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Expression and Production of Therapeutic Recombinant Human Platelet-Derived Growth Factor-BB in *Pleurotus eryngii*

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Abstract Recombinant human platelet-derived growth factor-BB (*rh*PDGF-BB) is widely used in many therapeutic applications. Until now, there has been no report on *rh*PDGF-BB expressed in fungi. In this study, we tested whether *Pleurotus eryngii* could support the expression of human therapeutic *rh*PDGF-BB protein. A binary vector pCAMBIA1304 containing the *h*PDGF-BB gene was constructed and introduced into *P. eryngii* via *Agrobacterium tumefaciens*-mediated transformation. The transformation of *h*PDGF-BB gene was confirmed by Southern blot and PCR, whereas the expression was confirmed by Western blot analysis. The recombinant *h*PDGF-BB reached a maximum expression level of 1.98% of total soluble protein in transgenic mycelia and was in dimeric form. A bioassay revealed that *h*PDGF-BB expressed in *P. eryngii* increased proliferation of NIH-3T3 cells similarly to standard material. These results suggest that *P. eryngii* can be a robust system for the production of human therapeutic proteins including the *h*PDGF-BB.

Keywords *Agrobacterium tumefaciens* · Human platelet-derived growth factor-BB · *Pleurotus eryngii* · Gene transfer · Gene expression

Introduction

In recent years, platelet-derived growth factor (PDGF) has attracted considerable interest because of its wide range of pharmaceutical applications. PDGF is one of the major mitogens for many cell types of mesenchymal origin and thought to play important roles in

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the pathogenesis of clinically important diseases such as cancer, atherosclerosis [1], and ulcers [2]. It is involved in bone formation, erythropoiesis, angiogenesis, wound healing [3], and in the normal development of the kidney, brain, cardiovascular, and respiratory system [4]. Structurally, PDGF is a disulfide-bonded dimer which consists of five different dimeric proteins, PDGF-AA, PDGF-AB, PDGF-BB, PDGF-CC, and PDGF-DD, built from four different peptide chains (A, B, C, and D) and exert their biological effects through their receptors, PDGFRα and PDGFRβ [5]. However, only recombinant human PDGF-BB (*rh*PDGF-BB) has therapeutically important applications. Human PDGF-BB is a 32-kDa B chain homodimer glycoprotein containing 109 amino acid residues. A gel formulation of recombinant human PDGF (rhPDGF-BB) is clinically approved to treat full-thickness ulcers in diabetic patients [6–8] and has shown efficacy for pressure ulcers [2], other chronic ulcers [9, 10], and non-healing wounds in previously irradiated tissues [11]. Recently, rhPDGF-BB has been introduced in the clinic for the treatment of skeletal disorders [12]. Moreover, it has been reported that rhPDGF-BB is involved in the production of proinflammatory cytokines [13], neuroprotection [14] as well as in regulation of orbital inflammation in Graves' ophthalmopathy [15].

Production of therapeutic proteins by recombinant DNA technology is one of the most important areas in the pharmaceutical industry. Recombinant protein expression systems have been developed in variety of organisms. Previously, several expression systems such as *Escherichia coli* [16–18], Chinese hamster ovary cells [19], *Saccharomyces cerevisiae* [20], and baculovirus [21] have been used to produce *rh*PDGF-BB. However, mushrooms have not been used as a production host for this therapeutic protein until now. Mushrooms play important roles in many human activities, including biotechnological processes, phytopathology, and biomedical research. They form part of the diet in many countries due to their good taste and nutritive value. They are attractive hosts for recombinant DNA technology because of their ability to secrete high levels of bioactive proteins with post-translational processing such as glycosylation [22]. Moreover, they offer several advantages over animal and plant production systems. For example, increased secretion, easy molecular and genetic manipulation, lack of endotoxins, stable storage, cost, and time efficient for marketing [23–25]. *Pleurotus eryngii*, also known as the king oyster mushroom, is one of the most popular and edible mushrooms in Asia, Europe, and North America [26]. The increasing popularity of *P. eryngii* among consumers is due to its flavor, texture, and shelf life [27]. Moreover, it is a higher eukaryotic organism which possesses the complete protein-translational modification and therefore is a good recipient for exogenous gene expression along-with great potential to produce pharmaceutical proteins.

Nevertheless, there is no report on *rh*PDGF-BB expressed in basidiomycete fungi. To investigate the feasibility of using *P. eryngii* for the mass production of *rh*PDGF-BB, we for the first time introduced *h*PDGF-BB gene into edible mushroom *P. eryngii*, using *Agrobacterium*-mediated transformation system and generated transgenic *P. eryngii* expressing the *h*PDGF-BB gene. Here, we report that *rh*PDGF-BB can be produced and expressed in transgenic *P. eryngii*.

Materials and Methods

Strains and Plasmids

E. coli strain DH5α (Invitrogen, Carlsbad, CA, USA) was used for the propagation of plasmids. pCMV-sport6 vector containing *h*PDGF-BB gene was purchased from 21C

Frontier Human Gene Bank (KRIBB, Daejeon, Republic of Korea). pCAMBIA1304 was obtained from BioForge, USA. *Agrobacterium tumefaciens* GV3101 was provided by NEXGEN Biotechnologies, Inc. (Seoul, Republic of Korea). *P. eryngii* was obtained from Rural Development Administration, Republic of Korea.

