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Full Length Research Paper

Polyethylene glycol (PEG)-mediated transformation of the fused *egfp-hph* gene into *Pleurotus ostreatus*

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A transformation system for the basidiomycete *Pleurotus ostreatus* was established using PEG-mediated transformation. The homologous glyceraldehyde-3-phosphate dehydrogenase gene (*gpd*) promoter and terminator regions of *P. ostreatus* were amplified and cloned onto the restriction site of the pUC19 vector. Enhanced green fluorescent protein-encoding gene (*egfp*) and a selection marker, the hygromycin phosphotransferase gene (*hph*), were then cloned and an expression vector, pUEGFP-*hph*, was constructed. Protoplasts of *P. ostreatus* were prepared and used as the recipients in transformation procedures. An improved transformation method was established in the present study and about 100 to 200 resistant colonies per microgram DNA per 10^7 viable protoplasts were obtained. After subculturing, putative transformants were screened by PCR and verified by Southern blot and RT-PCR analyses showing the correct insertion into the genomic DNA and transcription of the transformed DNA. Moreover, the quantitative PCR and green fluorescence observation were carried out, thus demonstrating expression of the *egfp* gene driven by the homologous *gpd* promoter. This was the first report of transformation of *P. ostreatus* using homologous *gpd* promoter by PEG-mediated methods. These data will be helpful in future investigations using PEG-mediated transformation for functional characterisation of genes in the edible fungi basidiomycete *P. ostreatus*.

Key words: *Pleurotus ostreatus,* PEG-mediated transformation, EGFP.

INTRODUCTION

Pleurotus ostreatus (Jacq.:Fr.) Kummer is widely cultivated globally and ranks as the second most cultivated edible fungus worldwide after *Agaricus bisporus* (Sánchez, 2010). Moreover, many factors render *P. ostreatus* a good model for studying biochemical and physiological processes. These include hydrophobins involved in hydrophobicity (Ma et al., 2008); medical effects (Gómez-Toribio et al., 2009); high lignin-cellulitic degrading activity (Marques et al., 2010; Ruiz-Duenas et al., 2011; Lettera et al., 2011; Piscitelli et al., 2011); agro-industrial waste bioconversion (Shabtay et al., 2009; Salvachúa et al., 2011); and toxic heavy metal biosorption activities (Pan et al., 2005) that have been applied to environmental protection. Furthermore, *P. ostreatus* can be cultivated in a short production cycle, which has created considerable interest in its genetic analysis. The raw sequence data from the *P. osttreatus* genome sequencing project was being made available by the Department of Energy Joint Genome Institute (JGI) before scientific publication (http://genome.jgi-psf.org/PleosPC15_1/PleosPC15_1.home.html). The understanding and functional analysis of genes of *P. ostreatus* would facilitate its use in various aspects. Thus the high efficient transformation system is needed urgently.

Many attempts have been tried in the transformation of *P. ostreatus*, such as polyethylene glycol/$CaCl_2$ (PEG/$CaCl_2$) (Peng et al., 1992; Koji et al., 1996; Kim et al., 1999; Honda et al., 2000), electroporation (Peng et al., 1993), restriction enzyme-mediated integration (REMI) (Irie et al., 2001) and *Agrobacterium tumefaciens*-mediated transfor- mation (ATMT) (Ding et al., 2010). Li et al. (2006) reported the improved transformation efficiency was about 100 to 150 transfor-

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mants emitting green fluorescence per µg of DNA per 10^7 viable protoplasts by using the addition of heparin, aurintricarboxylic acid (ATA) and spermidine, which was about 100 to 1800 times higher than that of Irie et al. (2001) and 40 to 90 times higher than that of Koji et al. (1996). However, the transformation efficiency was low and not up to the demands of commercial strain improvement. The main drawbacks of these methods were their low transformation efficiencies and heterogeneous integration into genomic loci (Peng et al., 1992). Ding et al. (2010) reported the first cloning of the *gpd* promoter of *P. ostreatus* and its use in the ATMT methods of *P. ostreatus*. The results demonstrated the efficiency of the homologous *gpd* promoter of *P. ostreatus* and myceilia as the more proper receipt materials in the transformation procedure. This cast light on the improvement of the PEG-mediated transformation by using homologous *gpd* promoter to enhance the transformation efficiency and possibility of the integration on the genomic DNA. Therefore, development is required of a reliable and highly efficient transformation system for *P. ostreatus* that may ultimately prove to be useful in the establishment of genetic model for basidiomycetes.

