Improvement of laccase activity by silencing PacC in *Ganoderma lucidum*

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Abstract

Ganoderma lucidum is a representative white-rot fungus that has great potential to degrade lignocellulose biomass. Laccase is recognized as a class of the most important lignin-degrading enzymes in *G. lucidum*. However, the comprehensive regulatory mechanisms of laccase are still lacking. Based on the genome sequence of *G. lucidum*, 15 laccase genes were identified and their encoding proteins were analyzed in this study. All of the laccase proteins are predicted to be multicopper oxidases with conserved copper-binding domains. Most laccase proteins were secreted enzymes in addition to Lac14 in which the signal peptide could not be predicted. The activity of all laccases showed the highest level at pH 3.0 or pH 7.0, with total laccase activity of approximately 200 U/mg protein. Silencing *PacC* resulted in a 5.2 fold increase in laccase activity compared with WT. Five laccase genes (*lac1*, *lac6*, *lac9*, *lac10* and *lac14*) showed an increased transcription levels (approximately 1.5-5.6 fold) in the *PacC*-silenced strains versus that in WT, while other laccase genes were downregulated or unchanged. The extracellular pH value was about 3.1, which was more acidic in the *PacC*-silenced strains than in the WT (pH 3.5). Moreover, maintaining the fermentation pH resulted in a downregulation of laccase activity which is induced by silencing *PacC* Our findings indicate that in addition to its function in acidification of environmental pH, PacC plays an important role in regulating laccase activity in fungi.

Introduction

Lignocellulose from plants is the largest organic renewable resource on earth, but it is also the most difficult to degrade due to its complex structure. Lignocellulose constructs cellulose, hemicellulose and lignin. In nature, fungi, especially white-rot basidiomycetes, could effectively degrade plant biomass (Kües 2015). Laccase, a class of the most important fungal enzymes from white-rot basidiomycetes, is considered to be vital for the degradation of plant biomass (Brijwani et al. 2010). Laccase was first found in the *Rhusvernificera*, and later was found to be widespread in plants, bacteria and insects, especially in white-rot basidiomycetes. Laccase is an oxygen oxidoreductase, that belongs to the multicopper-containing oxidases and contains copper ions in its catalytic centre. Like most fungal extracellular secreted enzymes, laccase is a type of glycoprotein, that can use many phenolic compounds as electron donors to catalyze the reduction of O_2 to H_2O. Based on its broad range of substrates, fungal laccases have been used as the potential enzymes in industrial applications, including environmental pollutant degradation, baking industry and fruit juice stabilization (Mayer and Staples 2002; Brijwani et al. 2010). Fungal laccases also function in many physiological events, such as fruiting body formation in *Laccaria bicolor* and *Volvariella volvacea* (Courty et al. 2009; Chen et al. 2004), conidial pigment biosynthesis in *Aspergillus fumigatus* (Tsai et al. 1999), and pathogenicity in *Cryptococcus neoformans* (Missal et al. 2005).

Fungal laccase shows a relatively wide range of temperature and pH adaptability (Brijwani et al. 2011). The optimal temperature of fungal laccase is usually between 50 and 70 °C (Schneider et al. 1999). Fungal laccase can also maintain activity in a wide acidic pH range (Schneider et al. 1999). In addition to these physical conditions, many studies have illustrated that the synthesis and secretion of laccase are

regulated by many factors including metal ions, nutrient concentrations and culture conditions (Piscitelli et al. 2011). Copper was found to be a widely used metal ion to enhance the activity and transcription levels of laccase in *Trametes versicolor* and *Pleurotus ostreatus* (Galhaup and Haltrich 2001; Giardina et al. 1999). The addition of organic nitrogen sources or depletion of nitrogen could increase fungal laccase activity (Janusz et al. 2007; Keyser et al. 1978). Traditional studies have revealed that the above factors could dramatically regulate laccase activity by changing the cultivation conditions. However, few studies have focused on the role of functional genes in regulating the laccase activity and secretion.

The Pal pathway is a well-known pathway that can regulate the fungal response to ambient pH (Peñalva and Arst 2004). PacC is considered the core transcription factor in the Pal pathway (Díez et al. 2002), which can activate the transcription of alkaline-expressed genes and repress acid-expressed genes (Peñalva et al. 2008). PacC was found to participate in many fungal physiological events, including fungal growth, hydrolases expression, secondary metabolism, reactive oxygen species production and pathogenesis (Wu et al. 2016; Nobile et al. 2008; Zhu et al. 2016; Huang et al. 2015). In addition to its role in response to ambient pH, PacC could also influence the external pH. The ability to acidify the environment of PacC is an important factor to overcome host defense in some pathogenic fungi (Barda et al. 2020; Zhu et al. 2016).

Ganoderma lucidum, which has been widely applied as a medicinal mushroom in many Asia countries, is a representative fungus that has strong enzymatic abilities to degrade lignocellulose biomass and has broad prospects for application (Zhou et al. 2013). Laccase is a lignin-modifying enzyme which has been widely studied in *G. lucidum*. Although most of the research work has focused on gene cloning, heterologous expression, isolation and purification of laccase isoenzymes (Fang et al. 2015b; Elisashvili et al. 2010; Ko et al. 2001), the detailed regulatory mechanism of laccase activity is still unknown. With the complete sequence of the *G. lucidum* genome (Chen et al. 2012), genes encoding laccase isoenzymes could be identified and the detailed mechanism of regulating laccase activity could be studied. Herein, based on the genome of *G. lucidum*, 15 laccase genes and their encoding proteins were analyzed. We found that not only could the laccase activity andits transcription levels be regulated by external pH, but also PacC, as an important transcription factor that responded to ambient pH (Wu et al. 2016), had a negative effect on laccase activity *in G. lucidum*. Moreover, this is the first study of the functional genes involved in regulating laccase activity. Our findings provide a new insight into the regulatory mechanism of laccase activity.

Materials And Methods

Strains and growth conditions

The wild-type strain of *Ganoderma lucidum* ACCC53264 was obtained from Shanghai Academy of Agricultural Science. The CK strains and *PacC*-silenced strains (PacCi-2 and PacCi-5) were constructed previously by Wu et al. (2016). The mycelia of all the tested strains were grown in the medium (2 g tryptone, 2 g yeast extract, 0.5 g $MgSO_4$, 0.46 g KH_2PO_4 and 1.5 % agar) buffered with 50 mM Na_2HPO_4-

25 mM citric buffer to pH 3.0, 4.0, 5.0, 6.0 or 7.0 at 28 °C. Two hundred micromolar 2, 2-azinobis-(3-ethylbenzthiazoline-6-sulphonate) (ABTS) was added into the medium to detect laccase activity.

