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Transformation of the Edible Basidiomycete, *Pleurotus ostreatus* to Phleomycin Resistance

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For transformation of *Pleurotus ostreatus*, two novel vectors, pPhKM1 and pPhKM2, were constructed, using the regulatory sequences of the *P. sajor-caju* β-tubulin gene (*TUB1*) and the *ble* gene encoding phleomycin binding protein. pPhKM1 contains *ble* fused to the *TUB1* promoter and the *Schizophyllum commune GPD* terminator. pPhKM2 contains *ble* fused to the promoter and terminator regions of *P. sajor-caju TUB1*. To confirm phleomycin-resistance activity, each vector was cotransformed with pTRura3-2 into the *P. ostreatus* homokaryotic *ura*⁻ strain. The transforming DNA was stably integrated into the genomic DNA. Subsequently, phleomycin resistance was conferred on wild-type dikaryotic *P. ostreatus* by transformation with pPhKM1 or pPhKM2. This transformation system generated stable phleomycin-resistant transformants.

KEYWORDS **Basidiomycete, Phleomycin resistance, *Pleurotus ostreatus*, Transformation, β-tublin**

Pleurotus ostreatus (Jacq.: Fr.) Kummer, the oyster mushroom, is one of the most widely cultivated and important edible mushrooms in the world (Chang and Miles, 1989). Recently, not only its edibility but also its ability to degrade various chemical compounds has attracted attention. The first example of transformation of *P. ostreatus*, using the vector pAN7-1 to confer hygromycin B resistance, was described in 1993 (Peng *et al.*, 1993). In that experiment, however, the introduced DNA was replicated extrachromosomally and was unstable. Carboxin and hygromycin resistance were subsequently reported as selection markers (Honda *et al.*, 2000; Irie *et al.*, 2001). In basidiomycetes the use of phleomycin resistance as a dominant selection marker for *Schizophyllum commune* basidiomycetes was first described in 1994 (Schuren and Wessels, 1994) using the pGPhT vector carrying the *ble* gene, which encodes a phleomycin-binding protein, under control of the regulatory sequences of the *S. commune* glyceraldehyde-3-phosphate dehydrogenase gene (*GPD*). Recently, a white-rot basidiomycete, *Trametes versicolor*, was transformed with this plasmid to express phleomycin resistance (Bartholomew *et al.*, 2001). However, this vector has not been effective in *P. ostreatus*. Because several kinds of selection markers are required in molecular genetic research, we developed another selectable marker, phleomycin resistance, in *P. ostreatus*. For efficient genetic transformation, we constructed two novel phleomycin-resistance vectors for *P. ostreatus*, using the promoter and transcription terminator regions of the β-tubulin (*TUB1*) gene from *P. sajor-caju*. We successfully transformed a wild-type dikaryotic strain of *P. ostreatus* to express phleomycin resistance, using both vectors.

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Materials and Methods

Strains and plasmids. The uracil auxotrophic mutant strain ASI2029-8 of *P. ostreatus* is obtained from a stock culture in the National Institute of Agricultural Science and Technology (NIAST) in Korea. *Pleurotus ostreatus* K01 is maintained in the Forestry and Forest Product Research Institute (FFPRI) in Japan. Plasmid pTRura3-2 containing the *Trichoderma reesei ura3* gene (Berges and Barreau, 1991) and pGPhT (Schuren and Wessels, 1994), carrying the phleomycin-resistance gene (*ble*) under control of the *S. commune GPD* promoter and terminator regions, were used. *TUB1* was cloned from genomic DNA of *P. sajor-caju* was cloned and sequenced as described previously (Kim *et al.*, 2001).

Construction of pPhKM1 and pPhKM2 vectors. The following vectors were constructed in this study (Fig. 1).

(1) pGPhT2: The *Hin*dIII and *Eco*RI fragment endcoding the phleomycin resistant gene, regulatory sequences and terminator sequences of pGPhT was ligated into *Hin*dIII-, *Eco*RI-digested pGEM3Zf (+) vector (Promega, WI, USA).

