Expression of the Laccase Gene from a White Rot Fungus in *Pichia pastoris* Can Enhance the Resistance of This Yeast to H₂O₂-Mediated Oxidative Stress by Stimulating the Glutathione-Based Antioxidative System

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Laccase is a copper-containing polyphenol oxidase that has great potential in industrial and biotechnological applications. Previous research has suggested that fungal laccase may be involved in the defense against oxidative stress, but there is little direct evidence supporting this hypothesis, and the mechanism by which laccase protects cells from oxidative stress also remains unclear. Here, we report that the expression of the laccase gene from white rot fungus in *Pichia pastoris* can significantly enhance the resistance of yeast to H₂O₂-mediated oxidative stress. The expression of laccase in yeast was found to confer a strong ability to scavenge intracellular H₂O₂ and to protect cells from lipid oxidative damage. The mechanism by which laccase gene expression increases resistance to oxidative stress was then investigated further. We found that laccase gene expression in *Pichia pastoris* could increase the level of glutathione-based antioxidative activity, including the intracellular glutathione levels and the enzymatic activity of glutathione peroxidase, glutathione reductase, and γ-glutamylcysteine synthetase. The transcription of the laccase gene in *Pichia pastoris* was found to be enhanced by the oxidative stress caused by exogenous H₂O₂. The stimulation of laccase gene expression in response to exogenous H₂O₂ stress further contributed to the transcriptional induction of the genes involved in the glutathione-dependent antioxidative system, including *PpYAP1*, *PpGPX1*, *PpPMP20*, *PpGLR1*, and *PpGSH1*. Taken together, these results suggest that the expression of the laccase gene in *Pichia pastoris* can enhance the resistance of yeast to H₂O₂-mediated oxidative stress by stimulating the glutathione-based antioxidative system to protect the cell from oxidative damage.

White rot fungus has a strong ability to degrade lignin because this kind of fungus can produce extracellular and nonspecific ligninolytic enzymes, which mainly include lignin peroxidase (LiP), manganese peroxidase (MnP), and laccase (10, 21, 22, 26, 46). Laccase belongs to a group of copper-containing polyphenol oxidases that can catalyze the four-electron reduction of O₂ to H₂O, with the concomitant oxidation of phenolic compounds. Due to its special characteristics, such as its wide range of substrates, its ability to oxidize many different phenolic compounds, and its use of molecular oxygen as the final electron acceptor, laccase has seen wide application in industry and biotechnology, including paper pulping and bleaching, bioremediation, and textile dye decolorization (1, 4, 8, 13, 25, 27, 31, 42, 44, 48, 59).

Although laccase has great potential for industrial and biotechnological applications, the biological function of laccase has not been fully determined or confirmed. Previous research has indicated that fungal laccase may play a role in lignin degradation, the development of fruiting bodies, fungal morphogenesis, fungal pathogenicity, and the synthesis of pigments (3, 11, 14, 45, 51, 52, 61).

Recent research has suggested that laccase may play an important role in fungal defense against oxidative stress, which acts as an element of the stress response. It has been observed that oxidative stress can induce the expression of ligninolytic enzymes in some basidiomycetes (6, 33, 41). The extracellular laccase activity of some white rot basidiomycetes such as *Fomes fomentarius*, *Tyromyces pubescens*, *Trametes versicolor*, and * Abortiporus biennis* can be stimulated by the oxidative stress caused by exogenous menadione and paraquat. Enhanced extracellular laccase activity is considered part of the system for the adaptive response of white rot fungus to paraquat- and menadione-caused oxidative stress conditions (33, 34). A notable increase in the laccase activity of two fungal species, *Trametes versicolor* and *Abortiporus biennis*, can be observed after treatment with oxidative stress factors such as menadione, paraquat, and hydrogen peroxide (12). The laccase activity of some other species such as *Cerrena unicolor*, *Abortiporus biennis*, *Ganoderma lucidum*, and *Cerioporiopsis subvermispora* can also be significantly induced by other oxidative stress factors, such as Cd ions (32), the herbicides bentazon and diuron (16), and hydroquinone (2). These stress factors, which induce oxidative stress, can increase extracellular laccase activity and enhance both superoxide dismutase (SOD) and catalase (CAT) activity. This implies that laccase can participate in the adaptive response to oxidative stress in white rot fungus (33, 34).

The study of plant-pathogenic fungi has also suggested that laccase is involved in defense against oxidative stress in other fungi besides white rot fungus. One study on the plant-pathogenic fung-
gus Rhizoctonia solani has revealed that copper, paraquat, and alcohol treatments, which are known to cause oxidative stress by promoting the formation of free radicals, can induce laccase activity and increase the level of lipid peroxidation. A straightforward link between oxidative stress and laccase induction was found in the case of paraquat treatment (20). A genetic study on another plant-pathogenic fungus, Fusarium oxysporum, has also suggested that laccase may have a role in protection against oxidative stress. Strains with null mutations in laccase genes showed higher sensitivity to oxidative stress than the wild-type strain, indicating the importance of laccase in the defense against oxidative stress (18, 19).

