Efficient expression of heterologous genes by the introduction of the endogenous glyceraldehyde-3-phosphate dehydrogenase gene intron 1 in *Ganoderma lucidum*

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**Abstract**

**Background:** *Ganoderma lucidum*, a well-known medicinal mushroom, has received wide attention as a promising cell factory for producing bioactive compounds. However, efficient expression of heterologous genes remains a major challenge in *Ganoderma*, hindering metabolic regulation research and molecular breeding of this species.

**Results:** We show that the presence of glyceraldehyde-3-phosphate dehydrogenase gene (*gpd*) intron 1 at the 5′ end of, the 3′ end of, or within the heterologous phosphinothricin-resistant gene (*bar*) is efficient for its expression in *G. lucidum*. The enhanced expression of *bar* is exhibited by the higher accumulation of mRNA and increased amounts of protein. Moreover, the insertion of the *gpd* intron 1 in the β-glucuronidase gene (*gus*) elevates its mRNA accumulation and enzyme activity, which facilitates the use of this reporter gene in *Ganoderma*.

**Conclusions:** This study has demonstrated the importance of the introduction of *gpd* intron 1 for the efficient expression of *bar* and *gus* in *G. lucidum*. The presence of the *gpd* intron 1 in heterologous genes increases levels of mRNA accumulation and protein expression in basidiomycete *Ganoderma*. The developed method may be utilized in upregulating the expression of other heterologous genes in *Ganoderma*.

**Keywords:** *Ganoderma*, Expression of heterologous genes, Intron, mRNA accumulation, Protein expression, Enzyme activity

**Background**

*Ganoderma lucidum*, a well-known medicinal mushroom, can synthesize a variety of bioactive products such as ganoderic acids, ganoderols, polysaccharides, immunomodulatory proteins, nucleotides, and sterols [1]. It has received wide attention as a promising cell factory for producing these valuable compounds in recent years [2–7]. The genome, transcriptome, and proteome of *G. lucidum* have been sequenced [8–10]. *G. lucidum* has been suggested as a model species for studying the biology of basidiomycetes and biosynthesis of secondary metabolites [8, 11].

Molecular genetic approaches tools such as genetic transformation, disruption, and deletion of target genes have been developed in *G. lucidum* [12–16]. However, efficient expression of heterologous genes remains a main challenge in *Ganoderma*, although it would be valuable for metabolic regulation and molecular breeding. Successful expression of heterologous genes in basidiomycetes may depend on several factors, including an effective promoter, codon optimization, and the presence of introns [12, 17, 18]. Among those elements, introns have a significant effect on the expression of heterologous genes.
genes. In some mushroom species such as Clitopilus passeckerianus, Coprinus cinereus, and Schizophyllum commune, the addition of an intron is required for the efficient expression of the green fluorescent protein gene (gfp), the hygromycin-resistant gene, and the phleomycin-resistant gene [19–21].

Intron-containing G. lucidum genes represent 85.4% of predicted genes (approximately 16,113) in its 43.3-Mb genome [8], indicating the importance of introns in Ganoderma. In a previous study, we found that an extra fragment, including gpd exon 1, intron 1, and 3-bp exon 2 at the 5' end of gfp and phosphinothricin-resistant gene (bar), was essential for their expression in G. lucidum [16]. However, the role of gpd intron 1 in regulating the expression of heterologous genes has not been yet thoroughly investigated in G. lucidum. Furthermore, how gpd intron 1 affects gene transcription, protein expression, and enzyme activity when heterologous genes are expressed in G. lucidum remains unclear.

In this study, we show that the presence of gpd intron 1 at different locations is effective in enhancing the expression of heterologous bar in G. lucidum. The efficient expression of heterologous genes is due to higher accumulation of mRNA and increased amount of protein. Moreover, the insertion of gpd intron 1 in the gus enhances its mRNA and enzyme activity, which facilitates the use of this reporter gene in Ganoderma.