(2) pBP: An exact fusion between the translation start site of *ble* and the *TUB1* promoter was generated by PCR (Fig. 1) (Stappert, 1994). First, the *TUB1* promoter region was amplified using the T7 primer and primer p1 (5'-GGTCAACTTGGCCATTCTACGGTAGAAAGGGTG-3')

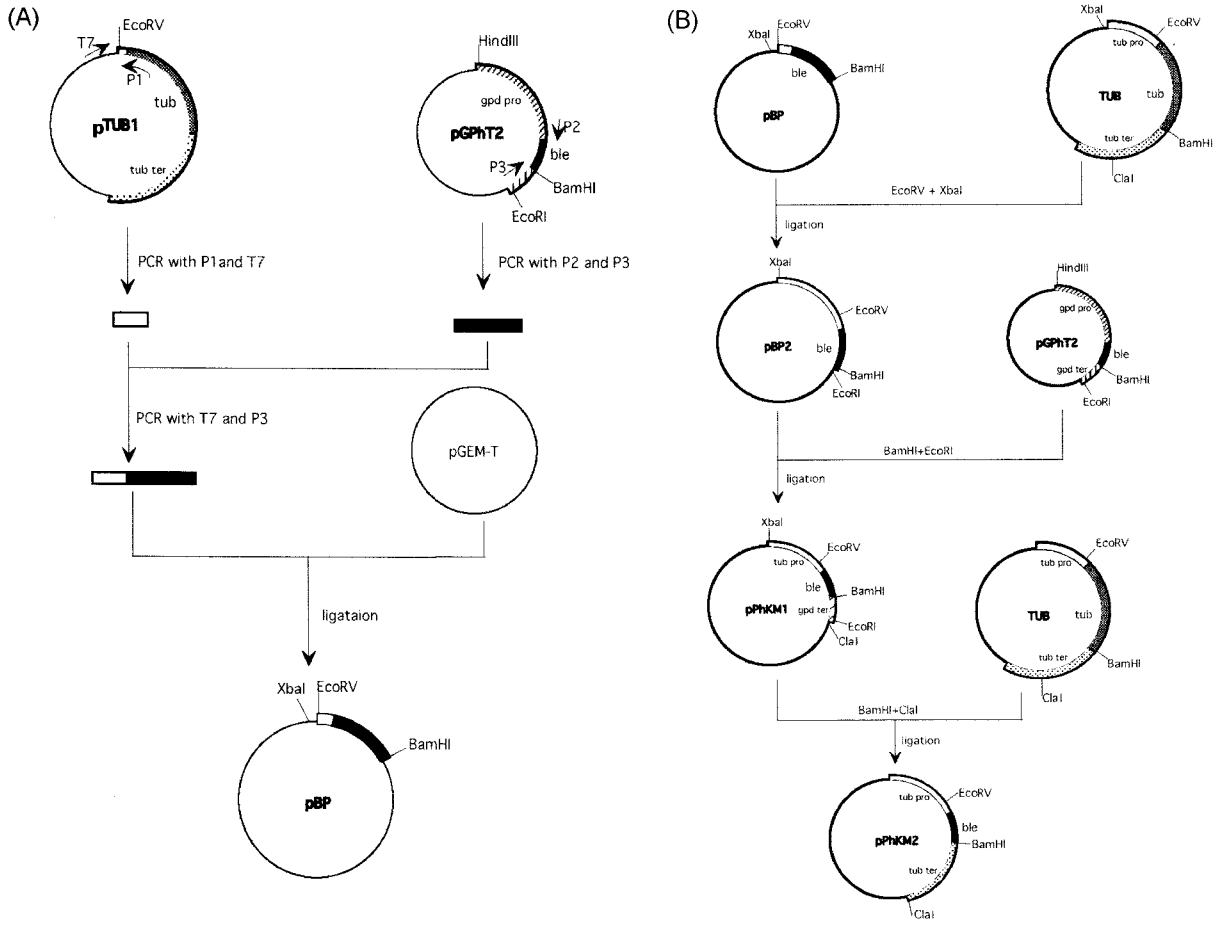

Fig. 1. Construction of the vetors pPhKM1 and pPhKM2. (A) pBP: Exact fusion between translation start site of *ble* and TUB1 promoter was generated by PCR. primer p1: 5'-GGTCAACTTGGCCATTCTACGGTAGAAAGGGTG-3', p2: 5'-AGAATGGCCAAGTTGACC-3', p3: 5'-GCTGATCGCGATGCCGG-3' (B) Construction of pPhKM1 and pPhkM2 vector using the pBP and pGPhT2 and -tubulin genomic clone.

from a *TUB*1 *Eco*RV subclone. *ble* was amplified with primers p2 (5'-AGAATGGCCAAGTTGACC-3') and p3 (5'-GCTGATCGCGATGCCGG-3') using pGPhT as the template. Both amplified fragments were mixed together and PCR-amplified again using primers T7 and p3. The final PCR product was ligated into the pGem-T vector (Promega).

(3) pPhKM1: After the rest of the *TUB*1 promoter was ligated into pBP, the *GPD* terminator region was cut from pGPhT2 with *Bam*HI and *Eco*RI and ligated into pBP.

(4) pPhKM2: The *GPD* terminator of pPhKM1 was replaced with the *TUB*1 terminator.

Transformations of *P. ostreatus*. Vegetative mycelia were incubated in MCM (0.2% yeast extract, 0.2% bactopeptone, 0.05% $MgSO_4 \cdot 7H_2O$, 0.05% KH_2PO_4, 0.1% K_2HPO_4) at 25°C for 5 days. Mycelia were recovered by filtration, washed in 0.6 M sucrose, and resuspended in 10 m*l* lysis buffer containing 20 mg Onozuka RS cellulase (Yakult Pharmaceutical Industries Co. Ltd.), 5 mg Zymolase 5000 (Kirin Brewery Co. Ltd.), 4 mg chitinase (Calbiochem-Behring Corp.), and 30 μl β-glucuronidase (Sigma Chemical Co.). The suspension was incubated at 25°C for 1~3 hrs with agitation. Protoplasts were recovered by filtration through a glass filter (pore size G3) and collected by centrifugation. The collected protoplasts were resuspended in 10 m*l* STC (0.6 M sucrose, 10 mM Tris-HCl [pH 7.5], 10 mM $CaCl_2$), centrifuged again, and resuspended to a final concentration of 5×10^7 protoplasts/m*l* in STC. Vector DNA (5 μg) and 50 μl PTC (60% polyethylene glycol 3350, 10 mM Tris-HCl [pH 7.5], 10 mM $CaCl_2$) were added to 200 μl of protoplast suspension and the reaction mixture was kept on ice for 20 min. Finally, 600 μl of PTC was added and the mixture was further incubated at room temperature for 20 min. To cotransform the *ura*$^-$ strain (Kim *et al.*, 1999), protoplasts were first plated on minimal medium and the regenerated colonies were transferred to medium containing 50 μg/m*l* phleomycin. The transformed protoplasts of wild-type strains were plated directly onto agar medium containing phleomycin (50 μg/m*l*).

Southern blot analysis. Genomic DNA was prepared according to the method described by Raeder and Broda (1985) using the lyophilized mycelia of the transformants.

Genomic DNA (5 μg) was separated electrophoretically on a 0.7% agarose gel. *Bam*HI restricts pPhKM1 and pPhKM2 at one site and pGPhT2 at two sites. The DNA was blotted onto nylon membrane (Hybond-N, Amersham Pharmacia Biotech, Buckinghamshire, England) according to the method of Sambrook *et al.* (1989).

The Southern blots were hybridized with a *ble* probe labeled with digoxigenin (Roche Molecular Biochemicals, Mannheim, Germany).