Construction of Binary Vector Harboring the hPDGF-BB Gene

To construct a binary vector, plasmids pCAMBIA1304 (Fig. 1b) and pCMV-SPORT6 (*h*PDGF-BB; Fig. 1a) were digested with *Afl* III and *Bbs* I, and the *h*PDGF-BB gene (2,718 bp) was inserted into pCAMBIA1304 at the same restriction-enzyme sites. The resulting plasmid was designated pCAMBIA1304 (*h*PDGF-BB; Fig. 1c). The binary vector (14,790 bp) designated pCAMBIA1304 (*h*PDGF-BB) consisted of a pCAMBIA1301 backbone containing the kanamycin resistance gene, hygromycin resistance, and GUS A genes under the control of cauliflower mosaic virus 35S (CaMV35S) promoters. The recombinant plasmid pCAMBIA1304 (*h*PDGF-BB) was transformed into *E. coli* DH5α for propagation. Transformed *h*PDGF-BB gene was confirmed by PCR using combination of primers (Table 1) and by DNA sequencing (090617KR-029, MACROGEN, Seoul, Republic of Korea).

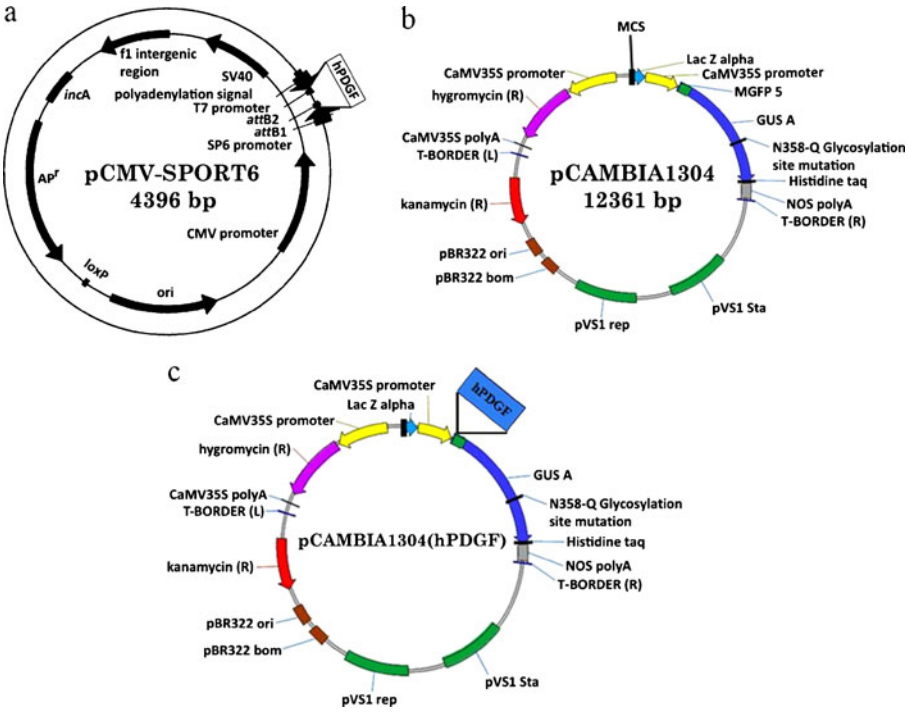

Fig. 1 **a** Plasmid map of pCMV-SPORT6 vector containing *h*PDGF-BB. **b** Plasmid map of pCAMBIA1304 binary vector. **c** Plasmid map of the constructed pCAMBIA1304 binary vector containing *h*PDGF-BB. pCAMBIA1304 is 12,361 bp in size and consists of a pCAMBIA1301 backbone containing the kanamycin resistance gene outside the right and left border sequences of *Agrobacterium* T-DNA. The hygromycin resistance and GUS A genes are located between the border sequences

Table 1 List of primers used for PCR

Genes	Primer sequences	Nucleotides position	PCR product (bp)	Gene reference
pCAMBIA1304	F1: CCC AGG CTT TAC ACT TTA TC	11453–11814	362	AF234300
	R1: CTA AAC TGA AGG CGG GAA AC			
*h*PDGF-BB	F2: GTT CCC TGA CCA TTG CTG AG	563–800	238	BC029822
	R2: GGC TTC TTC CGC ACA ATC TC			
	F3: CTT CCA AAA CCT GCT TCC TT	2558–2577	–	BC029822

Transformation of *Agrobacterium*

The recombinant plasmid pCAMBIA1304 (*h*PDGF-BB) was introduced into *A. tumefaciens* GV3101 by freeze–thaw method [28]. The cells were plated onto LB plates containing 50 μg μl^{-1} kanamycin to obtain positively transformed colonies. The positive colonies were confirmed by PCR (Fig. 2b).

