The Journal of Microbiology (2010) Vol. 48, No. 2, pp. 253-256
DOI 10.1007/s12275-010-9278-7
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NOTE

Genetic Introduction of Foreign Genes to *Pleurotus eryngii* by Restriction Enzyme-Mediated Integration

Won Noh[1], Sang-Woo Kim[1], Dong-Won Bae[2], Jae-Yean Kim[3], and Hyeon-Su Ro[1,3]*

[1]*Department of Microbiology and Research Institute of Life Sciences,*
[2]*Central Instrument Facility,* [3]*Division of Applied Life Science and Environmental Biotechnology National Core Research Center, Gyeongsang National University, Chinju 660-701, Republic of Korea*

(Received September 13, 2009 / Accepted October 15, 2009)

***Pleurotus eryngii* was transformed via restriction enzyme-mediated integration. In order to construct the transformation plasmid, the enhanced cyan fluorescent protein (*ECFP*) gene was ligated next to the *gpd* promoter of the plasmid pAN7-1. Transformation was facilitated via the heat treatment of a transformation mixture containing 1 μg of the *Hin*dIII-digested plasmid DNA and 10^6 mushroom protoplasts in 40% polyethyleneglycol solution, resulting in 10-40 hygromycin-resistant transformants. Successful transformation was evidenced by PCR, Southern blot, and confocal fluorescence microscopic analyses on the selected transformants. To date, this is the first report on the transformation of *P. eryngii* by REMI technique.**

***Keywords*:** hygromycin, *P. eryngii*, REMI, transformation

Pleurotus eryngii is a basidiomycete fungus, the fruiting body of which is one of the most popular edible mushrooms worldwide. This mushroom accounts for more than 30% of the Korean mushroom market. Additionally, *P. eryngii* has been sought for applications involving the bioremediation of heavy metal contamination and the bioconversion of organic compounds (Zorn *et al.*, 2003; Shin *et al.*, 2007; Gómez-Toribio *et al.*, 2009). However, despite its immense economical importance and broad variety of possible applications, there are currently very few available molecular biological tools that might accommodate a growing demand for its molecular breeding and biotechnological applications. In particular, a reliable transformation method for the introduction of a foreign gene into the mushroom cells has yet to be developed.

Mushroom transformation has been primarily assessed either by *Agrobacterium tumefaciens*-mediated transformation (ATMT) or via restriction enzyme-mediated integration (REMI) (Fan *et al.*, 2006). ATMT has been applied successfully to the transformation of a variety of filamentous fungi, including *Aspergillus*, *Fusarium*, and *Trichoderma* (de Groot *et al.*, 1998; Ruiz-Diez, 2002; Michielse *et al.*, 2004; Cardoza *et al.*, 2006) as well as the mushrooms *Agaricus bisporus* (Chen *et al.*, 2000) and *Flammulina velutipes* (Kong WS, personal communication). Meanwhile, REMI has also proven a powerful tool for the transformation of a variety of microorganisms, including *Ustilago maydis* (Bölker *et al.*, 1995), *Dictyostelium* (Kuspa and Loomis, 1992), and *Trichoderma* (Wang *et al.*, 2009). This method has been shown to be particularly efficient for the transformation of a variety of mushrooms, including *Pleurotus ostreatus* (Irie *et al.*, 2001; Joh *et al.*, 2003), *Trametes versicolor* (Kim *et al.*, 2002), *Ganoderma lucidum* (Kim *et al.*, 2004), and *Schizophyllum commune* (van Peer *et al.*, 2009). However, an efficient method for the introduction of foreign genes to the *P. eryngii* genome has yet to be developed. Our previous attempts to transform *P. eryngii* via ATMT proved completely unsuccessful. Similar conclusions were drawn by another mushroom study group, as well (Kong WS, personal communication). Accordingly, in this report, we demonstrated the first successful transformation of *P. eryngii* via a REMI-based transformation method using an enhanced cyan fluorescent protein (ECFP) as a reporter gene.