Analysis of transformants. To confirm the stable inheritance of transformed DNA, one cotransformant was mated with a compatible wild-type monokaryon. The resultant dikaryon was cultivated on sawdust and rice-bran medium until it produced fruiting bodies. Radial mycelial growth of dikaryotic transformants on malt extract agar (malt extract 2.0%, agar 1.8%) was measured after triplicate samples were grown for a week.

Results and Discussion

Cotransformation of *ura*⁻ monokaryon. To confirm the phleomycin-resistance activity of the constructed vectors, we first cotransformed a homokaryotic uracil auxotroph of *P. ostreatus* (Kim *et al.*, 1999). *Pleurotus ostreatus ura*⁻ strain was cotransformed with pTRura3-2 plus either of the constructs pPhKM1 or pPhKM2. *ura*⁺ transformants were selected on minimal medium, and then tested for phleomycin resistance. The transformation efficiency of pTRura3-2 was about 10 colonies/μg DNA. About 10% of *ura*⁺ transformed colonies were resistant to phleomycin (50 μg/ml). The transformation efficiencies of the pPhKM1 and pPhKM2 vectors were not significantly different.

When undigested genomic DNA of the *ura*⁺ phleomycin-resistant transformants was hybridized with the *ble* probe, a single high-molecular-weight band corresponding to the genomic DNA was observed (Fig. 2A). This result indicates that the vector DNA was integrated into the genome and did not exist as an extrachromosomal form. *Bam*HI digested-genomic DNA of phleomycin-resistant transformants showed that the vector DNA was integrated into the genome at multiple sites, whereas no hybridizing signal was detected in the original *ura*⁻ strain (Fig. 2B).

Although the same amounts of genomic DNA were loaded on the gel, hybridization signals were highly variable because of differences in copy number and integration of the vector into the chromosome. However, no particular integration patterns correlated with a specific vector.

Transformation of wild-type dikaryotic strain. After the phleomycin-resistance activity of the vectors was confirmed by cotransformation of uracil auxotrophic *P. ostreatus*, we transformed a wild-type dikaryotic strain directly with pGphT2, pPhKM1, or pPhKM2. Transformants were selected on phleomycin-containing medium. It is often difficult to use drug resistance as a selective marker for basidiomycetes because of high background signal. In this study, it was also impossible to avoid abortive colonies emerging on phleomycin medium. At a phleomycin concentration of 25 μg/ml, too many abortive colonies were regenerated on the medium, whereas at a concentration of 100 μg/ml, no transformants were recovered. At 50 μg/ml phleomycin, abortive transformants still regenerated on the agar medium, but the truly resistant colonies could be isolated after prolonged culture by their more vigorous growth. Finally, colonies were grown twice on phleomycin medium, to avoid the selection of false transformants. At this stage, a difference in colony size became apparent between the pPhKM1, pPhKM2, and pGPhT2 transformants (Fig. 4). After two weeks of growth on non-selective medium, vigorously growing transformants were selected.

