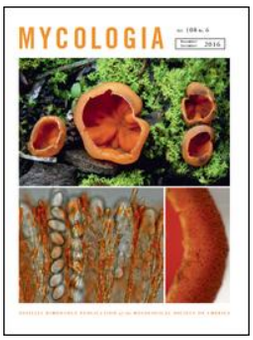

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# The pH-responsive transcription factor PacC regulates mycelial growth, fruiting body development, and ganoderic acid biosynthesis in *Ganoderma lucidum*

Feng-Li Wu
Guang Zhang
Ang Ren
Zhi-Hao Dang
Liang Shi
Ai-Liang Jiang
Ming-Wen Zhao[1]

*Microbiology Department, College of Life Sciences, Key Laboratory of Microbiological Engineering of Agricultural Environment, Ministry of Agriculture, Nanjing Agricultural University, Nanjing 210095, Jiangsu, People's Republic of China*

***Abstract:*** *Ganoderma lucidum* is a medicinal macrofungus that is widely used in traditional Chinese medicine. Nonetheless, the scarcity of basic biological studies of this organism has hindered the further development of its commercial value. The pH-responsive transcription factor PacC/Rim101 governs the adaptation to environmental pH, the development and the secondary metabolism of many fungi. In this study, a homologue of PacC/Rim101 that encodes GlPacC was identified in the higher basidiomycete *G. lucidum*. GlPacC is composed of 807 amino acids and contains three typical C2H2 zinc-finger domains, two potential PEST domains, a putative PKA phosphorylation site, and a putative nuclear localization signal (NLS). *GlPacC* was transcribed at a high level when the fungus was under neutral and alkaline conditions, and silencing of *GlPacC* impaired the fungal response to ambient pH. The distance between the hyphal branches (of vegetative hyphae and aerial hyphae) was significantly increased in the *GlPacC*-silenced strains. The *GlPacC*-silenced strains grew abnormally or became sickly on solid culture medium and were unable to form primordia and fruiting bodies. The ganoderic acid content, levels of the *sqs* and *ls* transcripts, and contents of the metabolic intermediates squalene and lanosterol were all up-regulated in the *GlPacC*-silenced strains. Our results indicate that *GlPacC* is functional and plays complex roles in mycelial growth, fruiting body development and ganoderic acid biosynthesis in *G. lucidum*.

***Key words:*** hyphal branching, macrofungi, RNA interference, secondary metabolism, sexual development, triterpenes

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[1]Corresponding author. E-mail: mwzhao@njau.edu.cn

## Introduction

All microorganisms, including fungi, must be able to sense and to respond to changes in their environment. They must protect themselves from low nutrient availability, varying pH or temperature, light, chemicals, competition among different species, the immune response of their host, and other factors. Therefore, they must coordinate the activity of multiple signaling pathways to control the appropriate cellular responses. One of the most common environmental stresses for fungi is a change in the extracellular pH. Alterations in ambient pH can affect a high number of cellular processes, including membrane and cell wall stability, morphogenesis, protein stability and function, and nutrient uptake (O'Meara et al. 2010). Many of the responses to pH alterations are regulated by Pal/Rim signaling pathway (Alkan et al. 2013, Franco-Frias et al. 2014). In ascomycetes, the Pal/Rim signaling pathway contains six Pal proteins (PalA/Rim20, PalB/Rim13, PalC/Rim23, PalF/Rim8, PalH/Rim21, PalI/Rim9), a zinc-finger transcription factor named PacC in filamentous fungi or Rim101 in yeasts, and some components of the endocytic system (Selvig and Alspaugh 2011, Cornet and Gaillardin 2014). But in basidiomycetes *Ustilago maydis* and *Cryptococcus neoformans*, only four (*PalA/Rim20, PalB/Rim13, PalC/Rim23, PalI/Rim9*) of the corresponding six *Pal/Rim* genes were identified in an in silico search (Cervantes-Chavez et al. 2010, Ost et al. 2015).

Under neutral and alkaline pH conditions, the Pal/Rim signaling pathway mediates the processing of full-length PacC via two proteolytic cleavages to yield a shorter functional form; the truncated form of PacC activates the transcription of the "alkaline-expressed" genes and at the same time, represses the transcription of the "acid-expressed" genes (Alkan et al. 2013, Penalva et al. 2014). *PacC/Rim101*-deletion mutants have difficulty adapting to changes in the ambient pH. In *Magnaporthe oryzae*, the deletion of *PacC* caused a progressive decrease in the growth rate and conidial production at pH 8 (Landraud et al. 2013). *MrpacC*-deletion strains of *Metarhizium robertsii* exhibited impaired growth and produced fewer spores under both acidic and alkaline conditions (Huang et al. 2015). In *Ustilago maydis Δrim101* mutants, mycelial morphology and the distribution of septa were altered at both pH 3 and 7 (Arechiga-Carvajal and Ruiz-Herrera 2005). Deletion of *PacC* also reduced the level of fungal virulence of the plant pathogen *Fusarium*

*oxysporum* (Caracuel et al. 2003), the mammalian pathogen *Aspergillus fumigatus* (Bignell et al. 2005), and the insect pathogen *M. robertsii* (Huang et al. 2015). In addition, *PacC/Rim101* affects the production of secondary metabolites in fungi. In *Fusarium graminearum*, Pac1 negatively regulates *Tri* gene expression and trichothecene production at an acidic pH (Merhej et al. 2011). A *PAC1*-disrupted strain of *Fusarium verticillioides* produced more fumonisin than did the wild type strain when grown on maize kernels and in a synthetic medium buffered to an acidic pH of 4.5 (Flaherty et al. 2003). Researches on *PacC/Rim101* in fungi have been concentrated on ascomycetes and a few basidiomycetes. However, the functions of *PacC/Rim101* in the higher basidiomycetes, particularly macrofungi, have not been investigated.