The KNR 2312 strain of *P. eryngii* (monokaryon) was utilized as a host strain for the transformation. The mycelia of the mushroom were cultivated in a potato dextrose broth (PDB, BD Diagnostic Systems, USA) for one week in darkness at 25°C. *Escherichia coli* DH5α was employed as a host strain in the gene manipulations. The pAN7-1 plasmid (Punt *et al.*, 1987), which harbors the *HPH* (hygromycin B phosphotransferase) gene as a selective marker, was employed as a mushroom-transforming vector. The gene for enhanced cyan fluorescent protein (ECFP) was obtained from the pECFP plasmid (Clontech, USA). The *ECFP* gene was PCR amplified using a primer set (forward: 5′-ctg gat cca cca tgg tga gca agg gcg agc tt-3′, reverse: 5′-cag gat cct cct cac ttg tac agc tcg tcc atg cc-3′,). PCR was performed for 30 cycles using a Pfu DNA polymerase (Solgent Co., Korea). Each cycle consisted of the following three steps: 94°C for 45 sec, 60°C for 45 sec, and 72°C for 2 min. The resultant PCR product was purified with a PCR Extraction kit (Solgent Co.). The purified DNA fragment was digested with the restriction enzyme *Bam*HI and ligated into the *Bam*HI site of pAN7-1 with a

* For correspondence. E-mail: rohyeon@gnu.ac.kr; Tel: +82-55-751-5946; Fax: +82-55-759-0187

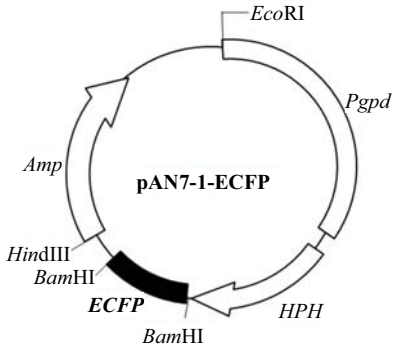

Fig. 1. Construction of the transformation vector DNA pAN7-1-ECFP. The *ECFP* gene was PCR-amplified from pECFP and ligated into the *Bam*HI site of pAN7-1.

ligation kit (TaKaRa, Japan). The start codon of the *ECFP* gene was located 27 base pairs after the stop codon of *HPH*. Therefore, both the *HPH* and *ECFP* genes were under the control of the *gpd* promoter.

In order to obtain protoplasts of *P. eryngii*, the mushroom mycelia were cultivated in PDB media (100 ml) for 5 days with a mild agitation at 160 rpm at 25°C. The mycelial pellets were homogenized using a Waring Blender (Waring Co.) for 30 sec to loosen the entangled mycelia and the cultivation was continued for an additional 3 days under the same culture conditions. The resultant mycelia were collected via centrifugation (3,000 rpm, 10 min). The mycelia were suspended in 1 M $MgSO_4$ solution and again collected via centrifugation. The collected mycelia (10 g wet weight) were treated with 20 ml of a lysing enzyme extract solution (25 μg/ml) from *Trichderma harzianum* (Sigma Co.) in 1 M $MgSO_4$ for 2 h at 30°C with mild agitation (70 rpm). When the reaction was completed, and confirmed via optical microscopy, the mycelial solution was filtered on filter paper (Whatman No. 3). The filtrate solution was then centrifuged for 10 min at 3,000 rpm. The pellet protoplasts were suspended in an STC buffer (pH 7.5) containing 0.6 M sucrose, 10 mM Tris-HCl, and 10 mM $CaCl_2$. The final concentration of the solution was adjusted to 1×10^7 protoplasts/ml. The survival of the protoplast was subsequently investigated in order to determine the optimal concentration of hygromycin B by spreading 1×10^6 protoplasts on PDA plates containing different concentrations of hygromycin B. As is shown in Fig. 2, the number of the mushroom mycelial colonies decreased along with increases in the concentration of hygromycin B. The mushroom essentially could not grow beyond 50 μg/ml of hygromycin B. The actual screening of the transformants was conducted with a higher concentration (100 μg/ml).