Characteristic analysis of laccase proteins

The sequences of 15 laccase genes were obtained from Chen et al. (2012). Signal peptides of each protein were predicted using SignalP 5.0 Server (http://www.cbs.dtu.dk/services/SignalP/). The molecules of proteins were analyzed by ExPASy (http://www.web.expasy.org/). The conserved domains of each laccase protein were analyzed by the NCBI conserved domains database (CDD). Multiple sequence alignment was analyzed using DNAMAN software. PacC binding sites were analyzed by online JASPAR database (http://jaspar.genereg.net/).

Laccase activity assay

The laccase activity was measured using the method described in the study by Fang et al. (2015b) with slight modifications. The mycelia were cultivated in liquid culture and shaken for 4 days. The supernatants were collected to detect the laccase activity. Reactions with 10 µl 200 µM ABTS, 40 µl supernatants and 150 µl 50 mM NaAc buffer (pH 5.0) were conducted. After reaction for 5 min at 25 °C, the mixtures were transferred to a water bath and boiled for 5 min. The absorbance of the reaction was measured at 420 nm. Reactions using heat-treated supernatants were used as the control. The concentration of proteins in the supernatants was detected by BCA protein assay kits (Sangong Biotech, Shanghai) according to the manufacturer's guidelines. One unit of laccase activity was calculated as the enzyme amount required for an OD increase of 1 after 1 min of reaction of each supernatant. The laccase activity was defined as units of laccase activity per mg protein.

Transcription analysis of target genes

The WT, CK, *PacCi-2* and *PacCi-5* strains were grown on medium (2 g tryptone, 2 g yeast extract, 0.5 g $MgSO_4$, 0.46 g KH_2PO_4) buffered with 50 mM Na_2HPO_4-citric buffer to pH 3.0, 4.0, 5.0, 6.0 or 7.0. After cultivation for 4 days, the mycelia of each strain were harvested. RNA was extracted with RNAiso^{TM} Plus Reagent (Takara, Dalian) according to the manufacturer's guidelines. The extracted RNA was immediately transferred to the cDNA using PrimeScript™ RT Master Mix (Takara, Dalian). The relative laccase genes transcript levels were determined using quantitative real-time PCR with paired primers (Table S2). The 18S rRNA of *G. lucidum* was used as the internal standard gene. The relative gene transcription level was calculated according to the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001).

Statistical analysis

The Duncan's multiple range test and Student's *t*-test were used for statistical analysis. All measurements herein were conducted in three biological replicates. The error bars represent the standard error of mean (SEM) from three replicates.

Results

Analysis of laccase genes and proteins in Ganoderma lucidum

According to the completed sequence information of the *G. lucidum* genome reported by Chen et al. (2012), we obtained a 15-member laccase gene family (Table S1). These 15 identified laccase genes encode proteins containing 496-614 amino acids, with predicted molecular weights ranging from 53.2 to 67.2 kDa. Bioinformatics analysis revealed that 14 laccase proteins possess a signal peptide in the N-terminus of their proteins, but only Lac14 was predicted to be an intracellular enzyme. The results of conserved domain database analysis showed that all of the laccase proteins identified herein were multicopper oxidases with cupredoxin domains. Amino acid sequence analysis by DNAMAN indicated that four copper-binding sites existed in the conserved domains, and each domain had conserved histidine residues (Fig. 1). These results indicated that 15 laccase genes were identified in *G. lucidum* genome and the proteins encoded by these genes could be classified as typical laccases in *G. lucidum*.

The effect of external pH on laccase activity and transcription levels

Previous studies have reported that the optimal pH condition of laccase activity was acidic pH, and laccases can adapt to a wide range of pH values to maintain their activities (Ko et al. 2001; Sitarz et al. 2013). We detected the laccase activity under different pH conditions. After cultivation for 4 days, when the cultivation pH was 5.0 or 6.0, the activity of laccase reached the lowest level, only approximately 80 U/mg protein (Fig. 2). When the cultivation pH was higher than 6.0 or lower than 5.0, the activity of laccase increased gradually. The activity of laccase reached the highest level at pH 3.0 or 7.0, approximately 200 U/mg protein. We next detected the transcription levels of 15 laccase genes under the control condition (medium without the addition of buffer, pH 5.4), pH 4.0 and pH 7.0 conditions. When mycelia were cultured in pH 4.0, most laccase genes showed an increasing tendency compared withthe tendency in the control condition (Fig. 3). The transcription levels of *lac4*, *lac5* and *lac14* showed no difference from the control (Fig. 3D, E, O), while only *lac15* showed a decreasing tendency, with an approximately 75% decrease compared with the decrease in the control (Fig. 3P). When mycelia were cultured at pH 7.0, the transcription levels of most laccase genes also increased more than 2 fold compared with the transcription levels under normal conditions (Fig. 3), but the transcription levels of *lac4*, *lac14* and *lac15* decreased dramatically under this condition (Fig. 3D, O, P). These results showed that laccase activity in *G. lucidum* could be detected over a wide range of pH values, and different pH levels could induce the transcription of different laccase genes.

Silenced of PacC could increase laccase activity

In our previous study, PacC was recognized as an important transcription factor for *G. lucidum* to adapt to ambient pH, but whether PacC could regulate laccase activity is relatively unknown in fungi. We detected laccase activity in the WT, CK1, CK2, *PacCi*-2 and *PacCi*-5 strains constructed by Wu et al. (2016) using ABTS as the substrate. First, the mycelia were grown on the plates containing 200 μM ABTS. A larger and darker halo formed around the mycelia of *PacC*-silenced strains compared with wild type

(Fig. 4A), which indicated that the laccase activity in the *PacC*-silenced strains was higher than the laccase activity in the wild type (WT). To further confirm this result, we measured the laccase activity in the supernatants of liquid culture using ABTS as a substrate. As shown in Fig. 4B, silencing *PacC* led to an increase (about 5.2 fold) in laccase activity compared with WT. These results indicated that PacC could negatively regulate the laccase activity in *G. lucidum*.