*Ganoderma lucidum* (Curtis:Fr.) P. Karst, which is known as "the symbol of traditional Chinese medicine", is one of the best-known medicinal macrofungi in the world and is a potential model system for the study of complicated growth patterns and the regulation of secondary metabolic pathways in medicinal mushrooms (Shi et al. 2010, Chen et al. 2012). *G. lucidum* contains many pharmacologically active compounds, and more than 400 different compounds have been identified in it, making this fungus a virtual cellular "factory" for the production of biologically useful compounds (Sanodiya et al. 2009, Baby et al. 2015). Ganoderic acids (GAs, also called triterpenoids or triterpenes) are the major secondary metabolites of *G. lucidum* (Boh et al. 2007, Xia et al. 2014). Modern pharmacological research has demonstrated that GAs exhibit many therapeutic activities, including anti-tumor, anti-inflammatory, anti-HIV-l and immunomodulatory activities (Boh et al. 2007, Shi et al. 2010). However, the low yield of GAs and the scarcity of basic biological studies of *G. lucidum* have hindered the further development of its commercial value. Due to the completion of its genomic sequence (Chen et al. 2012) and the development of methods for gene overexpression (Shi et al. 2012, Xu et al. 2012) and RNA interference (RNAi) (Mu et al. 2012) of *G. lucidum*, there are currently effective genetic tools with which to investigate this organism. Because gene deletion through homologous recombination with a disrupted version of the gene of interest has been rarely reported in higher basidiomycetes, it is a good choice to study the gene function through RNA interference.

In this study, we identified a homologue of the *PacC/Rim101* gene in the higher basidiomycete *G. lucidum* and designated it *GlPacC*. Next, *GlPacC* was functionally evaluated via an RNA interference-mediated gene silencing strategy. We found that *GlPacC* is required for mycelial growth, fruiting body development, and ganoderic acid biosynthesis in *G. lucidum*.

## MATERIALS AND METHODS

*Strains and growth conditions.*—The *Escherichia coli* DH5α strain, which was used for plasmid amplification, was grown in Luria-Bertani (LB) medium at 37 C. The *G. lucidum* HG strain was obtained from the culture collection of the Institute of Edible Fungi, Shanghai Academy of Agricultural Sciences, and was routinely maintained on potato dextrose agar (PDA) medium at 28 C. For the fungal growth and gene expression experiments at different pH values, the potato dextrose medium was buffered with 77 mM $Na_2HPO_4$ and 61 mM citric acid at pH 3 or 4; with 50 mM Tris-HCl and 125 mM NaCl at pH 7 or 8; and with 50 mM $Na_2HPO_4$ and 80 mM NaCl at pH 10 (Zou et al. 2010). For liquid fermentation, the *G. lucidum* strains were inoculated into mushroom complete medium (MCM) (2% glucose, 0.2% yeast extract, 0.2% tryptone, 0.05% $MgSO_4{\cdot}7H_2O$, 0.046% $KH_2PO_4$ and 0.1% $K_2HPO_4$) (Kim et al. 2006) and were maintained on a rotary shaker incubator at 28 C for 7 d, with agitation at 150 rpm. The solid culture medium for *G. lucidum* fruiting body growth was composed of 78% cottonseed shell, 20% wheat bran, 1% sucrose, and 1% gesso in 60% to 65% water (Mu et al. 2014). After inoculation, the cultivation bags containing 600 g of solid culture medium were placed in an incubator at 28 C until the bags were full of mycelia. Fruiting body growth was performed at 28 C with approximately 85% atmospheric humidity and a light intensity of 300 1x.

*Gene cloning and bioinformatic sequence analysis.*—The entire *GlPacC* coding sequence was amplified by polymerase chain reaction (PCR) using *G. lucidum* cDNAs as a template and the primers listed (SUPPLEMENTARY TABLE I), which were designed based on the *G. lucidum* genomic sequence (Chen et al. 2012). The fragments were cloned into the pMD18-T vector (Takara, Dalian, China) for sequencing. The open reading frame (ORF) and exon/intron positions were deduced by comparing the genomic and cDNA sequences. The *PacC/Rim101* protein sequences were aligned using ClustalW and were then analyzed using the phylogenetic package MEGA5 (Tamura et al. 2011). The phylogenetic tree was obtained using the neighbor-joining (NJ) method, with 1000 bootstrap replications. The theoretical molecular weight of the protein was calculated using ExPASy (http://expasy.org/tools/pi_tool.html). Searches of epestfind (http://emboss.bioinformatics.nl/cgi-bin/emboss/epestfind), SMART (http://smart.embl-heidelberg.de/), and PSORT II (http://psort.hgc.jp/form2.html) were conducted to identify potential domains in *GlPacC*.

*Construction of the* GlPacC*-silencing plasmid and the silenced strains.*—The construction of the *GlPacC*-silencing vector and the transformation of the *G. lucidum* HG strain were performed as previously described (Mu et al. 2012). After digestion using the restriction endonucleases, *Kpn* I and *Xba* I (Takara, Dalian, China), the GlPacCi sense fragment was inserted into the plasmid pAN7-dual vector at the corresponding restriction sites, yielding the final silencing vector pGlPacCi (SUPPLEMENTARY FIG. 2A). This vector was used to produce the *GlPacC*-silenced strains. The *G. lucidum* HG strain was transformed by electroporation as previously described (Mu et al. 2012).

