-mediated integration (REMI), Uracil auxotrophic marker

Pleurotus ostreatus known as the oyster mushroom is commercially important in the world mushroom market. In addition to importance for food production, *P. ostreatus* has, recently, received increasing attention for use in biobleaching, catalysis of difficult chemical conversion, and the pharmaceutical industry (Vyas, 1994).

So far, in many number of basidiomycetes including edible mushrooms such as *Agaricus bisporus* (Rhee, 1996), *Agrocybe aegerita* (Noel, 1994), *Lentinus edodes* (Sato, 1998), *Volvariella volvacea* (Jia, 1998) and *P. ostreatus* (Kim, 1999; Peng, 1993; Yanai, 1996), DNA-mediated transformation systems have been developed. In the previous studies, *P. ostreatus* was also transformed to drug-resistance, but the transformation efficiencies were too low (Honda, 2000). Recently, efficient transformation of *P. ostreatus* has been reported to increase the transformantion frequency using carrier DNA, but this procedure have a disadvantage that carrier DNA was incorperated in the transformants (Irie, 2001).

For the developement of efficient transformation system we tried the restriction enzyme-mediated integration (REMI) procedure. REMI method as first described in *Saccharomyces cerevisiae* (Schiestl, 1991) involves in the introduction of plasmid DNA along with a restriction enzyme into a cell during transformation and offers the prospect of introducing random tagged mutations into the host genome with high frequency. This technique has been employed for the disruption, tagging, and identification of genes in *Dictyostelium discoideum* (Kuspa, 1992), *Cochliobolus heterostrophus* (Lu, 1994), *Ustilago maydis* (Bölker, 1995), *Aspergillus nidulans* (Sanchez, 1998) and *Coprinus cinereus* (Cummings, 1999).

In this study, we report the increase of transformation efficiency and the possibility of efficient tagging mutagenesis in *P. ostreatus* using REMI method.

Materials and Methods

Strains, plasmids and culture conditions. Auxotrophic *P. ostreatus* strain ASI2029-8 was obtained from the National Institute of Agricultural Science and Technology, and the plasmid, pTRura3-2 was obtained from Dr. Christian Barreau (Berges, 1991). This plasmid contains the orotidine monophosphate decarboxylase (*ura3*) gene of *Trichoderma reesei*. Vegetative cultures of monokaryotic mycelia were made on MCM (mushroom complete media: 0.2% yeast extract, 0.2% bacto-peptone, 2% glucose, 0.05% $MgSO_4{\cdot}7H_2O$, 0.05% KH_2PO_4, and 0.1% KH_2PO_4) with uridine (50 μg/ml) at 28°C.

REMI procedures. The mycelia were harvested from the liquid cultures by filtration. After being washed in 0.6 M sucrose, the mycelia were resuspended in 10 ml lysis buffer (0.5 mg/ml Novozyme, NovoBiolabs, 0.5 μl/ml Glucanase, BDH chemicals, 0.5 μl/ml Glucuronidase, Sigma), and gently shaken at 28°C for 4 hours. The protoplasts were filtered through a nylon filter, centrifuged at 2000×g, and resuspended in 10 ml of STC (0.6 M sucrose, 10 mM Tris-HCl, pH 7.5, and 10 mM $CaCl_2$). The protoplasts were then centrifuged again at 2000×g and resuspended in STC to a concentration of 1×10^8 protoplasts per ml.

For REMI transformation, circular plasmid DNA (0.1~5 μg), restriction enzyme of *Bam*HI (10~200 units) or *Hin*dIII (10~200 units) and 50 μl PTC (60% PEG M.W.

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3350, 10 mM Tris-HCl, pH 7.5, and 10 mM $CaCl_2$) were added to 100 μl of the protoplast suspension. The mixture was kept on ice for 20 min. A further 600 μl of PTC was then added to the mixture, followed by incubation for 20 min at room temperature. The protoplasts were directly plated on MMM (mushroom minimal media: 2% glucose, 0.05% $MgSO_4 \cdot 7H_2O$, 0.05% KH_2PO_4, and 0.1% KH_2PO_4) for regeneration.