As mentioned above, fungal laccase may be involved in the adaptive response to oxidative stress. However, the hypothesis that laccase plays an important role in defense against oxidative stress is mainly based on the phenomenon that laccase activity can be induced by various oxidative stress factors (2, 12, 16, 20, 32–34). To our knowledge, there is little direct evidence supporting the involvement of laccase in the defense against oxidative stress. Therefore, additional efforts are needed to prove that laccase contributes to defense against oxidative stress. More direct experimental data are required to confirm that the induction of laccase is an element of the oxidative stress response. In addition, the mechanism by which laccase protects cells from oxidative stress has yet to be elucidated. Recently Kim et al. reported that expression of the laccase gene from the fungus Coprinellus congregatus in Saccharomyces cerevisiae could increase the survival rate of yeast under the oxidative stress caused by H₂O₂. Laccase expression was found to increase the survival rate of yeast exposed to oxidative stress. This study provides evidence that laccase is involved in resistance to oxidative stress (37). However, the mechanism underlying this protection remains unclear.

In order to determine the function and mechanism of laccase in the defense against oxidative stress, we expressed the laccase gene from white rot fungus in the heterologous yeast host Pichia pastoris and investigated the mechanism of resistance to oxidative stress conferred. Pichia pastoris has seen widespread use as a protein expression system because it has the advantages of higher protein expression activity and increased recombinant laccase activity was defined as the amount of enzyme required to oxidize 1 mol ABTS per minute at room temperature, and 100 μl culture fluid. Oxidation of ABTS was monitored by determining the increase in A₄₂₀ (ε = 36,000 M⁻¹ cm⁻¹). One unit of enzyme activity was defined as the amount of enzyme required to oxidize 1 μmol of ABTS per min (17). The transcription of the laccase gene lac5930-1 in Pichia pastoris transformants was detected by reverse transcription-PCR (RT-PCR) as follows. Total RNA was isolated from the cultures of yeast transformants using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions, followed by RNase-free DNase (Promega) digestion to remove the genomic DNA (gDNA) contamination. Then RT-PCR was performed to detect the transcription of the laccase gene in yeast using a PrimeScript RT-PCR kit (TaKaRa) according to the manufacturer’s instructions. The PpGPD gene, encoding Pichia pastoris glyceraldehyde-3-phosphate dehydrogenase, was used as the internal control. The sequences of laccase gene-specific primers (lac5930-1-Fw and lac5930-1-Rv) and PpGPD gene-specific primers (PpGPD-Fw and PpGPD-Rv) were used for RT-PCR. All experiments were performed in triplicate.

Detection of the level of resistance to H₂O₂-mediated oxidative stress of two yeast transformants. The Pichia pastoris transformant pPIC3.5K/lac5930-1/GS115 and pPIC3.5K/GS115 were inoculated into separate 20-ml volumes of BMG medium in 250-ml Erlenmeyer flasks and incubated at 30°C to an optical density at 600 nm (OD600) of 10 with shaking at 200 rpm. The cultures were centrifuged at 3,000 × g for 5 min, and the cell pellets were suspended to an OD600 of 2.0 with 30 ml BMG medium (pH 6.0). The cultures were grown at 20°C with shaking at 200 rpm, with 0.5% (vol/vol) methanol being added daily.
(ii) Measurement of intracellular reduced GSH. The amount of intracellular glutathione (GSH) was determined using a GSH detection kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) based on the method reported by Jollow et al. (35). 5,5’-Dithiobis-2-nitrobenzoic acid (DTNB) was used to develop color. The development of yellow color was monitored at 412 nm on a spectrophotometer. GSH content is expressed as μmol/g of dry yeast cells. All experiments were performed in triplicate.

(iii) Measurement of glutathione peroxidase activity. The activity of glutathione peroxidase (GPx) was measured using a GSH-Px detection kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer’s instructions; the kit was designed based on principles described by Haefeman et al. and Banni et al. (5, 29). Glutathione peroxidase degraded H$_2$O$_2$ in the presence of GSH, decreasing GSH levels. The remaining GSH was then measured using the reaction with DTNB. Absorbance was recorded at 412 nm. One unit of GPx enzyme activity was defined as the amount of enzyme capable of synthesizing 1 μmol of GSH per minute. The activity of glutathione peroxidase is expressed as U/mg of dry yeast cells. All experiments were performed in triplicate.

(iv) Measurement of γ-GCS activity. The activity of γ-glutamylcysteine synthetase (γ-GCS) was measured using a γ-glutamylcysteine synthetase detection kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer’s instructions; the kit was designed using principles described by Chung et al. and Kenchappa et al. (15, 36). The amount of inorganic phosphate (P$_i$) released by γ-GCS was calculated from the standard curve. One unit of γ-GCS activity was defined as the amount of enzyme capable of synthesizing 1 μmol of P$_i$ per hour under assay conditions. The activity of γ-GCS is expressed as U/mg of dry yeast cells. All experiments were performed in triplicate.

