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. ostreatus 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-
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 mycelia 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 plates 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 strain 2106 was obtained from the fruiting body were distributed on the potato dextrose agar (PDA) plates. 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 pPgpT- and pTgpT, respectively.

A 1055-bp fragment of the gpd gene promoter region was obtained by digesting pPgpT- with *Sph* I and *Sal* I, and it was ligated into plasmid pUC19 digested with the same enzymes, yielding pUC19-PgpT. A 1001-bp fragment of the gpd gene terminator region was obtained by digesting pTgpT- with *BamH* I and *EcoR* I, and it was ligated into plasmid pUC19-PgpT digested with the same enzymes, yielding pUC19-Pgpd-T. 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 *BamH* I and then cloned into pEGFP-C1, which was also digested with *Sal* I and *BamH* I, resulting in the recombinant 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 ampiclon was digested with *Sal* I and *BamH* I, and the resulting fragment was ligated into pUC19-Pgpd-TgpT, 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% D-glucose, 0.05% MgSO4·7H2O, 0.05% KH2PO4, and 0.1% K2HPO4] (Kim et al., 1999) was collected by filtration through gauze and rinsed three times with 0.6 M mannitol. Mycelia were then incubated for 20 min in 5 ml of 1.5% lywalzyme (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 1000 x 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 CaCl2) to a concentration of 106 to 108 protoplasts per ml for storage at 4°C.

**Highly efficient PEG transformation of protoplasts**

Protoplasts (~106) in 70 µ1 MTC (0.5 M mannitol, 10 mM Tris-HCl (pH7.5), 5 mM CaCl2) buffer were mixed thoroughly with 10 µl of 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 CaCl2; 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 x g) and resuspended in 500 µl STC buffer (1 M.
sorbitol, 100 mM CaCl₂, 10 mM Tris-HCl (pH 7.5)). Protoplasts were then centrifuged (4°C, 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°C 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°C 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°C, followed by 30 cycles of 30 s denaturation (95°C), 1 min annealing (56°C) and 40 s elongation (72°C).

**Table 1. Primers used in the present study.**

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequences (5′→3′)</th>
<th>Restriction site</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pgpd-F</td>
<td>ATTAGCATGCCGAATTGGTTGTT</td>
<td>Sph I</td>
<td>This work</td>
</tr>
<tr>
<td>Pgpd-R</td>
<td>AAGTCGACATTCAAGCAGTCATGGAT</td>
<td>Sal I</td>
<td>This work</td>
</tr>
<tr>
<td>Tgpd-F</td>
<td>TAAGGATCCGAAAGGGCTGTGACCTCAGACTCT</td>
<td>BamH I</td>
<td>This work</td>
</tr>
<tr>
<td>Tgpd-R</td>
<td>TACAGGAGTCATCATACACCCCTACCGACATCT</td>
<td>Sac I</td>
<td>This work</td>
</tr>
<tr>
<td>hph-F</td>
<td>ACCGTCCAGTTTCTGGAAGCTC</td>
<td>Xho I</td>
<td>This work</td>
</tr>
<tr>
<td>hph-R</td>
<td>AAGTCGACATTCAAGCAGTCATGGAT</td>
<td>Sal I</td>
<td>This work</td>
</tr>
<tr>
<td>EGFP-F</td>
<td>AAGTCGACATTCAAGCAGTCATGGAT</td>
<td>Sal I</td>
<td>This work</td>
</tr>
<tr>
<td>EGFP-R</td>
<td>AATGGGATCCGAAAGGGCTGTGACCTCAGACTCT</td>
<td>BamH I</td>
<td>This work</td>
</tr>
</tbody>
</table>

Underlined letters corresponding to the restriction site.

**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.
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.

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

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 EcoRV as negative control; lane 3, Genomic DNA (10 µg) of randomly selected positive transformants of P. ostreatus digested with EcoRV.

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...
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...
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 digestion 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 integration and exonucleolytic 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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