**Results and discussion**

**Endogenous gpd intron 1 increases the expression of the heterologous bar in G. lucidum**

Our previous study has shown that the insertion of the gpd fragment containing the first exon (6 bp), the first intron (67 bp), and part of the second exon (3 bp) at the 5' end of the bar and gfp exerts a significant influence on protein expression. To investigate the effect of the endogenous gpd intron 1 on protein expression in G. lucidum, we constructed plasmids in which the heterologous codon-optimized phosphinothricin-resistance gene (opbar)-flag was regulated by the endogenous gpd promoter and the succinate dehydrogenase gene (sdh) terminator of G. lucidum (pJW-EXP-opbar-flag) (Fig. 1). Alternatively, the opbar-flag was cloned in similar plasmids that contained the gpd intron 1 directly upstream of the start codon (pJW-EXP-in-opbar-flag), 6 bp downstream of the translation start site of bar (pJW-EXP-in (M)-opbar-flag), and directly downstream of the stop codon (pJW-EXP-opbar-flag-in) (Fig. 1). These plasmids also contained a carboxin-resistance cassette, allowing selection of transformants on CYM plates with carboxin. After G. lucidum protoplasts were transformed with intron-containing and intronless opbar-flag plasmids (pJW-EXP-opbar-flag), we obtained carboxin-resistant colonies with all used plasmids. However, when these carboxin-resistance transformants were re-screened

![Fig. 1](https://example.com/figure1.png)  
**Fig. 1** Structure of opbar-flag plasmids used for G. lucidum transformation. See “Methods” for details on plasmid construction.
on CYM plates including phosphinothrin, no phosphinothrin-resistant colonies were obtained with plasmid pJW-EXP-opbar-flag. The phosphinothrin-resistance transformants were obtained with intron-containing plasmids pJW-EXP-in-opbar-flag, pJW-EXP-in (M)-opbar-flag, and pJW-EXP-opbar-flag-in (Fig. 2A, Additional file 1: Figs. S1A and S2A). PCR analysis showed that the opbar-flag has integrated into the genomes of transformants pJW-EXP-opbar-flag, transformants pJW-EXP-in-opbar-flag, pJW-EXP-opbar-in (M)-flag, and pJW-EXP-opbar-flag-in (Fig. 2B, Additional file 1: Figs. S1B and S2B). These results indicated that the introduction of the endogenous gpd intron 1 is important for efficient expression of heterologous bar in G. lucidum. Moreover, this effect was independent of the position of the gpd intron 1. Positive effects of intron on transgene expression have also been observed in other basidiomycetes such as Agaricus bisporus, C. cinereus, Phanerochaete chrysosporium, S. commune, C. passeckerianus and Trichoderma viride [20–24]. Enhancement of gene expression by introns may play a role in those organisms that generally possess introns in their genes [22].

Enhancement is associated with increased levels of mRNA

The transformant pJW-EXP-in-opbar-flag was chosen with the transformant pJW-EXP-opbar-flag to study how the gpd intron 1 affects gene transcription and protein expression. The presence of opbar-flag in the genome of transformants pJW-EXP-opbar-flag and pJW-EXP-in-opbar-flag was further determined by molecular hybridization. The genome of transformants pJW-EXP-opbar-flag and pJW-EXP-in-opbar-flag were digested with NheI and probed with an opbar fragment (Fig. 3A, B). Southern blot analysis verified that these transformants had insertions of opbar, with single or multiple copies in their genomes. Single-copy integration of opbar-flag event was detected in the genomes of transformants pJW-EXP-opbar-flag T1, T4, and T5, and pJW-EXP-in-opbar-flag T1, T3, and T5. To minimize copy-number effect, the transcription level of opbar was analyzed in transformants pJW-EXP-opbar-flag T1 and T5, and pJW-EXP-in-opbar-flag T1 and T3 carrying a single-copy opbar by real-time qRT-PCR analysis. The results showed high transcription levels in transformants pJW-EXP-in-opbar-flag T1 and T3 bearing gpd intron 1 in opbar and low levels for transformants pJW-EXP-opbar-flag T1, and T5 containing opbar without gpd intron 1. No significant transcription difference was observed between pJW-EXP-in-opbar-flag T1 and T5. The transcription level of opbar in transformants pJW-EXP-in-opbar-flag T1 and T3 was, respectively, 12.3- and 10.1-fold higher than in transformant pJW-EXP-in-opbar-flag T1 (Fig. 3C). Our results indicated that gpd intron 1 increased the transcription level of heterologous opbar in G. lucidum. Previous studies have suggested that introns increased the level of mRNA possibly by enhancing maturation and stability of transcripts [25]. The difference in transcription level between pJW-EXP-in-opbar-flag T1 and T3 might be relevant to integration position effects [26].

