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An Efficient PEG/$CaCl_2$-Mediated Transformation Approach for the Medicinal Fungus *Wolfiporia cocos*

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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/$CaCl_2$-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 10 μg DNA when the protoplast suspension density was 10^6 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 mushrooms [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/$CaCl_2$-mediated protoplast transformation method for *W. cocos*.

The *W. cocos* strain AS5.78 and *Ganoderma lucidium* 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 ≥5 μg/ml hygromycin B (Fig. 1). In order to reduce the possibility of false positives, 20 μg/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 *Apa*I and *Sal*I. 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 *Cla*I and *Xba*I. The *hph-TtrpC* fragment from pTFCM was ligated with pBluescript II KS(+) to generate the vector KS *hph-TtrpC*. The KS *hph-TtrpC* vector was then digested by *Apa*I and *Sal*I, and the P_{gpd} fragment was ligated with KS *hph-TtrpC* to generate the vector KS P_{gpd}-*hph-TtrpC*. The KS P_{gpd}-*hph-TtrpC* and the pCAMBIA3300 were then digested by *Apa*I and *Xba*I, and the P_{gpd}-*hph-TtrpC* fragment was ligated with pCAMBIA3300 to generate the vector P_{gpd}-*hph-TtrpC* 3300.

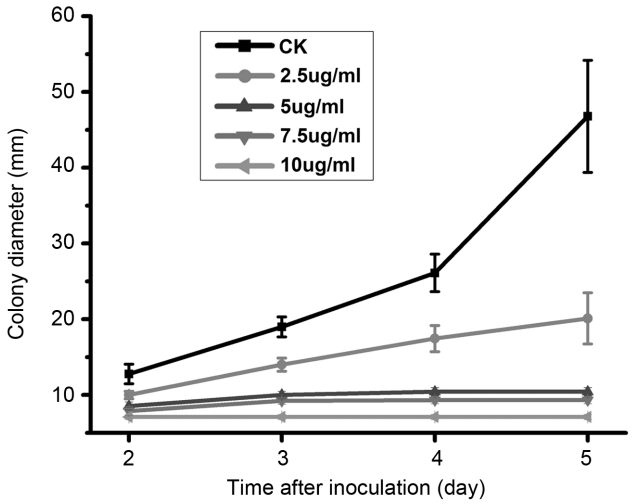

Fig. 1. The minimum inhibitory concentration of hygromycin B to *W. cocos*.

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 μg/ml) at 25°C, and then the colony diameter was measured.

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 μl protoplasts suspension (10^4, 10^5, 10^6, or 10^7 protoplasts/ml, respectively) and 10 μl vector (pTFCM or P_{gpd}-*hph-TtrpC* 3300, 1 μg/μl), 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 3,000 rpm 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 μg/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_{gpd} 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 μg DNA. No colonies were obtained when the *PtrpC* promoter was used for transformation. However, the yield of transformants was less than 10 per 10 μg DNA at a 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.

Table 1. Primers used in this study.

Primer	Sequence
P_{gpd}-L	5' GGGCCCTCCAAAGCCGCTCTCATGGCAT 3'
P_{gpd}-R	5' GTCGACGTTGAGAGGGGGATGAAGAGTGAG 3'
hph-L	5' TCTGCGGGCGATTTGT 3'
hph-R	5' ATCAGGTCGGAGACGCTG 3'
18S rRNA-L	5' GTTCGGTGATTCATGATAACTTCT 3'
18S rRNA-R	5' CTTTGATTTCTCGTAAGGTGCC 3'

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 μg/ml hygromycin B. Seventeen of the putative transformants showed resistance to 20 μg/ml hygromycin B and to a high concentration of hygromycin B (50 μg/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 880 bp *hph* gene 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