We analyzed the promoters of 15 laccase genes in *G. lucidum* and found that 8 of 15 laccase genes have PacC-binding sites in their promoters (Table S1), which suggested that PacC might regulate the expression of laccase genes. The transcription levels of 15 laccase genes were detected in the controls and *PacC*-silenced strains. Using qRT-PCR, we found that five laccase genes (*lac1*, *lac6*, *lac9*, *lac10* and *lac14*) showed higher transcription levels in the two *PacC*-silenced strains than in the control strains (Fig. 4C). Four laccase genes (*lac3*, *lac4*, *lac8* and *lac11*) were downregulated in the *PacC*-silenced strains. The expression of the remaining six laccase genes (*lac2*, *lac5*, *lac7*, *lac12* and *lac13*) showed no difference among all the tested strains (Fig. 4C). These results showed that PacC might regulate laccase activity by up or downregulating the transcription levels of laccase genes

Silencing PacC **resulted in an acidification of the external pH**

Many fungi favor an acidic environment. During the liquid fermentation of *G. lucidum*, the pH of the fermentation broth gradually acidified (Fang and Zhong 2002; Kim et al. 2006). Additionally, PacC was reported to have the ability to influence the extracellular pH in some species (Zhu et al. 2016; Barda et a. 2020). To further explore the mechanism of PacC in regulating laccase activity, we next detected the pH values in the supernatants after fermentation of all the tested strains. The pH of fermentation broth cultured with *PacC*-silenced strains was more acidic than that the pH of fermentation broth cultured with control strains after fermentation for 4 days. The pH value was approximately3.1 in the *PacC*-silenced strains, but was maintained at approximately 3.5 in the control strains (Fig. 5). This result showed that silencing *PacC* resulted in accelerated acidification of fermentation broth.

Maintaining the external pH could affect the increase in laccase activity which is caused by silencing PacC

As the above results showed that extracellular pH could dramatically influence laccase activity and that silencing *PacC* could result in accelerated acidification of fermentation broth, maintaining the extracellular pH could recover laccase activity when *PacC* was silenced. After maintaining the pH of the liquid medium, we detected the laccase activity in all test strains. The mycelia of the tested strains were respectively cultivated in the media adjusted to pH 4.0 or 7.0 using 50 mM Na_2HPO_4-citric buffer. After cultivation for 4 days, the pH was detected. All the media could maintain the initial pH due to the addition of buffer. As shown in Fig. 6A, the *PacC* silenced strains still showed an increased activity compared with the control strains under different pH conditions. Under normal fermentation conditions (without maintaining the pH of the medium), the laccase activity increased by about 5.2 fold in the *PacC*-silenced strains versus the laccase activity in the WT (Fig. 4B; Fig S1). However, the laccase activity increased by

about 1.5 fold in the *PacC*-silenced strains at pH 3.0, 1.6 fold at pH 4.0, 2.0 fold at pH 5.0, 1.9 fold at pH 6.0 and 1.8 folds at pH 7.0, respectively, versus that in WT (Fig. 6B). Although the laccase activity still increased in *PacC*- silenced strains, maintaining the external pH could partially decrease the laccase activity induced by silencing of *PacC*. These results indicated that maintaining the external pH could partially restore the increase in laccase activity which is caused by silencing PacC.

PacC could influence the expression of different laccase genes at different pH conditions

The transcription levels of 15 laccase genes were detected in the control and *PacC*-silenced strains at pH 4.0 and 7.0 (Fig. 7). The expression of *lac2* and *lac9* showed an increase in the *PacC*-silenced strains versus the increase in WT at both pH 4.0 and pH 7.0 (Fig. 7B, I). Silencing *PacC* led to a significant increase of approximately 2-3 fold of *lac 8* and *lac9* compared with the increase in WT at pH 4.0, and an increase of *lac1*, lac*12* and lac*15* at pH 7.0. Silencing of *PacC* led to decreased transcription levels of *lac3*, *lac4*, *lac11* and *lac13* at both pH 4.0 and pH 7.0 (Fig. 7C, D, K, M). The transcription levels of *lac14* showed no difference among the tested strains at pH 4.0 and pH 7.0. These results indicated that PacC could affect laccase expression at different pH values.

Discussion

Fungal laccases are important oxidases that have been widely studied due to their roles in fungal physiological functions, degradation of plant biomass and several industrial applications. Recent studies have focused on revealing the components and mechanisms involved in improving the productivity and activity of laccases in fungi (Piscitelli et al. 2011). As demonstrated herein, the laccase activity and their transcription levels could be regulated by environmental pH conditions, as well as a transcription factor PacC in *Ganoderma lucidum*. As an important transcription factor, PacC could not only respond to ambient pH (Wu et al. 2016), but also influenceenvironmental pH. Moreover, PacC could regulate the laccase activity and their transcriptional levels in *G. lucidum*, suggesting an important role of PacC in regulating laccase activity in filamentous fungi. These findings provide an understanding of the regulatory mechanism of laccase activity from the perspective of functional gene regulation.

Studies have revealed that the synthesis and secretion of fungal laccases could be regulated by many inducers such as physical conditions (temperature and pH), chemical compounds and cultural nutrient status (Liu et al. 2017; Piscitelli et al. 2011). Metals involving Cu^{2+}, Ag^{+} and Mn^{2+} are widely accepted as important inducers to regulate the laccase activity. Adding optimal Cu^{2+} during fermentation of fungi, the highest values of laccase activity could be obtained in *Trametes pubescens* (Galhaup and Haltrich 2001), *Ganoderma appanatum*, *Peniophora sp*. (Fonseca et al. 2010). Some phenolic and aromatic compounds have been reported to participate in increasing laccase activity due to their structure similar to lignin (Elisashvili et al. 2010; de Souza et al. 2004). The concentration of nitrogen or carbon also plays key roles in laccase activity and production (Levin et al. 2010). Our study revealed that external pH could be an inducer to influence the laccase activity in *G. lucidum* (Fig. 2).

Many fungi favor acidic environment and the extracellular pH could decrease gradually during their growth. To adapt to the environment, fungi have developed several mechanisms to respond to extracellular pH appropriately. As an important transcription factor that responds to the external pH (Peñalva et al. 2008), PacC plays key roles in fungal growth, stress resistance, secondary metabolism and pathogenesis in many previous studies (Barda et al. 2020; Zhu et al. 2019; Zhu et al. 2016). In addition to its classical roles in response to ambient pH for fungal survival, recent studies in some pathogenic fungi have also found its other role in influencing the extracellular pH during their infection process. Deletion of *PacC* resulted in decreased virulence due to a smaller reduction of pH in *A. carbonarius* (Barda et a. 2020). Deletion of *PacC* also slowed cultural acidification in *Beauveria bassiana* (Zhu et al. 2016). However, our study showed an opposite result compared with these pathogenic fungi, and the pH of fermentation broth showed an accelerated acidification in *G. lucidum* (Fig. 5). The different abilities to change the external pH indicated the different functions of PacC in fungi. In addition, silencing *PacC* leads to accelerated acidification, which is also an important factor to improve the laccase activity.