Fusion of target and selective marker genes is an efficient method of enhancing expression (Nyyssonen and Keranen, 1995). Ding et al. (2010) reported that the fused *egfp-hph* gene was efficiently expressed in *P. ostreatus* using the ATMT method. This represented a novel approach for the efficient transformation of *P. ostreatus*. Thus, the present study aimed to use the fused *egfp* and *hph* selective marker genes driven by the *gpd* promoter from *P. ostreatus*. The PEG-mediated transformation protocol was improved in the present study. Furthermore, Southern blot, reverse transcriptase-polymerase chain reaction (RT-PCR) and fluorescence visualization were performed to confirm the validity of the transformation. This study laid the foundation of biotechnological applications of *P. ostreatus* as a beneficial resource.

MATERIALS AND METHODS

Strains and plasmids

The *P. ostreatus* monokaryotic strain 2106 was obtained from the Institute of Plant and Environmental Protection, Beijing Academy of Agriculture and Forestry Science (Beijing, China) Basidiospores from the fruiting body were distributed on the potato dextrose agar (PDA) plates. Then the plated were incubated at 25°C till small colony appeared. The colonies were transferred to PDA slants and then validated by the clamp connections by visualizing under a microscope (BX51; Olympus, Tokyo, Japan). The monokaryotic strains were maintained on PDA slants at 25°C and then were used for isolation of genomic DNA. *Escherichia coli* DH10B cells and pGEM-T (Promega, Madison, USA) were used as the host cell and cloning vector, respectively. *E. coli* DH10B harbouring the recombined vectors was cultured at 37°C on Luria-Bertani (LB) agar plates supplemented with ampicillin (100 µg/ml; Sigma, St. Louis, MO, USA). Plasmid pUC19 was purchased from Takara Co. Ltd. (Japan). Plasmid pEGFP-C1 was purchased from Beijing Dingguochangsheng Biotechnology Co. Ltd. (Beijing, China). Plasmid pAN7-1 was a gift from Dr. P. J. Punt from the Department of Applied Microbiology and Gene Technology, TNO Voeding, the Netherlands (Punt et al., 1987).

Plasmid construction

To clone the promoter and terminator regions of the *P. ostreatus gpd* gene, two pairs of primers were designed based on the *Lentinula edodes* sequences published by Hirano et al. (2000) (Table 1). Genomic DNA of *P. ostreatus* was isolated using a DNeasy Plant Mini Kit (Qiagen, Germany). The *gpd* promoter and terminator regions were amplified using genomic DNA of the monokaryotic strain 2106 as the template. The PCR products were purified and cloned into pGEM-T, yielding pPgpd-T and pTgpd-T, respectively.

A 1055-bp fragment of the *gpd* gene promoter region was obtained by digesting pPgpd-T with *Sph* I and *Sal* I, and it was ligated into plasmid pUC19 digested with the same enzymes, yielding pUC19-Pgpd. A 1001-bp fragment of the *gpd* gene terminator region was obtained by digesting pTgpd-T with *Bam*H I and *Eco*R I, and it was ligated into plasmid pUC19-Pgpd digested with the same enzymes, yielding pUC19-Pgpd-Tgpd. A 1031-bp *hph* gene coding region was PCR-amplified using pAN7-1 as template. The primers used for amplification of the *hph* gene are shown in Table 1. The amplified fragment was digested by *Xho* I and *Bam*H I and then cloned into pEGFP-C1, which was also digested with *Sal* I and *Bam*H I, resulting in the recombinat plasmid pEGFP-*hph*-C1. Then the 1788-bp fragment containing *egfp* and *hph* was PCR-amplified using plasmid pEGFP-*hph*-C1 as the template and EGFP-F and *hph*-R as primers (Table 1). The PCR amplicon was digested with *Sal* I and *Bam*H I, and the resulting fragment was ligated into pUC19-Pgpd-Tgpd, which was digested with the same restriction enzymes, generating pUEGFP-*hph* (Figure 1).

Preparation of protoplasts from *Pleurotus ostreatus*

Mycelia cultured in 100 ml of MCM [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% K_2HPO_4] (Kim et al., 1999) was collected by filtration through gauze and rinsed three times with 0.6 M mannitol. Mycelia were then incubated for 30 min in 5 ml of 1.5% lywallzyme (Guangdong Institute of Microbiology, China) in 0.6 M mannitol at 32°C with gentle shaking. Protoplasts were separated from hyphal debris by filtration through a G3 glass filter, collected by centrifugation at 1 800 × g for 10 min and washed twice with MM buffer [0.5 M mannitol and 50 mM maleic acid buffer (pH 5.5)]. Protoplasts were finally resuspended in 100 µl of MMC buffer [0.5 M mannitol, 50 mM maleic acid buffer (pH 5.5), 5 mM $CaCl_2$] to a concentration of 10^8 to 10^9 protoplasts per ml for storage at 4°C.