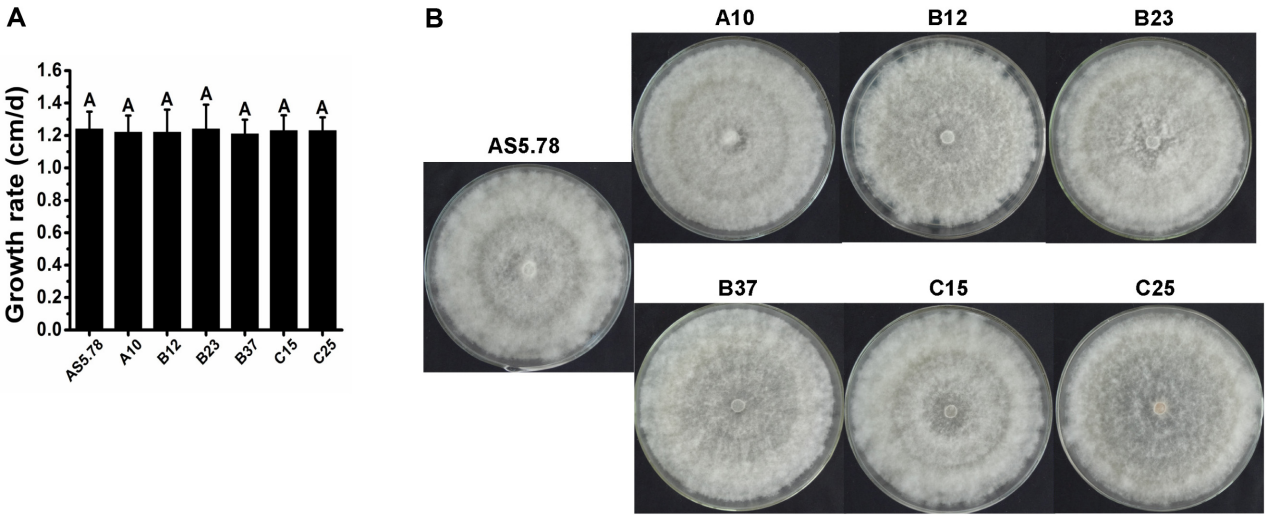

Fig. 2. Comparison of growth rate and colony morphology of *W. cocos* among wild-type strain AS5.78 and the randomly selected transformants.

(**A**) Hyphal growth rates of *W. cocos* based on colony diameter of cultures. Growth rates were examined on PDA at 25°C. The vertical bars represent standard errors based on 10 replicates. Within columns, means followed by the same upper case letter within each chart are not significantly different ($p < 0.05$) according to the least-significant-difference test. (**B**) The colony morphology produced by AS5.78 and transformants. Colonies were grown on PDA for 15 days at 25°C.

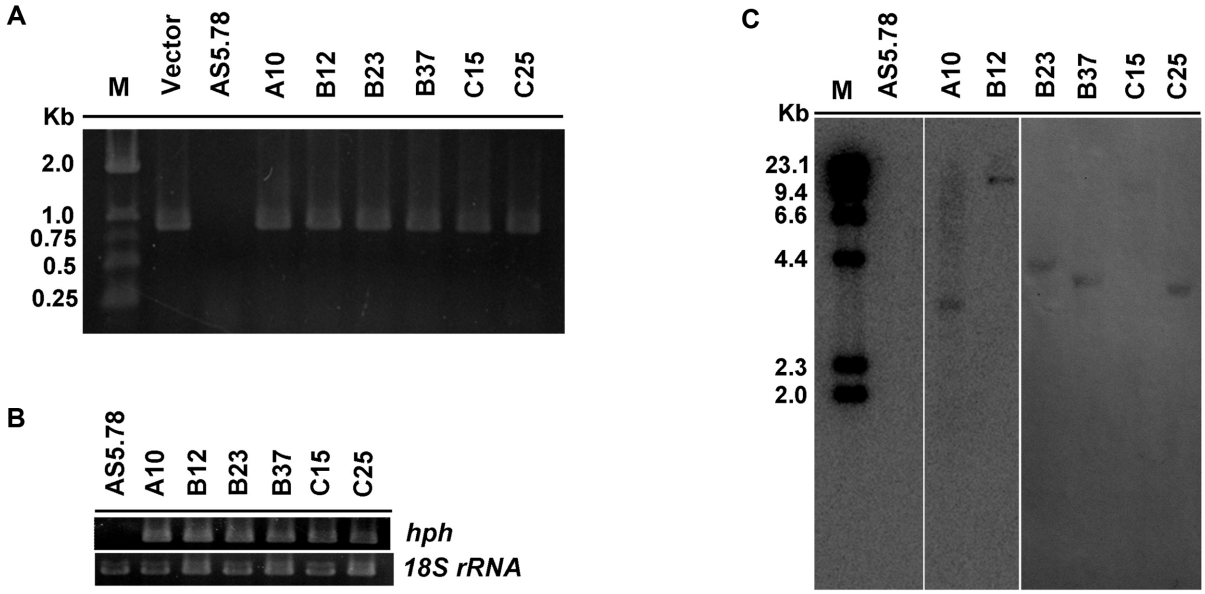

Fig. 3. Hybridization analysis of *hph* gene in *W. cocos* transformants and its expression.

(**A**) PCR analysis of *W. cocos* transformants by amplifying the *hph* fragment. Vector P_{gpd}-*hph*-*TtrpC* 3300 and strain AS5.78 were used as positive and negative control, respectively. (**B**) RT-PCR analysis of the transcription of *hph* in wild-type strain AS5.78 and transformants. The *W. cocos* 18S rRNA was used as the reference gene in this study. (**C**) Southern blot analysis, displaying only one copy of gene *hph* in the transformants but not AS5.78. All genomic DNA samples were digested with *Bam*HI. The *hph* gene PCR fragment was used as a probe.

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 *PtrpC*

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

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