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# 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/CaCl$_2$-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.

**Competing Interests:** The authors have declared that no competing interests exist.

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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 ferment 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 *Lentinula 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 Zhengzhou 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, $P_{gpd\ 1035}$, *hph*, $P_{gpd}$, and $GLS_{1025}$ (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 UF-R (Table 1), and its GeneBank 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 UF-R sequence, the last 15 nts (highlighted with underline) were complementary to the 5′ end sequence of $P_{gpd\ 1035}$.

The 1,035 bp $P_{gpd\ 1035}$ served as the upstream FLP recognition target (FRT) sequence; it was the partial 5′ end sequence of $P_{gpd}$, the promoter of *gpd* gene in *Aspergillus nidulans*. $P_{gpd\ 1035}$ was generated from plasmid PAN7-1 by PCR using primers $P_{gpd\ 1048}$-F and $P_{gpd\ 1048}$-R (Table 1). The first 15 nts of $P_{gpd\ 1048}$-F and last 16 nts (with underline) of $P_{gpd\ 1048}$-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 *Escherichia coli* controlled by the *gpd* promoter and the transcription termination signal of tryptophan synthetase gene (*trp*C) from *A. nidulans* [19].

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 $P_{gpd\ 1035}$ and 5′ end sequence of $P_{gpd}$, respectively.

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

| Primers | Nucleotide sequence |
| --- | --- |
| UH-F | 5′-TCCCCCGGGACTCGTTATCGTATTC-3′ |
| UH-R | 5′-TAGTCGTTGGCAGGCCAGTTTCCATATC-3′ |
| $P_{gpd\ 1048}$-F | 5′-GATATGGAAACTGGCGAATTCCCTTGTATCT-3′ |
| $P_{gpd\ 1048}$-R | 5′-GTAATCGTTGGCAGAAATCCTTACAGCTTG-3′ |
| hph-F | 5′-CAAGCTGTAAGGATTTCTGCCAACGACTA-3′ |
| hph-R | 5′-AGATACAAGGGAATTCATCAACATGCTACC-3′ |
| $P_{gpd}$-F | 5′-GGTAGCATGTTGATGAATTCCCTTGTATCT-3′ |
| $P_{gpd}$-R | 5′-GCTGTGTGAGTCCAATGGAG TATGGATGTAG-3′ |
| $GLS_{1025}$-F | 5′-CTACATCCATACTCCATTGGACTCACACAGC-3′ |
| $GLS_{1025}$-R | 5′-CGGGATCCAGTAAGTCTTGAAGAAAA-3′ |
| hph-1 | 5′-GGAAGTGCTTGACATTGGGGAGTT-3′ |
| hph-2 | 5′-TACTTCTACACAGCCATCGGTCCAG-3′ |
| $P_{gpd}$-1 | 5′-CAAGGTCGTTGCGTCAGTCC-3′ |
| GLS-1 | 5′-CCAAGTCATAGGTGCAGAAGGA-3′ |
| GLS-F | 5′-AATTTGGATTCCAACGGGA-3′ |
| GLS-R | 5′-GGGCTATTGATCGCTTCTC-3′ |
| AC-1 | 5′-GATAGAACCACCAATCCAAAC-3′ |
| AC-2 | 5′-AAGTCATCACCATCGGTAACG-3′ |

The 2,206 bp $P_{gpd}$ was also amplified from plasmid PAN7-1 by PCR using primers $P_{gpd}$-F and $P_{gpd}$-R (Table 1). The first 14 nts of $P_{gpd}$-F and last 17 nts (with underline) of $P_{gpd}$-R were complementary to the 3′-end sequence of *hph* and 5′-end sequence of $GLS_{1025}$, respectively.

