β-Glucan Synthase Gene Overexpression and β-Glucans Overproduction in *Pleurotus ostreatus* Using Promoter Swapping

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**Abstract**

Mushroom β-glucans are potent immunological stimulators in medicine, but their productivities are very low. In this study, we successfully improved its production by promoter engineering in *Pleurotus ostreatus*. The promoter for β-1,3-glucan synthase gene (GLS) was replaced by the promoter of glyceraldehyde-3-phosphate dehydrogenase gene of *Aspergillus nidulans*. The homologous recombination fragment for swapping GLS promoter comprised five segments, which were fused by two rounds of combined touchdown PCR and overlap extension PCR (TD-OE PCR), and was introduced into *P. ostreatus* through PEG/CaCl₂-mediated protoplast transformation. The transformants exhibited one to three fold higher transcription of GLS gene and produced 32% to 131% higher yield of β-glucans than the wild type. The polysaccharide yields had a significant positive correlation to the GLS gene expression. The infrared spectra of the polysaccharides all displayed the typical absorption peaks of β-glucans. This is the first report of successful swapping of promoters in filamentous fungi.

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**Introduction**

Mushroom β-glucans are the major structural constituents of the mushroom cell wall. They also provide antioxidant activity [1,2] and can be used as immunological stimulators in medicine, such as antitumor, immunomodulating, antioxidant, radical scavenging, cardiovascular, antihypercholesterolemia, antiviral, antibacterial, antiparasitic, antifungal, detoxification, hepatoprotective, and antidiabetic effects [3]. Therefore, there has been growing popularity in developing mushroom β-glucans as drugs or dietary supplements and scientifically investigating their functions [4]. However, controlled by metabolic regulation, the production of β-glucans in mushroom is low. Furthermore, due to enzymatic degradation by glucanase activity during storage, the production from fruiting body or submerged fermentation liquid is only 20–50 mg/100 g dry matter of fruiting body [5] or 0.15–5.3 g/l fermented broth [6,7]. Accordingly, it is of great value to improve β-glucans productivity in mushrooms by genetic engineering.

Most mushroom β-glucans with immunological stimulation are β-(1→6)-branched β-(1→3)-linked [8], and are synthesized by β-1,3-glucan synthase (GLS) (UDP-glucose 1,3-β-D-glucan 3-β-D-glucosyl transferase, EC 2.4.1.34). The GLS complex is composed of a catalytic subunit FKS and a regulatory subunit RHO. FKS is activated by RHO, a G protein, through GTP-dephosphorylation, and synthesizes the polymer of glucose monomers. RHO activity is regulated by a wall GDP-GTP exchange factor protein [9]. In fungi genomes, FKS and RHO genes are highly conserved and usually have only one or two copies [10,11]. The information about the transcriptional regulation of GLS has been scant [11].

In yeast, GLS activity, both at transcriptional and enzymatic level, is stimulated by stress-inducing compounds present in the media [12]. A similar mechanism is also present in *Leptinula edodes* [13–15]. Nevertheless, the expression of the GLS genes in *Ustilago maydis*, a fungus causing smut disease on maize, was constitutive during its infection in maize and in response to ionic and osmotic stress [11].

Oyster mushroom *Pleurotus ostreatus* is a widely cultivated edible and medicinal mushroom in China and East Asia due to its short growth time, high adaptability and productivity. Its β-glucans demonstrated efficacy in promoting the survival of mice susceptible to bacterial infections [16], high SOD-like activity and antitumor activity [17]. In this study, we obtained the sequences of its only GLS gene and the promoter from *P. ostreatus* PC15 v2.0 in JGI Genome Portal (http://genome.jgi-psf.org). Using homologous recombination, the GLS gene promoter of *P. ostreatus* was replaced by the promoter of glyceraldehyde-3-phosphate dehydrogenase (*gpd*) gene of *Aspergillus nidulans*; the transformants displayed high expression of GLS gene and high production of β-glucans.
Materials and Methods

Strains and DNAs

*Pleurotus ostreatus* TD300, often used as a commercial cultivation strain in China, was obtained from Zhongzhou Composite Experiment station, China Edible Fungi Research System (Zhengzhou, China), and cultivated on PDA medium at 28°C for six days as described elsewhere [18].

The homologous recombination fragment for the GLS promoter swap comprised five segments: UH, Pgpd1035, hph, Pgphb and GLS1025 (Fig. 1).

