An Efficient PEG/CaCl₂-Mediated Transformation Approach for the Medicinal Fungus Wolfiporia cocos

Qiao Sun¹, Wei Wei², Juan Zhao¹, Jia Song¹, Fang Peng¹, Shaopeng Zhang¹, Yonglian Zheng¹, Ping Chen¹, and Wenjun Zhu*¹

¹College of Biology and Pharmaceutical Engineering, Wuhan Polytechnic University, Wuhan 430023, P.R. China
²Institute for Interdisciplinary Research, Jianghan University, Wuhan 430056, P.R. China

Sclerotia of Wolfiporia cocos are of medicinal and culinary value. The genes and molecular mechanisms involved in W. cocos sclerotial formation are poorly investigated because of the lack of a suitable and reproducible transformation system for W. cocos. In this study, a PEG/CaCl₂-mediated genetic transformation system for W. cocos was developed. The promoter P_gpd from Ganoderma lucidum effectively drove expression of the hygromycin B phosphotransferase gene in W. cocos, and approximately 30 transformants were obtained per 10μg DNA when the protoplast suspension density was 10⁶ protoplasts/mL. However, no transformants were obtained under the regulation of the PtrpC promoter from Aspergillus nidulans.

Keywords: Genetic transformation, medicinal and culinary value, promoter, Wolfiporia cocos

The sclerotia of Wolfiporia cocos, also known as Fuling in China, have been used as an edible mushroom and in traditional Chinese medicines for centuries [3, 4, 7]. W. cocos sclerotial formation is dependent on parasitism of the wood of Pinus species [8, 13]. Studying the parasitic mechanisms and the genetic basis of sclerotial development will improve our understanding of the overall biology of the fungus and may facilitate its commercial production. However, compared with ascomycota fungi, genetic transformation is generally more difficult for basidiomycota fungi [12] and the efforts to understand the sclerotial formation of W. cocos sclerotia have been limited by the lack of suitable, easily reproducible transformation methods. Therefore, we developed a stable and reproducible PEG/CaCl₂-mediated protoplast transformation method for W. cocos.

The W. cocos strain AS5.78 and Ganoderma lucidum strain ZM5.29 were obtained from the Agricultural Culture Collection of China, Institute of Soil and Fertilization, Chinese Academy of Agricultural Sciences, Beijing, China. Growth of W. cocos was almost completely inhibited when inoculated on PDA medium containing ≥5μg/ml hygromycin B (Fig. 1). In order to reduce the possibility of false positives, 20μg/ml hygromycin B was used for selection of W. cocos transformants in further experiments.

G. lucidum P_gpd (NCBI Accession No.: DQ404345.1) [5] was amplified by PCR with a pair of specific primers P_gpd-L 5’ and P_gpd-R. Primers sequences are listed in Table 1. The products were digested by Apal and SalI. To construct a hph expression vector driven by P_gpd, the vectors pBluescript II KS(+) and pTFCM [10], carrying hygromycin B phosphotransferase gene (hph) under the control of the Aspergillus nidulans PtrpC promoter, were both digested by Clal and XbaI. The hph-TrrpC fragment from pTFCM was ligated with pBluescript II KS(+) to generate the vector KS hph-TrrpC. The KS hph-TrrpC vector was then digested by Apal and SalI, and the P_gpd fragment was ligated with KS hph-TrrpC to generate the vector KS P_gpd-hph-TrrpC. The KS P_gpd-hph-TrrpC and the pCAMBIA3300 were then digested by Apal and XbaI, and the P_gpd-hph-TrrpC fragment was ligated with pCAMBIA3300 to generate the vector P_gpd-hph-TrrpC 3300.
The present study showed that the use of an appropriate suspension density of $10^7$ protoplasts/ml, probably because of nutritional and/or space limitations. In addition, as described previously [2], a high density of protoplasts may also lead to fungal overgrowth, which renders subsequent isolation of single-colony transformants difficult.

Twenty putative W. cocos transformants were selected randomly for the transformant stability analysis. After culturing on PDA medium without antibiotics for five generations, all of the transformants were subcultured on PDA medium supplemented with 20 $\mu$g/ml hygromycin B. Seventeen of the putative transformants showed resistance to 20 $\mu$g/ml hygromycin B and to a high concentration of hygromycin B (50 $\mu$g/ml), suggesting that the hph gene was stably maintained and expressed in the transformants. Six of the putative transformants (A10, B12, B23, B37, C15, and C25) were selected randomly for further research. Phenotype analysis of the transformants indicated that, compared with the wild-type strain, there was no obvious change in growth rate (Fig. 2A) or colony morphology (Fig. 2B).

Genomic DNA of the wild-type strain AS5.78 and six randomly selected transformants subcultured on PDA medium for five generations was extracted for identification. PCR amplification using the primer pair hph-L and hph-R (see primers in Table 1) showed that a 880bp fragment was amplified from the genomic DNA of the transformants but not from the wild-type strain AS5.78 (Fig. 3A). To analyze the expression of the hph gene, total RNAs of fungal strains were isolated with TriZOL reagent (Invitrogen, Carlsbad, CA, USA). W. cocos 18S rRNA (NCBI Accession No.: AB022188.1) (see primers in Table 1) was used to normalize the different samples. The analysis indicated that the hph gene was expressed in all transformants (Fig. 3B). Southern blot analysis was performed following the method described by Liu et al. [9] and demonstrated that the hph gene was successfully integrated into the genome of the W. cocos transformants and that all of the randomly selected transformants possessed a single-copy hph gene insertion (Fig. 3C).