*Quantitative real-time PCR analysis of gene expression.*—The total RNA was extracted using RNAiso plus (Takara, Dalian, China) according to the manufacturer's instructions. Approximately 2.0 μg of total RNA was used for first-strand cDNA synthesis using 5× All-In-One RT MasterMix with AccuRT Genomic DNA Removal Kit (ABM, Richmond, Canada) according to the manufacturer's instructions. Quantitative real-time PCR (qRT-PCR) was conducted in a Mastercycler® ep realplex$^2$ System (Eppendorf, Hamburg, Germany) using EvaGreen 2× qPCR MasterMix-S (ABM, Richmond, Canada) according to previously reported procedures (Ren et al. 2010). Relative quantification of gene expression levels was performed based on the level of the housekeeping gene *18S* rRNA (Mu et al. 2012). The gene expression levels were evaluated by calculating the difference between the Ct value of the gene analyzed and the Ct value of *18S* rRNA, which was used as a normalizer. The post-qRT-PCR calculations to determine the relative gene expression levels were performed using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001). The primers used are listed (SUPPLEMENTARY TABLE I).

*Measurement of the distance between* G. lucidum *hyphal branches.*—For measurements of vegetative hyphae, the strains were inoculated on very thin PDA medium and were incubated at 28 C for 3 d. The vegetative hyphae and the PDA medium were stained with 10 μL of a solution of 2.5 μg/mL Calcofluor White M2R (CFW) (Sigma, St. Louis, Missouri) for 5 min. For measurements of aerial hyphae, the strains were inoculated on PDA medium, and sterile glass slides were obliquely embedded into the PDA medium adjacent to the mycelia. When mycelia had grown onto the slides, the slides were suspended in 10 μL of a solution of 2.5 μg/mL CFW for 5 min. A Zeiss Axio Imager A1 fluorescence microscope (Carl Zeiss, Jena, Germany) was used to detect fluorescence. The distance between hyphal branches was measured as previously described (Mu et al. 2014).

*Measurement of ganoderic acid, squalene, and lanosterol levels.*—GAs were extracted from 200 mg of dried mycelia using 10 mL of 95% (v/v) ethanol and were quantitated as previously described (Shi et al. 2015). Squalene and lanosterol were extracted from cells and were fractionated according to a previously reported method (Li et al. 2015). A 30 mg sample of dried mycelia was saponified in 1.5 mL of 10% (w/v) KOH/75% (v/v) ethanol at 50 C for 2 h. The supernatant was extracted three times using an equal volume of hexane. The hexane layers were combined, and the solvent was removed under $N_2$. For subsequent analysis by ultraperformance liquid chromatography (UPLC), the product was dissolved in 0.5 mL of methanol and was analyzed using an Agilent 1290 Infinity UPLC System (Agilent Technologies, Santa Clara, California) equipped with an Agilent ZORBAX Eclipse Plus C18 column (Rapid Resolution HT 2.1× 50 mm 1.8 μm 600 Bar) and an Agilent 1290 Diode Array Detector. Squalene and lanosterol were separated using a mobile phase of 100% methanol at a constant flow rate of 0.5 mL/min. Squalene and lanosterol were monitored at the wavelength of 210 nm. The peaks of squalene and lanosterol were identified based on their retention times and comparisons of their UV spectra with those obtained using squalene and lanosterol standards (Sigma, St. Louis, Missouri). Quantification was performed according to external calibration graphs of the peak areas versus different concentrations of the squalene and lanosterol standards.

*Statistical analysis.*—The mean values obtained in individual experiments were expressed as the mean values ± SD. To determine the significance of differences, a one-way ANOVA was performed using GraphPad Prism 6 (GraphPad Software, San Diego, California). Statistical significance was expressed as $P < 0.05$ or $P < 0.01$.

## RESULTS

*Cloning and sequence analysis of the* PacC *of* G. lucidum.—To determine whether *G. lucidum* possesses a homologue of the *PacC/Rim101* gene, the sequences of *Saccharomyces cerevisiae RIM101* and *Aspergillus nidulans PacC* were used to blast search the *G. lucidum* genomic database (NCBI taxid: 1077286), and the corresponding gene (GL30073) was designated *GlPacC*. The full-length sequence of *GlPacC* cDNA was obtained and submitted to GenBank (KX016022). The genomic sequence of *GlPacC* included 2647 bp and contained 4 exons (SUPPLEMENTARY FIG. 1A), encoding a protein of 807 amino acid residues (SUPPLEMENTARY FIG. 1A) with a theoretical molecular mass of 86.15 kDa.

Searches of protein databases revealed that GlPacC contains three conserved C2H2 zinc-finger domains, two potential proteolytic cleavage sites called PEST domains (enriched in proline [P], glutamic acid [E], serine [S], and threonine [T], 10 SSP…APG 49 and 670 SPS…PSP 682), a putative PKA phosphorylation site (78 RKST 81), and a putative pat4 nuclear localization signal (NLS) (271 KKRR 274) (FIG. 1A). The three typical C2H2 zinc-finger domains conforming to the consensus sequence $CX_{2\text{-}4}CX_{12}HX_{3\text{-}5}H$ (Jacobs 1992, Tilburn et al. 1995) are indicated by underlining and the Cys (C) and His (H) sites are marked with black dots (SUPPLEMENTARY FIG. 1B). The two potential PEST domains of GlPacC are located in its N- and C-termini (FIG. 1A). The PacC transcription factor of *A. nidulans* undergoes two C-terminal proteolytic processes in response to alkaline pH (Penalva et al. 2014). In *S. cerevisiae*, proteolytic removal of the C-terminal ~100-residue PEST-like region yields active Rim101p (Xu and Mitchell 2001). Therefore, we speculate that the C-terminal PEST domain of GlPacC might be a functional cleavage site, and the other is nonfunctional. These results indicated that the protein domains of PacC/Rim101 are relatively conserved in fungi.