Highly efficient PEG transformation of protoplasts

Protoplasts (~10^8 in 70 µl MTC [0.5 M mannitol, 10 mM Tris-HCl (pH7.5), 5 mM $CaCl_2$] buffer) were mixed thoroughly with 10 µl pUEGFP-*hph* (2 µg/µl), 10 µl 20 mM aurintricarboxylic acid (ATA; Sigma-Aldrich), 5 µl 50 mM spermidine and 5 µl heparin (20 µg/µl). This mixture was then vortexed 10 times for 1 s at maximum speed. Then 50 µl of room temperature, freshly filtered PTC buffer [40% PEG3350, 100 mM $CaCl_2$, 10 mM Tris-HCl (pH7.5)] was added to the mixture, which was then vortexed five times for 1 s at maximum speed, followed by incubation on ice for 25 min. One millilitre of room temperature, freshly filtered PTC buffer was added to the mixture and then was incubated for an additional 25 min at room temperature. Protoplasts were recovered by centrifugation (4°C, 10 min at 1 800 × g) and resuspended in 500 µl STC buffer (1 M

Table 1. Primers used in the present study.

Primer name	Sequences (5′→3′)	Restriction site	Reference
Pgpd-F	ATTAGCATGCCGAAGTTTGAGGTGGTT	*Sph* I	This work
Pgpd-R	AAGTCGACATTCAAGCAGTCAATGGAT	*Sal* I	This work
Tgpd-F	TAAGGATCCGAAAGGGCTGTGCATCTCGAACT	*Bam*H I	This work
Tgpd-R	TCAGAGCTCTCATCATACCCCCTACCGACATCT	*Sac* I	This work
hph-F	ACGCTCGAGCTATGAAAAAGCCTGAACTC	*Xho* I	This work
hph-R	AATGGATCCCGGTCGGCATCTACTCTAT	*Bam*H I	This work
EGFP-F	AAGTCGACATGGTGAGCAAGGGC	*Sal* I	This work
EGFP-R	AAGGATCCTTAACCATCGACTG CAGAATT	*Bam*H I	This work
RV-M	GAGCGGATAACAATTTCACACAGG	-	TaKaRa, Code No. D3880.
M13-47	CGCCAGGGTTTTCCCAGTCACGAC	-	TaKaRa, Code No. D3887.
qEGFP-F	CGACCACTACCAGCAGAA	-	This study
qEGFP-R	GAACTCCAGCAGGACCAT	-	This study
q18s-F	ATTCACTATGGAGTTGTTGCTG	-	This study
q18s-R	AACGACTTGAGAGACTT	-	This study

Underlined letters corresponding to the restriction site.

sorbitol, 100 mM $CaCl_2$, 10 mM Tris-HCl (pH7.5)). Protoplasts were then centrifuged (4℃, 10 min at 1 800 × g) and resuspended in 1 ml liquid RM medium [MCM medium with 1 M sorbitol as osmotic stabilizer]. Subsequently, protoplasts were allowed to regenerate for 18 to 24 h without hygromycin B. The mixture was poured onto an RM plate containing 100 μg/ml hygromycin B and incubated at 28℃ for 10 to 14 days. Colonies were sub-cultured for 5 times individually onto fresh MCM plates containing 100 μg/ml hygromycin B and incubated at 28℃ for 5-7 days for further use.

PCR amplification of the transformants of *Pleurotus ostreatus*

Genomic DNA was extracted from the mycelia of each hygromycin B-resistant colony using a DNeasy Plant mini kit (Qiagen, Germany). PCR for detecting the *egfp* gene in putative transformants was performed using EGFP-F/EGFP-R as primers (Table 1). Positive controls of plasmid pUEGFP-*hph* were also subjected to PCR amplification. The amplification protocol consisted of an initial denaturing cycle of 5 min at 95℃, followed by 30 cycles of 30 s denaturation (95℃), 1 min annealing (56℃) and 40 s elongation (72℃).

Southern blot analysis of transgenic *Pleurotus ostreatus*

To prepare genomic DNA for southern blot, genomic DNA was extracted following the method of Doyle and Doyle (1987). Approximately 10 μg genomic DNA of putative transformants and negative control strains was digested with *Eco*R V (Takara, Japan) and separated by electrophoresis on a 1% (w/v) agarose gel. The 750-bp fragment containing the *egfp* gene, amplified with primers EGFP-F/EGFP-R from recombinant plasmid DNA was used as the probe. Hybridization and detection were carried out using the DIG system (Roche, Germany).