(v) Measurement of glutathione reductase activity. The activity of glutathione reductase (GR) was measured using a GR detection kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer’s instructions; the kit was designed based on the method described by Di Ilio et al. and Casalone et al. (9, 24). GR activity was determined by following the decrease in absorbance at 340 nm due to the oxidation of NADPH to NADP$^+$. The activity of GR is expressed as mU/mg of dry yeast cells. All experiments were performed in triplicate.

(vi) Measurement of catalase activity. The activity of CAT was measured using a CAT detection kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer’s instructions. CAT activity was measured using the ammonium molybdate spectrophotometric method, which is based on the fact that ammonium molybdate can rapidly terminate the H$_2$O$_2$ degradation reaction catalyzed by CAT and react with the residual H$_2$O$_2$ to generate a yellow complex, which could be monitored by the absorbance at 405 nm (30). All experiments were performed in triplicate.

qRT-PCR detection of the transcription of various genes in yeast transformants under H$_2$O$_2$-mediated oxidative stress. Total RNA was isolated from yeast cells using the TRizol reagent (Invitrogen) according to the manufacturer’s instructions. First-strand cDNA was then synthesized using a PrimeScript RT reagent kit with gDNA eraser (TaKaRa). Two microliters of RT product was used as a template for quantitative real-time RT-PCR (qRT-PCR). qRT-PCR was performed using an iCycler iQ5 real-time PCR system (Bio-Rad) and a SYBR Premix Ex Taq II kit (Tli RNaseH Plus; TaKaRa) according to the manufacturer’s instructions. The primer pairs used for quantitative measurement of the transcription of the laccase gene (lac5930-1) and other genes related to the glutathione redox system (PpGPX1, PpPMP20, PpGLR1, PpGSH1, and PpYAP1) are listed in Table 1. The qRT-PCR mixture (25 μl) contained 2.0 μl of cDNA and 0.4 μl of each gene-specific primer as well as 1× SYBR Premix Ex Taq II (TaKaRa). The qRT-PCR was performed as follows: 10 min at 95°C followed by 30 cycles of 30 s at 95°C, 30 s at 55°C, and 30 s at 72°C, followed by a melting cycle from 55°C to 95°C to check for amplification specificity. The PpACT1 gene, encoding actin, and PpGPD gene, encoding glyceraldehyde-3-phosphate dehydrogenase, were used as internal controls. The

<table>
<thead>
<tr>
<th>Primer</th>
<th>Nucleotide sequence</th>
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<tr>
<td>lac5930-1-Fw</td>
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</tr>
<tr>
<td>lac5930-1-Rv</td>
<td>CTATCGGTCCGTCAGCGAACC</td>
</tr>
<tr>
<td>PpGPD-Fw</td>
<td>TTGGATGTCGCTCTCAATGG</td>
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<td>PpGPD-Rv</td>
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</tr>
<tr>
<td>qRT-PpACT1-Rv</td>
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Preparation of yeast cell extracts. Preparation of whole-cell homogenate for measurement of the physiological indexes related to yeast oxidative stress and antioxidative activity was performed as follows. Yeast cells were collected by centrifugation and washed three times with distilled water to remove the external H$_2$O$_2$ (used for exerting H$_2$O$_2$-mediated oxidative stress) and any traces of growth medium. Then cells were disrupted by using the method enclosed with the multicopy Pichia expression kit (Invitrogen). Cell homogenates were clarified by centrifugation. The resulting supernatant was collected and used as cell extract for further analysis. Protein concentration was determined using the method described by Bradford (7).

Measurement of the physiological indexes related to the yeast oxidative stress and antioxidative activity. (i) Measurement of MDA and intracellular H$_2$O$_2$. The level of malondialdehyde (MDA), an end product of the peroxidation of polyunsaturated fatty acids, was measured using an MDA spectrophotometric detection kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the method described previously (43). MDA was determined using the thiobarbituric acid (TBA) method based on its reaction with TBA to form thiobarbituric acid-reactive substances (TBARS). The MDA level is expressed as μmol/g of dry yeast cells. The level of intracellular H$_2$O$_2$ was measured according to the method described previously (23). All experiments were performed in triplicate.
relative abundance of mRNAs was normalized against the levels of \textit{PpACT1}. Each sample was amplified in triplicate in each experiment. Two independent experiments were performed, and they showed the same results.