The phylogenetic analysis indicated that the PacC/Rim101 of different fungi could be sorted into two distinct groups, the ascomycota and basidiomycota groups. GlPacC is most homologous to the PacC of

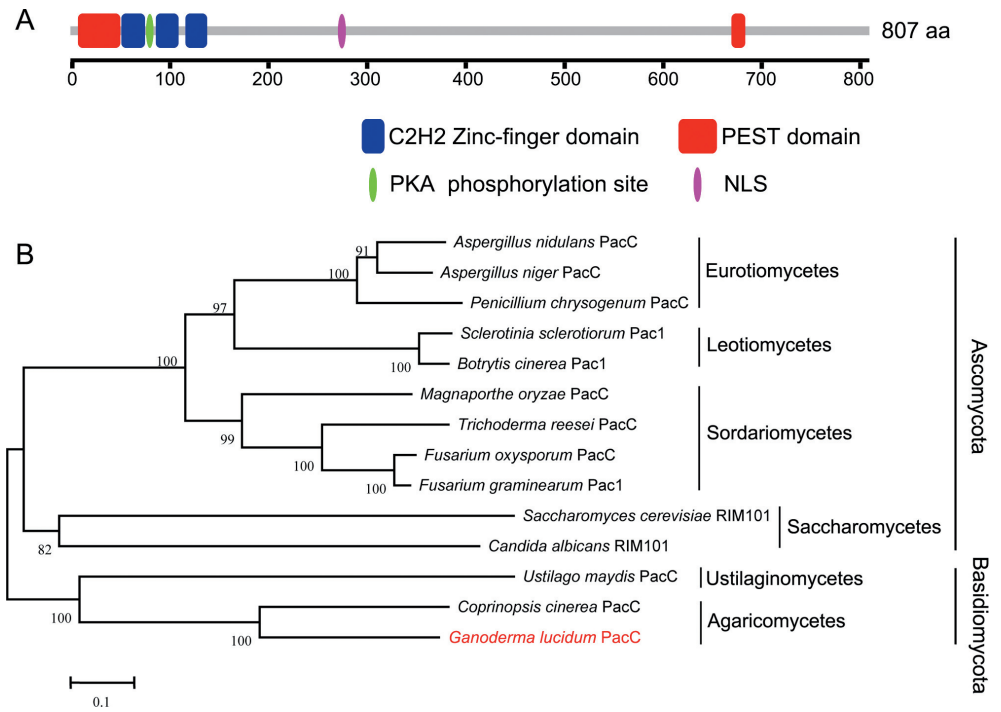


FIG. 1. Sequence analysis of the PacC of *G. lucidum*. A. Domain architecture of the GlPacC. The number under the line indicates the number of amino acids. B. Phylogenetic analysis of PacC/Rim101 from *G. lucidum* and other species. The other PacC/Rim101 proteins used for phylogenetic tree analysis were *A. nidulans* PacC (Q00202), *A. niger* PacC (CAA67063), *Penicillium chrysogenum* PacC (AAC36492), *S. sclerotiorum* Pac1 (AAF93178), *Botrytis cinerea* Pac1 (AAV54519), *M. oryzae* PacC (AEK70425), *Trichoderma reesei* PacC (ETS05643), *F. oxysporum* PacC (AAM95700), *F. graminearum* Pac1 (ADO60821), *S. cerevisiae* RIM101 (P33400), *Candida albicans* RIM101 (Q9UW14), *U. maydis* PacC (Q6H8R9), and *C. cinerea* PacC (XP_001829114). The horizontal bar indicates the relative distance in the phylogenetic tree.

the higher basidiomycete *Coprinopsis cinerea* and is separate from those of the ascomycota (FIG. 1B). These results implied that the gene that we cloned most likely belongs to the *PacC/Rim101* family.

*Analysis of* GlPacC *gene expression under different pH conditions.*—Considering the role of *PacC/Rim101* as a modulator of gene expression in response to ambient pH, the expression of which is up-regulated in response to neutral and alkaline pH conditions (MacCabe et al. 1996, Arechiga-Carvajal and Ruiz-Herrera 2005), we investigated the expression of *GlPacC* in *G. lucidum* grown on potato dextrose broth (PDB) medium buffered to pH 4, 7, or 10. As reported for other fungi, the expression level of *GlPacC* was low at pH 4 and higher under neutral and alkaline pH conditions. The *GlPacC* expression level reached a maximal value at 3 h of exposure to these conditions and was up-regulated by approximately 25- and 60-fold at pH 7 and 10, respectively, compared with the level at pH 4 (FIG. 2). This result indicated that the expression of *GlPacC* in *G. lucidum* is repressed by acidic pH conditions and induced by neutral and alkaline pH conditions and that the gene that we cloned most likely belongs to the *PacC/Rim101* family.