Reverse transcription-polymerase chain reaction (RT-PCR) analysis

Total RNA was prepared from the powdered mycelia of *P. ostreatus* positive transformants using the RNA/DNA Mini Kit (Qiagen, Germany). The total RNA was then reverse-transcribed using an oligo (dT) primer and Moloney murine leukemia virus reverse transcriptase (Fermentas, UK). Synthesised cDNA was subjected to PCR amplification with primers EGFP-F/EGFP-R (Table 1). PCR was performed as described above.

Real-time quantitative RT-PCR analysis

RNA was extracted as described above. Real-time quantitative RT-PCR was performed using SYBR FAST qPCR Master Mix Universal (Kapa Biosystems, Woburn, MA, USA) and a real-time PCR system (ABI 7500; Applied Biosystems) according to the manufacturer's instructions. Primers were designed using Beacon Designer software (Premier Biosoft International, Palo Alto, CA, USA), and the EGFP-specific primer pair qEGFP-F and qEGFP-R (Table 1), which generated only one amplicon (133 bp), was used. The level of 18S rRNA was used as an internal control for which the primers q18S-F and q18S-R (Table 1) generated a 108-bp amplicon.

Fluorescence microscopy

Analysis of EGFP expression was carried out by affixing the hyphae of random *P. ostreatus* transformants to glass slides after 5 day growth on agar plates with hygromycin B. Samples were then visualized under a fluorescence microscope (IX71; Olympus, Tokyo, Japan) with excitation at 455 to 490 nm and emission at 515 to 560 nm. Images were taken under the 40 × objective for random transformed selected colonies and processed with DP2-BSW mapping software (Olympus).

GenBank accession numbers

The GenBank accession numbers of the Pgpd and Tgpd sequences were HQ286597 and HQ286598, respectively.