The present study showed that the use of an appropriate

---

**Fig. 1.** The minimum inhibitory concentration of hygromycin B to W. cocos.

The mycelial plugs from the active colony edge of wild-type strain AS5.78 was inoculated onto PDA plates supplemented with different concentrations of hygromycin B (0, 2.5, 5, 7.5, and 10 $\mu$g/ml) at 25°C, and then the colony diameter was measured.

---

**Fig. 2.** Colonies of transformants isolated from PDA plates supplemented with different concentrations of hygromycin B (0, 2.5, 5, 7.5, and 10 $\mu$g/ml).

---

**Fig. 3.** Southern blot analysis of transformants. (A) Southern blot analysis of genomic DNA of the wild-type strain AS5.78 and six randomly selected transformants subcultured on PDA medium for five generations. (B) PCR amplification using the primer pair hph-L and hph-R (see primers in Table 1) showed that a 880bp fragment was amplified from the genomic DNA of the transformants but not from the wild-type strain AS5.78 (Fig. 3A). (C) Southern blot analysis of transformants. (D) PCR amplification using the primer pair hph-L and hph-R (see primers in Table 1) showed that a 880bp fragment was amplified from the genomic DNA of the transformants but not from the wild-type strain AS5.78 (Fig. 3A). (E) Southern blot analysis of transformants. (F) PCR amplification using the primer pair hph-L and hph-R (see primers in Table 1) showed that a 880bp fragment was amplified from the genomic DNA of the transformants but not from the wild-type strain AS5.78 (Fig. 3A).

---

**Table 1.** Primers used in this study.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>P$_{ptr}$-L</td>
<td>5' GGCCGCTTCAAGCCCGCTCATGAGCAT 3'</td>
</tr>
<tr>
<td>P$_{ptr}$-R</td>
<td>5' GTCCGCTTGAAGCCGGATTGAGCTG 3'</td>
</tr>
<tr>
<td>hph-L</td>
<td>5' TCTGCCGCGAGTTGCT 3'</td>
</tr>
<tr>
<td>hph-R</td>
<td>5' ATCAGGGTCTCGAGGCTG 3'</td>
</tr>
<tr>
<td>18S rRNA-L</td>
<td>5' GTTCGGTATCTCATGAACTTCT 3'</td>
</tr>
<tr>
<td>18S rRNA-R</td>
<td>5' CTGGATTTTCICCTAGGTGCC 3'</td>
</tr>
</tbody>
</table>

The preparation of W. cocos protoplasts was performed as previously described [1]. The PEG/CaCl$_2$-mediated transformation was performed as follows: after mixing the 100 $\mu$l protoplasts suspension (10$^4$, 10$^5$, 10$^6$, or 10$^7$ protoplasts/ml, respectively) and 10 $\mu$l vector (pTFCM or P$_{ptr}$-hph-TtrpC 3300, 1 $\mu$g/ml), the mixture was kept on ice for 5 min. Then 2 ml of PTC (60% (w/v) PEG3350, 10 mM Tris-HCl (pH 7.5), and 50 mM CaCl$_2$) was added. The mixture was kept at room temperature for 30 min, and 30 ml of MTC (0.6 M mannitol, 10 mM Tris-HCl (pH 7.5), and 50 mM CaCl$_2$) was added. The mixture was centrifuged at 3,000rpm for 5 min. The pelleted protoplasts were suspended in 2 ml of RCM (0.2% tryptone, 0.2% yeast extract, 2% glucose, and 0.6 M mannitol) and incubated at 25°C for 48 h. The suspension was spread on 10 RCM agar plates (20 ml/plate) containing 20 $\mu$g/ml hygromycin B and incubated at 25°C for 10 days until the colonies appeared. Each transformation with different protoplasts density was repeated at least twice.

Transformation results showed that by using the P$_{ptr}$ promoter, an increase in the number of putative W. cocos transformants was obtained with increasing protoplast suspension density (10$^4$, 10$^5$, and 10$^6$ protoplasts/ml, respectively), ranging from 5 to 30 transformants/10 $\mu$g DNA. No colonies were obtained when the PtrpC promoter was used for transformation. However, the yield of transformants was less than 10 per 10 $\mu$g DNA at a
The promoter was an additional important factor that affects the transformation efficiency of filamentous fungi [6, 11]. The \( P_{gpd} \) promoter from a closely related member of the Polyporaceae, \( G. lucidum \), was able to effectively and stably drive expression of \( hph \) in \( W. cocos \). Despite five repeat attempts, no transformant was obtained when the \( P_{tr public\text{domainiddy}C} \)
promoter from the ascomycetous fungus *A. nidulans* was used, which indicated that promoters from ascomycetous fungi may be unsuitable for driving gene expression in the basidiomycete *W. cocos*. Meanwhile, we also tried to optimize the transformation system by altering other factors, such as the amount of vector used and the incubation time of protoplasts in RCM after treating with PTC and MTC solutions. However, no obvious enhancement in transformation frequency was observed. More optimization for other factors, including the use of different osmosis reagents, could be explored in a further study.

In summary, we developed a convenient and stable genetic transformation system for the economically important fungus *W. cocos* and highlighted the importance of using an appropriate promoter to drive expression of the antibiotic resistance gene hph during transformation. The yield of *W. cocos* transformants could be improved by using promoters isolated from *W. cocos*. The transformation method will facilitate further gene functional studies of *W. cocos*.

**Acknowledgments**

We are grateful to Pro Daohong Jiang, College of Plant Science and Technology, Huazhong Agricultural University, for the vectors donation. This research was supported by the State of Traditional Chinese Medicine Industry Research Program (No. 201107009).

**References**