The 1,015 bp UH was the upstream homologous sequence which matched to the 3’ end partial sequence preceding the GLS promoter of *P. ostreatus*. It was cloned from the genomic DNA of *P. ostreatus* by PCR using primers UH-F and UH-R (Table 1), and its accession number was JX889617. The primers were designed based on the genome sequence of *P. ostreatus* PC15 v2.0 in JGI Genome Portal (http://genome.jgi-psf.org). In the primer UP-R sequence, the last 15 nts (highlighted with underline) were complementary to the 5’ end sequence of Pgd1035.

The 1,035 bp Pgpd1035 served as the upstream FLP recognition target (FRT) sequence; it was the partial 5’ end sequence of Pgd which the promoter of gpd gene in *Aspergillus nidulans*. Pgd1035 was generated from plasmid PAN7-1 by PCR using primers Pgd1048-F and Pgd1048-R (Table 1). The first 15 nts of Pgd1048-F and last 16 nts (with underline) of Pgd1048-R were complementary to the 3’ end sequence of UH and 5’ end sequence of hph, respectively. The plasmid pAN7-1 (kindly provided by Prof. van den Hondel, Leiden University, Netherlands) contains the hygromycin B resistance gene of *E. coli* and the transcription termination signal of tryptophan synthetase, respectively. It was cloned from the genomic DNA of *P. ostreatus* by PCR using primers Pgd1048-F and Pgd1048-R (Table 1). The first 14 nts of Pgd1048-F and last 17 nts (with underline) of Pgd1048-R were complementary to the 3’-end sequence of hph and 5’-end sequence of GLS1025, respectively.

The 2,822 bp hph was hygromycin B resistance gene of *E. coli* expression cassette, and was amplified from plasmid PAN7-1 by PCR using primers hph-F and hph-R (Table 1). The first 16 nts of hph-F and last 14 nts (with underline) of hph-R were complementary to the 3’ end sequence of Pgd1035, and 5’ end sequence of Pgpdh respectively.

The 2,206 bp Pgd was also amplified from plasmid PAN7-1 by PCR using primers Pgd-F and Pgd-R (Table 1). The first 14 nts of Pgd-F and last 17 nts (with underline) of Pgd-R were complementary to the 3’-end sequence of hph and 5’-end sequence of GLS1025, respectively.

The 1,025 bp GLS1025 was the downstream homologous sequence in the 5’ end partial sequence of *P. ostreatus* GLS. It was cloned from the genomic DNA of *P. ostreatus* by PCR using primers GLS1025-F and GLS1025-R (Table 1), and its accession number in GeneBank was JX889617. The primers were designed

![Diagram](https://example.com/diagram.png)

**Figure 1.** The outline of homologous recombination for promoter swapping. The homologous recombination fragment consisting of five DNA segments (A): the integration of the fragment into *P. ostreatus* chromosome via homologous recombination (B); the intramolecular homologous replacement of Pgd1035 by Pgpd (C); the deletion of selection marker hph (D). UH: upstream homology sequence; Pgd1035: the partial 5’ end sequence of the gpd promoter in *Aspergillus nidulans*; hph: hygromycin B resistance gene (hph) of *E. coli* expression cassette; Pgd: the gpd promoter; GLS1025: the downstream homologous sequence to the 5’ end partial sequence of GLS.

Table 1. Primers used in this study.

| Primers     | Nucleotide sequence                        |
|-------------|--------------------------------------------|
| UH-F        | 5'-TCCTCCCCGGACCGTGTATTGATTTAC-3'          |
| UH-R        | 5'-CAGCGTGTATTGATTTACATTACATAC-3'          |
| Pgd 1048-F  | 5'-GATATGGAAACTGGCGGAATTTCCCTTGTATCT-3'   |
| Pgd 1048-R  | 5'-GAATGCCGTAAGGTTCTGCAACGACTA-3'         |
| hph-F       | 5'-CACTCATGCACTCATGACATCCACACAGC-3'       |
| hph-R       | 5'-AGATCAAGGATGGAAATCACCACGCTTACCC-3'     |
| Pgph-F      | 5'-GCTGTGATGCACTTTGAGTATGATGATGAG-3'      |
| Pgph-R      | 5'-CTACATCCATACCTCATTGGACTCACACAGC-3'     |
| GLS-F       | 5'-CTACATCCATACCTCATTGGACTCACACAGC-3'     |
| GLS-R       | 5'-CCCGGATCTAGTGTCAGAAGAAA-3'             |
| GLS-F       | 5'-CTACATCCATACCTCATTGGACTCACACAGC-3'     |
| GLS-R       | 5'-ATTTGGACTGATGGGAAGGT-3'                |
| AC-1        | 5'-ATTTGGACTGATGGGAAGGT-3'                |
| AC-2        | 5'-CTACATCCATACCTCATTGGACTCACACAGC-3'     |