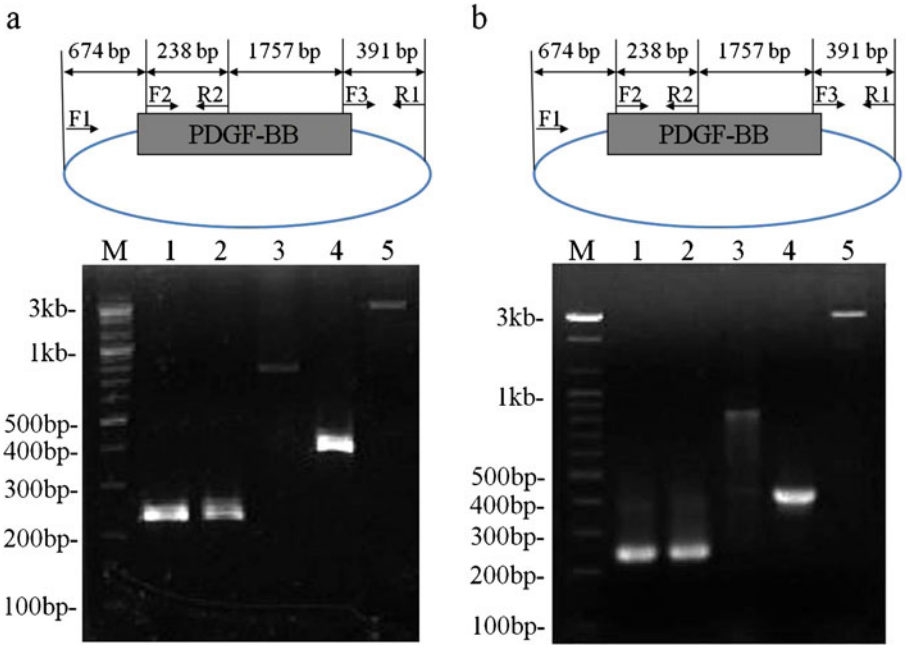

Fig. 2 Identification of the recombinant pCAMBIA1304 containing the *h*PDGF-BB gene, after transformation into *E. coli* and detection of the *h*PDGF-BB from transformed *A. tumefaciens*. PCR analysis of the *h*PDGF-BB from **a** transformed *E. coli* and **b** *A. tumefaciens* GV3101 using the primer combination. *M* 100 bp marker; *lane 1* plasmid pCMV-SPORT6 (*h*PDGF-BB) using primer F2, R2 (control); *lane 2* transformed DNA using primer F2, R2; *lane 3* transformed DNA using primer F1, R2; *lane 4* transformed DNA using primer F3, R1; *lane 5* transformed DNA using primer F1, R1

Explant Preparation and Transformation by Vacuum Infiltration

A. tumefaciens GV3101 carrying plasmid pCAMBIA1304 (*h*PDGF-BB) were grown in 50 ml LB medium supplemented with 50 μg μl^{-1} kanamycin at 28 °C for 2 days to an optical density 1.6 at 600 nm. Bacteria were collected by centrifugation for 30 min at 4,000×*g* and then washed once with 50 ml washing solution containing 10 mM $MgCl_2$ and 100 μM acetosyringone. After centrifugation at 4,000×*g* for another 30 min, pellet of bacteria was resuspended in washing solution to an optical density 1 at 600 nm.

After the veil was cut from the fruiting body of *P. eryngii*, the exposed gill tissue was aseptically excised and sectioned into 1.0×0.5 cm pieces. These pieces were vacuum-infiltrated with the *Agrobacterium* suspension culture for 10 min two times. The evacuated tissues were washed with triple distilled water and dried on sterile Whatman filter paper under aseptic condition for 10 min. The tissues were then transferred to a sterile Petri dish without medium and incubated for 7–14 days in the dark at 25 °C. For selection, the dark cultured active tissues were transferred to PDA medium (20% potato extract, 2% dextrose, and 1.5% Agar) containing 50 μg ml^{-1} hygromycin, 100 μg ml^{-1} cefotaxime, and cultured for 2 to 3 weeks in the dark condition at 25 °C. This dark culture method is highly effective for growing mycelium and inhibiting *Agrobacterium*. Overgrowing putative transformants were then subcultured onto PDA medium at 25 °C for 1 week in the dark. Finally, the mycelia were subcultured on liquid medium containing PDB (20% potato extract, 2% dextrose) for 2 weeks in the shaker at 25 °C, 130 rpm. Mycelia were then separated by filtration through Whatman filter paper and used for further processing.

GUS Histochemical Assay

The pieces of transformed and untransformed mushroom were incubated in cold 90% acetone on ice for 15 min. The acetone was removed and the samples were washed by rinse solution containing 50 mM sodium phosphate buffer (pH 7.2), 0.5 mM potassium ferrocyanide, and 0.5 mM potassium ferricyanide. After removing the rinse solution from samples, staining solution containing 50 mM sodium phosphate buffer (pH 7.2), 0.5 mM potassium ferrocyanide, 0.5 mM potassium ferricyanide, and 2 mM X-Gluc was added and kept on ice. The samples were then subjected to vacuum infiltration on ice for 15 min. The vacuum treatment was repeated two times. The samples were incubated at 37 °C and photographs were taken.

Genomic DNA Extraction and Southern Blot Analysis

To verify the integration of *h*PDGF-BB in *P. eryngii* genome, putative transgenic mycelium was analyzed by PCR using the *h*PDGF-BB specific primers. Twenty microliters of PCR reaction mixture contained 10 pM of the each primers, 1× PCR buffer, 2.5 mM dNTPs, 1 U *Tag* DNA polymerase (TaKara, Kyoto, Japan), and 1 μl of each transformant lysate as the template. PCR was performed by denaturing at 94 °C for 5 min, followed by 35 cycles of amplification (95 °C for 1 min, 55 °C for 30 s, and 72 °C for 10 s), and final extension at 72 °C for 7 min. PCR products were analyzed on 1% agarose gel and visualized by ethidium bromide.

For Southern blot analysis, mycelia were collected from putative transgenic and untransformed mushroom and ground in liquid nitrogen using a pre-chilled mortar and pestle. DNA was then isolated from mycelia following the cetyltrimethylammonium

bromide method by Scott and Bendich [29]. The genomic DNA (5 μg) from putative transgenic mushroom and untransformed mushroom was digested with *Hind* III. The digested DNA was electrophoresed on a 1% agarose gel and then transferred to a Hybond-N membrane (Amersham Biosciences, Buckinghamshire, UK). Digoxigenin (Dig)-labeled probe was generated by PCR using Dig labeling Mix (Roche Diagnostics GMbH, Mannheim, Germany) with the specific primer set for the *h*PDGF-BB gene. DNA gel blot was hybridized at 42 °C in a DIG easy hybridization buffer. After hybridization overnight, the membrane was washed with SSC buffer series and then detected using the Dig Detection Kit following the manufacturer's instructions (Roche Diagnostics GMbH, Mannheim, Germany).

Total Protein Extraction and Western Blot Analysis

Mycelia were collected and subsequently ground in liquid nitrogen using mortar and pestle. A total of 50 mg mycelia powder was mixed with 1 ml protein extraction buffer containing 50 mM sodium phosphate, 300 mM NaCl, and 10 mM imidazole (pH 8.0) and vortexed vigorously for 15 min. Following centrifuging at 13,000×*g* for 20 min, supernatant was collected as total cellular protein.