RESULTS

Selection of *Pleurotus ostreatus* transformation by PCR

P.ostreatus protoplasts treated with plasmid pUEGFP-hph

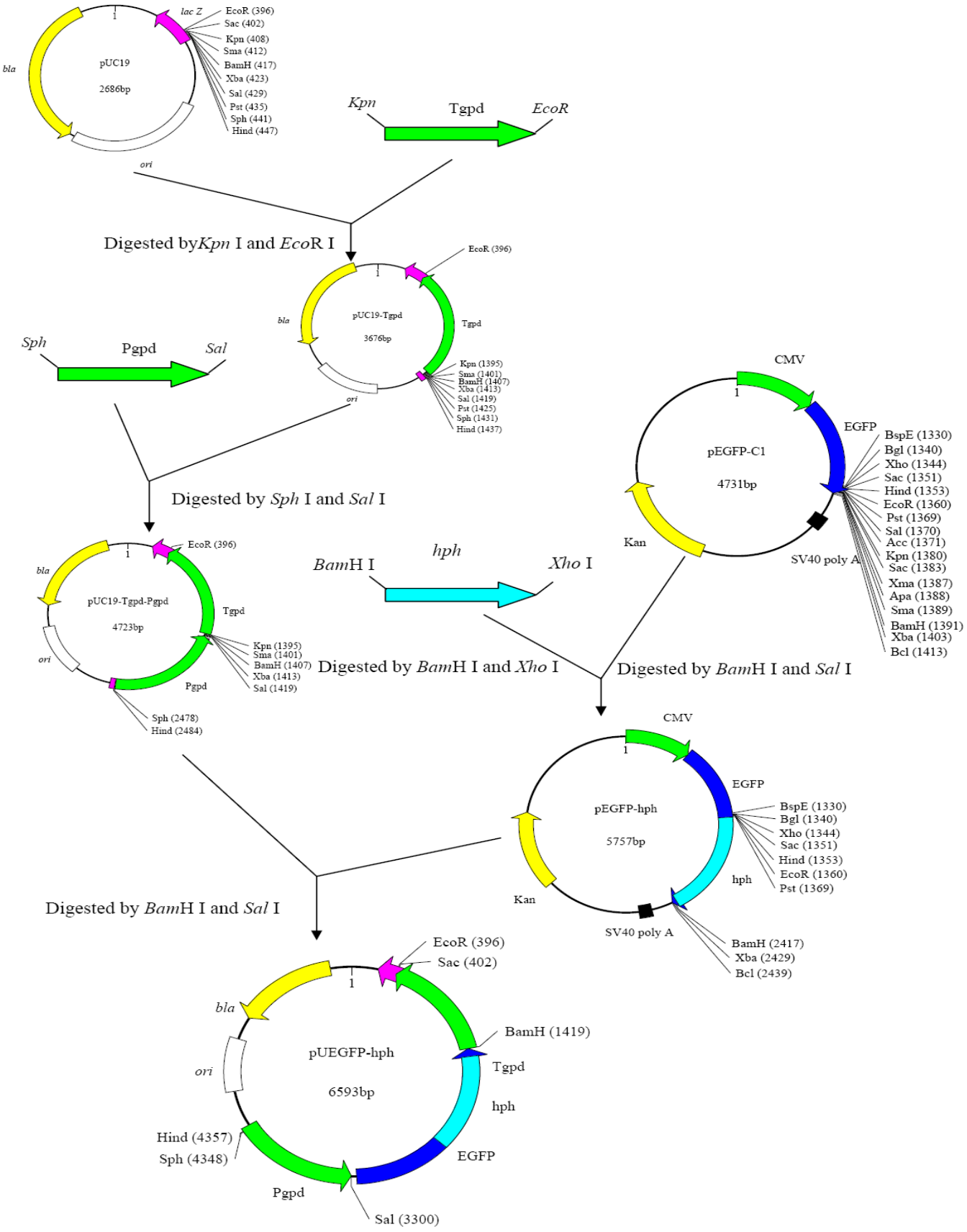

Figure 1. Construction of expression vector.

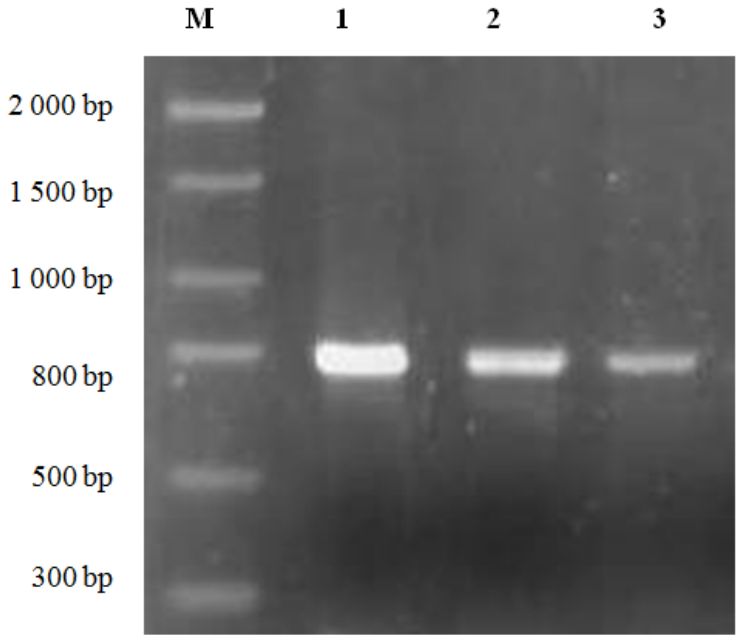

Figure 2. PCR analysis of *egfp* gene in putative transformants of *P. ostreatus*. Lane M, 1 kb plus DNA ladder; lane 1, PCR product amplified from plasmid pEGFP-C1 as positive control; lanes 2 and 3, PCR products amplified from the randomly selected transformants.

in the presence of PEG were first incubated in liquid RM without hygromycin B for 24 h at 25°C for the purpose of recovery growth and *hph* gene expression. Then recovering protoplasts were subjected to screening on RM plates containing 100 µg/ml hygromycin B. After incubation at 25°C, the colonies, which could grow to large size were transferred to selective MCM plates containing hygromycin B for 5 rounds.

Through PCR amplification, the 756 bp for *egfp* gene were amplified from the putative transformant DNA templates (Figure 2). This demonstrated that *egfp* was successfully integrated into the genomes of all transformants. The average transformation efficiency was about 100 to 200 transformants per microgram of plasmid pEGFP-*hph* DNA. This transformation efficiency was higher than that reported by Peng et al. (1992, 1993), Kim et al. (1998, 2003), Joh et al. (2003) and Li et al. (2006) previously (Table 2).

Southern blot and RT-PCR analysis of transformants

To ascertain the presence of *egfp* in the *P. ostreatus* transformants, southern blot was carried out on randomly selected positive transformants. No specific hybridization signal was detected in the non-transformed negative control, while specific strong hybridization signals were clearly visible in the transformants (Figure 3). These results further demonstrated the integration of *egfp* into the chromosomal DNA. Furthermore, the results obtained via southern blot indicated that a single copy of *egfp* was integrated into the genomes of the tested transformants.

To confirm the transcription of *egfp* gene in transformants under the control of *P. ostreatus gpd* promoters, detection of mRNA was performed by RT-PCR. The 756-bp signal was detected from the RNA of transformants (Figure 4). In contrast, no such amplification was observed in the negative control (non-transformants).

Real-time quantitative RT-PCR analysis of the transformants

To determine the level of *egfp* gene transcription, 2 positive transformants were randomly selected for real-time quantitative RT-PCR analysis. As shown in Figure 5, the *egfp* gene was transcribed in all transformants, but the levels of transcription were different in these two transformants. Transformants PoPUGH2 showed 1.21-fold transcription levels than that of PoPUGH24 (Figure 5).