*Construction of* GlPacC*-silenced strains.*—To further investigate the function of *GlPacC* in *G. lucidum*, we constructed *GlPacC*-silenced strains using our previously established dual-promoter system (Mu et al. 2012). We characterized mutants in which *GlPacC* expression was silenced via an RNAi-silencing vector, pAN7-dual, which was used to express the sense and antisense strands of *GlPacC*. To accomplish this goal, we constructed a *GlPacC*-silencing vector called pGlPacCi (SUPPLEMENTARY FIG. 2A) and then characterized the relative phenotypes of the mutants produced using electroporation. We first screened the silenced transformants using qRT-PCR to determine the levels of the *GlPacC* transcript in the transformants and obtained four *GlPacC*-silenced strains (PacCi-2, 3, 5, 7) that expressed the gene at a level of 55–75% lower than those of the wild-type (WT) and empty-vector control (CK, which does not include GlPacCi) strains (SUPPLEMENTARY FIG. 2B). Multiplex PCR analyses were also conducted to confirm the presence of the hygromycin B phosphotransferase (*hph*) gene and a fusion fragment containing the glyceraldehyde-3-phosphate dehydrogenase (*gpd*) gene promoter and *GlPacC* gene-silencing fragment (GlPacCi) in the genomic DNA of the four transformants (SUPPLEMENTARY FIG. 2C).

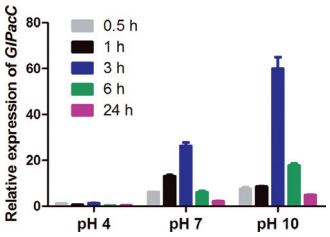


FIG. 2. qRT-PCR analysis of the expression of *GlPacC* in *G. lucidum* strains responding to different pH values. The WT strain was cultivated in PDB at 28 C with shaking for 4 d and then was inoculated into PDB buffered to pH 4, pH 7, or pH 10. Samples were collected at 0.5, 1, 3, 6, and 24 h. The relative abundance of the *GlPacC* transcript in cells grown at different pH values was normalized by comparison with the level in untreated 4 d old mycelia (relative transcript level = 1). Three independent biological replicates were performed. The error bars represent the standard deviations.

These data suggested that the four transformants obtained were *GlPacC*-silenced strains. Two of these *GlPacC*-silenced strains (PacCi-2 and 5) were randomly selected for further analysis.

GlPacC *is required for* G. lucidum *to adapt to the ambient pH.*—It has been reported that PacC/Rim101 regulates fungal adaptation to the ambient pH and that *PacC/Rim101*-deletion mutants have some difficulties in growing under neutral or alkaline pH conditions (Rollins 2003, Arechiga-Carvajal and Ruiz-Herrera 2005, Lou et al. 2015). We investigated the effect of the ambient pH on the growth of the *GlPacC*-silenced strains. The results of disc-diffusion assays on PDA medium buffered to pH 3, 4, and 7 demonstrated that the PacCi strains had impaired growth under both acidic and neutral conditions compared with the WT and CK strains, which was particularly apparent under neutral conditions (FIG. 3A, B). When inoculated on PDA medium buffered to pH 7, the PacCi strains hardly grew, exhibiting an average of 23% greater growth inhibition compared with those of the WT and CK strains (FIG. 3A, B). All of the strains barely grew on PDA medium that was buffered to pH 8 or higher (data not shown). These results suggested that *GlPacC* is required for the mycelial growth of *G. lucidum* under different pH conditions.

*Analysis of the expression of some downstream genes regulated by* PacC/Rim101.—In other fungi, *PacC/Rim101* usually regulates the expression of many genes, such as the genes encoding permeases, proteases and ion membrane transporters (Alkan et al. 2013, Franco-Frias et al. 2014). To investigate whether the regulation patterns of these genes were conserved in *G. lucidum*, five permease genes (maltose permease: GL21866, GL21815; amino acid

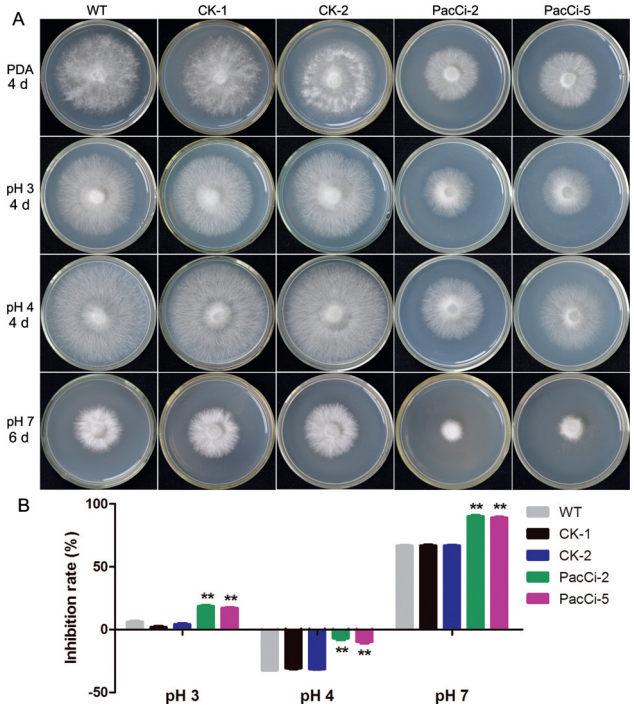


FIG. 3. *GlPacC* is required for *G. lucidum* to adapt to the ambient pH. A. Phenotypes of the WT, CK, and PacCi strains grown for 4 or 6 d at 28 C on PDA or on PDA buffered to pH 3, 4, or 7. B. The diameters of colonies of the tested strains were measured and were subjected to statistical analysis. The growth inhibition rate was expressed relative to the growth rate of each untreated control (Inhibition rate = [the diameter of the untreated strain–the diameter of the treated strain]/the diameter of untreated strain $\times 100\%$). Three independent biological replicates were performed. The error bars represent the standard deviations, and the asterisks indicate significant differences (**, $p < 0.01$).