Southern blot analysis. Genomic DNA was prepared from lyophilized mycelia according to the method described previously (Reader, 1985). Southern blot analysis was performed with 5 μg DNA per sample. The probe, *Bam*HI-digested pTRura3-2 vector, was labelled by random primer DNA labeling kit (TaKaRa) with α-^{32}P-dCTP (Amersharm). The signals were subjected to autoradiography using Kodak X-ray film.

Results

Optimal conditions of REMI transformation in *P. ostreatus*. Transformation of *P. ostreatus* uracil auxotroph (ASI2029-8) was attempted using the REMI with pTRura3-2 vector. Transformed protoplasts were screened on regenerating MMM plate without uridine. About 98.5% of regenerated colonies in MMM plate grew on MMM plate after three continuous transfers. These strains were regarded as transformants. No regenerating protoplasts were observed on the plates when they were not treated with DNA. Protoplasts of *P. ostreatus* were transformed by either the conventional PEG transformation or by REMI. The PEG transformation with circular pTRura3-2 DNA yielded 4.5 transformants per 1 μg of DNA in average. As shown in Fig. 1, REMI transformation with 10 units of *Bam*HI gave 64 transformants per 1 μg of circular pTRura3-2 DNA, demonstrating that the frequency of REMI transformation was increased by about 14.2 times than that of the PEG transformation.

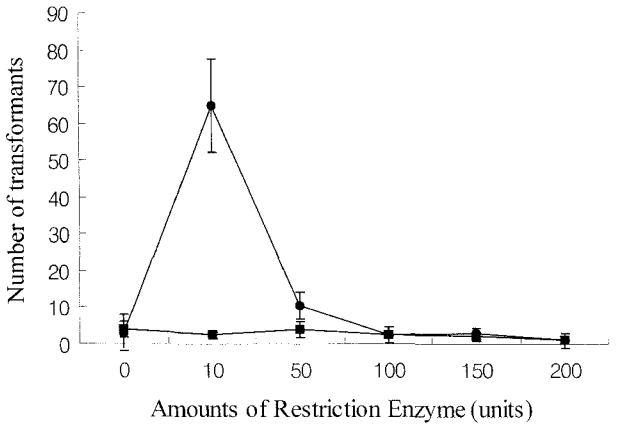

Fig. 1. Effects of restriction enzymes on REMI transformation of *Pleurotus ostreatus*. Protoplasts of auxotrophic *P. ostreatrus* were transformed with a range of amounts of *Bam*HI (●) or *Hin*dIII (■). Transformation was performed with 1 μg of circular plasmid DNA for 1×10^7 protoplasts. Values represent the average of three independent experiments.

Plasmid pTRura3-2 has unique restriction site for *Bam*HI and *Hin*dIII. The effects of enzyme amounts on transformation efficiency were determined using these restriction enzymes when 1 μg of circular pTRura3-2 DNA was added into 1×10^7 protoplasts. The maximum number of REMI transformants was obtained when 10 units of *Bam*HI was used (Fig. 1) and transfromation frequency was dramatically reduced when higher amounts of *Bam*HI were used. However, the amounts of *Hin*dIII showed no significant changes in the number of transformants.

As a next step, we examined the effects of the DNA concentration (Fig. 2), and the number of protoplasts on transformation frequency (data not shown). All plasmids used in this experiment were concentrated by ethanol precipitation and used in the same volume (1 μl). In conventional PEG transformation, almost the same number of transformants (2-7) was obtained with all of concentrations examined (5 μg, 4 μg, 2 μg, 1 μg or 0.5 μg) of circular pTRura3-2 DNA. However, the transformation efficiency for DNA concentration in the REMI transformation was proportionally increased, and the maximum number of transfromants was obtained with 5 μg of pTRura3-2 DNA. High concentration of plasmid DNA raised the number of transformants but the efficiency of