The effect of the gpd intron 1 on protein yield of heterologous opbar

Proteins isolated from transformants pJW-EXP-opbar-flag T1, and T5, and pJW-EXP-in-opbar-flag T1 and T3 were analyzed by western blotting (Fig. 4). A protein of \( \approx 22 \) kDa was detectable in the transformants pJW-EXP-in-opbar-flag T1 and T3. However, it was not observed in transformants pJW-EXP-opbar-flag T1 and T5 containing opbar without the gpd intron.
1. The band at 22 kDa that was recognized by flag-specific antibodies was the opbar-flag protein, as the molecular weight was similar to the predicted value. As shown in Fig. 4, the amount of opbar-flag protein also increased with the introduction of gpd intron 1. These results coincided with those of qRT-PCR analysis and phosphinothrin-resistance screening. Gpd intron 1 enhanced protein expression both at the RNA and protein levels. Opbar protein levels did not correlate with opbar mRNA levels in the intronless G. lucidum transformants, indicating that gpd intron 1 may also enhance the efficiency of translation besides increasing mRNA content by affecting transcription and stability of mRNA [27, 28]. The detailed mechanism of enhancement of introns on the translation of heterologous genes requires further investigation.

Introduction of gpd intron1 enhances β-glucuronidase (GUS) expression
To confirm the positive effect of gpd intron 1 on the expression of heterologous genes, we also constructed plasmids pJW-EXP-opgus and pJW-EXP-in-opgus (Fig. 5A) and transformed these into Ganoderma protoplasts. These plasmids contain a carboxin-resistant cassette for transformation selection and the full codon-optimized gus (opgus) cassette (pJW-EXP-opgus) or the intron-opgus cassette. Carboxin-resistant colonies were screened for transgenes by genome PCR (Additional file 1: Fig. S3) and subsequently were further analyzed by Southern blotting. The results showed that opgus and intron-opgus had integrated into the genome of the recipient as single copies in transformants pJW-EXP-opgus T1, T2, T3, and T4, and transformants pJW-EXP-in-opgus T1 (Fig. 5B). Real-time
qRT-PCR analysis showed that the transcription level of \textit{opgus} in transformant pJW-EXP-in-opgus T1 was 3.8-fold higher than that in transformants pJW-EXP-opgus T1 and T2 without \textit{gpd} intron 1 (Fig. 6A). The increase in transcription level relative to the intronless control ranged from 3.8-fold for \textit{opgus} to 12.3-fold for \textit{opbar}, which may be dependent on the cDNA sequence and the transcription level of the intronless heterologous gene [27, 29]. Histochemical staining analysis was also performed to detect expression of \textit{opgus} in wild-type (WT), and transformants pJW-EXP-opgus T2 and pJW-EXP-in-opgus T1. Figure 6B shows that GUS activity was observed in the mycelia of transformants pJW-EXP-opgus T2 and pJW-EXP-in-opgus T1, while no blue staining was detected in the WT mycelia. Moreover, GUS activity in transformant pJW-EXP-in-opgus T1 was higher than that in transformant pJW-EXP-opgus T2. Again, our results showed that \textit{gpd} intron 1 enhanced the \textit{opgus} transcription and GUS enzyme activity in \textit{G. lucidum}. It may thus be possible...
to efficiently express other heterologous genes in *Ganoderma* by introducing the *gpd* intron 1.

**Conclusions**

This work demonstrated the importance of introducing *gpd* intron 1 for the efficient expression of *opbar* and *gus* in *G. lucidum*. The presence of *gpd* intron 1 in heterologous genes enhances mRNA accumulation and protein expression in basidiomycete *Ganoderma*. The developed method can be applicable to upregulate the expression of other heterologous genes in *Ganoderma*.

**Methods**

**Strains and media**

Monokaryotic *G. lucidum* 5.616-1 strain [30] was cultured in CYM plates (10 g/L maltose, 20 g/L glucose, 2 g/L tryptone, 2 g/L yeast extract, 0.5 g/L MgSO₄, 4.6 g/L KH₂PO₄ and 10 g/L agar) and in transformations. *Escherichia coli* strain DH5α was used in the construction and transformation of plasmids. The mycelia of *G. lucidum* were cultured in fermentation medium (35 g/L glucose, 1 g/L KH₂PO₄, H₂O, 0.5 g/L MgSO₄, 7H₂O, 5 g/L peptone, 2.5 g/L yeast extract, and 0.05 g/L vitamin B1, pH 5.5) in the dark at 30 °C [31, 32].