**RESULTS**

**Expression of laccase gene from \textit{Trametes} sp. 5930 in \textit{Pichia pastoris}**. The cDNA of the laccase gene from white rot fungus \textit{Trametes} sp. 5930, \textit{lacc}5930-1 (54), was cloned into the expression vector of \textit{Pichia pastoris} pPIC3.5K, producing the recombinant plasmid pPIC3.5K-lac5930-1. Then pPIC3.5K-lac5930-1 and the empty vector pPIC3.5K were transformed into \textit{Pichia pastoris} GS115, generating two yeast transformants, pPIC3.5K-lac5930-1/GS115 and pPIC3.5K/GS115. Successful expression of the lac5930-1 gene in \textit{Pichia pastoris} was confirmed by measuring the secreted laccase activity of these yeast transformants (Fig. 1A) and detecting the transcription level of the \textit{lac}5930-1 gene by RT-PCR (Fig. 1B).

Expression of the laccase gene in \textit{Pichia pastoris} could increase the level of resistance to H\textsubscript{2}O\textsubscript{2}-mediated oxidative stress and protect the yeast cells from lipid oxidative damage caused by exogenous H\textsubscript{2}O\textsubscript{2}. The level of resistance to H\textsubscript{2}O\textsubscript{2}-mediated oxidative stress was analyzed by detection of the growth rate of pPIC3.5K-lac5930-1/GS115 and pPIC3.5K/GS115 subjected to various concentrations of exogenous H\textsubscript{2}O\textsubscript{2}. As shown in Fig. 2A, the growth of pPIC3.5K-lac5930-1/GS115 and that of pPIC3.5K/GS115 were very similar in the absence of exogenous H\textsubscript{2}O\textsubscript{2}. As shown in Fig. 2B, the growth of pPIC3.5K-lac5930-1 was strongly inhibited under oxidative stress caused by exogenous H\textsubscript{2}O\textsubscript{2} when no laccase was expressed. However, the growth of pPIC3.5K-lac5930-1/GS115, in which laccase was successfully expressed, was not influenced by exogenous H\textsubscript{2}O\textsubscript{2}. pPIC3.5K-lac5930-1/GS115 was more resistant to exogenous H\textsubscript{2}O\textsubscript{2} (50 mM) than pPIC3.5K/GS115 (Fig. 2B). We also found that pPIC3.5K-lac5930-1/GS115 could be resistant to higher concentrations of exogenous H\textsubscript{2}O\textsubscript{2} (100 and 200 mM) (data not shown). These results indicate that the expression of laccase in \textit{Pichia pastoris} can increase the resistance of yeast to H\textsubscript{2}O\textsubscript{2}-mediated oxidative stress.

The oxidative damage to the two yeast transformants exposed to different concentrations of H\textsubscript{2}O\textsubscript{2} was evaluated further. The degree of oxidative damage to lipids was assessed by determining the levels of oxidized lipids. These were determined by measuring levels of malondialdehyde (MDA), an end product of the peroxidation of polyunsaturated fatty acids. As shown in Fig. 3A, the MDA levels of pPIC3.5K/GS115 exposed to 50 mM and 100 mM H\textsubscript{2}O\textsubscript{2} became significantly higher than those of control cells not exposed to exogenous H\textsubscript{2}O\textsubscript{2}. For example, after 12 h, the MDA levels of pPIC3.5K/GS115 exposed to 50 mM and 100 mM H\textsubscript{2}O\textsubscript{2} were found to be 1.06 and 1.85 \textmu mol/g dry cells but the MDA content of pPIC3.5K/GS115 exposed to 0 mM H\textsubscript{2}O\textsubscript{2} was only 0.13 \textmu mol/g dry cells. The MDA level of pPIC3.5K/GS115 increased as exogenous H\textsubscript{2}O\textsubscript{2} stress increased. The high level of oxidized lipids confirmed the development of severe oxidative stress in the yeast cultures subjected to exogenous H\textsubscript{2}O\textsubscript{2} (Fig. 3A). As shown in Fig. 3A, the MDA levels of pPIC3.5K-lac5930-1/GS115 exposed to 50 mM and 100 mM H\textsubscript{2}O\textsubscript{2} were much lower than those of pPIC3.5K/GS115 under the same concentrations of H\textsubscript{2}O\textsubscript{2} for the same time. For example, the MDA contents of pPIC3.5K-lac5930-1/GS115 exposed to 50 mM and 100 mM H\textsubscript{2}O\textsubscript{2} were found to be only 0.36 and 0.45 \textmu mol/g dry cells, respectively, much lower than those of pPIC3.5K/GS115 (Fig. 3A). These results suggest that the degree of lipid oxidative damage to pPIC3.5K-lac5930-1/GS115 upon exposure to exogenous H\textsubscript{2}O\textsubscript{2} is much less than that to pPIC3.5K/GS115. Laccase gene expression in pPIC3.5K-lac5930-1/GS115 (Fig. 2B).
Enhanced Resistance to Oxidative Stress by Laccase Gene

1/GS115 can protect the yeast cells from lipid oxidative damage caused by exogenous H$_2$O$_2$.