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The homologous recombination fragment consisting of five DNA segments (A): the integration of the fragment into *P. ostreatus* chromosome via homologous recombination (B); the intramolecular homologous replacement of Pgd1035 by Pgpd (C); the deletion of selection marker hph (D). UH: upstream homology sequence; Pgd1035: the partial 5’ end sequence of the gpd promoter in *Aspergillus nidulans*; hph: hygromycin B resistance gene (hph) of *E. coli* expression cassette; Pgd: the gpd promoter; GLS1025: the downstream homologous sequence to the 5’ end partial sequence of GLS.

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based on the only GLS gene sequence of *P. ostreatus* PC15 v2.0 collected by visual inspection using JGI Genome Portal (http://genome.jgi-psf.org). In the primer GLS$_{1025}$-F sequence, the first 15 nts (with underline) was complementary to the 3' end sequence of *P. ostreatus* TD900 was 99.6% and 56.3% identical to that of *P. ostreatus* PC15 v2.0 and *Laccaria bicolor* S238N-H82, and the GLS amino acid sequences from *P. ostreatus* PC15 v2.0 and *Laccaria bicolor* S238N-H82 shared 89.3% identity (Fig. 2).

**Touchdown-overlap extension PCR**

Two rounds of combined touchdown PCR and overlap extension PCR (TD-OE PCR) were performed to fuse the above five long DNA segments.

The first round of TD-OE PCR was carried out for the fusion of the three segments: *hph*, *P*$_{gpd}$, and GLS$_{1025}$; it produced a long fusion segment hPG. This round includes two steps. In step I, 47 mL reaction solution contains 1 mL of each DNA segment at 0.5 mM, 5 mL of 10× PCR buffer, 8.0 mL of 2.5 mM of dNTP, and 0.5 mL of 5 U/mL LA Taq. Amplification started at 94°C for 40 sec; the annealing temperature of the reaction decreased from 61.5°C to a touchdown 57.5°C at the cooling rate of 0.5°C every cycle, followed by five cycles at 57.5°C, 4 min at 68°C, and 10 min at 72°C. In step II, the reaction solution from Step I was added to 1.0 mL of 0.1 mM hph-F and GLS$_{1025}$-R separately, and 0.5 mL of 5 U/mL LA Taq. PCR conditions are similar to that of step I amplification: 94°C for 40 sec; annealation: 60°C to 35°C by 0.5°C per cycle, then 20 cycles at 56°C, 7 min at 68°C, and 10 min at 72°C. After completion, 5 mL of the PCR reaction aliquots were analyzed on 1% agarose gels stained with ethidium bromide.

The second round of TD-OE PCR was carried out to fuse the three segments, i.e., UH, *P*$_{gpd}$, and hPG, to generate the homologous recombination fragment. The PCR procedure was similar to that in the first round except for the annealing temperature and primers. In step I, the annealing temperature decreased from 62°C to a touchdown 58°C; in step II, added primers were UH-F and GLS$_{1025}$-R, the annealing temperature decreased from 58°C to 35°C.

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**Figure 2.** Comparison of predicted GLS amino acid sequences among *P. ostreatus* TD300, *Laccaria bicolor* S238N-H82, and *P. ostreatus* PC15 v2.0. Lb: GLS from *Laccaria bicolor* S238N-H82, GeneBank accession numbers is XM_001875351. Po PC: GLS from *P. ostreatus* PC15 v2.0, collected by visual inspection using the JGI Genome Portal for the *P. ostreatus* PC15 v2.0 genome (http://genome.jgi-psf.org); Po TD: GLS from *P. ostreatus* TD300, GeneBank accession numbers is JX889617. doi:10.1371/journal.pone.0061693.g002

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PEG/CaCl₂-mediated protoplast transformation and transformant identification

Protoplasts preparation and PEG/CaCl₂-mediated transformation of *P. ostreatus* TD 300 were performed as described previously [20,21]. The introduced foreign DNA was the homologous recombination fragment for swapping GLS promoter. Protoplasts of *P. ostreatus* were suspended in MTC buffer at 10⁶ protoplasts/mL, and 100 μL suspensions were mixed with 10 μg the DNA fragment. Transformants were subcultured on PDA with or without hygromycin B. A full description of this method is given in the Extended Methods S1. To verify the replacement of GLS promoter by the introduced fragment, two PCR reactions were performed using the genomic DNA of the transformants as template, primers hph-1 and hph-2 for the amplification of the *hph* gene, and primers P₁₀₂₅-1 and GLS-1 (Table 1) for amplifying the combination of P₁₀₂₅ and GLS. Both PCR conditions were 94°C for 1 min, 56°C for 1 min, and 72°C for 1 min in 30 cycles. 5 μL of the PCR reaction aliquots were analyzed on 1% agarose gels stained with ethidium bromide.