The 1,025 bp $GLS_{1025}$ 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 $GLS_{1025}$-F and $GLS_{1025}$-R (Table 1), and its accession number in GeneBank was JX889617. The primers were designed
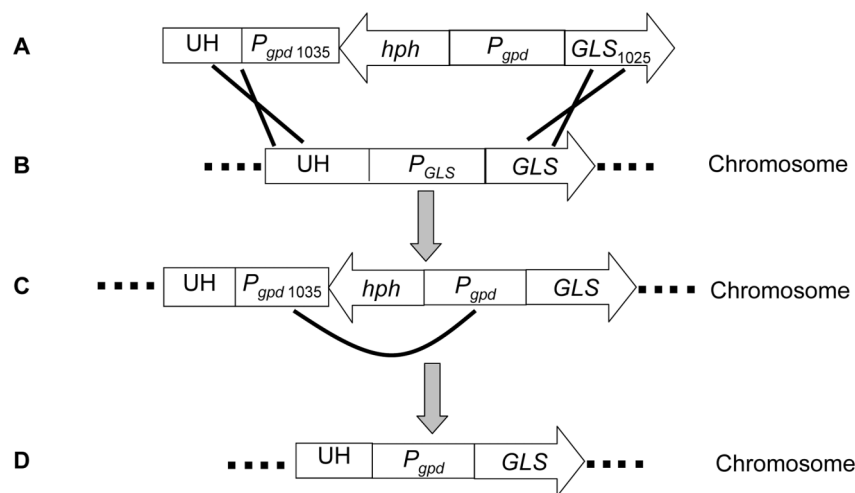


**Figure 1. The outline of homologous recombination for promoter swapping.** The homologous recombination fragment consisting of five DNA segments (A); the integration of the fragment into *P. ostreatus* chromosome via homologous recombination (B); the intramolecular homologous replacement of $P_{gpd\ 1035}$ by $P_{gpd}$ (C); the deletion of selection marker *hph* (D). UH: upstream homology sequence; $P_{gpd\ 1035}$: the partial 5′ end sequence of the *gpd* promoter in *Aspergillus nidulans*; *hph*: hygromycin B resistance gene (*hph*) of *E. coli* expression cassette; $P_{gpd}$: the *gpd* promoter; $GLS_{1025}$: 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 $P_{gpd}$. The predicted amino acid sequences of GLS$_{1025}$ from *P. ostreatus* TD300 was 99.6% and 86.3% 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 µL reaction solution contains 1 µL of each DNA segment at 0.5 µM, 5 µL of 10× PCR buffer, 8.0 µL of 2.5 mM of dNTP,