The intracellular H$_2$O$_2$ concentrations of two yeast transformants exposed to different concentrations of H$_2$O$_2$ were also determined. As shown in Fig. 3B, the intracellular H$_2$O$_2$ concentrations of pPIC3.5K/GS115 exposed to 50 mM and 100 mM exogenous H$_2$O$_2$ were much higher than those in pPIC3.5K-lac5930-1/GS115. For example, the intracellular H$_2$O$_2$ concentrations in pPIC3.5K/GS115 exposed to 50 mM and 100 mM H$_2$O$_2$ for 12 h were found to be 21.1 and 32.0 mmol/g dry cells, respectively, but the intracellular H$_2$O$_2$ concentrations in pPIC3.5K-lac5930-1/GS115 under the same conditions were only 6.9 and 7.2 mmol/g dry cells (Fig. 3B). The concentration of intracellular H$_2$O$_2$ in pPIC3.5K/GS115 increased as exogenous H$_2$O$_2$ stress increased. These results indicate that pPIC3.5K-lac5930-1/GS115 has a greater ability to scavenge intracellular H$_2$O$_2$ than pPIC3.5K/GS115. This implies that laccase gene expression in pPIC3.5K-lac5930-1/GS115 can contribute to the scavenging of intracellular H$_2$O$_2$ and protect cells against H$_2$O$_2$-mediated oxidative stress.

Expression of the laccase gene in *Pichia pastoris* could enhance the level of resistance to H$_2$O$_2$-mediated oxidative stress by stimulating the glutathione redox system of yeast. The above results indicated that the expression of the laccase gene in *Pichia pastoris* could enhance resistance to H$_2$O$_2$-mediated oxidative stress. pPIC3.5K-lac5930-1/GS115 had greater ability to resist this oxidative stress than pPIC3.5K/GS115. Based on this finding, we studied the mechanism of how laccase gene expression increased the resistance of yeast to H$_2$O$_2$-mediated oxidative stress. In order to determine the mechanism underlying the differences in resistance to H$_2$O$_2$-mediated oxidative stress between pPIC3.5K-lac5930-1/GS115 and pPIC3.5K/GS115, the levels of antioxidant defense activity of these two yeast transformants in the presence of H$_2$O$_2$ were evaluated.

First, the levels of glutathione-based antioxidative activity of the two yeast transformants exposed to exogenous H$_2$O$_2$ were investigated. As shown in Fig. 4 and 5, the levels of glutathione redox activity of pPIC3.5K-lac5930-1/GS115 and pPIC3.5K/GS115, including the intracellular GSH content and the activities of glutathione peroxidase, glutathione reductase, and γ-glutamylcysteine synthetase, were very similar in the absence of exogenous H$_2$O$_2$. In contrast, the level of glutathione redox activity of pPIC3.5K-lac5930-1/GS115 was much higher than that of pPIC3.5K/GS115 when the yeast transformants were exposed to exogenous H$_2$O$_2$. A significant increase in the level of glutathione redox activity was observed when pPIC3.5K-lac5930-1/GS115 was exposed to exogenous H$_2$O$_2$. However, the activity of the glutathione redox system of pPIC3.5K/GS115 remained very low under H$_2$O$_2$ stress conditions (Fig. 4 and 5). As shown in Fig. 4A, the intracellular GSH content of pPIC3.5K-lac5930-1/GS115 was significantly higher than that of pPIC3.5K/GS115 when the yeast transformants were exposed to exogenous H$_2$O$_2$. The activities of glutathione peroxidase (Fig. 4B), γ-GCS (Fig. 5A), and glutathione reductase (Fig. 5B) of pPIC3.5K-lac5930-1/GS115 were also always higher than those of pPIC3.5K/GS115 when the yeast transformants were exposed to exogenous H$_2$O$_2$ for 12 h. We also observed that the levels of intracellular GSH and the activities of glutathione peroxidase, γ-GCS, and glutathione reductase in pPIC3.5K-lac5930-1/GS115 all increased as the concentration of exogenous H$_2$O$_2$ increased (Fig. 4 and 5).

The activity of another antioxidative enzyme, catalase, was also
The catalase activity of pPIC3.5K-lac5930-1/GS115 exposed to exogenous H$_2$O$_2$ was similar to that of pPIC3.5K/GS115 (data not shown). The transcription of the laccase gene in pPIC3.5K-lac5930-1/GS115 could be stimulated by the oxidative stress caused by exogenous H$_2$O$_2$. The effects of the level of oxidative stress on laccase gene transcription in pPIC3.5K-lac5930-1/GS115 were investigated. A time course detection of laccase gene transcription in pPIC3.5K-lac5930-1/GS115 exposed to different concentrations of H$_2$O$_2$ was performed. As shown in Fig. 6A and B, the transcription of the laccase gene increased over time when pPIC3.5K-lac5930-1/GS115 was exposed to 50 or 100 mM exogenous H$_2$O$_2$ but not when it was exposed to 0 mM H$_2$O$_2$. It instead remained low throughout the incubation (Fig. 6A and B). This suggested that the transcription of the laccase gene in pPIC3.5K-lac5930-1/GS115 was significantly increased upon exposure to exogenous H$_2$O$_2$. The transcription levels of the laccase gene in pPIC3.5K-lac5930-1/GS115 exposed to 50 mM and 100 mM H$_2$O$_2$ for 12 h were about 5.3 and 9.2 times higher than the level of transcription in pPIC3.5K-GS115 exposed to 0 and 100 mM H$_2$O$_2$ respectively. This suggested that the transcription of laccase genes in yeast increased as the level of oxidative stress increased.