permease: GL19746, GL29937, GL23068), two aspartic protease genes (GL18287, GL21546) and a P-Type $Na^+$ ATPase gene *Ena1* (GL29594) were selected, and their transcriptional levels were determined by qRT-PCR. The transcriptional levels of permease and aspartic protease were not altered at pH 4, but the transcriptional levels of maltose permease (GL21866) and aspartic protease (GL18287) were down-regulated by approximately 2.5- and 2-fold and that of amino acid permease (GL29937) was up-regulated by approximately 2~3-fold in the PacCi strains at pH 7 (SUPPLEMENTARY FIG. 3A–C). The expression level of *Ena1* (GL29594) was very low at pH 7 and was much higher when treated with 10 mM LiCl in WT and CK strains, but that was significantly down-regulated in the PacCi strains (SUPPLEMENTARY FIG. 3D). The expression of other selected genes were not influenced (data not shown). These results further verified that regulation patterns of permease, protease, and *Ena1* by *PacC/Rim101* were conserved in *G. lucidum*.

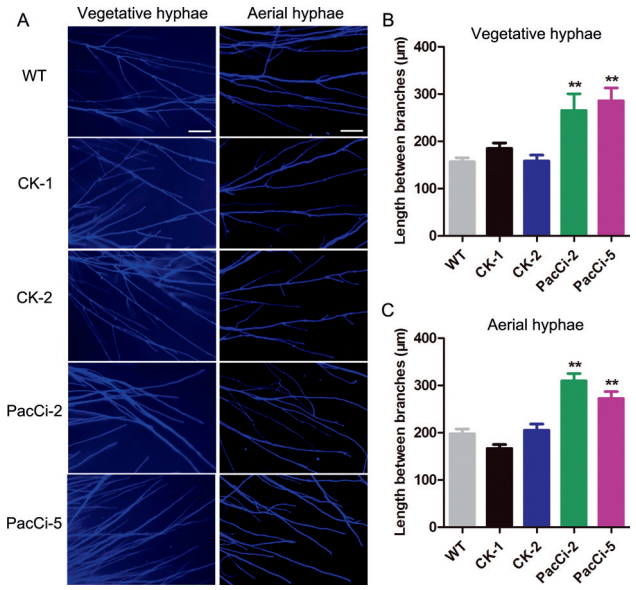


FIG. 4. *GlPacC* regulates hyphal branching. A. Vegetative and aerial hyphae were photographed using a fluorescence microscope. *G. lucidum* strains were cultivated on PDA plates for 4 d and then were stained using a solution of 2.5 μg/mL CFW (scale bar = 100 μm). B. The distances between the vegetative hyphal branches of the WT, CK, and PacCi strains. C. The distances between the aerial hyphal branches of these strains. Three independent biological replicates were performed, and at least 50 measurements were made for each colony. The error bars represent the standard deviations, and the asterisks indicate significant differences (**, $p < 0.01$).

*The effect of* GlPacC *on the hyphal branching of* G. lucidum.—Hyphal branching is an important indicator of the mycelial growth process of filamentous fungi. Because *GlPacC* plays an important role in the mycelial growth of *G. lucidum*, the hyphal branching phenotype upon growth on solid plates was investigated using fluorescence microscopy. Compared with those of the WT and CK strains, the distance between the vegetative hyphal branches in the PacCi strains was increased by approximately 75% (FIG. 4A, B) and the distance between their aerial hyphal branches was increased by approximately 45% (FIG. 4A, C). These results suggested that reducing the level of the *GlPacC* transcript increased the distance between the vegetative and aerial hyphal branches and further indicated that *GlPacC* plays an important role in the mycelial growth of *G. lucidum*.

GlPacC *is required for the development of* G. lucidum.—Several studies have demonstrated that *PacC/Rim101* plays a central role in the fungal meiotic process (Su and Mitchell 1993, Lambert et al. 1997). To investigate the function of *GlPacC* in the fruiting body development of *G. lucidum*, we monitored strains grown on solid culture medium. The PacCi strains grew more slowly than did the WT or CK strains and their vegetative mycelia secreted the yellow liquid during the late growth stage (FIG. 5A). In addition to the PacCi strains being unable to form primordia or fruiting bodies during the normal cultivation period (FIG. 5A), no primordia or fruiting bodies were detected even when the PacCi strains were cultivated for many more days (data not shown). In contrast, the WT and CK strains formed normal primordia and fruiting bodies (FIG. 5A).

Next, we evaluated the level of *GlPacC* expression at different developmental stages and found that the expression level of *GlPacC* was relatively low in liquid-fermented mycelia, was up-regulated in petri dish-grown mycelia, peaked during solid cultivation and at the primordium formation stage, and then was slightly down-regulated during the fruiting body development stage (FIG. 5B). These results suggested that *GlPacC* plays an important role in the mycelial growth and sexual differentiation of *G. lucidum*.