The concentration of total protein extracted from putative transgenic and untransformed mushroom was determined using a bicinchoninic acid (BCA) protein assay kit (Pierce Co. Rockford, IL, USA). Twenty micrograms of protein extract from each sample was mixed with 5× sample loading buffer containing 1 M Tris–HCl (pH 6.8), 50% glycerol, and 1% bromophenol blue. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the procedure described by Laemmli [30] using 12% (*w*/*v*) polyacrylamide gel. Proteins were stained with Coomassie brilliant blue R-250. For Western blot analysis, proteins were transferred to a polyvinylidene fluoride membrane, and then the membrane was blocked for 3 h with TBST (3% BSA in TBS with 0.05% Tween-20) on ice to avoid non-specific binding of the antibody. Incubation with polyclonal rabbit anti-human PDGF-BB antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) with 1:2,000 dilution was performed in TBST solution for overnight on ice. The membrane was then washed three times for 10 min with TBST followed by the incubation of horseradish peroxidase-labeled goat anti-rabbit IgG antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), 1:5,000 for 3 h at room temperature. After washing three times for 15 min with TBST, the membrane was incubated with West-Zol (Plus) Western blot detection system (Intron Biotechnology Inc, Seongnam, Republic of Korea) and exposed on X-ray film (Kodak, New York, NY, USA).

Purification of Recombinant *h*PDGF-BB

For purification of the His-tag containing recombinant *h*PDGF-BB under native conditions, the Ni-NTA Spin kit (Qiagen, Hilden, Germany) was employed. Mycelia were ground in liquid nitrogen using mortar and pestle. After thawing for 15 min, the pellets were resuspended in lysis buffer containing 50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole (pH 8.0), and incubated with agitation for 1 h at room temperature (RT). After centrifugation (30 min at 10,000×*g* at RT), 600 μl of the crude lysate was loaded onto the Ni-NTA Spin column pre-equilibrated with lysis buffer. The Ni-NTA Spin column was washed twice with 600 μl of washing buffer containing 50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole (pH 8.0), and the target protein was eluted with 200 μl elution buffer containing 50 mM NaH_2PO_4, 300 mM NaCl, and 250 mM imidazole (pH 8.0). After

elution, the samples were analyzed by SDS-PAGE and Western blot. Protein concentrations were determined using the BCA protein assay with BSA as a standard.

Mass Spectrometry

To measure the molecular mass of the purified protein, each protein sample was subjected to SDS-PAGE, and then the band of interest was cut from the gel. The gel fraction was treated for the removal of the dye. The peptides obtained were extracted and taken to dry in a vacuum microcentrifuge. The sample was acidified by the addition of 0.1% (*v*/*v*) trifluoroacetic acid (TFA) and was left at room temperature for a few minutes, to reduce the droplet volume through evaporation. The matrix (1% α-cyano-4-hydroxycinnamic acid in 1:1 (*v*/*v*) H_2O/ACN solution containing 0.1% (*v*/*v*) TFA) was added, and the sample was allowed to dry at room temperature. All measurement were done by Korea Basic Science Institute (Jeonju, Republic of Korea) on a matrix-assisted laser/desorption ionization combined with time-of-flight mass spectrometry (MALDI-TOF-MS) using a Voyager Biospectrometry Workstation (Applied Biosystems, Foster City, CA, USA).

Bioassay

Biological activity of *rh*PDGF-BB was performed using the NIH-3T3 fibroblast cell line (ATCC, Rockville, MD, USA). Cell proliferation was assayed by measuring bromodeoxyuridine (BrdU) incorporation (Roche Diagnostics, Mannheim, Germany). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; GIBCO, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (FBS), 100 U ml^{-1} penicillin, and 100 μg ml^{-1} streptomycin at 37 °C in 5% CO_2, and media were changed every 2 days. For bioassay, cells were seeded in 96-well plates at a density of 1×10^4 cell/well in DMEM containing 10% FBS. The cells were serum starved for 48 h in DMEM without FBS. The cells were then subjected to the treatments of *rh*PDGF-BB in a concentration ranging from 0.1 to 100 ng ml^{-1} for 24 h. Cells of the control group were treated with the same volume of vehicle (culture medium) only. The assay was carried out in parallel with *rh*PDGF-BB standard (Koma Biotech Inc, Seoul, Republic of Korea). After 24 h incubation, cells were labeled with BrdU for 2 h at 37°C. Cells were then fixed (FixDenat, Roche) and incubated with peroxidase conjugated anti-BrdU antibody. Then the peroxidase substrate 3,3′,5,5′,-tetramethylbenzidine was added, and BrdU incorporation was quantitated by OD_{370}–OD_{492} using a VERSAmax microplate reader (Molecular Devices, Sunnyvale, CA, USA). EC_{50} value was calculated using the following Hill equation: $E_{rh\mathrm{PDGF}} = E_{\max} \times rh\mathrm{PDGF}^{\mathrm{nH}} / \left(rh\mathrm{PDGF}^{\mathrm{nH}} + \mathrm{EC}_{50}\right)$, where $E_{rh\mathrm{PDGF}}$ is the response measured after stimulation with *rh*PDGF-BB, and nH is the Hill coefficient.