The transcription of the laccase gene in pPIC3.5K-lac5930-1/GS115 could be stimulated by the oxidative stress caused by exogenous H$_2$O$_2$. The effects of the level of oxidative stress on laccase gene transcription in pPIC3.5K-lac5930-1/GS115 were investigated. A time course detection of laccase gene transcription in pPIC3.5K-lac5930-1/GS115 exposed to different concentrations of H$_2$O$_2$ was performed. As shown in Fig. 6A and B, the transcription of the laccase gene increased over time when pPIC3.5K-lac5930-1/GS115 was exposed to 50 or 100 mM exogenous H$_2$O$_2$ but not when it was exposed to 0 mM H$_2$O$_2$. It instead remained low throughout the incubation (Fig. 6A and B). This suggested that the transcription of the laccase gene in pPIC3.5K-lac5930-1/GS115 was significantly increased upon exposure to exogenous H$_2$O$_2$. The transcription levels of the laccase gene in pPIC3.5K-lac5930-1/GS115 exposed to 50 mM and 100 mM H$_2$O$_2$ for 12 h were about 5.3 and 9.2 times higher than the level of transcription in pPIC3.5K-GS115 exposed to 0 and 100 mM H$_2$O$_2$. This suggested that the transcription of laccase genes in yeast increased as the level of oxidative stress increased.

The effects of phases of growth on the transcription of the housekeeping gene $PpACT1$ in pPIC3.5K-lac5930-1/GS115 were also detected. Another housekeeping gene, $PpGPD$, encoding glyceraldehyde-3-phosphate dehydrogenase, was used as an internal control. The transcription levels of $PpACT1$ (used as the negative control in the quantitative real-time RT-PCR in Fig. 6A and B) in different phases of growth were measured by qRT-PCR. As shown in Fig. 6C, the transcription levels of $PpACT1$ did not differ across phases.

Expression of the laccase gene in *Pichia pastoris* could stimulate the transcription of genes involved in the glutathione-based antioxidative system in response to H$_2$O$_2$-mediated oxidative stress. The above results suggested that the expression of...
the laccase gene in *Pichia pastoris* could stimulate the level of glutathione-based antioxidative activity. Based on this, the transcription levels of genes related to the glutathione-based antioxidative system, such as *PpGPX1*, which encodes glutathione peroxidase, *PpPMP20*, which encodes peroxisome glutathione peroxidase, *PpGLR1*, which encodes glutathione reductase, *PpGSH1*, which encodes γ-glutamylcysteine synthetase, and *PpYAP1*, which encodes the *PpYAP1* transcription factor (55), were also measured by qRT-PCR under H2O2-mediated oxidative stress.

First, the transcription levels of genes related to the glutathione redox systems of the two yeast transformants exposed to exogenous H2O2 were compared. As shown in Fig. 7F, the transcription levels of *PpGPX1*, *PpPMP20*, *PpGLR1*, *PpGSH1*, and *PpYAP1* in pPIC3.5K-lac5930-1/GS115 exposed to 50 mM H2O2 for 12 h were much higher than those in pPIC3.5K/GS115 exposed to H2O2 for the same amount of time (Fig. 7F). The transcription levels of *PpGPX1*, *PpPMP20*, *PpGLR1*, *PpGSH1*, and *PpYAP1* in pPIC3.5K-lac5930-1/GS115 exposed to 50 mM H2O2 for 12 h were increased to about 7.8, 5.7, 5.5, 10.2, and 12.1 times, respectively, those of the corresponding genes in pPIC3.5K/GS115 (Fig. 7F).

The time course of the transcription of genes involved in the glutathione-based antioxidative system was determined in two yeast transformants subjected to exogenous H2O2 stress. Figure 7A to E show the change in the transcription levels of *PpGPX1*, *PpGLR1*, *PpGSH1*, and *PpYAP1* between pPIC3.5K-lac5930-1/GS115 and pPIC3.5K/GS115 exposed to 50 mM H2O2 for 12 h. The transcription levels of various genes in pPIC3.5K/GS115 were set as 1-fold. *PpACT1* is the housekeeping gene that encodes the actin of *Pichia pastoris*. *PpGPX1* encodes glutathione peroxidase. *PpPMP20* encodes peroxisome glutathione peroxidase. *PpGLR1* encodes glutathione reductase. *PpGSH1* encodes γ-glutamylcysteine synthetase. *PpYAP1* encodes the *PpYAP1* transcription factor. Results are means ± standard deviations (*n* = 3).