PacC was found to have effects on regulating the activity of secretase in many fungi. In *Metarhizium robertsii*, deletion of *PacC* led to a loss of ability to degrade chitin, which showed that PacC positively regulates chitinase activity (Huang et al. 2015). During fruit colonization, cell wall degrading enzymes and pectin lyases were downregulated in *PacC* mutant strains, which suggested an interaction of PacC with these hydrolases in *Penicillium digitatum* and *Colletotrichum gloeosporioides* (Zhang et al. 2013; Miyara et al. 2008). As few studies have focused on the functional genes involved in the regulation of laccase activity, we detected the effect of PacC on regulating laccase activity. During the growth of *G. lucidum*, the laccase activity could be detected, and the transcription levels of most laccase genes could be downregulated or upregulated by PacC (Fig. 4C). Furthermore, PacC could regulate the expression of different laccase genes at different pH values (Fig. 7). However, which laccase genes and how laccase activity could be regulated by PacC at different pH conditions are important works for the future. However, the detailed mechanisms of the function of PacC in regulating laccase activity need to be further explored.

Recently, based on the development of bioinformatics and the completion of genome sequence of many fungi, the promoters of laccase genes were analyzed and the molecular regulation mechanism of laccase activity was illustrated. Motifs involving the TATA box, CreA binding sites, metal responsive elements, heat shock elements and stress responsive elements were found in the promoters of some laccase genes in *C. cinerea*, *Trametes* and *Gaeumannomyces graminis* (Hoegger et al. 2004; Xiao et al. 2006; Galhaup et al. 2002; Litvintseva and Henson 2002). The findings of these motifs provide an important clue to reveal the genetic mechanism of laccase activity and productivity regulation under metal stress, heat shock stress and other environmental stresses. We also analyzed the promoters of 15 laccase genes in *G. lucidum* using the online JASPAR database, and several PacC binding sites were found (Table S1). However, as illustrated in Fig. 7, the transcription levels of some laccase genes were not changed although there were several predicted binding sites found in their promoters, such as *lac5*, *lac7* and *lac14*. For those genes that have no predicted binding sites, their transcript levels were also changed such as

lac2, *lac3*, *lac10* and *lac13*. These results indicated that PacC could up or downregulated the laccase gene expression, not only by directly binding to their promotors, but also by other unknown mechanisms.

Additionally, twelve putative laccase genes were identified in *Pleurotus ostreatus*, eleven were identified in *Flammulina velutipes* and *Laccaria bicolor* genomes, and seventeen were identified in *C. cinerea* (Jiao et al. 2018; Courty et al. 2009; Kilaru et al. 2006). We identified 15 laccase genes in the genome of *G. lucidum*, among which 14 laccase genes have signal peptides and only one did not contain the signal peptide (Table S1). Although most laccases were secreted to the outside of cells, studies also found that few laccases were reported to beintracellular enzymes in some mushrooms, such as *F. velutipes*, *P. ostreatusis*, and *Suillus granulatus* (Wang et al. 2015; Palmieri et al. 2000; Gunther et al. 1998). Many secreted laccase isoenzymes have been identified and some of which were cloned, extracted and purified for lignocellulosic biomass conversion (Sitarz et al. 2013; Ko et al. 2001). In addition to their classical roles in utilizing lignin, laccases are also involved in fruiting body formation, conidial pigment biosynthesis and pathogenesis. The anthocyanin degradation enzyme was identified as an intracellular laccase in *Litchi chinensis*, which has functions in coupled enzymatic anthocyanin degradation (Fang et al. 2015a). The Lcc4 of *Lentinula edodes* is recognized as an intracellular laccase that is an industrially potential enzyme (Kurose et al. 2014). Since the detailed functions of intracellular laccase have rarely been reported in the large basidiomycetes, the function of lac14 in *G. lucidum* which was predicted as an intracellular laccase is a new research direction.

Declarations

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Conflict of interest

The authors declare that they have no conflict of interest.

Ethical approval

This article does not contain any studies with human participants or animals performed.