*The effect of* GlPacC *on ganoderic acid biosynthesis in* G. lucidum.—GA, one of important pharmacologically active compounds of *G. lucidum*, is synthesized via the mevalonate (MVA) pathway (Shi et al. 2010). To assess the function of *GlPacC* in ganoderic acid biosynthesis, we determined the GA content in the WT, CK, and PacCi strains. As shown (FIG. 6A), the content of GA was increased to approximately 1.35-fold in the PacCi strains compared with those of the WT and CK strains. The *sqs* (encoding squalene synthase) (Zhao et al. 2007) and *ls* (encoding lanosterol synthase) (Shang et al. 2010) are two critical genes in the ganoderic acid biosynthetic pathway, and their transcription levels were up-regulated by approximately 3.5- and 2.5-fold, respectively, in the PacCi strains (FIG. 6B). The contents of squalene and lanosterol, two metabolic intermediates in the MVA pathway, were also increased significantly in the PacCi strains compared with those in the WT and CK strains (FIG. 6C, D). These results indicated that *GlPacC* negatively regulates ganoderic acid biosynthesis in *G. lucidum*.

## DISCUSSION

pH-mediated signal regulation is an important mechanism for the survival of fungi in nature. The pH regulator PacC/Rim101 plays pivotal roles in fungal adaptation to the environmental pH through controlling the transcription of many genes involved in various cellular processes (Alkan et al. 2013, Franco-Frias et al. 2014). In this study, a *PacC/Rim101* homologue, *GlPacC*, was found to be functionally important for the mycelial growth and fruiting body development of *G. lucidum*. The expression of *GlPacC* was up-regulated in response to neutral and

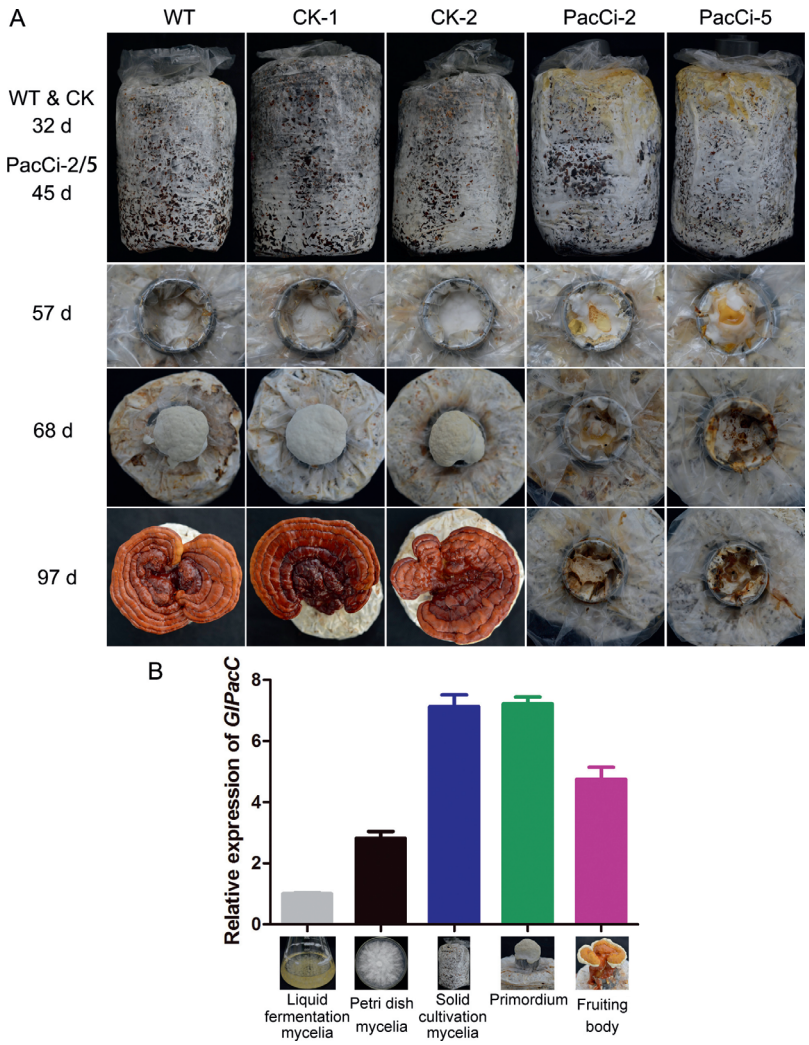


FIG. 5. The function of *GlPacC* in the development of *G. lucidum*. A. The three developmental stages in the life cycle of the WT, CK, and PacCi strains (dikaryotic mycelia, primordial, fruiting bodies stages). Ten independent biological replicates were performed. B. qRT-PCR analysis of the *GlPacC* expression levels in *G. lucidum* at different cultivation stages. The relative abundance of the *GlPacC* transcript at different stages was normalized by comparison with its level in liquid-fermented mycelia (relative transcript level = 1). The mean values and standard errors were determined using data obtained from three independent biological replicates.

alkaline pH conditions. Moreover, *GlPacC* negatively regulated ganoderic acid biosynthesis in *G. lucidum*.

It is known that *PacC/Rim101* mRNA is present in fungi under all physiological pH conditions (Arechiga-Carvajal and Ruiz-Herrera 2005, Huang et al. 2015). The expression of *GlPacC* was strongly induced in response to neutral and alkaline pH conditions but was also detected under acidic pH conditions (FIG. 2). The occurrence of *GlPacC* expression under all pH conditions implied that it may be a functional gene under all of the above-mentioned conditions. The PacCi strains displayed impaired growth on PDA medium under both acidic and neutral pH conditions, particularly under neutral conditions (FIG. 3). This result is consistent with the findings in *M. robertsii*, in which *ΔMrpacC* strains exhibited impaired growth under both acidic and alkaline pH conditions (Huang et al. 2015). However, it was difficult for the *ΔPacC/Rim101* mutants of other fungi to grow under neutral or alkaline pH conditions but not under acidic pH conditions (Merhej et al. 2011, Landraud et al. 2013). Thus, it is likely that *PacC/Rim101* plays different roles in the mycelial growth of different fungi.