FIG 7 qRT-PCR for detecting the transcription levels of genes related to the glutathione-based antioxidative system, including *PpGPX1*, *PpGLR1*, *PpGSH1*, *PpYAP1*, and *PpPMP20*, in the *Pichia pastoris* transformants pPIC3.5K-lac5930-1/GS115 and pPIC3.5K/GS115 when exposed to 50 mM H2O2. (A to E) Time course detection of the transcription level of *PpGPX1* (A), *PpGLR1* (B), *PpGSH1* (C), *PpYAP1* (D), and *PpPMP20* (E) in the *Pichia pastoris* transformants pPIC3.5K-lac5930-1/GS115 and pPIC3.5K/GS115 when exposed to 50 mM H2O2. To evaluate the change of gene transcription over time, the transcription levels of various genes at 0 h were set as 1-fold. (F) Comparison of the transcription levels of *PpGPX1*, *PpPMP20*, *PpGLR1*, *PpGSH1*, and *PpYAP1* between pPIC3.5K-lac5930-1/GS115 and pPIC3.5K/GS115 exposed to 50 mM H2O2 for 12 h. The transcription levels of various genes in pPIC3.5K/GS115 were set as 1-fold. *PpACT1* is the housekeeping gene that encodes the actin of *Pichia pastoris*. *PpGPX1* encodes glutathione peroxidase. *PpPMP20* encodes peroxisome glutathione peroxidase. *PpGLR1* encodes glutathione reductase. *PpGSH1* encodes γ-glutamylcysteine synthetase. *PpYAP1* encodes the *PpYAP1* transcription factor. Results are means ± standard deviations (*n* = 3).
DISCUSSION

Our work suggests that the expression of the laccase gene in *Pichia pastoris* can enhance resistance to H$_2$O$_2$-mediated oxidative stress. This is consistent with the results of a previous study that showed that expression of the laccase gene from the fungus *Coprinellus congregatus* in Saccharomyces cerevisiae increased yeast survival under the oxidative stress caused by H$_2$O$_2$ (37). There have been few reports of increased resistance to oxidative stress induced by heterologous expression of fungal genes in yeast. Zhang et al. have reported that the expression of the superoxide dismutase (SOD) gene from the thermophilic fungus *Chaetomium thermophilum* in *Pichia pastoris* could increase the yeast resistance to paraquat and menadione-mediated oxidative stress (60). Yoo et al. have found that the overexpression of the human Cu/Zn superoxide dismutase gene in *Saccharomyces cerevisiae* increased the resistance to oxidative stresses caused by paraquat, menadione, and heat shock (57). Superoxide dismutase can catalyze the removal of superoxide radicals. It is thought to be the first line of cellular defense against oxidative damage caused by superoxide anion radicals (57). This shows that the expression of SOD genes from other species in yeast can increase the resistance of yeast to paraquat and menadione, both of which generate superoxide radicals. Laccase is a group of copper-containing polyphenol oxidases that can catalyze the four-electron reduction of O$_2$ to H$_2$O with the concomitant oxidation of phenolic compounds. The mechanism of the antioxidative role of laccase may be quite different from that found in previous studies using the SOD gene to increase the resistance of yeast to oxidative stress (57, 60).

One previous study only showed that the expression of the laccase gene from the fungus *Coprinellus congregatus* in *Saccharomyces cerevisiae* could increase the survival rate of yeast subjected to oxidative stress caused by H$_2$O$_2$. However, the mechanism by which yeast resistance to H$_2$O$_2$ stress was increased and its connection to the laccase gene remained unknown (37). In our research, the mechanism by which laccase protects yeast from H$_2$O$_2$ stress was further investigated. This study is the first to reveal the mechanism by which laccase gene expression increases the resistance of yeast to oxidative stress. Our results indicate that the expression of laccase in *Pichia pastoris* can increase the resistance of yeast to H$_2$O$_2$-mediated oxidative stress by stimulating the activity of the glutathione-based antioxidative system (including GSH, glutathione peroxidase, γ-glutamylcysteine synthetase, and glutathione reductase). The high level of glutathione-based antioxidative activity can increase the cell’s ability to detoxify H$_2$O$_2$ and protect itself from oxidative damage.