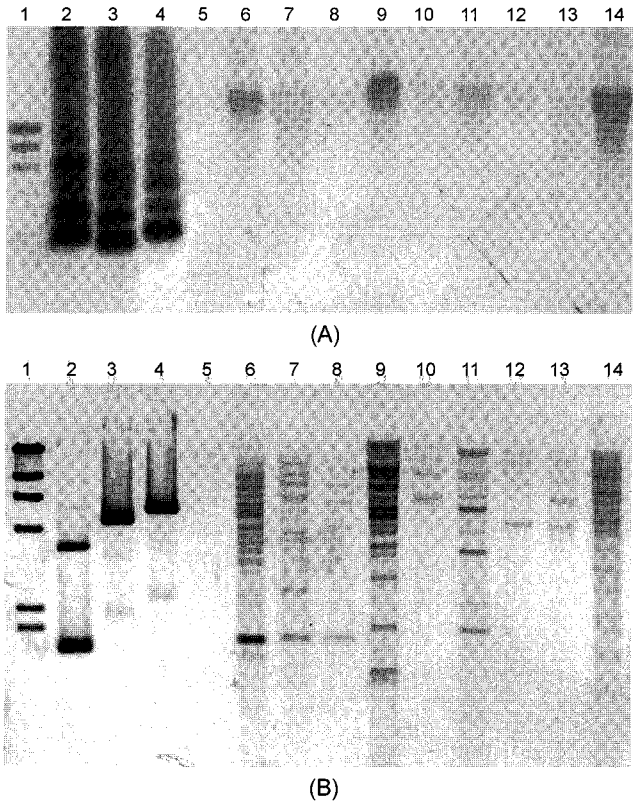

Fig. 2. Southern hybridization analysis of co-transformants. (A) Undigested genomic DNA was hybridized with Dig-labelled *ble*. Lane 1: λ/*Hin*dIII DNA marker, Lane 2: undigested pGphT2 vector, Lane 3: undigested pPhKM1, Lane 4: undigested pPhKM2, Lane 5: *ura*⁻ strain ASI2029, Lanes 6-8: pGphT2 co-transformants, Lanes 9-11: pPhKM1 co-transformants, Lanes 12-14: pPhKM2 co-transformants. (B) *Bam*HI digested genomic DNA was hybridized with Dig-labeled *ble*. Lane no. are the same as in Fig. 2A.

Analysis of dikaryotic transformants. The mycelial growth of transformants differed when vectors included

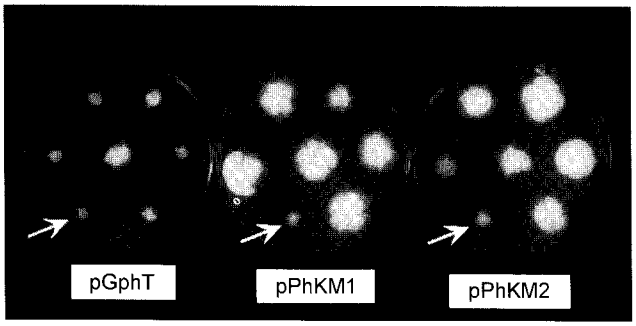

Fig. 3. Colonies of pGphT2, pPhKM1 and pPhKM2 dikaryotic transformants. Transformant colonies of each vector were picked up randomley and plated on malt extract agar containing 50 μg/ml of phleomyin. Arrow indicates untransformed control colonies.

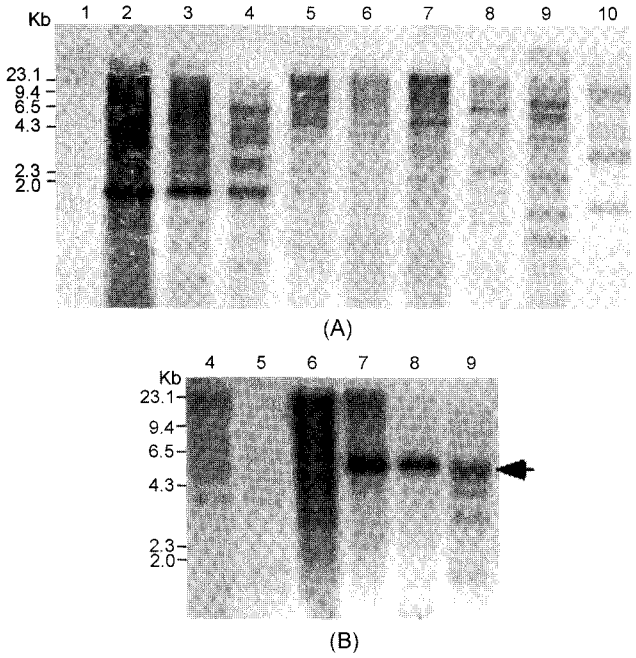

Fig. 4. Southern hybridization analysis of dikaryotic transformants. A: Dig-labeled *ble* was hybridized with *Bam*HI digested genomic DNA of each transformants. Lane 1: untransformed Original strain 2-4: pGphT2 transformants, 5-7: pPhKM1 transformants, 8-10: pPhKM2 transformants. B: After one year of propagation on non-selective medium. Lane no. are the same as in Fig. 4A.

the *S. commune GPD* promoter or the *P. sajor-caju TUB1* promoter. The construct pGPhT2, which contains the *S. commune GPD* promoter, showed slower colony growth than did pPhKM1 or pPhKM2 (Fig. 3). Transformants with high-copy-number integrated pGphT2 showed the slowest growth.