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Figures

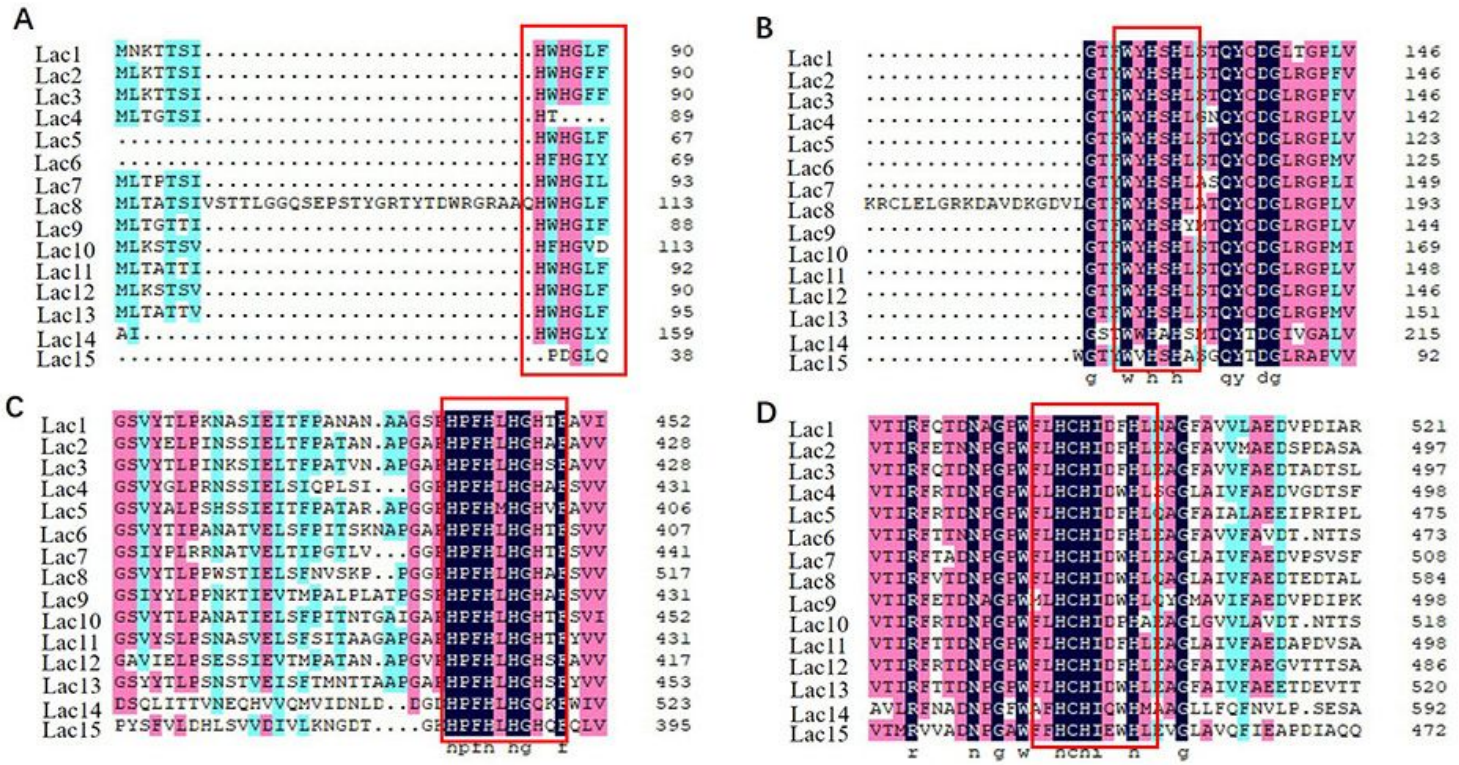

Figure 1

Characteristics of laccase protein. Multiple sequence alignment was analyzed by DNAMAN software. The sequences in the red box represent the copper-binding conserved domains with histidine and cysteine residues.

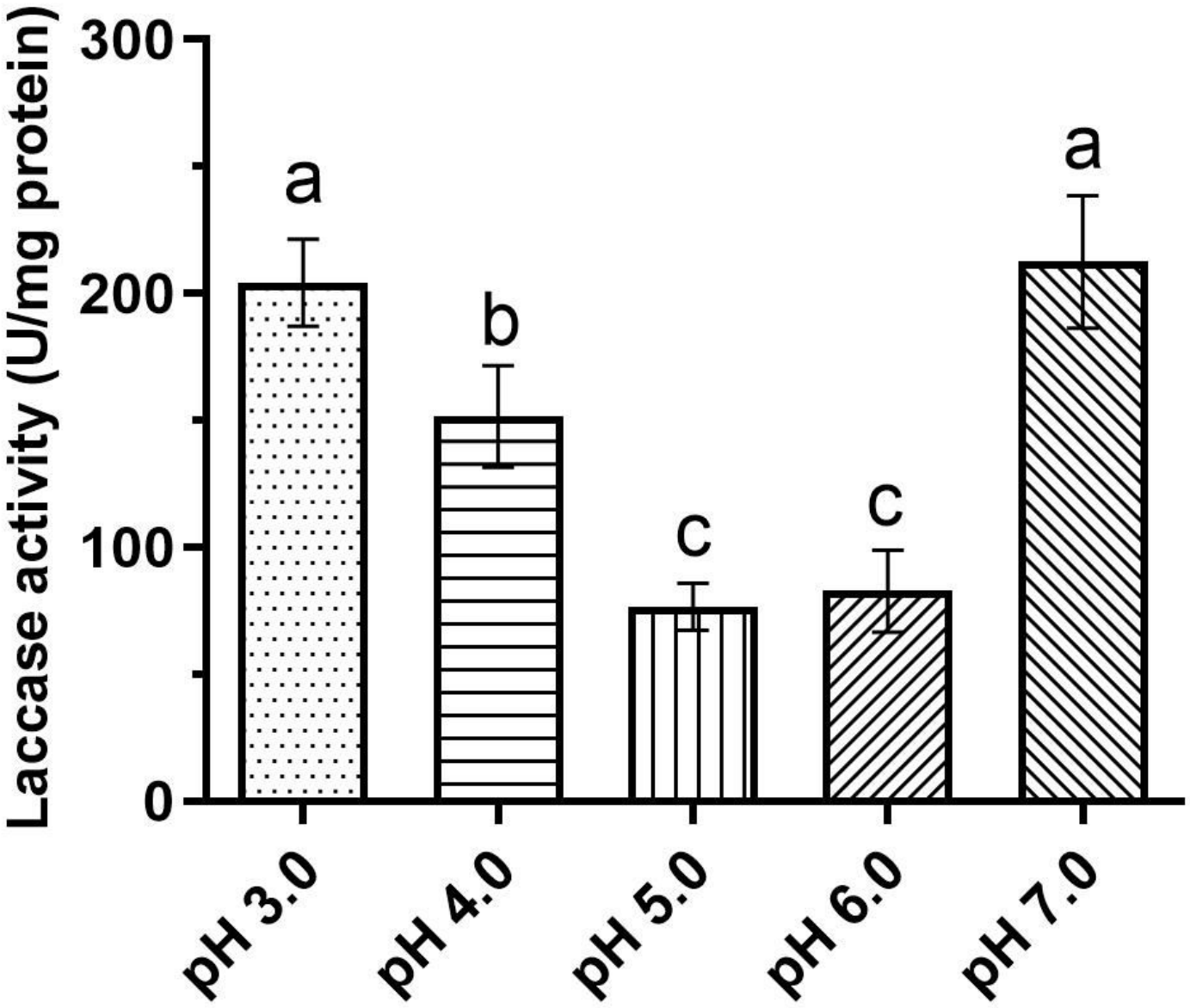

Figure 2

Detect the laccase activity at different pH values. The mycelia were cultivated in liquid MCM medium in which the pH value was adjusted to 3.0, 4.0, 5.0, 6.0 or 7.0. After cultivation for 4 days, laccase activity was detected using ABTS in the supernatant of the medium. Different letters represent significant differences between different groups according to Duncan's multiple range test.