In order to transform *P. eryngii* via the REMI method, the pAN7-1-ECFP plasmid (1 μg) was digested for 2 h with 200 units of *Hin*dIII. The linearized DNA solution (100 μl), in which the restriction enzyme was retained, was mixed with 100 μl of the protoplast solution. The mixture was then incubated for 10 min on ice and mixed with 20 μl of 40% polyethylene glycol (PEG, molecular weight 3,350, Sigma Co.) solution. The mixture was incubated for 10 min on ice. An additional 220 μl of 40% PEG solution was subsequently added to the reaction mixture and incubated for 30 min at 35°C. After the incubation, the solution was maintained for 1 h at room temperature. The resultant protoplasts were harvested via centrifugation for 10 min at 3,000 rpm and then suspended in 200 μl of STC buffer. To screen the transformants, 5 ml of mushroom complete medium (MCM, containing yeast extract 2 g/L, peptone 2 g/L, $MgSO_4$ 0.5 g/L, KH_2PO_4 0.46 g/L, K_2HPO_4 1 g/L, glucose 20 g/L, agar 15 g/L) were layered and solidified on a petri dish. The remaining MCM was maintained in a water bath at 45°C to maintain its liquid state. When the petri dish was ready, 100 μl of the protoplast

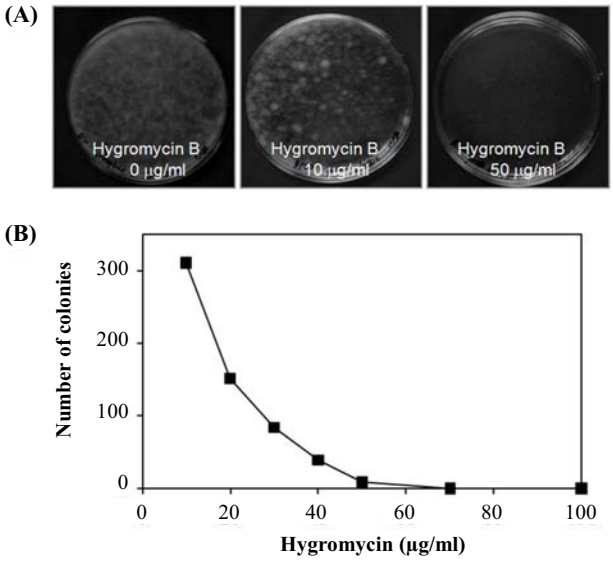

Fig. 2. Effect of hygromycin B on the growth of mushroom protoplast. Mushroom protoplasts were spread on MCM agar plates containing various concentrations of hygromycin B (A). The number of colonies on the plates was counted according to the concentration of hygromycin B (B).

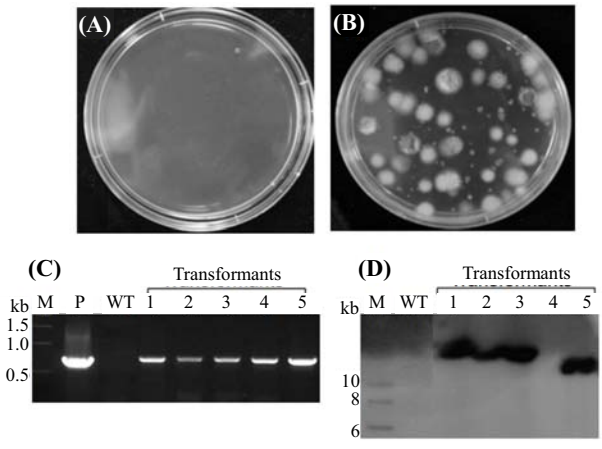

Fig. 3. Transformation of *P. eryngii*. (A) Protoplasts without plasmid. (B) Protoplasts with 1 μg *Hin*dIII-digested pAN7-1-ECFP. (C) PCR analysis targeting the *ECFP* gene. The total cellular DNAs of the wild type (WT) and the transformants (lane 1-5) were subjected to PCR analysis. Lane P is a positive control containing pAN7-1-ECFP as a template DNA. (D) Southern blot analysis. *Eco*RI-digested chromosomal DNAs were subjected to Southern blot analysis with a ^{32}P-labelled *HPH* as a probe DNA.