Expression of EGFP by the transformants

Expression of the modified *egfp* gene in *P. ostreatus* was determined by fluorescence microscopy. All *P. ostreatus* transformants were found to fluoresce, suggesting strong expression of EGFP in their mycelia (Figure 6). EGFP expression was not detected in the negative control under the same conditions (data not shown). Thus, the protein

Table 2. Transformation efficiency of this improved methods comparison with previously reported results for *Pleurotus ostreatus*.

Method	Promoter	Transformation efficiency (transformants per mg DNA per 10^7 protoplasts)	Reference
PEG-mediated	*Pleurotus ostreatus gpd* gene promotor	100–200	This study
PEG-mediated	*Aspergillus nidulans gpd* gene promotor	80–180	Li et al. 2006
REMI	*Trichoderma ressei ura3* gene promotor	64	Joh et al., 2003
Electroporation and PEG-mediated	*Aspergillus nidulans gpd* gene promotor	3–48	Peng et al., 1993
PEG-mediated	*Aspergillus nidulans gpd* gene promotor	5–46	Peng et al., 1992
PEG-mediated	*Trichoderma ressei ura3* gene promotor	15	Kim et al., 1998
PEG-mediated	*Pleurotus sajor-caju β-tubulin* gene promotor	10	Kim et al., 2003

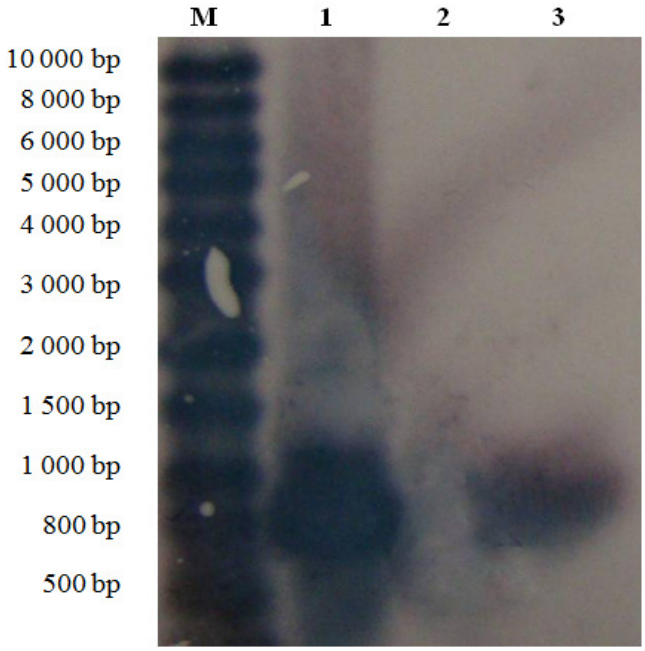

Figure 3. Southern blot analysis of DNA isolated from putative transformants. Lane M, 1 kb plus DNA ladder; lane 1, PCR product amplified from plasmid pEGFP-C1 as positive control; lane 2, Genomic DNA (10 µg) of non-transformed *P. ostreatus* digested with *Eco*RV as negative control; lane 3, Genomic DNA (10 µg) of randomly selected positive transformants of *P. ostreatus* digested with *Eco*RV.

fusion was sufficient to allow both hygromycin resistance and EGFP fluorescence.

DISCUSSION

The quality of protoplasts and DNA was crucial for successful transformation. Additionally, the transformation method used had a marked effect on transformation frequency. Thus, we focused on these factors in this study, and their effects are discussed in detail below.

Firstly, three multiplex enzymes, a combination of snailase (Sigma, St. Louis, MO, USA) and cellulase (Yakult Honska Co., Nishinomiya, Japan), lysing enzyme (Sigma, St. Louis, MO, USA) and lywallzyme (Guangdong, China) were examined for their ability to efficiently convert mycelia to protoplasts. Lywallzyme produced protoplasts efficiently; more than 10^7 protoplasts per mg fresh weight mycelia in a total of 5 ml osmotic stabilized solution were obtained within 30 min. Lysing enzyme also reached this