and 0.5 µL of 5 U/µL 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 µL of 0.1 mM hph-F and GLS$_{1025}$-R separately, and 0.5 µL LATaq. PCR conditions are similar to that of step I: amplification: 94°C for 40 sec; annealation: 60°C to 56°C decreasing 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 µL 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, $P_{gpd\ 1035}$, 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 56°C.

```
Lb     MSTEEIEDIFLDLTQKFGFQRDSMRNMFDFTMHLLDSRASRMSPNQALITLHADYIGGQHAKSLDSALNRWRNAMNNMSQYDRLRQIALYLLCWGEGGNV
Po PC  --A----------------------Y--L-Q---------T-----L-V--------------------S------H---I--L---------A---
Po TD  --A----------------------Y--L-Q---------T-----L-V--------------------S------H---I--L---------A---

Lb     RFVPETLCFIFKCADDYYRSPECQNRVEPVPEGLYLNTVIKPLYRFMRDQGYEVVDGKFVRKEKDHADIIGYDDVNQLFWYPEGLAKIVLSDNTRLVDVP
Po PC  -----C----------------------R-----ENI--------------------R-----Q------I---------I-R-----------AA
Po TD  -----C----------------------R-----ENI--------------------R-----Q------I---------I-R-----------AA

Lb     PSQRFMKFSKIEWNRVFFKTYFEKRSTAHLLVNFNRIWILHVSVFYFYTAFNSPKVYAPRTKATPSAPMTWSAVALGGAVSTIIMLLATIAEFSYIPTTW
Po PC  -A-----LM-VD--------------I-----------V---A----------------N-NN---------T------A-G--I---L----------
Po TD  -A-----LM-VDC------------I

Lb     NNASHLTTRFIFLLVILALTAGPTFYIAMVDDRPAQTQIPLIIGIVQFFISVVVTIAFGLIPSGRMFGDRVAGKSRKYMASQTFTASYPALARSARSASI
Po PC  ---------L--------------------G--N-GNV-----------A---V---I-----------R--N--F-----------T-PGT------

Lb     SLWLLIFGCKFAESYFFLTSSFSSPIAVMARTKVQGCNDKLFGNALCTNQVPFTLAIMYVMDLVLFFLDTYLWYIIWIVIFSIGRSFSLGLSIWTPWKDI
Po PC  GI-----T---V---------------------------S--F--S---S-----A---------I--------------V---------------

Lb     FTRLPKRIYAKLLATAEMEVKYKPKVLVSQIWNAVIISMYREHLLSIDHVQRLLYHQVDGADGRRTLRAPPFFTNQNGFKGNFFPPGGEAERRISFFASS
Po PC  Y---------------------------------------------------N---------SP--------------E-..-----S-------------

Lb     LTTALPEPLPVDAMPTFTVLVPHYSEKILLSLREIIREEDQNTRVTLLEYLKQLHPVEWDNFVKDTKILAEEGADSTTSQANEKTSKTDDLPFYCIGFKT
Po PC  ----------------------------------------------------------------SEGMDGATSTNIEK-------------