solution was mixed with 15 ml of MCM containing hygromycin B (100 μg/ml). The mixture was then poured into the MCM-layered petri dish and the resulting agar plate was incubated for 10 days at 25°C. As is shown in Figs. 3A and B, the mushroom mycelial colonies appeared with different sizes only in the plate containing the linearized plasmid DNA. The tiny colonies around the big colonies turned out to be satellite colonies that could not grow on new hygromycin plates, thereby indicating that they had originated from untransformed host protoplasts. The transformation yield was 10-40 transformants per μg of plasmid DNA, which was coincident with previous reports concerning mushroom transformation (Kim *et al.*, 2002, 2004; Joh *et al.*, 2003). In order to verify whether the transformants harbored the plasmid-originated DNA, the genomic DNAs of the 5 selected transformants and the original strain as a control were extracted and subjected to PCR analysis targeting the *ECFP* gene using the primer set utilized for the construction of pAN7-1-ECFP. As per our expectations, all the transformants were shown to harbor the *ECFP* gene, whereas the control genomic DNA failed to generate the targeted gene, thereby indicating that the REMI could be successfully utilized for the transformation of *P. eryngii* (Fig. 3C). The genomic integration was further confirmed by a series of transfer cultures on PDA without hygromycin B. Southern blot analysis was conducted in order to determine the frequency of the vector plasmid integration event. The chromosomal DNAs of the transformants and host strain were fragmented with *Eco*RI and the fragmented DNAs were subjected to Southern blot analysis using a ^{32}P-labelled *HPH* as a probe DNA. Single chromosomal DNA fragments for 4 out of 5 transformants evidenced strong radioactivity with an approximate size in a range between 10 and 15 kb, thereby indicating that the integration was principally single-event in nature. The transformant failed to hybridize with the probe DNA (Fig. 3D, lane 4), and this is conceivably because it maintained the target vector as plasmid DNA, rather than as a chromosomal integrant. Finally, we evaluated the expression of the integrated *ECFP* gene in the mushroom mycelia using a confocal microscope (Olympus FV1000). The transformant No. 5 in Fig. 3C was subjected to CFP imaging with excitation at 458 nm and emission at 475-500 nm. As is shown in Fig. 4A, the CFP protein was expressed throughout the mycelia, whereas the control mycelia evidenced faint background fluorescence (Fig. 4B). A magnified image (400×) of the mycelia revealed that the expressed CFP was distributed inside the cytoplasm (Figs. 4C, D, and E).

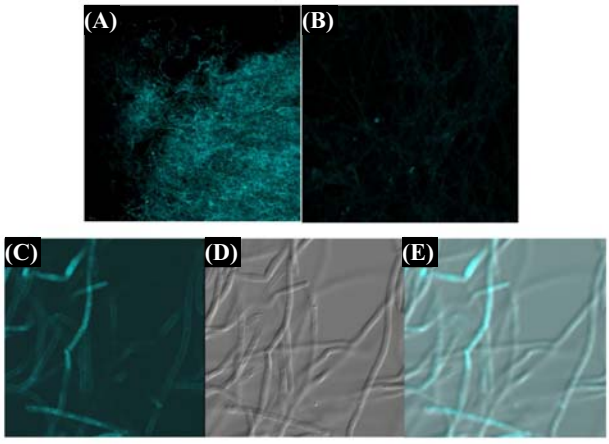

Fig. 4. Expression of the *ECFP* gene. The expression of the *ECFP* gene was analyzed with a fluorescence confocal microscope with excitation at 458 nm and emission at 475-500 nm. Fluorescence images (40x) of the transformant No.5 (A) and the wild type strain (B) of *P. eryngii*. Enlarged images (400x) of the transformant No.5: (A) Fluorescence, (B) Phase contrast, (C) Merged image.

In conclusion, we were able to transform *P. eryngii* via the REMI technique with a transformation yield of 10-40 transformants/10^6 protoplasts. The successful integration of the foreign DNA fragments was confirmed via PCR and Southern blot analyses. The integration of the enzyme-digested plasmid was found to occur primarily as a single event. Finally, the expression of the integrated gene at the protein level was monitored by the fluorescence light emitted by the expressed ECFP protein. Our REMI-based transformation method is expected to contribute to the development of genetically modified *P. eryngii* variants that can be used in a range of applications, including enhanced heavy metal and oil absorption capability, live vaccines for animal feeds and human health, and functional foods containing high contents of essential nutrients.

We are grateful to Prof. H. T. Choi at Kangwon National University for his helpful advises on REMI technique. This study was carried out with the support of Mushroom Export Research Program and Technology Development Program for Agriculture and Forestry, Ministry of Agriculture and Forestry, Republic of Korea.

References

Bölker, M., H.U. Böhnert, K.H. Braun, J. Görl, and R. Kahmann. 1995. Tagging pathogenicity genes in *Ustilago maydis* by restriction enzyme-mediated integration (REMI). *Mol. Gen. Genet*. 248, 547-552.