Results

Expression Vector Construction and Generation of Transgenic Mushroom Carrying the *rh*PDGF-BB

The binary vector pCAMBIA1304 was used for the plasmid construction; 2,718-bp fragment of the *h*PDGF-BB gene was inserted into pCAMBIA1304. The resultant plasmid pCAMBIA1304 (*h*PDGF-BB) was subsequently introduced into fruiting bodies of *P. eryngii* using *A. tumefaciens* strain GV3101 by vacuum infiltration. After cultivation of infiltrated samples in the dark for 7–14 days, histochemical GUS assay

was carried out. GUS enzyme activity was not detected in untransformed control sample; on the other hand, the samples transformed with *A. tumefaciens* showed higher level of GUS activity than untransformed control sample (Fig. 3a). Following culture in the dark, the tissues infected with *Agrobacterium* were placed on the selection media containing 50 μg ml^{-1} hygromycin and 100 μg ml^{-1} cefotaxime. Growth was observed in the transformed mycelia but not in nontransformed mycelia. Out of 20 tissues, only eight to ten tissue pieces regenerated colonies on hygromycin medium. After final selection with hygromycin, a stable hygromycin-resistant culture was typically maintained for weeks to months on a PDA medium without antibiotic selection (Fig. 3b). After sub-culturing on PDB medium (Fig. 3c), we obtained the putative transgenic mycelium for further analysis.

Detection of *h*PDGF-BB Gene Integration in Transformed Mushroom

Genomic DNAs of putative transformant and nontransformed culture were used as templates for PCR detection with *h*PDGF-BB-specific primers to confirm the integration of *h*PDGF-BB gene, and amplified product was expected to be 238 bp. The results showed that *P. eryngii* mycelia contained the *h*PDGF-BB insert. Figure 4a showed the PCR results of transformed mycelium which showed the specific band as expected and untransformed mycelium which lacked the specific band. Plasmid pCAMBIA1304 (*h*PDGF-BB) was used as a positive control.

Southern blot analysis was performed on PCR-positive transformant to confirm the *h*PDGF-BB gene was integrated into the genome of transformant. DNA sample from transformant gave specific hybridization signal (Fig. 4b), indicating incorporation of *h*PDGF-BB gene into its respective genome.

Expression of *h*PDGF-BB in Transgenic Mushroom

To investigate the expression of *rh*PDGF-BB protein in transgenic mushroom, Western blot analysis was carried out using *h*PDGF-BB specific polyclonal antibody. The selected

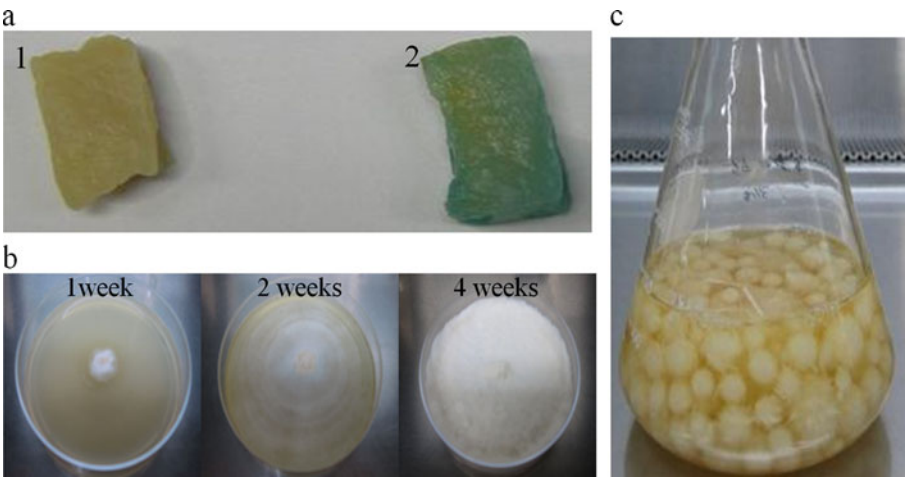

Fig. 3 **a** GUS histochemical result of transformation assay after vacuum infiltration and incubation in the dark at 25 °C for 10 days. Wild-type (*1*) and transformed *P. eryngii* tissues (*2*) with *A. tumefaciens* GV3101 harboring the vector pCAMBIA1304 (*h*PDGF-BB) gene. **b** Transgenic hygromycin-resistant cultures of *P. eryngii* on a PDA medium without antibiotic. **c** Putative transgenic mycelia subcultured on PDB medium

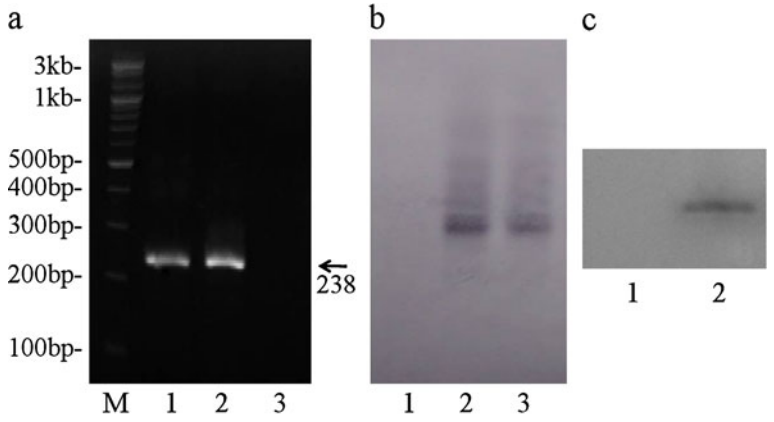

Fig. 4 Detection of the *rh*PDGF-BB gene in transgenic mushroom *P. eryngii*. **a** PCR analysis of genomic DNA from putative hygromycin-resistant transformant of *P. eryngii*. *M* DNA size marker, *lane 1* plasmid pCAMBIA1304 (*h*PDGF-BB; positive control), *lane 2* putative transformant # p36, *lane 3* untransformed negative control. **b** Southern blot hybridization of genomic DNA isolated from the transgenic mushroom. Genomic DNA was digested with *Hind* III, and the digested DNA samples were probed with Dig-labeled *h*PDGF-BB fragment. *Lane 1* untransformed negative control, *lane 2* plasmid *h*PDGF pCAMBIA1304 (*h*PDGF-BB; positive control), *lane 3* putative transformant # p36. **c** Western blot analysis of *rh*PDGF-BB from *P. eryngii. Lane 1* protein extracts from untransformed mushroom, *lane 2* protein extracts from transformed mushroom sample # p36

mycelia were found to express *h*PDGF-BB protein with identical molecular mass of 32 kDa. Recombinant *h*PDGF-BB protein synthesized in the transgenic mycelia is shown in Fig. 4c. The results showed that a molecular weight of 32-kDa specific band was observed in transgenic mushroom samples, which was equivalent to the full-length commercial *h*PDGF-BB dimer protein. Untransformed mycelia protein extract did not react with the anti- *h*PDGF-BB (lane 1). This result confirmed that the *h*PDGF-BB in mushroom cells was correctly translated.