Semi-quantitative RT-PCR

To analyze the GLS expression in transformants, semi-quantitative RT-PCR was carried out as described elsewhere [21] with slight modifications. Total RNA was extracted from transformants. Primers were GLS-F and GLS-R for reverse transcription and amplification of GLS, and AC-1 and AC-2 for reverse transcription and amplification of housekeeping gene β-actin. Reverse transcription of the mRNA was carried out at 42°C for 60 mins by using MMLV Reverse Transcriptase 1st-Strand cDNA Synthesis Kit (Epicentre, Madison, WI, USA). The PCR conditions were 94°C for 40 sec, 49°C for 40 sec, and 72°C for 1 min in 30 cycles.

5 μL of the PCR reaction aliquots was analyzed on 1% agarose gels stained with ethidium bromide. The electrophoresis bands of RT-PCR reaction were photographed and the density of each band was quantified using image analysis software, UVI band V. 97 (UVI Tech, Cambridge, UK).

Analysis of β-glucan content and infrared spectrum

To measure the β-glucan yield of the transformants, the transformants were cultivated in PD broth (150 mL in a 500 mL flask) at 25°C for 12 d under 150 rpm shaking. The culture broth was extracted in 98°C water bath for 3 h and then filtered; the supernatant was concentrated 10-fold via evaporation. Four volume 95% ethanol was added and placed overnight, centrifuged at 5,000-g for 15 min. The precipitate was washed 2 times with 85% ethanol, then dissolved in hot water, and de-proteinized by Sevag method [22]. Equal volume of Sevag reagent (chloroform/butanol 4:1, v/v) was added, vigorously shaken for 30 min, the mixture was centrifuged at 5,000-g for 10 min. The upper layer was separated and Sevag reagent was again added. This process was repeated 3 times. The polysaccharide content was measured using phenol-sulfuric acid method [23]. The polysaccharide characteristic was determined by infrared spectroscopy (Tensor 37, Bruker, Ettlingen, Germany).

Results

Construction of the homologous recombination fragment for swapping GLS promoter

The three segments of *hph*, P₁₀₂₅, and GLS₁₀₂₅ were fused successfully, and the expected fusion product hPG (5,873 bp) was produced by only one round TD-OE PCR (Fig. 3A); hPG was subsequently fused with the two upstream segments UH and P₁₀₂₅ in the second round of TD-OE PCR (Fig. 3B), and produced the homologous recombination fragment for swapping GLS promoter (8,103 bp). The sequencing result of the fusion product confirmed that all of the five segments were fused correctly in accordance with the design order.

Identification of homologous recombination transformant by PCR

The integration and the promoter swapping in the transformants were verified by PCR. Six transformants, A1, A4, A9, A15, A17, and A21, were randomly selected. The expected PCR amplifications were obtained from all the DNA samples of the second generation transformants using the primer pair hph-1 and hph-2, but not from the untransformed original strain (wild type) and the next generation transformants (Fig. 4A). The segment could be amplified from the DNA samples of the transformants from generation three to five by using the primer pair P₁₀₂₅-1 and GLS-1, which was 1,320 bp in length and spanned the 3’ end of P₁₀₂₅ and the downstream of the 5’ end of GLS₁₀₂₅ (Fig. 4B). The result revealed that *hph* gene in the homologous recombination fragment was deleted by the homologous recombination between P₁₀₂₅-1 and P₁₀₂₅ from the third generation of the transformants. In addition, the introduced P₁₀₂₅ replaced the GLS promoter and remained genetically stable.

GLS gene overexpression and β-glucan overproduction of the transformants

To determine whether GLS gene over-expressed after the swap of its promoter by P₁₀₂₅ its mRNA expression level in the transformants was measured by semi-quantitative RT-PCR and was found to be two to four folds higher than that of wild type (Fig. 5).

The polysaccharide yields of the six transformants were determined and they were 32% to 131% higher than that of wild type (Fig. 6); including the wild type, the yield had positive correlation to the GLS gene expression (*p*<0.05). The result indicated that the GLS gene expression may correspond with its enzymatic activity and protein level.