**Plasmid construction**

The codon optimized phosphinothricin-resistance gene (opbar)-flag, *gpd* gene intron 1 (in)-opbar-flag, *in*-(M)-opbar-flag, *opbar*-flag-in, *opgus*, and in-*opgus* (Additional file 1) were synthesized by Sangon Co., Ltd. (Shanghai, China). These genes were ligated into pUC57 (Sangon) to produce plasmids, pUC57-opbar-flag, pUC57-in-opbar-flag, pUC57-opgus, and pUC57-in-opgus, respectively.

The opbar-flag, in-opbar-flag, in-(M)-opbar-flag, and opbar-flag-in genes were PCR amplified from plasmids pUC57-opbar-flag, pUC57-in-opbar-flag, pUC57-in (M)-opbar-flag, and pUC57-opgus using primers bar probe F/bar probe F and opgus probe F/opgus probe R were used as probes. Southern blot analysis was conducted under conditions recommended for the digoxigenin (DIG) hybridization system by Mylab™ (Beijing, China).

**Nucleic acid isolation**

*Ganoderma lucidum* mycelia were harvested, frozen, and powdered in liquid nitrogen. Genomic DNA was isolated employing the cetyltrimethylammonium bromide (CTAB) method. Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s protocols and treated with RNase-free DNase I before use.

**Quantitative real-time (qRT)-PCR analysis**

Approximately 1 mg total RNA was used as template, and reverse transcription was performed using the PrimeScript™ RT reagent Kit (Takara, China) following the manufacturer’s instructions. The transcription levels of *opbar* and *opgus* were determined by qRT-PCR as previously described [6, 16]. The 18S-rRNA transcript was used as internal control to normalize relative transcription levels. For *opbar* and *opgus*, the transcription level in
opbar-flag and opgus strains was set to 1.0, and the relative transcription levels in other strains were presented as fold changes relative to the reference level. The primers qRT-bar-F/qRT-bar-R, qRT-gus-F/qRT-gus-R, and qRT-18S-F/qRT-18S-R (Additional file 1: Table S1) were used for amplification of the opbar, opgus, and 18S rRNA genes, respectively.

Western blot analysis
*Ganoderma lucidum* mycelia were washed and homogenized in 1 mL of lysis buffer (25 mM Tris base (pH 7.4), 200 mM glycine, 5 mM EDTA, 1 mM phenylmethyl sulfonyl fluoride, and 1 mM 2-mercaptoethanol). The homogenates were centrifuged at 12,000×g for 20 min at 4 °C, then protein concentration in the supernatants was determined using the Bradford method. For western bolt analysis, 30 µg of protein was separated on a 12% SDS-polyacrylamide gel and electro-transferred to a polyvinylidene difluoride membrane in TBST buffer [10 mM Tris/HCl (pH 7.4), 100 mM NaCl, 0.1% Tween 20] for 1 h. Membranes were blocked with non-fat milk dissolved in TBSF buffer at room temperature for 2 h, followed by incubation with primary antibodies (anti-Flag, PROTEINTECH, Cat No. 20543-1-AP at 1:5000 dilution; anti-tubulin rabbit polyclonal antibody, BBI, Cat No. D110015 at 1:5000 dilution). Membranes were washed with TBSF and incubated with an HRP-conjugated goat anti-rabbit IgG (BBI, Cat No. D110058 at 1:5000 dilution). The blots were washed with TBSF buffer again, and the bands were visualized using an enhanced chemiluminescence method.

GUS activity assay
*Ganoderma lucidum* mycelia were scraped from CYM plate on a glass slide for 20 and 40 min, respectively. After washing with PB buffer (10 mM sodium phosphate, pH 7.0), the mycelia were observed under a Nikon Coolpix 900 camera.

Statistical analysis
Data were generated in three independent sample measurements, and all data are presented as the mean ± standard deviation. The results were considered significant for \( p < 0.05 \) in a two-tailed analysis.

Supplementary Information
The online version contains supplementary material available at https://doi.org/10.1186/s12934-021-01654-8.
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