Lb     SSPEYTLRTRIWASLRAQTLYRTVSGMMNYSKAIKLLYRVENPDIVHNFGGNTERLERELERMARRKFKFAISMQRFSKFNKEEQENAEFLLRAYPDLQI
Po PC  --------------------------------------------QT---------------------------------D----------------

Lb     AYLDEEPGPKGGESKLFSALIDGHSEIDEKTGKRKPKFRVELPGNPILGDGKSDNQNHAMIFYRGEYLQLIDANQDNYLEECLKIRNILGEFEELSISSQ
Po PC  -------------AR---T------------------I------------------IV---------------------------------Y-C---

Lb     SPYAQWGHKEFSKSPVAIVGTREYIFSENIGVLGDIAAGKEQTFGTLTARALAWIGGKLHYGHPDFLNATFMNTRGGVSKAQKGLHLNEDIFAGMNAFGR
Po PC  ----------------------------------------------M--------------------T---------------------

Lb     GGRIKHSEYYQCGKGRDLGFGTILNFQTKIGTGMGEQMLSREYYYLGTQLPIDRFLTFYYGHPGFHINNILVIKSIQIFMVTLLFIGTLNKQLAICRVDS
Po PC  ---------------------------------------------------------------------Y------L---YL---------

Lb     QGNVIGGQPGCYNLIPVFDWIRRCIVSIFLVFGIAFLPLFLQELVERGTGKALLRLGKHFLSLSPIFEVFSTQIYSNSILSNLSFGGARYIATGRGFATT
Po PC  S------------V--------------FV-----------------------------------------Q-----T---------------

Lb     RISFSILYSRFAGPSIYMGMRNLLILLYATMSIWIPHLIYFWLSVLSLCIAPFLFNPHQFSYADFIIDYREFLRWMSRGNSRTKASSWYGYCRLSRTMIT
Po PC  --N-------------------L-------L-M-------A----------V------F--------Y-----------------------

Lb     GYKKKKLGHPSEKLSGDVPRASWRSVIFVEVLWPICSSIIFVVAYMFVKSFPDKDGNQHPSPLIRIAVIAIGPVVWNAAILISFFFISLFLGPMMESWAK
Po PC  -------------------T------S--VF-V-VA-V------------AN-DLP-------------------V-MAL---A-------D--I-

Lb     FGSVMATVAHTLCLFGLVAFFEFFWFLELWDASHAVLGVIAIIAVQRTVQKILIAVFLTREFKHDETNRAWWTGKWYGRGLGNSAMSQPAREFIVKIVEM
Po PC  ------AI--V-G-V----------------------CVV-I--AI--F--S-V---Y---------------------------------

Lb     SLWGSDFLLGHVLLLILTPPILIPYANSLHSTMLFWLRPSKQIRAPLFSTKQKRQRRWIVMKYTIVYVVVIAFLAALIVLPALMRDRITFDCSICRNI
Po PC  ---T----I--I--I----------F-R------------------------T-----I---F--M--VSCI---------F------E----KS-
```
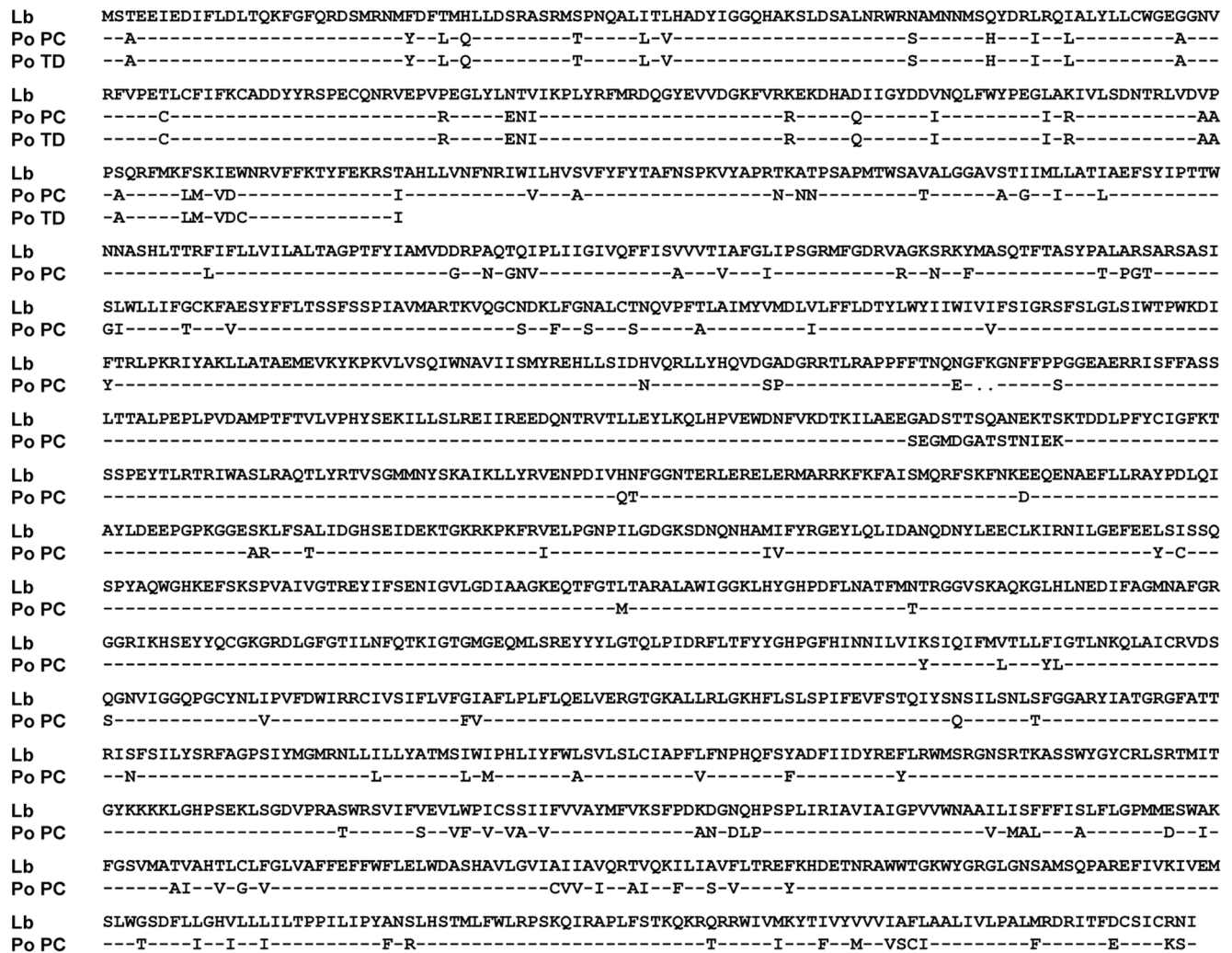
**Figure 2. Comparison of predicted GLS amino acid sequences among *P. ostreatus* TD300, *Laccaria bicolor* S238N-H82, and *P. ostreatus* PC15 v2.0.** **Lb**: GLS from *Laccaria bicolor* S238N-H82, GeneBank accession numbers is XM_001875351. **Po PC**: GLS from *P. ostreatus* PC15 v2.0, collected by 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