Purification of Recombinant *h*PDGF-BB from *P. eryngii*

To become a viable recombinant protein production system, *P. eryngii* must not only express recombinant protein but that protein must be biologically active in a highly purified state. *rh*PDGF-BB was purified by affinity chromatography with Ni-NTA Spin columns under native conditions. As shown in Table 2, the yield of the purified *rh*PDGF-BB was 40.4 μg ml^{-1} of culture processed from transformed mushroom (sample # P36), which was approximately 1.98% of total soluble protein. The identity and purity of *rh*PDGF-BB was established through SDS-PAGE, Western blot, and MALDI-TOF mass spectrometry analysis. Coomassie blue staining of purified *rh*PDGF-BB revealed a single homogenous band at approximately 32 kDa (Fig. 5a), the expected mass of the *h*PDGF-BB homodimer. The *rh*PDGF-BB B chain dimer was also confirmed by immunoreactivity using a specific and commercially available polyclonal antibody (Fig. 5b). Native *rh*PDGF-BB is active as a homodimer. *P. eryngii* expressed *h*PDGF-BB appears to show homodimerization suggesting proper protein folding. These results were also confirmed by mass spectrometry analysis. MALDI-TOF-MS analysis showed an apparent molecular mass of 32,329 Da as expected for *rh*PDGF-BB (Fig. 5c).

Table 2 Production of recombinant human PDGF-BB in *P. eryngii*

Sample	Total soluble protein (mg ml^{-1} of culture processed)	Recombinant *h*PDGF-BB (μg ml^{-1} of culture processed)
P36	2.04±0.02	40.4±1.1
WT	1.86±0.04	–

The data are expressed as means±SD of three independent measurements

Biological Activity

To examine the biological activity of the purified *rh*PDGF-BB, mouse fibroblast NIH-3T3 cells growing in 96-well plates were maintained in medium containing increasing concentrations of *rh*PDGF-BB, and the cell proliferation was quantified using BrdU incorporation assay. As shown in Fig. 6, *rh*PDGF-BB stimulated the proliferation of fibroblast cells in a dose-dependent manner, which is indistinguishable from that of the *rh*PDGF-BB reference standard. All concentrations of *rh*PDGF-BB resulted in proliferation compared to untreated cells. The calculated EC_{50} value of *P. eryngii* expressed *h*PDGF-BB was similar to standard material (data not shown).

Discussion

In this study, we investigated whether an edible mushroom can be a potential system for the therapeutic protein production by examining the expression of human protein in the *P. eryngii*. Fungi are robust industrial fermentation organisms for the expression of therapeutic proteins as compared to other eukaryotic expression systems like *Baculovirus* and

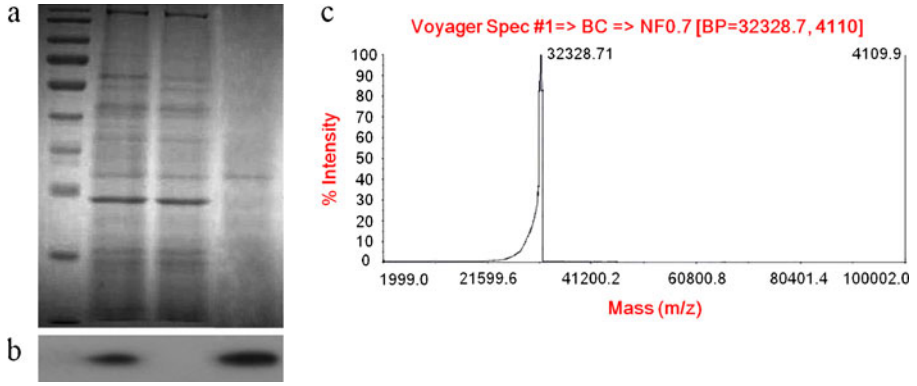

Fig. 5 Purification of mushroom-expressed *h*PDGF-BB. The protein was purified by affinity chromatography with Ni-NTA Spin columns under native conditions. The product was analyzed by **a** SDS-PAGE with Coomassie brilliant blue staining and **b** Western blotting. *M* protein size marker, *lane 1* total soluble protein, *lane 2* column flow-through, *lane 3* elute. **c** MALDI-TOF mass spectrometry analysis of purified *rh*PDGF-BB

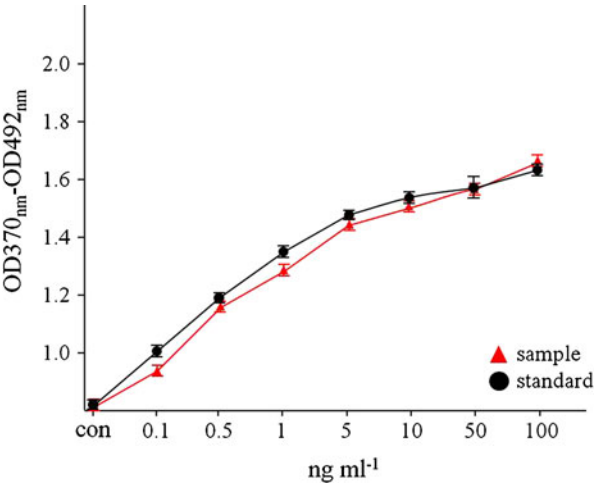

Fig. 6 Biological assay of purified and standard *rh*PDGF-BB. NIH-3T3 cells were treated with increasing concentrations of *rh*PDGF-BB from transgenic mushroom and standard *rh*PDGF-BB. To analyze the proliferation of NIH-3T3 cells, after 24 h treatment, cells were labeled with BrdU for 2 h. BrdU incorporation was determined by ELISA and the OD_{370}–OD_{492} was determined using a VERSAmax micro plate reader (Molecular Devices, Sunnyvale, CA, USA). The data are expressed as means±SD of three independent measurements