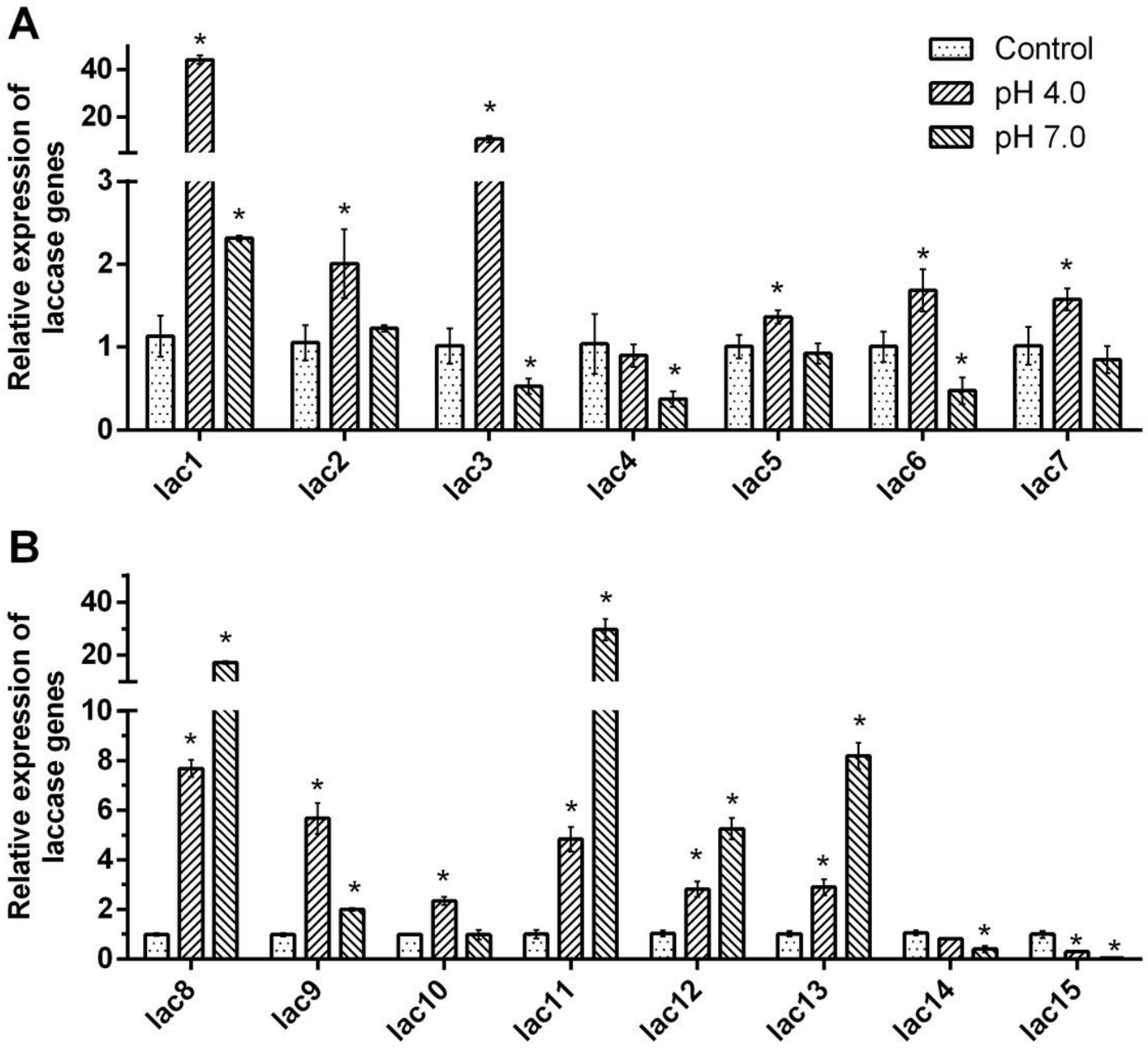

Figure 3

Detection of the expression levels of 15 laccase genes at different pH values. qRT-PCR analysis was used to examine the transcription of 15 laccase genes in the WT in the G. lucidum. The relative transcription levels of different laccase genes were calculated as the ratio of each laccase transcription level to 18S at pH 4.0 or 7.0. The Control represents the cultivation condition without adding buffer to maintain the pH value. The relative mRNA levels of controlwere taken as the value of 1. Asterisks represent significant differences versus the control group according to Student's t-test (*P□0.05, **P□0.01).