## PEG/CaCl$_2$-mediated protoplast transformation and transformant identification

Protoplasts preparation and PEG/CaCl$_2$-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 $10^6$ 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 $P_{gpd}$-1 and GLS-1 (Table 1) for amplifying the combination of $P_{gpd}$ 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).

## Statistical analysis

Statistical analysis was performed using standard ANOVA techniques. Comparisons among the means of three independent experiments were made using the least significant difference (LSD) test.

# Results

## Construction of the homologous recombination fragment for swapping GLS promoter

The three segments of *hph*, $P_{gpd}$, and $GLS_{1025}$ 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 $P_{gpd\ 1035}$ 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 $P_{gpd}$-1 and GLS-1, which was 1,320 bp in length and spanned the 3′ end of $P_{gpd}$ and the downstream of the 5′ end of $GLS_{1025}$ (Fig. 4B). The result revealed that *hph* gene in the homologous recombination fragment was deleted by the homologous recombination between $P_{gpd\ 1035}$ and $P_{gpd}$ from the third generation of the transformants. In addition, the introduced $P_{gpd}$ 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 $P_{gpd}$, 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.
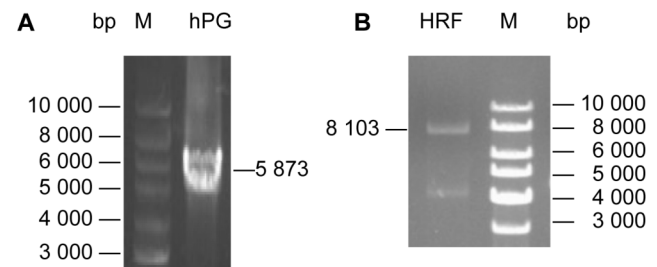


**Figure 3. Agarose gel analysis of TD-OE PCR products for the construction of the homologous recombination fragment for promoter swapping.** hPG: the fusion product of *hph*, $P_{gpd}$, and $GLS_{1025}$; HRF: the homologous recombination fragment which was the fusion product of UH, $P_{gpd\ 1035}$, and hPG.
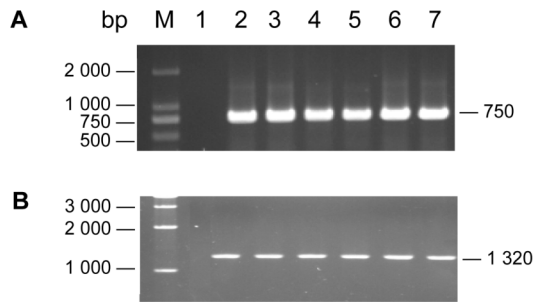doi:10.1371/journal.pone.0061693.g003

**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*$_{1025}$ (B). Lane 1: WT; Lane 2–7: transformant A1, A4, A9, A15, A17, and A21.
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The infrared spectra (IR) of the polysaccharides produced by the six transformants and wild type had the same characteristic with close resemblance to each other as shown in Fig. 7. The absorption peaks at $3,457\ cm^{-1}$ were ascribed to hydroxyl stretching vibration of the polysaccharide; peaks at $3,151\ cm^{-1}$ were CH (CH3, CH2, or CH) stretching vibration; those at $1,626\ cm^{-1}$ attributed to $C=O$ asymmetric stretching vibration [24]; those at $1,398\ cm^{-1}$ were due to C-H variable-angle vibration; peaks at $1,143$ to $1,088\ cm^{-1}$ were characteristics of C-O-C asymmetric vibration of pyranose ring [25]; finally the bands at $856\ cm^{-1}$ mapped to β-glucan [26], suggesting that β-glycosidic bond was present in all samples.

## 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*AOX1*) in methanol-grown cells of *Pichia pastoris* [35]. 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]. The other
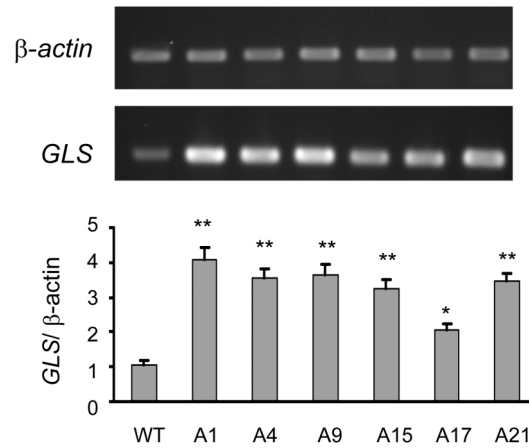


**Figure 5. Semi-quantitative RT-PCR analysis of *GLS* mRNA in the fifth generation transformants.** The amount of *GLS* mRNA, expressed as the ratio of densitometric measurement of the sample to the corresponding internal standard (β-actin), is shown in the upper panels. * $p<0.05$ comparing to WT; ** $p<0.01$ comparing to WT.
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constitutive promoters used in filamentous fungi included *adhA*, *gdhA*, *oliC*, *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.