[Figure 3. Agarose gel analysis of TD-OE PCR products for the construction of the homologous recombination fragment for promoter swapping. hPG: the fusion product of *hph*, P₁₀₂₅, and GLS₁₀₂₅; HRF: the homologous recombination fragment which was the fusion product of UH, P₁₀₂₅, and hPG. doi:10.1371/journal.pone.0061693.g003]
**Discussion**

Promoter engineering is the principal strategy for metabolic engineering. It employs mutagenic endogenous promoters or introduced heterologous promoters to increase the expression of key pathway genes and maximize target production, and has been successfully applied to bacteria and yeast [27–30]. Nevertheless, the application of promoter engineering in filamentous fungi has not been reported. In this study, we introduced a heterologous promoter to increase the GLS expression and significantly improved β-glucan production.

In previous studies, the promoters for replacing native promoters could be constitutive or inducible [28]. Compared with constitutive promoters, inducible promoters tightly control their downstream gene to achieve high level expression, maximize protein production and reduce toxicity during growth phase [31]; but it is limited in practice due to inducer cost and cell hypersensitivity to inducer concentration [32]. 

$P_{gpd}$ is a constitutive promoter used across fungal species and provides high levels of constitutive gene expression [33,34]; for example, the expression of genes under the control of $P_{gpd}$ was significantly higher than that by the commonly used alcohol oxidase 1 promoter ($P_{AOX}$) in methanol-grown cells of Pichia pastoris [33]. In this study, we employed $P_{gpd}$ from A. nidulans to swap the native promoter of GLS in P. ostreatus, and consequently GLS expression was improved by up to two folds and β-glucan production increased by up to 32% compared to the wild type strain. CaMV 35S is another constitutive promoter often used in filamentous fungi, which is considerably weaker than $P_{gpd}$ in P. ostreatus [21].

**Conclusions**

By our knowledge, this is the first report of successful swapping of promoters using TD-OE PCR in filamentous fungi through constitutive promoters used in filamentous fungi included adhA, gdhA, bC, pgkA, etc [36], but the comparison among them has not been reported.

Construction of the homologous recombination fragment for swapping promoter requires five to six segments fusion [29,37]. Multiple segments fusion is usually performed by overlap extension PCR (OE-PCR) [38,39]. However, OE-PCR cannot fuse more than two DNA segments simultaneously [40]. Several modified OE-PCR procedures can fuse multiple segments at the same time, but require chimeric primers and high and close annealing temperatures in order to minimize mispriming [41,42]. Touchdown PCR (TD-PCR) is an efficacious solution to reduce mispriming and rapidly optimize PCR to increase specificity, sensitivity, and yield [43,44]. In this study, we combined TD-PCR and OE-PCR and used only two rounds to fuse the five segments. Our technique produced the homologous recombination fragment for swapping GLS promoter without sedulously adjusting the annealing temperature of primers. It showed that touchdown-overlap extension PCR (TD-OE PCR) was a fast and highly efficient method for promoter swapping and metabolic engineering.

**Figure 4.** PCR for the identification of the recombinant hph and $P_{gpd}$ sequences in the transformants of P. ostreatus. PCR amplification on total DNA from the second generation transformants using primers hph-1 and hph-2 which defined a 750 bp sequence across the hph gene (A), PCR amplification on total DNA from the fifth generation transformants using primers $P_{gpd}$-1 and GLS-1 which defined a 1,320 bp sequence spanning the $P_{gpd}$ and GLS gene located far from GLS$_{5q2s}$ (B). Lane 1: WT; Lane 2–7: transformant A1, A4, A9, A15, A17, and A21.

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**Figure 5.** Semi-quantitative RT-PCR analysis of GLS mRNA in the fifth generation transformants. The amount of GLS mRNA, expressed as the ratio of densitometric measurement of the sample to the corresponding internal standard ($\beta$-actin), is shown in the upper panels. * $p<0.05$ comparing to WT; ** $p<0.01$ comparing to WT.

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**Figure 6.** The polysaccharide yield of the fifth generation transformants. * $p<0.05$ comparing to WT; ** $p<0.01$ comparing to WT.

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rapid construction of homologous recombination fragment. The polysaccharide yields of the transformants were 32% to 131% higher than that of wild type, and had significantly positive correlation to the GLS gene expression levels. TD-OE PCR, a novel procedure combining touchdown and overlap extension PCR, was carried out for the fusion of five segments to construct the homologous recombination fragment for swapping GLS promoter. Our study supports that TD-OE PCR was a fast and highly efficient method for promoter swapping and metabolic engineering.

Supporting Information
Methods S1  Extended Methods.

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