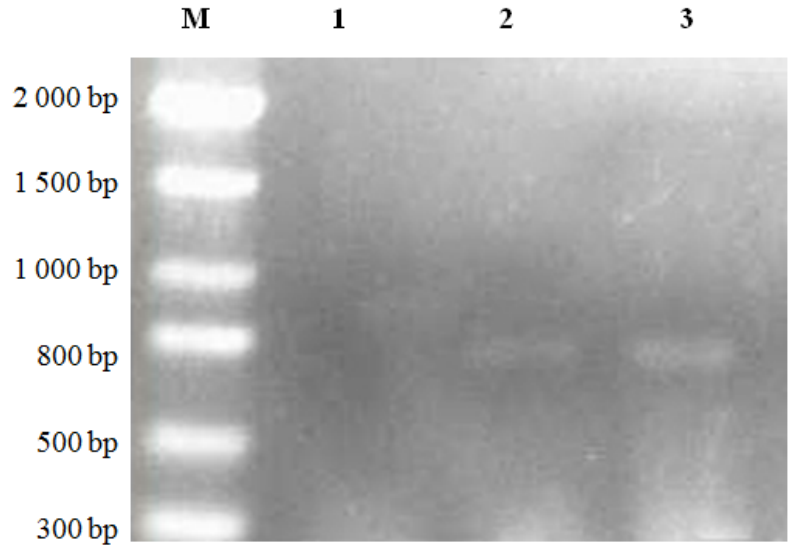

Figure 4. RT-PCR analysis of cDNA isolated from putative transformants. Lane M, 1 kb plus DNA ladder; lane 1, PCR product amplified using cDNA of non-transformed *P. ostreatus* as negative control; lanes 2 and 3, RT-PCR products using cDNA of the selected positive transformants.

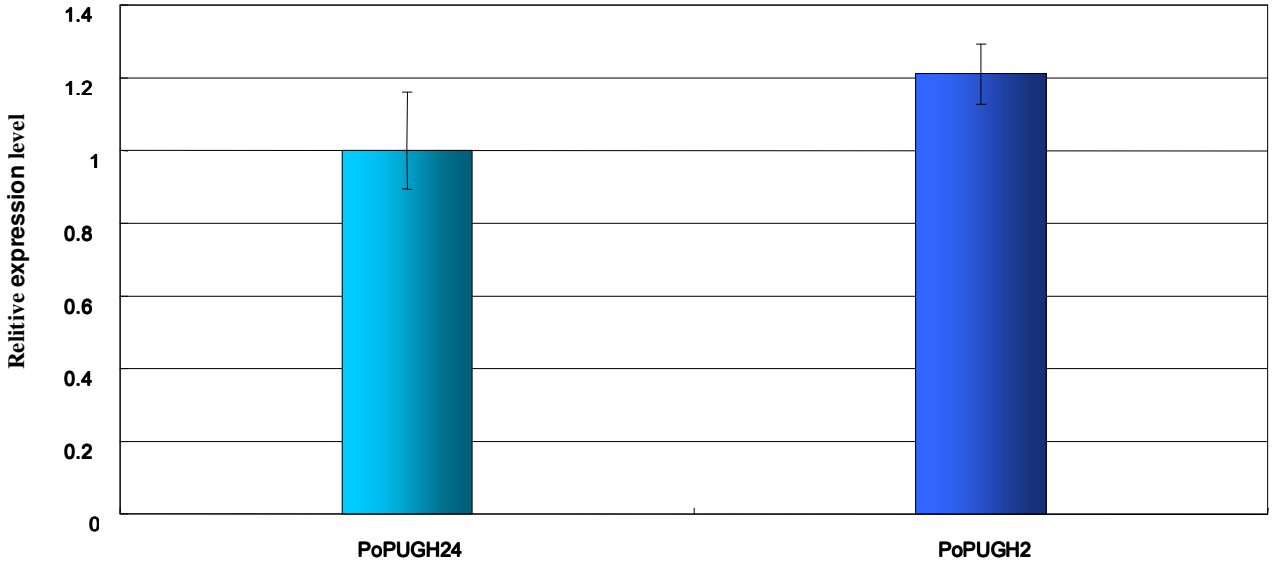

Figure 5. Real-Time quantitative reverse transcriptase-polymerase reaction (RT-PCR) analysis of *egfp* expression. Data represent the means and error bars for the experiment. Relative expression levels during the mycelium of 2 *P. ostreatus* transfromants.

efficiency after more than 2 h. In contrast, the combination of snailase and cellulase did not efficiently degrade the cell wall of *P. ostreatus*. We found that the age of mycelia was critical for efficient production of protoplasts. The maximum protoplast yield was obtained from fast-growing mycelia (usually 3 to 4 days after inoculation to MCM). Moreover, the effect of three osmotic stabilizers, sorbitol, $MgSO_4$ and mannitol, on the protoplast production frequency of *P. ostreatus* was investigated. The highest production frequency was obtained using 0.6 M mannitol as the lywallzyme solvent.