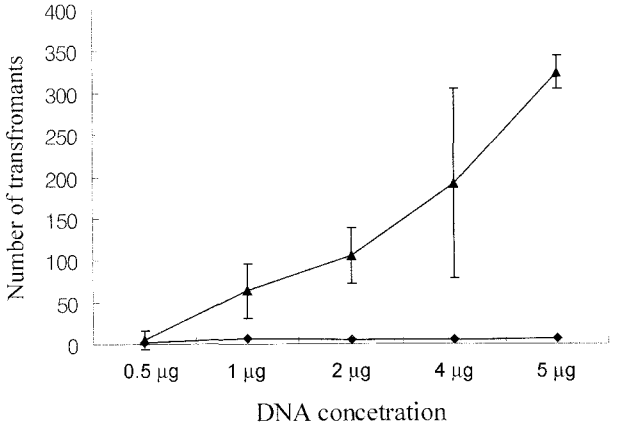

Fig. 2. Effects of DNA concentration on transformation of *Pleurotus ostreatus*. Conventional PEG (◆) and REMI (▲) transformation was performed with circular plasmid DNA for 1×10^7 protoplasts and, in REMI transformation, using 10 units of *Bam*HI. All plasmid DNA was concentrated by Et-OH precipitation and was used in the same volume (1 μl). Values represent the average of three independent experiments in REMI transformation, but the numbers of transformants by PEG transformation were obtained in one or two experiments.

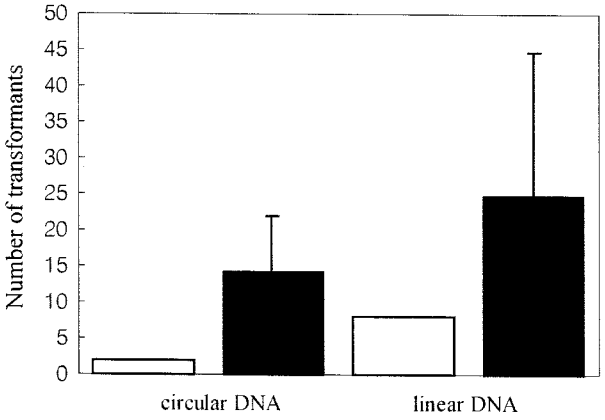

Fig. 3. Effects of DNA conformation on REMI transformation of *Pleurotus ostreatus*. Conventional PEG (open column) and REMI (closed column) transformation was performed using 1 μg of circular or linear plasmid DNA and 1×10^7 protoplasts using the circular and linear DNA. For REMI, 10 units of *Bam*HI were used. Values represent the average of three independent experiments in REMI transformation, but the numbers of transformants by PEG method were obtained in one experiment.

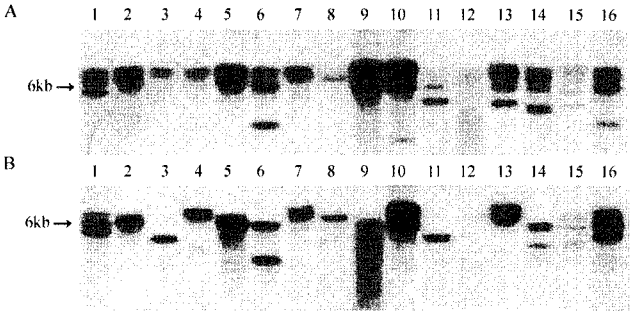

Fig. 4. Southern hybridization analysis of *Bam*HI-mediated transformants. The probe DNA labelled with α-^{32}P-dCTP was a 0.8 kb *Bam*HI or *Eco*RI-digested DNA fragment, containing *ura3* gene of pTRura3-2. (A) The genomic DNAs extracted from uracil auxotroph mutant and transformants in *P. ostreatus* were digested with *Bam*HI to analyze the rate of *Bam*HI-mediated integration. Lane 1: transformant by PEG method, lanes 2-16: T1-T15 transformants by REMI. (B) The genomic DNAs extracted from *P. ostreatus* transformants were digested with *Eco*RI to analyze the copy number of integration DNA. Lane 1: transformants by PEG method, lanes 2-16: T1-T15 transformants by REMI.

transformation per 1 μg plasmid DNA was similar in all concentrations examined. The average number of transformants is 57.3 per 1 μg DNA. Then, we examined the effects of plasmid DNA conformation on transformation frequency. In the case of the conventional PEG transformation, the linearized DNA increased the transformation efficiency by about 4 times compared with that of circular plasmid DNA. And, in the REMI transformation using the linearized DNA, the efficiency of transformation was increased by about 1.7 times compared with that of circular plasmid DNA (Fig. 3).