A heterologous promoter should work inefficiently, which explains why pGPhT2 vector was not generally used in homobasidiomycetes. The origin of the terminator did not affect the apparent growth of the colony. DNA obtained from transformants was digested with *Bam*HI, which cuts once in the pPhKM1 and pPhKM2 vectors and twice in pGPhT2. Southern hybridization patterns indicated that the DNA was integrated at various sites in the genome. pGPhT2 produced the most uniform integration patterns for *ble*, whereas most variation was observed with pPhKM2 (Fig. 4A).

After one year of propagation on non-selective medium, Southern blotting was analysed again. Southern blot patterns were not changed with pGPhT2 (Data not shown) and pPhKM1 (Fig. 4B). But with pPhKM2, it was different from the previous result. Although low molecular weight bands were still visible, a strong hybridization signal run at about 4.8 kb, which was not present in genome of pPhKM2 transformants previously. The size corresponds to the length of the transforming plasmid. This indicates that pPhKM2 initially integrated at multiple sites of the genome and later was duplicated as tandem repeats in one locus. pGPhT2 caused the repressed growth of the mycelium although the vector DNA was stable after the integration into the genome. As a conclusion, pPhKM1 showed the most stable and suitable character for transforming *P. ostreatus* to phleomycin resistance.

References

Bartholomew, K., Santos, G. D., Dumonceaux, T., Charles, T. and Archibald, F. 2001. Genetic transformation of *Trametes versicolor* to phleomycin resistance with the dominant selectable marker shble. *Appl. Microbiol. Biotechnol.* **56**: 201-204.

Berges, T. and Barreau, C. 1991. Isolation of uridine auxotrophs from Trichoderma reesei and efficient transformation with the cloned *ura3* and *ura5* genes. *Curr. Genet.* **19**: 359-365.

Chang, S.-T. and Miles, P. G. 1989. Edible mushrooms and their cultivation CRC Press Inc, pp. 165-166.

Honda, Y., Matsuyama, T., Irie, T., Watanabe, T. and Kuwahara, M. 2000 Carboxin resistance transformation of the homobasidiomycete fungus *Pleurotus ostreatus*. *Curr. Genet.* **37**: 209-212.

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

Kim, B.-G., Magae, Y., Yoo, Y.-B. and Kwon, S.-T. 1999. Isolation and transformation of uracil auxotrophs of the edible basidiomycete *Pleurotus ostreatus*. *EFMS Microbiol. Lett.* **181**: 225-228.

_____, Yoo, Y.-B., Kwon, S.-T. and Magae, Y. 2001. Molecular characterization of -tubulin gene from *Pleurotus sajor-caju*. *Biosci. Biotechnol. Biochem.* **65**: 2280-2283.

Peng, M., Lemke, P. A. and Singh, N. K. 1993. A nucleotide sequence involved in replicative transformation of a filamentous fungus. *Curr. Genet.* **24**: 114-121.

Raeder, U. and Broda, P. 1985. Rapid preparation of DNA from filamentous fungi. *Lett. Appl. Microbiol.* **1**: 17-20.

Schuren, F. H. and Wessels, J. G. 1994. Highly-efficient transformation of the homobasidiomycete *Schizophyllum commune* to phleomycin resistance. *Curr. Genet.* **26**: 179-183.

Stappert, J. 1994. Methods for generating multiple site-directed mutations *in vitro*. In: Griffin, H. G., Griffin, A. M. PCR Technology Current Innovations. CRC Press Inc, pp. 59-65.