## Conclusion

By our knowledge, this is the first report of successful swapping of promoters using TD-OE PCR in filamentous fungi through
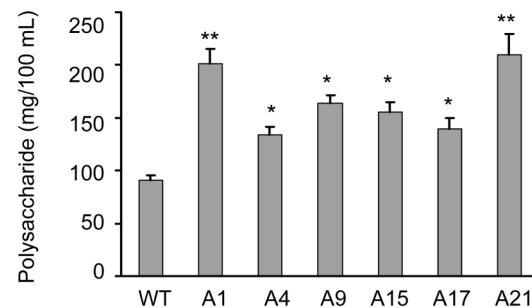


**Figure 6. The polysaccharide yield of the fifth generation transformants.** * $p<0.05$ comparing to WT; ** $p<0.01$ comparing to WT.
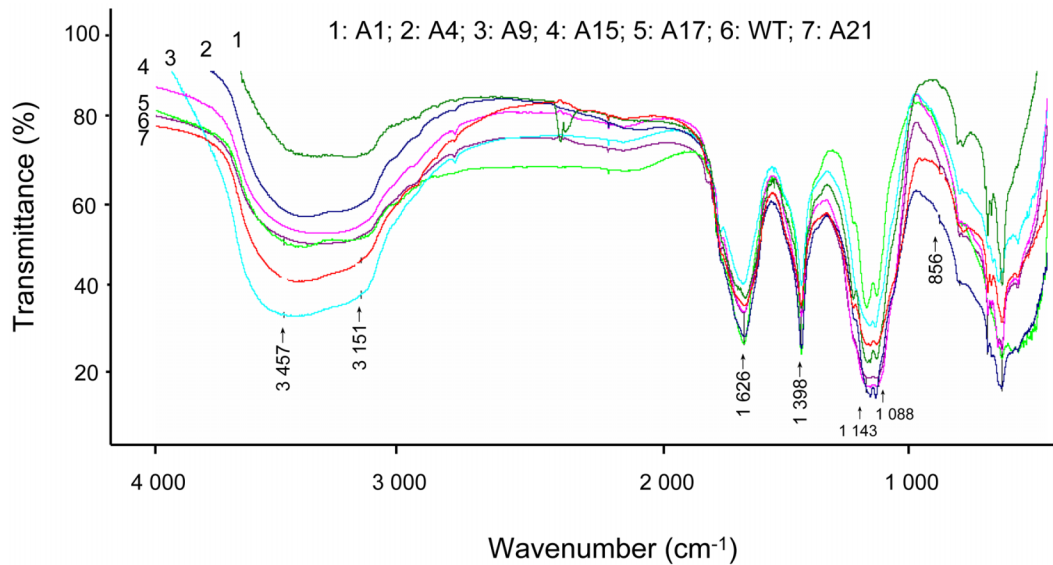doi:10.1371/journal.pone.0061693.g006

**Figure 7. The infrared spectroscopy of the β-glucans produced by the fifth generation transformants.**
doi:10.1371/journal.pone.0061693.g007

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.**

(DOC)

## Author Contributions

Conceived and designed the experiments: LQ JS. Performed the experiments: RC CQ DL. Analyzed the data: RC CQ DL YQ YG. Contributed reagents/materials/analysis tools: YQ YG. Wrote the paper: RC CQ LQ.