To determine whether expression of the laccase gene is really linked to defense mechanisms like enhanced glutathione production, we expressed gene *cdh*, encoding cellobiose dehydrogenase, which was cloned from white rot fungus *Trametes* sp. 5930, in *Pichia pastoris* and then determined whether the heterologous expression of cellobiose dehydrogenase could also enhance yeast resistance to H$_2$O$_2$-mediated oxidative stress. Our results showed that *cdh* could be successfully expressed in *Pichia pastoris*. However, the growth of the yeast transformant expressing *cdh* was inhibited under the oxidative stress caused by exogenous H$_2$O$_2$. Our results indicate that the expression of the *cdh* gene in *Pichia pastoris* cannot increase the resistance of the yeast to H$_2$O$_2$-mediated oxidative stress by enhancing the glutathione-dependent antioxidative system. Our work also suggested that it was not a co-incidence that the heterologous expression of the laccase gene in *Pichia pastoris* could enhance the resistance of the yeast to H$_2$O$_2$-mediated oxidative stress by stimulating the glutathione-dependent antioxidative system. The heterologous expression of another enzyme, such as cellobiose dehydrogenase, did not lead to effects similar to those observed in cells expressing laccase.

Although our work has suggested that a real link between laccase expression and enhancement of the glutathione-dependent antioxidative system exists, the question of how laccase expression is linked to defense mechanisms like enhanced glutathione production remains unclear. The mechanism by which laccase expression in *Pichia pastoris* can stimulate the glutathione-dependent antioxidative system remains unknown. We here put forward one hypothesis that may answer this question based on the following findings. In this study, we found that laccase gene expression in *Pichia pastoris* could significantly stimulate the transcription of the *PpYAPI* gene in response to the oxidative stress caused by exogenous H$_2$O$_2$. Time course analysis revealed that the transcription of various genes related to the glutathione-dependent antioxidative system in *pPIC3.5Klac5930-1/GS115* was stimulated over time under the oxidative stress caused by exogenous H$_2$O$_2$ (Fig. 7A to E). Out of the five genes studied here, *PpYAPI* was the first to be induced. The transcription of *PpYAPI* in *pPIC3.5Klac5930-1/GS115* exposed to 50 mM H$_2$O$_2$ was stimulated after only 2 h, increasing 5.4-fold over the baseline (0 h) level (Fig. 7D). After 12 h of exposure to 50 mM H$_2$O$_2$, *PpYAPI* showed the highest level of transcriptional induction among the five genes related to the glutathione-dependent antioxidative system (Fig. 7F). In *Pichia pastoris*, the *PpYAPI* gene encodes the *PpYAPI* transcription factor, which is the *Pichia pastoris* homologue of ScYAPI, which was first discovered in *Saccharomyces cerevisiae*. Previous research has revealed that ScYAPI is an important transcription factor in *Saccharomyces cerevisiae*, in which it can stimulate the expression of several genes of the glutathione-dependent antioxidative system, such as *GLR1, GPX2*, and *GSH1* (28, 38–40, 53, 55). Recent studies have shown that the PpYAPI-regulated glutathione reductase system plays an important role in the detoxification of reactive oxygen species in the methanol metabolism of *Pichia pastoris* (55). *PpYAPI* can act as a regulator of the redox system in *Pichia pastoris*. It plays an important role in the defense against oxidative stress by activating the expression of other antioxidative genes involved in the glutathione-based antioxidant system (55, 56). Based on our results and previous research, we propose the following hypothesis to address the issue of how laccase expression is linked to defense mechanisms like enhanced glutathione production. The transcription of the laccase gene can be stimulated by the oxidative stress caused by exogenous H$_2$O$_2$. The stimulation of laccase gene expression in response to exogenous H$_2$O$_2$ stress may further activate some transcriptional activator that can specially stimulate the transcription of the *PpYAPI* gene. Laccase expression first contributes to the transcriptional induction of the *PpYAPI* gene, increasing the production of the *PpYAPI* transcription regulator. Then *PpYAPI* induces the expression of other important genes involved in the glutathione-dependent antioxidative system, including *PpGPX1, PpGLR1, PpGSH1*, and *PpPMP20*. In this way, the level of glutathione-based antioxidative activity in yeast (including the intracellular GSH level and the enzymatic activities of glutathione peroxidase, glutathione reductase, and γ-GCS) is elevated correspondingly, which confers a strong ability to scavenge intracellu-
lar H₂O₂ and protect against oxidative stress. Further studies need to be performed to validate this hypothesis. Research into the molecular mechanism underlying the connection between laccase expression and stimulation of the glutathione-dependent antioxidative system in *Pichia pastoris* is ongoing in our laboratory.

In conclusion, we found that the heterologous expression of laccase gene in *Pichia pastoris* can enhance the resistance of yeast to H₂O₂-mediated oxidative stress by stimulating the glutathione-dependent antioxidative system. Our work will shed light on the function and mechanism of laccase in the defense against oxidative stress. This study may be of interest especially for the understanding of laccase’s physiological role and function. *Pichia pastoris* has been widely applied as a heterologous expression system for eukaryotic proteins. Our findings may help increase the efficiency of *Pichia pastoris* systems expressing useful proteins by enhancing the resistance of yeast to oxidative stress.

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