We earned that the optimal condition for REMI transformation of *P. ostreatus* was the addition of 10 units *Bam*HI with 1 μg of linear pTRura3-2 DNA for 1×10^7 protoplasts.

Southern analysis of DNA isolated from transformants. To analyze the integration events of transforming vectors at the genomes, genomic DNAs of the transformants obtained by REMI using *Bam*HI were digested with *Bam*HI or *Eco*RI, and subjected to southern blotting probed with the 0.8 kb DNA fragments containing the *ura3* gene.

The genomic DNAs of *Bam*HI-derived transformants were digested with *Bam*HI, in order to release the integrated pTRura3-2. Southern hybridization analysis revealed that 22 of 45 transformants (48.8%) yielded a hybridizing fragment of the size of 6 kb, indicating that insertions at *Bam*HI recognition site were restored upon integration. These clones provide the possibility for *Bam*HI site mediated chromosome integration but true REMI event was not shown because there was not 6 kb single DNA fragement. To assess the frequency of single copy integration of pTRura3-2, genomic DNAs were digested with *Eco*RI which cuts once within pTRura3-2. In the previous report, conventional PEG transformation of *P. ostreatus* results in multi-copy integration at different genomic sites (Kim, 1999), and identical result was observed in this study (Fig. 4B, lane 1). Of the 45 REMI transformants analyzed, 14 transformants (31.1%) carried one copy of the integrated plasmid (Fig. 4B, lanes 3, 8, and 11).

Discussion

Efficiency of REMI transformation in auxotrophic *P. ostreatus* was increased by about 14.2 times compared with conventional PEG transformation. we also knew that it is possible to increase the number of transformants in proportion to DNA contents in REMI method. Similar results were obtained by REMI transformation in *Magnaporthe grisea* (Shi, 1995), *Ustilago maydis* (Bolker, 1995), *C. cinereus* (Granado, 1997), *Alternaria alternata* (Akamatsu, 1997) and *Lentinus edodes* (Sato, 1998). Although an efficient transformation of *P. ostreatus* was reported using carrier DNA (Irie, 2001), the efficiency of REMI transformation in this study is the higher than those previously reported.

In the test of optimal concentration of *Hin*dIII, the results indicate that the optimal amounts of restriction enzyme for increased transformaion are dependent on the kinds of enzyme. As in *M. grisea* (Shi, 1995), and *L. edodes* (Sato, 1998), the REMI transformation using the linearized DNA was more efficient in *P. ostreatus*, Therefore, this result suggests that linear DNA integrates more

easily into the fungal genome than circular DNA, and the restriction enzyme helps the integration of linearized plasmid DNA into the genome. The maximum increase was observed in REMI transformation with linear plasmid DNA.

When the integration of plasmid DNA was mediated by the enzyme used for REMI transformation, either end of integrated plasmid DNA was recognized by the same enzyme. The digestion of genomic DNA by the enzyme used for REMI transformation should produce a fragment identical in size to linear plasmid DNA (Schiestl, 1991; Granado, 1997).

In the Southern analysis, transformants didn't showed true REMI event but some transformants provided the possibility of *Bam*HI mediated chromosome integration and 31.1% of transformants carried one copy of the integrated plasmid. This REMI event efficiency and single copy transformation efficiency was lower than that of yeast (Schiestl, 1991; Brown, 1996), but was similar to those of basidiomycete, *U. maydis* (Bolker, 1995), *C. cinereus* (Granado, 1997), and *L. edodes* (Sato, 1998).

So far, traditional UV or chemical mutagenesis was usually used for genetic analysis and tagging mutagenesis method was not applied in *Pleurotus ostreatus*. However, REMI method dramatically increased transformation efficiency and the single-copy integration event compared with conventional PEG transformation method. The high efficiency of transformation and single-copy integration event suggests that the REMI transformation could be used for tagging mutagenesis of *P. ostreatus*.

Acknowledgements

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