Secondly, to increase the transformation efficiency, several transformation methods were attempted and optimized. In our improved transformation method, vortexing the protoplast and plasmid DNA thoroughly was critical. Although this might cause some loss of protoplasts, it was

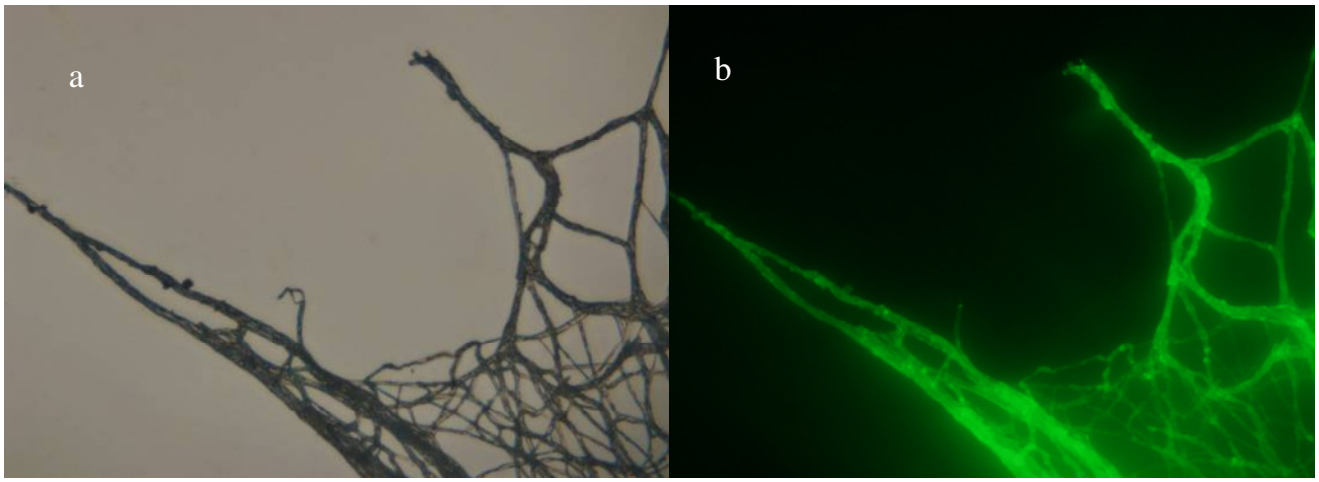

Figure 6. Expression of EGFP in *P. ostreatus*. (a), Detection of the fluorescence expressed by EGFP in the mycelia of the positive transformants under the phase-contrast invrographs; (b), detection of the fluorescence expressed by EGFP in the mycelium of the positive transformants, which was excitated by the blue light . Images were taken with 40× fields of view by Olympus DP72 fluorescence microscope.

better to lose some protoplasts than to fail to mix the DNA with the protoplasts thoroughly (Szewczyk et al., 2006). The negative control indicated that vortexing did not cause the anticipated loss of the regeneration ability of the protoplasts.

Thirdly, Nadine et al. (1987) reported that failure to remove certain restriction enzymes after diges- tion reduced the transforming ability of DNA from 10 to 50-fold.

This might because those certain restriction enzymes remained bound to the DNA ends after digestion, thus generating a substrate unfavorable both for inte- gration and exonucleolyric degradation (Nadine et al., 1987). While, Garnand and Nelson (1995) also reported that the use of linear instead of circular transforming DNA did not significantly increase the generation of stable transformants (Garnand and Nelson, 1995). Moreover, in the present study, linear plasmid DNA produced fewer positive transformants. These findings strongly argued for the report by Wang et al. (2010) that linear plasmid DNA enhanced integration into host genomic DNA. In our opinion, except the possible reasons mentioned above, this might also have been due to the reduced DNA concentration because of the digestion by restriction enzyme and recycling procedure. On the contrary, circular plasmid DNA could produce more positive transformants for the reason of not-reduced concentration of circular plasmids DNA.

In summary, a reliable, integrative and highly efficient transformation system for the *P. ostreatus* was developed. The fused *hph* and *egfp* genes were transformed into *P. ostreatus* using hygromycin B resistance gene as dominant marker. The data show that the fused *egfp-hph* gene was transformed into the genomic DNA of *P. ostreatus*. Southern blot, RT-PCR and fluorescence visualization confirmed the efficiency of this improved transformation method. Further investigation revealed that these transformants were stable and integrated.

The transformation efficiency of this improved method is higher than that reported by other authors (Irie et al., 2001; Li et al., 2006). These results indicate that the improved method represents a marked increase in transformation efficiency over other PEG-mediated methods. This will not only accelerate the transformation of useful gene into *P. ostreatus*, but also facilitate the functional analysis of the gene based on the genomic sequence of this genus and provide an efficient method for the establishment of transformation system of the other edible fungi.

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