The mycelia of the *Δrim101* mutants of *U. maydis* displayed a significantly higher number of septa compared with that of the wild-type strain, and the *Δrim101* mutant yeast cells were significantly longer, with some of them containing septa (Arechiga-Carvajal and Ruiz-Herrera

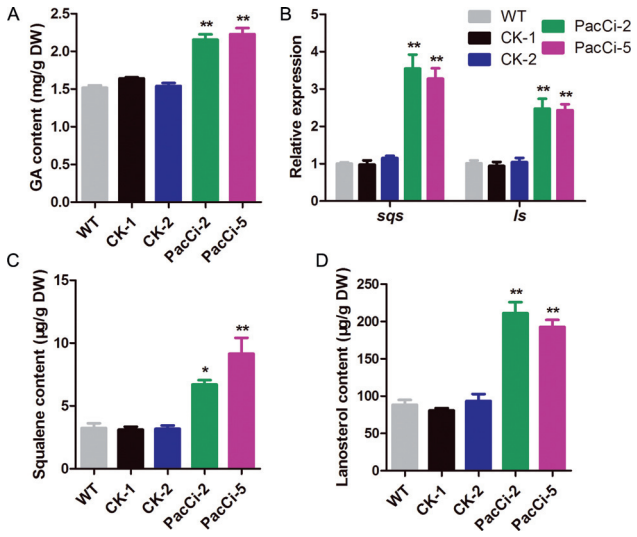


FIG. 6. *GlPacC* regulates ganoderic acid biosynthesis. A. The GA content of the WT, CK, and PacCi strains. B. The relative expression levels of key genes (*sqs* and *ls*) in the ganoderic acid biosynthetic pathway. C. The content of cellular squalene in the WT, CK, and PacCi strains. D. The content of cellular lanosterol in these strains. Three independent biological replicates were performed. The error bars represent the standard deviations, and the asterisks indicate significant differences (*, $p < 0.05$; **, $p < 0.01$).

2005). The *ΔTrpac1* mutant of *T. reesei* had sparse aerial hyphae under neutral and alkaline pH conditions, unlike the dense hyphae that the parent strain produced under these conditions (He et al. 2014). In our study, the distance between the hyphal branches (of vegetative hyphae and aerial hyphae) was significantly increased in the PacCi strains (FIG. 4). These results support the conclusion that *GlPacC* plays an important role in the mycelial growth of *G. lucidum*.

*Rim101* was initially cloned from *S. cerevisiae* and was reported to be a positive regulator of the expression of meiotic genes in this organism (Su and Mitchell 1993). In *Yarrowia lipolytica*, YlRim101p is essential for mating and sporulation, and the truncated form of YlRim101p fulfilled all of the YlRim101p requirements for mating and sporulation (Lambert et al. 1997). *PacC*-deletion mutants of *Neurospora crassa* could form protoperithecia when grown on cellophane but were unable to produce ascospores and complete female development (Chinnici et al. 2014). These results indicate that *PacC/Rim101* plays a central role in fungal sexual development. In our study, the expression of *GlPacC* peaked during the solid cultivation and primordium formation stages and the PacCi strains were unable to form primordia and fruiting bodies (FIG. 5). These results suggest that *GlPacC* is required for fruiting body development in *G. lucidum*. Moreover, the PacCi strains secreted the yellow liquid from their vegetative mycelia during the late growth stage and the mycelia appeared to lose viability and entered into a stage of decline earlier than did the WT and CK mycelia.

In several ascomycetes, *PacC/Rim101* negatively regulates the biosynthesis and secretion of secondary metabolites, including sterigmatocystin and aflatoxin in *Aspergillus* spp. (Keller et al. 1997), trichothecene in *F. graminearum* (Merhej et al. 2011) and fumonisin in *F. verticillioides* (Flaherty et al. 2003). The initial step of the trichothecene biosynthetic pathway is the catalysis of farnesyl pyrophosphate (FPP) by trichodiene synthase, which is encoded by *Tri5* (Kimura et al. 2007), and *Pac1* negatively regulates *Tri* gene expression and trichothecene production in *F. graminearum* under acidic pH conditions (Merhej et al. 2011). Moreover, FPP is one of important intermediates in the MVA pathway and GA is synthesized via the MVA pathway (Shi et al. 2010). Interestingly, in our study, the content of GA, levels of *sqs* and *ls* transcripts, and contents of the metabolic intermediates squalene and lanosterol were all up-regulated in the PacCi strains (FIG. 6). These results indicate that *GlPacC* negatively regulates ganoderic acid biosynthesis in *G. lucidum*, which is similar to the findings in *F. graminearum* (Merhej et al. 2011). Further studies are required to determine whether *GlPacC* is directly involved in regulating the transcription of the critical genes in the ganoderic acid biosynthetic pathway. However, the results of transcriptomic analysis indicated that terpene metabolism is independent of the Pal/Rim pathway because the transcription of genes involved in that process was not affected in *Rim101*-deletion mutants of *U. maydis* (Franco-Frias et al. 2014). This result might be due to *PacC/Rim101* playing different roles in regulating the secondary metabolism of different species.

In conclusion, we evaluated the function of the pH-responsive transcription factor GlPacC in the medicinal macrofungus *G. lucidum*. We found that *GlPacC* is important for mycelial growth, fruiting body development and ganoderic acid biosynthesis in *G. lucidum*. The results obtained may facilitate investigation of the mechanism underlying Pal/Rim pathway regulation in *G. lucidum* and other higher basidiomycetes.

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