mammalian cells [31]. Fungi are becoming an option of choice for applications in biotechnology for heterologous gene expression. For recombinant protein production, use of fungi, as compared to that of other systems is much safer and less expensive, requires less time and is superior in terms of storage and distribution issue [22–25].

Basidiomycetes mushrooms have not been considered as a platform for the production of therapeutic proteins until recently [25]. This is the first report to express *h*PDGF-BB in *P. eryngii* by the *Agrobacterium*-mediated transformation system and demonstrated a possibility to produce pharmaceutically important gene in transgenic mushroom. Previously, expression of *h*PDGF-BB has been attempted by various researchers using variety of heterologous systems such as bacteria, yeast, insect, and mammalian cells [17–21, 32]. The expression level in these systems is a key issue that has led to high production cost. Wang et al. [20] reported the yield of 32 mg l^{-1} from *S. cerevisiae* whereas Karumuri et al. [18] could achieve about 10–12 mg g^{-1} from *E. coli*. In the current study using *A. tumefaciens*, significantly higher yield of recombinant *h*PDGF-BB (40.4 μg ml^{-1} of culture processed) was achieved from *P. eryngii*. The yield reported here is close to the yields reported from for the therapeutic and other industrial proteins from the mushrooms. For example, the yields of heterologous proteins production in filamentous fungi was reported up to the gram per liter level [22]. Human interleukin-6 was reported to accumulate 0.1 mg to 100 mg l^{-1} from *Aspergillus niger* [33], and industrial protein from *Aspergillus* and *Trichoderma reesei* was reported to be as high as 30 g l^{-1} [25]. Although yeasts have been extensively used for industrial protein production, only a few examples have been reported of high level expression of complex heterogeneous multimeric proteins [34]. Moreover, the aberrant *N*-glycosylation poses many problems with respect to therapeutic applications. In spite of the low expression level than yeast, the basidiomycete fungus *P. eryngii* has several advantages to use it as a platform to produce the therapeutic proteins. For instance, this mushroom has the ability to secrete high levels of proteins with post-translational processing. Its *N*-glycosylation patterns provide an excellent possibility to produce human-like, glycosylated proteins and also offers other advantages over currently used systems, such as scale and flexibility of production, safety, contained growth, and storage strategies [25].

*rh*PDGF-BB is recently sold as therapeutics for various diseases. Importantly, *rh*PDGF-BB produced from *P. eryngii* is a homodimer in its native and biologically active form. SDS-PAGE, Western blot, and MALDI-TOF-MS analysis confirm the maintenance of its

active structure after purification from native condition. Homodimerization suggests the presence of an active protein capable of executing its biological function. These data confirm that *P. eryngii* is able to produce bioactive protein and that the protein can be easily purified from mushroom extracts. Although there is plenty of room to improve the expression level, we believe that production of *rh*PDGF-BB in transgenic mushroom is economically feasible even at the current expression level.

Up to now, there was no available method for the efficient transformation of human gene into mushroom using *Agrobacterium*. Pioneering efforts were made by De Groot et al. [35] and Chen et al. [36] for *Agrobacterium*-mediated transformation of fungi. In this study, we explored a different approach to transform human gene into *P. eryngii* mediated by *Agrobacterium*. Our observation suggested that incubation of bacterial suspension prior to vacuum infiltration is not necessary. Similarly, we avoided the incubation of evacuated tissues on filter paper for 3 days as described by Chen et al. [36], instead only used 10 min to dry on filter paper under aseptic condition. Our study suggested that incubation for 7–14 days in the dark in a Petri dish without medium gives the good result and has the following advantages: (a) kills bacteria, (b) increases transformation efficiency, and (c) quickly adapts in selection media. However, various parameters such as nature of fungal starting material, the *Agrobacterium* strain, condition of co-cultivation, and selection agent affect the transformation frequency [36, 37]. Vacuum infiltration is important factor in this experiment, which led to a very high number of bacteria getting evenly distributed in the intercellular space. However, prolonged exposure to the vacuum rapidly decreased the temperature of the suspension of *Agrobacterium*, which may have additionally reduced the expression [38]. Ten-minute vacuum infiltration for two times was perfect in our condition. Our transformation system provided 40–50% efficiency, which is higher than previous study [36]. However, each fungus requires its own optimal conditions to obtain optimal transformation frequencies [37].

In conclusion, we have engineered the *h*PDGF-BB gene yet reported in edible mushroom and demonstrated the biological activity of mushroom-expressed *h*PDGF-BB. Aside from improving the quality of mushroom, the results of this study indicate the suitability of basidiomycete fungi as a production host for the expression of human therapeutic protein. Although more research will be needed to optimize the transformation techniques which we have developed, this work could contribute substantially to pharmaceutical production of bioactive proteins for combating human diseases and maintenance and promotion of good health.

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References

1. Habenicht, A. J., Salbach, P., Janssen-Timmen, U., Blattner, C., & Schettler, G. (1992). *Klinische Wochenschrift, 8*, 53–59.
2. Kallianinen, L. K., Hirshberg, J., Marchant, B., & Rees, R. S. (2000). *Plastic and Reconstructive Surgery, 6*, 1243–1248.
3. Li, W. L., Yamada, Y., Ueno, M., Nishikawa, S., Nishikawa, S. I., & Takakura, N. (2006). *Journal of Biochemistry, 140*, 267–273.
4. Board, R., & Jayson, G. C. (2005). *Drug Resistance Updates: Reviews and Commentaries in Antimicrobial and Anticancer Chemotherapy, 8*, 75–83.