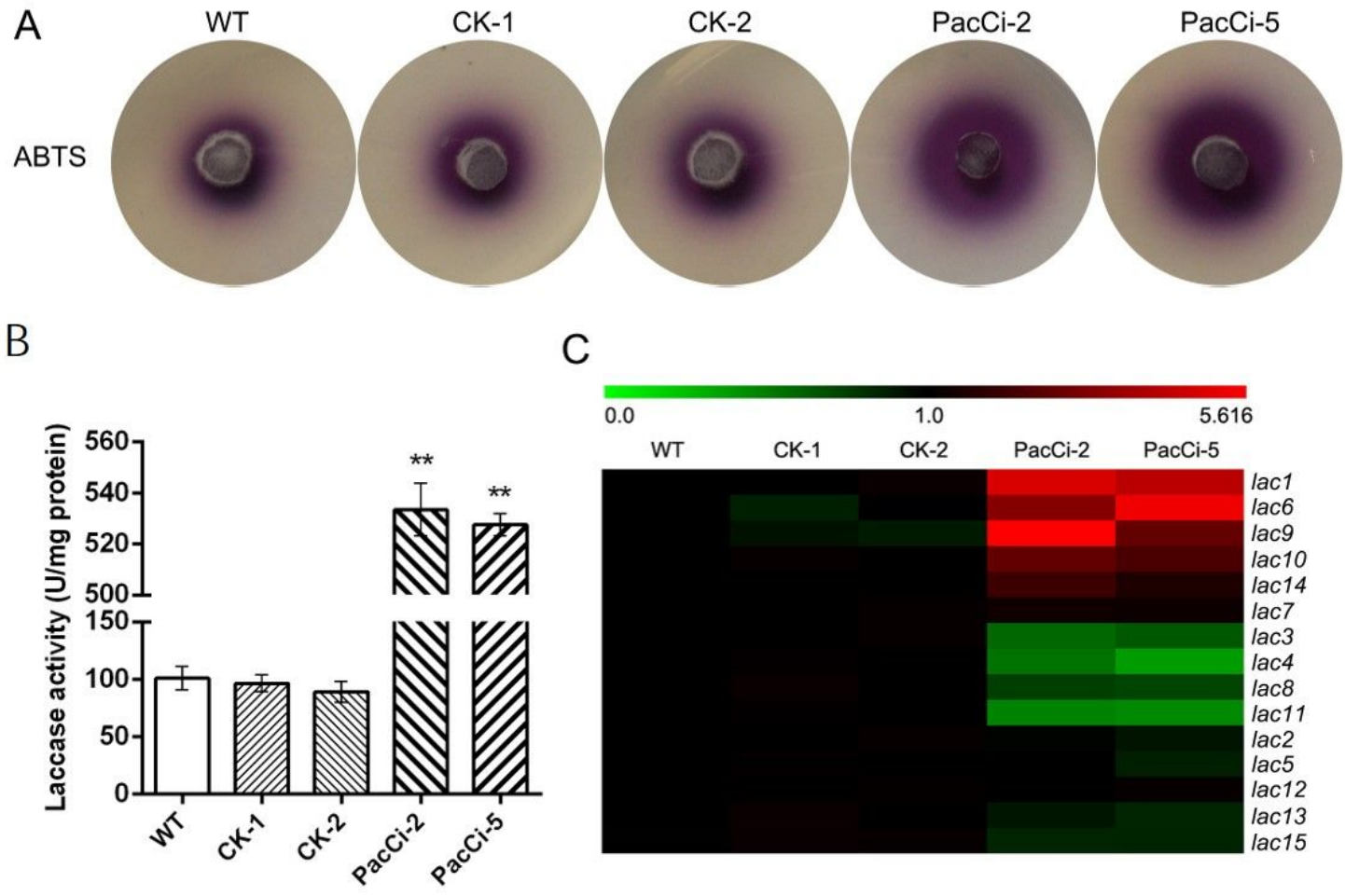

Figure 4

Silencing PacC led to an increase in laccase activity. (A) WT and mutant strains were cultivated on the plates with 200 μM ABTS used as the substrate of laccase. (B)The laccase activity in the fermentation broth cultivated with WT, CK and PacC-silenced strains was measured. Asterisks represent significant differences versus the control according to Student's t-test (**P□0.01). (C) qRT-PCR analysis was used to examine the expression of 15 laccase genes in all the tested strains under normal cultivation conditions. The relative mRNA levels of different laccase genes were calculated as the ratio of each laccase mRNA to 18S mRNA. The transcription levels of WT were calculated as the value of 1. MeV software was used to show the gene expression.

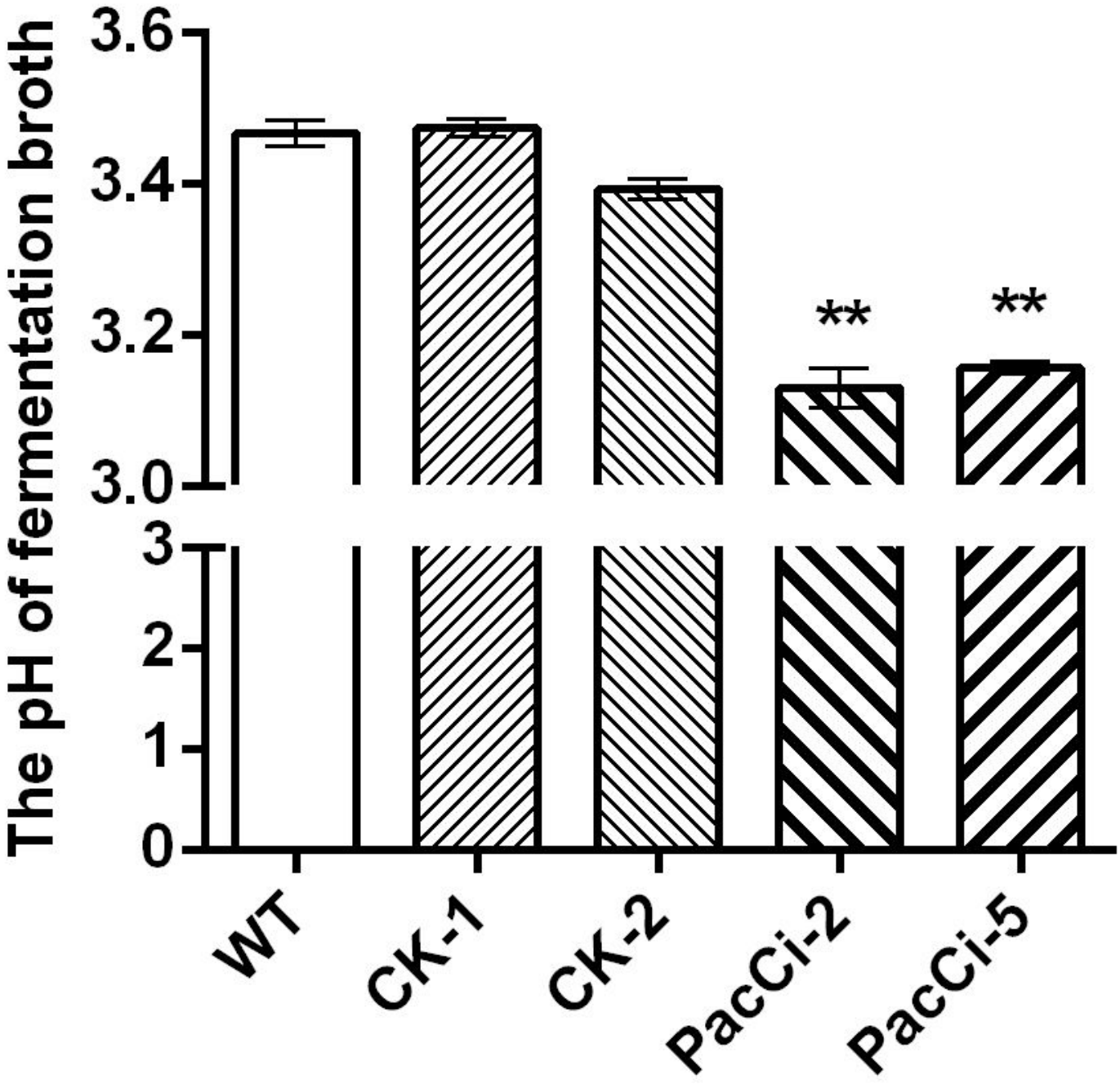

Figure 5

Detection of the pH value of fermentation broth. The mycelia of WT, CK and PacC-silenced strains were grown under normal fermentation conditions. After cultivation for 7 days, the pH values of each supernatant of medium were measured using a pH meter. Asterisks represent significant differences versus WT according to Student's t-test (**P□0.01)