## References

1. Popov I, Lewin G (1999) Antioxidant homeostasis: characterization by means of chemiluminescent technique. Methods Enzymol 300: 437–456.
2. Chen J, Seviour R (2007) Medicinal importance of fungal beta-(1→3), (1→6)-glucans. Mycol Res 111: 635–652.
3. Wasser SP (2010) Medicinal mushroom science: history, current status, future trends, and unsolved problems. Int J Med Mushr 12: 1–16.
4. De Silva DD, Rapior S, Fons F, Bahkali AH, Hyde KD (2012) Medicinal mushrooms in supportive cancer therapies: an approach to anti-cancer effects and putative mechanisms of action. Fungal Divers 55: 1–35.
5. Rop O, Mlcek J, Jurikova T (2009) Beta-glucans in higher fungi and their health effects. Nutr Rev 67: 624–631.
6. Tang YJ, Zhu LW, Li HM, Li DS (2007) Submerged culture of mushrooms in bioreactors-challenges, current state-of-the-art, and future prospects. Food Technol Biotechnol 45: 221–229.
7. Da Silva NA, Srikrishnan S (2012) Introduction and expression of genes for metabolic engineering applications in *Saccharomyces cerevisiae*. FEMS Yeast Res 12: 197–214.
8. Borchers AT, Keen CL, Gershwin ME (2004) Mushrooms, tumors, and immunity: an update. Exp Biol Med 229: 393–406.
9. Reverberi M, Di Mario F, Tomati U (2004) β-Glucan synthase induction in mushrooms grown on olive mill wastewaters. Appl Microbiol Biotechnol 66: 217–225.
10. Bowman SM, Free SJ (2006) The structure and synthesis of the fungal cell wall. BioEssays 28: 799–808.
11. Robledo-Briones M, Ruiz-Herrera J (2012) Transcriptional regulation of the genes encoding chitin and β-1,3-glucan synthases from *Ustilago maydis*. Cur Microbiol 65: 85–90.
12. Smits GJ, Van der Ende H, Klis FM (2001) Differential regulation of cell wall biogenesis during growth and development in yeast. Microbiology 147: 781–794.
13. Schultz TP, Hubbard TF, Jin L, Fisher TH, Nicholas DD (1990) Role of stilbenes in the natural durability of wood: fungicidal structure-activity relationships. Phytochemistry 29: 1501–1507.
14. Giovannozzi-Sermanni G, Perani C, Porri A, De Angelis F, Barbaruto MV, et al. (1991) Plant cell wall degradation by means of *L. edodes* and chemical characterization of produced soluble lignocellulose. Agrochimica 35: 174–189.
15. Hammel KE, Kapich AN, Jensen KA, Ryan ZC (2002) Reactive oxygen species as agents of wood decay by fungi. Enzym Microb Technol 30: 445–453.
16. Karácsonyi Š, Kuniak Ľ (1994) Polysaccharides of *Pleurotus ostreatus*: isolation and structure of pleuran, an alkali-insoluble β-d-glucan. Carbohydr Polym 24: 107–111.
17. Silva S, Martins S, Karmali A, Rosa E (2012) Production, purification and characterisation of polysaccharides from *Pleurotus ostreatus* with antitumour activity. J Sci Food Agric 92: 1826–1832.
18. Wang L, Li Y, Liu D, Zhang C, Qi Y, et al. (2011) Immobilization of mycelial pellets from liquid spawn of oyster mushroom based on carrier adsorption. Horttechnology 21: 82–86.
19. Punt PJ, Oliver RP, Dingemanse MA, Pouwels PH, van den Hondel CAMJJ (1987) Transformation of *Aspergillius* based on the hygromycin-B resistance gene from *Escherichia coli*. Gene 56: 117–124.
20. Qiu L, Li Y, Liu Y, Gao Y, Qi Y, et al. (2010) Particle and naked RNA mycoviruses in industrially cultivated mushroom *Pleurotus ostreatus* in China. Fungal Biol 114: 507–513.
21. Dong X, Zhang K, Gao Y, Qi Y, Shen J, et al. (2012) Expression of hygromycin B resistance in oyster culinary-medicinal mushroom, *Pleurotus ostreatus* (Jacq.:Fr.)P. Kumm. using three gene expression systems. Int J Med Mushr 14: 21–26.
22. Staub AM (1965) Removal of proteins: Sevag method. In: Methods in carbohydrate chemistry, vol. 5. Whistler RL, editors. New York: Academic Press Inc, 5–6 p.

23. Kochert G (1978) Carbohydrate determination by the phenol-sulphuric acid method. In: Handbook of Phycological Methods, Physiological and Biochemical Methods. Hellebust JA, Craigie, JS, editors. Cambridge: Cambridge University Press, 95–97 p.

24. Ge Y, Duan Y, Fang G, Wang S (2009) Polysaccharides from fruit calyx of *Physalis alkekengi* var. *Francheti*: isolation, purification, structural features and antioxidant activities. Carbohydr Polym 77: 188–193.