Cardoza, R.L., J.A. Vizcaino, M.R. Hermosa, E. Monte, and S. Gutierrez. 2006. A comparison of the phenotypic and genetic stability of recombinant *Trichoderma* spp. Generated by protoplast- and *Agrobacterium*-mediated transformation. *J. Microbiol.* 44, 383-385.

Chen, X., M. Stone, C. Schlagnhaufer, and C.P. Romaine. 2000. A fruiting body tissue method for efficient Agrobacterium-mediated transformation of *Agaricus bisporus*. *Appl. Environ. Microbiol*. 66, 4510-4513.

de Groot, M.J., P. Bundock, P.J. Hooykass, and A.G. Beijersbergen. 1998. *Agrobacterium tumefaciens*-mediated transformation of filamentous fungi. *Nature Biotechnol*. 16, 839-842.

Fan, L., H. Pan, A.T. Soccol, A. Pandey, and C.R. Soccol. 2006. Advances in mushroom research in the last decade. *Food Technol. Biotechnol.* 44, 303-311.

Gómez-Toribio, V., A.B. García-Martín, M.J. Martínez, A.T. Martínez, and F. Guillén. 2009. Induction of extracellular hydroxyl radical production by white-rot fungi through quinone redox cycling. *Appl. Environ. Microbiol*. 75, 3944-3953.

Irie, T., Y. Honda, T. Hirano, T. Sato, H. Enei, T. Watanabe, and M. Kuwahara. 2001. Stable transformation of *Pleurotus ostreatus* to hygromycin B resistance using *Lentinus edodes* GPD expression signals. *Appl. Microbiol. Biotechnol*. 56, 707-709.

Joh, J.H., B.G. Kim, K.S. Chu, W.S. Kong, Y.B. Yoo, and C.S. Lee. 2003. The efficient transformation of *Pleurotus ostreatus* using REMI method. *Mycobiology* 31, 32-35.

Kim, K., Y. Leem, K. Kim, K. Kim, and H.T. Choi. 2002. Transformation of the medicinal basidiomycete *Trametes versicolor* to hygromycin B resistance by restriction enzyme mediated integration. *FEMS Microbiol. Lett.* 209, 273-276.

Kim, S., J. Song, and H.T. Choi. 2004. Genetic transformation and mutant isolation in *Ganoderma lucidum* by restriction enzyme-mediated integration. *FEMS Microbiol. Lett*. 233, 201-204.

Kuspa, A. and W.F. Loomis. 1992. Tagging developmental genes in *Dictyostelium* by restriction enzyme-mediated integration of plasmid DNA. *Proc. Natl. Acad. Sci. USA* 89, 8803-8807.

Michielse, C.B., A.F. Ram, P.J. Hooykaas, and C.A. Hodel. 2004. Role of bacterial virulence proteins in *Agrobacterium*-mediated transformation of *Aspergillus awamori*. *Fungal Genet. Biol.* 41, 571-578.

Punt, P.J., R.P. Oliver, M.A. Dingemanse, P.H. Pouwels, and C.A. van den Hondel. 1987. Transformation of *Aspergillus* based on the hygromycin B resistance marker from *Escherichia coli*. *Gene* 56, 117-124.

Ruiz-Diez, B. 2002. Strategies for the transformation of filamentous fungi. *J. Appl. Microbiol*. 92 , 189-195.

Shin, E.H., H.T. Choi, and H.G. Song. 2007. Biodegradation of endocrine-disrupting bisphenol A by white rot fungus *Irpex lacteus*. *J. Microbiol. Biotechnol.* 17, 1147-1151.

van Peer, A.F., C. de Bekker, A. Vinck, H.A. Wösten, and L.G. Lugones. 2009. Phleomycin increases transformation efficiency and promotes single integrations in *Schizophyllum commune*. *Appl. Environ. Microbiol*. 75, 1243-1247.

Wang, B., L. Liu, Y. Gao, and J. Chen. 2009. Improved phytoremediation of oilseed rape (*Brassica napus*) by *Trichoderma* mutant constructed by restriction enzyme-mediated integration (REMI) in cadmium polluted soil. *Chemosphere* 74, 1400-1403.

Zorn, H., S. Langhoff, M. Scheibner, M. Nimtz, and R.G. Berger. 2003. A peroxidase from *Lepista irina* cleaves beta,beta-carotene to flavor compounds. *Biol. Chem.* 384, 1049-1056.