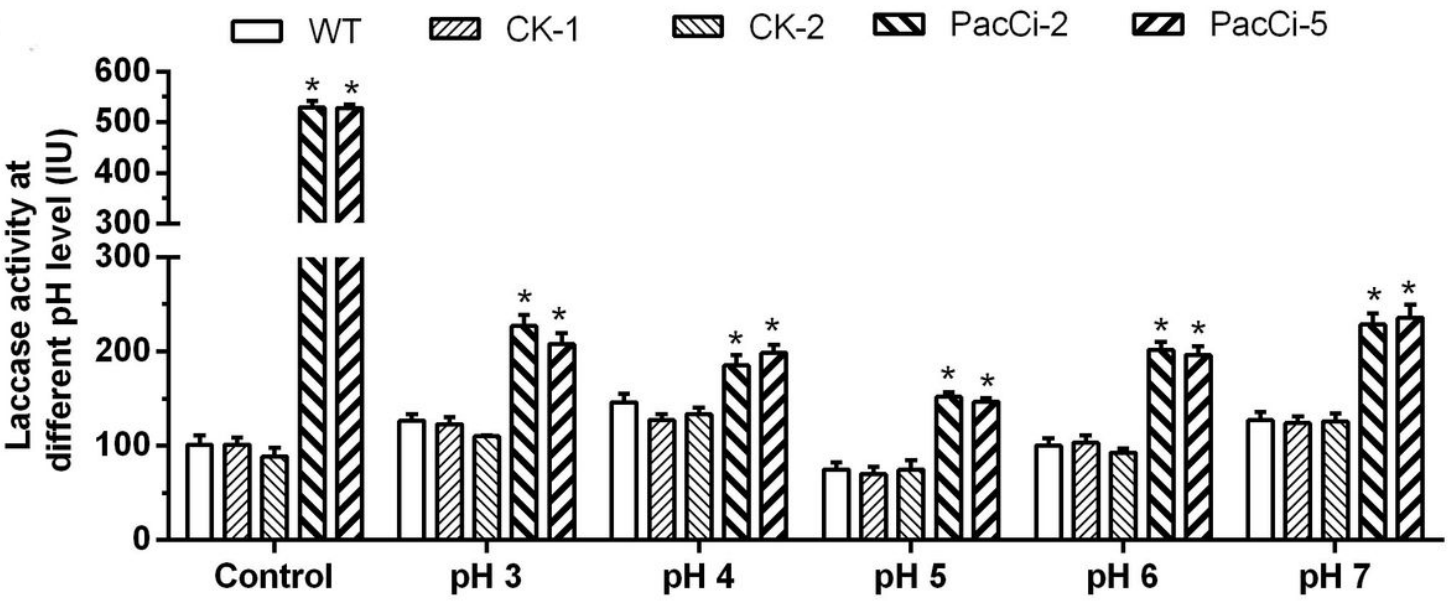

Figure 6

Detection of laccase activity in WT and mutant strains at different pH values. The mycelia of all the tested strains were grown in medium buffered with 50 mM Na2HPO4-citric acid to adjust the pH to 3.0, 4.0, 5.0, 6.0 or 7.0. After cultivation for 4 days, the laccase activity of each supernatant of medium was measured. Asterisks represent significant differences versus the controls according to Student's t-test (*P 0.05).

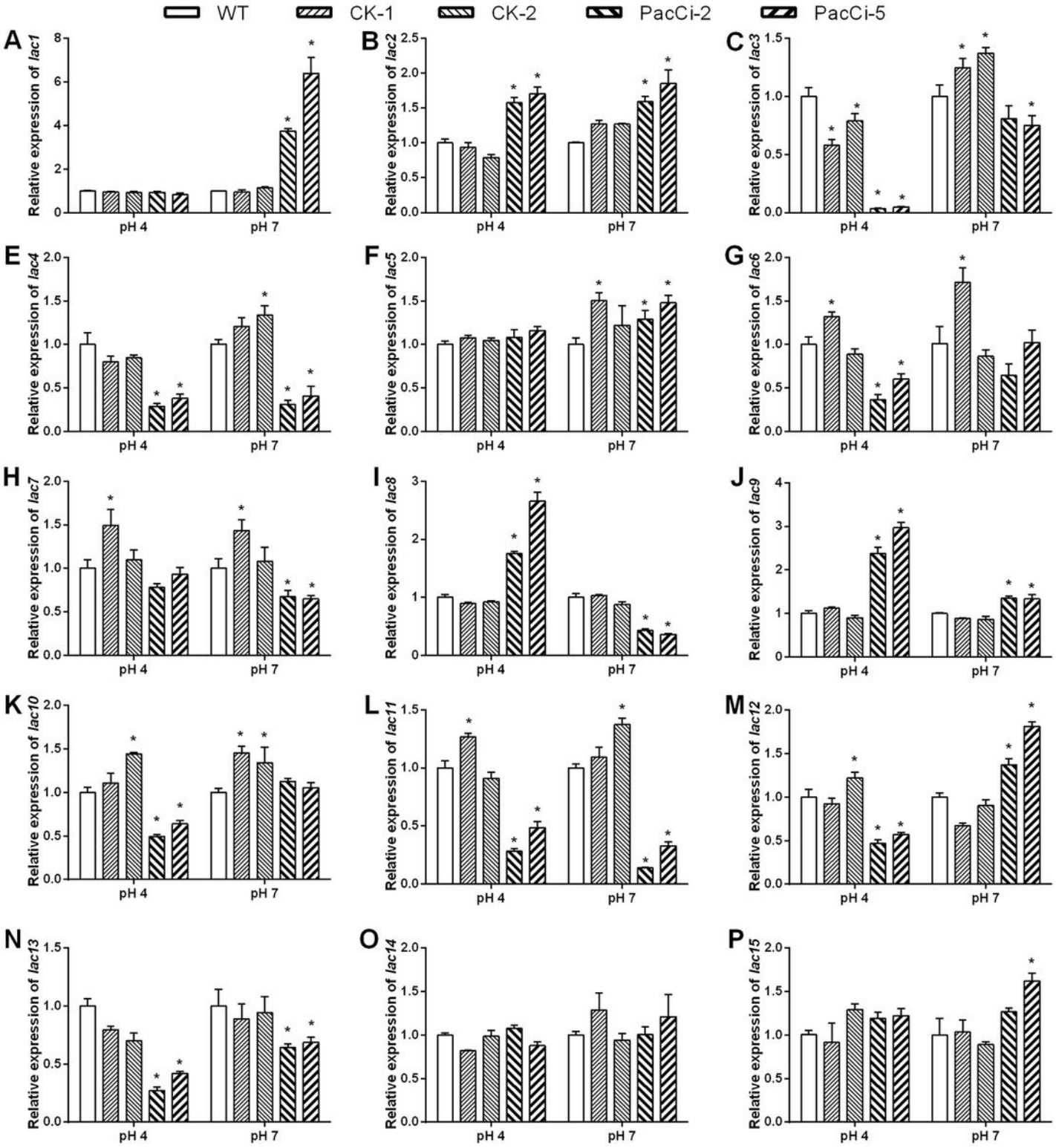

Figure 7

Detection of transcription levels of 15 laccase genes in the WT and mutant strains at different pH values. The mycelia were cultivated in liquid MCM medium in which the pH value was adjusted to 4.0 or 7.0. The expression of 15 laccase genes at pH 4.0 or pH 7.0 in all the tested strains were detected. The relative transcription levels of different laccase genes were calculated as the ratio of each laccase transcription

level to 18S. The relative transcription levels of WT at pH 4.0 or pH 7.0 were taken as the value of 1. Asterisks represent significant differences versus the controls according to Student's t-test (*P□0.05).

Supplementary Files

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