25. Zhao G, Kan J, Li Z, Chen Z (2005) Structural features and immunological activity of a polysaccharide from *Dioscorea opposita* Thunb roots. Carbohydr Polym 61: 125–131.

26. Kiho T, Sakushima M, Wang SR, Nagai K, Ukai S (1991) Polysaccharides in fungi. XXVI. Two branched (1→3)-beta-D-glucans from hot water extract of Yu r. Chem Pharm Bull (Tokyo) 39: 798–800.

27. Alper H, Fischer C, Nevoigt E, Stephanopoulos G (2005) Tuning genetic control through promoter engineering. Proc Natl Acad Sci U S A 102: 12678–12683.

28. Blazeck J, Alper HS (2013) Promoter engineering: Recent advances in controlling transcription at the most fundamental level. Biotechnol J 8: 46–58.

29. McCleary WR (2009) Application of promoter swapping techniques to control expression of chromosomal genes. Appl Microbiol Biotechnol 84: 641–648.

30. Yadav VG, De Mey M, Lim CG, Ajikumar PK, Stephanopoulos G (2012) The future of metabolic engineering and synthetic biology: towards a systematic practice. Metab Eng 14: 233–241.

31. Terpe K (2006) Overview of bacterial expression systems for heterologous protein production: From molecular and biochemical fundamentals to commercial systems. Appl Microbiol Biotechnol 72: 211–222.

32. Hahn S, Young ET (2011) Transcriptional regulation in *Saccharomyces cerevisiae*: Transcription factor regulation and function, mechanisms of initiation, and roles of activators and coactivators. Genetics 189: 705–736.

33. Punt PJ, Zegers ND, Busscher M, Pouwels PH, van den Hondel CA (1991) Intracellular and extracellular production of proteins in *Aspergillus* under the control of expression signals of the highly expressed *Aspergillus nidulans gpdA* gene. J Biotechnol 17: 19–33.

34. Jensen DF, Schulz A (2003) Exploitation of GFP-technology with filamentous fungi. In: Handbook of Fungal Biotechnology 2nd edition. Arora, DK, Bridge PD, Bhatnagar D, Bharat R, Mukerji KG, editors. New York: Marcel Dekker, 441–451 p.

35. Waterham HR, Digan ME, Koutz PJ, Lair SV, Cregg JM (1997) Isolation of the *Pichia pastoris* glyceraldehyde-3-phosphate dehydrogenase gene and regulation and use of its promoter. Gene 186: 37–44.

36. Fleissner A, Dersch P (2010) Expression and export: recombinant protein production systems for *Aspergillus*. Appl Microbiol Biotechnol 87: 1255–1270.

37. Gu P, Yang F, Kang J, Wang Q, Qi Q (2012) One-step of tryptophan attenuator inactivation and promoter swapping to improve the production of L-tryptophan in *Escherichia coli*. Microb Cell Fact 11: 30.

38. Horton RM, Hunt HD, Ho SN, Pullen JK, Pease LR (1989) Engineering hybrid genes without the use of restriction enzymes: gene splicing by overlap extension. Gene 77: 61–68.

39. Dillon PJ, Rosen CA (1990) A rapid method for the construction of synthetic genes using the polymerase chain reaction. BioTechniques 9: 298–299.

40. Horton RM, Pease LR (1991) Recombination and mutagenesis of DNA sequences using PCR. In: Directed Mutagenesis: A Practical Approach. McPherson MJ, editors. Oxford: IRL Press, 217–247 p.

41. Shevchuk NA, Bryksin AV, Nusinovich YA, Cabello FC, Sutherland M, et al. (2004) Construction of long DNA molecules using long PCR-based fusion of several fragments simultaneously. Nucleic Acids Res 32: e19.

42. Heckman KL, Pease LR (2007) Gene splicing and mutagenesis by PCR-driven overlap extension. Nat Protoc 2: 924–932.

43. Don RH, Cox PT, Wainwright BJ, Baker K, Mattick JS (1991) 'Touchdown' PCR to circumvent spurious priming during gene amplification. Nucleic Acids Res 19: 4008.

44. Korbie DJ, Mattick JS (2008) Touchdown PCR for increased specificity and sensitivity in PCR amplification. Nat Protoc 3: 1452–1456.