5. Frederiksson, L., Li, H., & Eriksson, U. (2004). *Cytokine & Growth Factor Reviews, 15*, 197–204.
6. Senet, P. (2004). Becaplermin gel (Regranex gel). *Annales de Dermatologie et de Vénéréologie, 131*, 351–358.
7. Wieman, T. J. (1997). *American Journal of Surgery, 176*, 74S–79S.
8. Steed, D. L. (1995). *Journal of Vascular Surgery, 1*, 71–81.
9. Mandracchia, V. J., Sanders, S. M., & Frerichs, J. A. (2001). *Clinics in Podiatric Medicine and Surgery, 18*, 189–209.
10. Harrison-Balestra, C., Eaglstein, W. H., Falabela, A. F., & Kirsner, R. S. (2002). *Dermatologic Surgery, 28*, 755–759.
11. Hom, D. B., & Manivel, J. C. (2003). *The Laryngoscope, 113*, 1566–1571.
12. Hollinger, J. O., Hart, C. E., Hirsch, S. N., Lynch, S., & Friedlaender, G. E. (2007). *The Journal of Bone and Joint Surgery. American Volume, 90*, 48–54.
13. Pahl, H. L. (1999). Activators and target genes of Rel/NF-kappaB transcription factors. *Oncogene, 18*, 6853–6866.
14. Peng, F., Dhillon, N. K., Yao, H., Zhu, X., Williams, R., & Buch, S. (2008). *The European Journal of Neuroscience, 7*, 1255–1264.
15. van Steensel, L., Paridaens, D., Dingjan, G. M., van Daele, P. L., van Hagen, P. M., Kuijpers, R. W., et al. (2010). *Investigative Ophthalmology & Visual Science, 2*, 1002–1007.
16. Hoppe, J., Weich, H. A., & Eichner, W. (1989). *Biochemistry, 28*, 2956–2960.
17. Alexander, D. M., Hesson, T., Mannarino, A., Cable, M., & Dalie, B. L. (1992). *Protein Expression and Purification, 3*, 204–211.
18. Karumuri, N. N., Gangireddy, S. R., Narala, V. R., Majee, S. S., Gunwar, S., & Reddy, R. C. (2007). *Biotechnology Letters, 29*, 1333–1339.
19. Ostman, A., Rall, L., Hammacher, A., Wormstead, M. A., Coti, D., Valenzuela, P., et al. (1988). *The Journal of Biological Chemistry, 31*, 16202–16208.
20. Wang, Y., Xue, L., Li, Y., Zhu, Y., Yang, B., & Wang, X. (2009). *Prikladnaia Biokhimiia i Mikrobiologiia, 2*, 176–180.
21. Giese, N., May-Siroff, M., LaRochelle, W. J., van Wyke Coelingh, K., & Aaronson, S. A. (1989). *Journal of Virology, 7*, 3080–3086.
22. Nevalainen, K. M., Teo, V. S., & Bergquist, P. L. (2005). *Trends in Biotechnology, 23*, 468–474.
23. Fernandez, J. M., & Hoeffler, J. P. (1999). *Gene expression systems*. San Diego: Academic.
24. Demain, A. L., & Vaishnav, P. (2009). *Biotechnology Advances, 27*, 297–306.
25. Berends, E., Scholtmeijer, K., Wosten, H. A., Bosch, D., & Lugones, L. G. (2009). *Trends in Microbiology, 10*, 439–443.
26. Rodriguez Estrada, A. E., & Royse, D. J. (2007). *Bioresource Technology, 98*, 1898–1906.
27. Royse, D. J. (1999). *Mushroom News, 47*, 4–8.
28. Holsters, M., De Waele, D., Depicker, A., Messens, E., Van Montague, M., & Schell, J. (1978). *Molecular & General Genetics: MGG, 163*, 168–181.
29. Scott, O. R., & Bendich, A. J. (1997). Extraction of total cellular DNA from plant, algae and fungi. In S. B. Gelvin & R. A. Schilperoort (Eds.), *Plant molecular biology manual, D1* (pp. 1–8). Dordrecht: Kluwer Academic.
30. Laemmli, U. K. (1970). *Nature, 227*, 680–685.
31. Apte-Deshpande, A., Rewanwar, S., Kotwal, P., Raiker, V. A., & Padmanabhan, S. (2009). *Biotechnology and Applied Biochemistry, 54*, 197–205.
32. Calderon-cacia, M., Tekamp-Olson, P., Allen, J., & George-Nascimento, C. (1992). *Biochemical and Biophysical Research Communications, 187*, 1193–1199.
33. Punt, P. J., van Biezen, N., Conesa, A., Albers, A., Mangnus, J., & van den Hondel, C. (2002). *Trends in Biotechnology, 5*, 200–206.
34. Gasser, B., & Mattanovich, D. (2007). *Biotechnology Letters, 29*, 201–212.
35. De Groot, M. J. A., Bundock, P., Hooykaas, P. J. J., & Beijersbergen, A. G. M. (1998). *Nature Biotechnology, 16*, 839–842.
36. Chen, X., Stone, M., Schlagnhaufer, C., & Romaine, C. P. (2000). *Applied and Environmental Microbiology, 664*, 510–513.
37. Michielse, C. B., Hookaas, P. J., van den Hondel, C. A., & Ram, A. F. (2005). *Current Genetics, 48*, 1–17.
38. Wroblewski, T., Tomczakt, A., & Michelmore, R. (2005). *Plant Biotechnology Journal, 3*, 259–723.