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Rational design for fungal laccase production in the model host *Aspergillus nidulans*

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Laccases, multicopper oxidoreductases, are mainly produced in white-rot fungi and are considered as ideal green catalysts in industrial and biotechnological applications. However, the development of laccases is limited due to the slow growth of natural laccase producing strains and the low expression levels of laccases. In this study, we designed three regulation strategies for laccase gene expression in the model fungus *Aspergillus nidulans*. By introducing various promoters in front of the laccase gene *pslcc* from the white-rot fungus *Pycnoporus sanguineus*, we found that the laccase gene with the original promoter had effective expression in *A. nidulans*. Using the previously identified transcription factor RsmA regulatory mechanism, the *aflR* promoter was inserted into the *pslcc* expression vectors, and the laccase production was 15-fold higher in the strain overexpressing of RsmA compared to the control strain. To improve the laccase yield, the dipeptidyl-peptidase DppV, aspartic protease PepA and mannosyltransferase Mnn9 were successfully deleted in the *A. nidulans* host. The laccase activities were increased approximately 8-fold and 13-fold in the double deletions strains of *ΔmnnΔpepA* and *ΔdppVΔpepA* over the control strains, respectively. Taken together, these results not only demonstrate an efficient system for heterologous protein production in the model fungus *A. nidulans* but also provide a general approach to applying regulatory methods to control gene expression.

laccase, heterologous expression, promoter, *Aspergillus nidulans*

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INTRODUCTION

Laccases (benzenediol: oxygen oxidoreductase, EC 1.10.3.2) belong to the multicopper polyphenol oxidase family and catalyze the reduction of oxygen to water accompanied by the oxidation of a substrate, typically a *p*-dihydroxy phenol or another phenolic compound (Baldrian, 2006). Due to their wide substrate specificity, laccases have been applied in a broad array of fields, including paper pulping, wood composite production, bioremediation, dye decolorization, textile cleaning, juice clarification, biosensors and biofuel materials pretreatment (Kudanga et al., 2011; Liu et al., 2018). Laccases are mainly found in plants and white-rot fungi. Plant laccases participate in the radical-based mechanisms of lignin polymer formation (Hoopes and Dean, 2004), whereas fungal laccases play important roles in morphogenesis, fungal plant-pathogen/host interactions, stress defenses and lignin degradation (Yaropolov et al., 1994). In nature, basidiomycetes are the main sources of laccases, but the enzymes produced by the natural fungal strains are not suitable for industrial applications. Higher yields of active laccases can be achieved after optimized production (Majeau et al., 2010), and although many white-rot fungi produce several laccase isoforms with similar properties, purifying the individual enzymes is difficult (Colao et al., 2006).

To overcome these obstacles, laccase genes have been

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overexpressed in different heterologous hosts, including bacteria (Nicolini et al., 2013; Salony et al., 2008) and yeast (Antošová and Sychrová, 2016; Iimura et al., 2018; Na et al., 2017; Yang et al., 2015). However, due to large differences in glycosylation, such recombinant enzymes often altered the properties of the enzyme (Otterbein et al., 2000; Sigoillot et al., 2004), which makes them less suitable for biotechnological applications. Moreover, the expression level of the laccase gene is different and is dependent on the yeast hosts, laccase subtype expression vector and cultivation scale (Pezzella et al., 2009). In addition, several filamentous fungal hosts have been used to express white-rot fungal laccases. For example, laccase genes from *Stachybotrys chartarum*, *Pycnoporus cinnabarinus*, *Pleurotus sajor-caju*, and *Phanerochaete flavido-alba* were expressed in *Aspergillus niger* (Benghazi et al., 2014; Mander et al., 2006; Record et al., 2002; Soden and Dobson, 2003). Laccase genes from *Melanocarpus albomyces* and *Trametes* sp. AH28-2 were expressed in *Trichoderma reesei* (Kiiskinen et al., 2004; Zhang et al., 2012). The yields of laccases were approximately 592–774,000 U L^{-1} or 8–920 mg L^{-1} by heterologous expression in filamentous fungi after optimizing fermentation (Antošová and Sychrová, 2016).

To increase laccase gene expression in filamentous fungal hosts, efficient promoters were used, such as the *cbh1* (exoglycodextran glycohydrolytic enzyme gene) promoter from *T. reesei*, the *glaA* (glucoamylase gene) promoter from *A. niger* (Nevalainen et al., 2005), the *gpdA* (glyceraldehyde-3-phosphate dehydrogenase gene) promoter from *A. nidulans* and the *SC3* hydrophobin gene promoter from *Schizophyllum commune* (Alves et al., 2004). In addition, downregulation of protease expression is another method to reduce the degradation of laccase in the heterologous host (van den Hombergh et al., 1997). For example, disruption of the extracellular acid protease PEPA in both *Aspergillus awamori* and *A. niger* reduced the extracellular proteolytic activity by 20% compared to the wild type (van den Hombergh et al., 1997). Double disruption of the proteinase genes *tppA* and *pepE* increased the production of human lysozyme by *Aspergillus oryzae* (Jin et al., 2007). Disruption of ten protease genes or quintuple proteases in *A. oryzae* improved the production of heterologous proteins (Kitamoto et al., 2015; Yoon et al., 2011).

Aspergillus nidulans, an important model fungus, has been used as a host for enzyme production and the study of secondary metabolites (SM) in recent years (Ma et al., 2018; van Dijk and Wang, 2016; Yin et al., 2013a). It was also used for laccase gene expression from *Ceriporiopsis subvermispora* (Larrondo et al., 2003). In this study, we engineered *A. nidulans* host by the disruption of proteases or mannosyltransferase. Then, we successfully expressed the laccase gene *pslcc* from *Pycnoporus sanguineus* and tested laccase production by assessing different regulatory elements (Figure 1).

RESULTS

Identification and cloning of the laccase gene in *Pycnoporus sanguineus*

Using the inverse PCR method, we obtained the laccase gene with its original promoter and terminator sequences (*pslcc*, MF198440) from the gDNA of *Pycnoporus sanguineus* mk528. The *pslcc* gene consists of a promoter sequence (1,179 bp upstream of ATG), open reading frame sequence (2,153 bp) and terminator sequence (1,554 bp downstream of TAG). By analyzing the characteristics of the *pslcc* gene promoter with the Gene2Promoter software (Gershenzon and Ioshikhes, 2005), we found that there are multiple regulatory sites, including one Tsp transcriptional start site (−49 bp), five CAAT boxes (−306, −634, −1,006 bp, 1,034 and −115 bp), one TATA box (−255 bp), two CT-rich regions (−34 and −193 bp), two MRE core regions (metal responsive elements: −954 and −1,166 bp), two XREs (xenobiotic responsive elements: −204 and −751 bp), one HSE (heat shock element: −131 bp) and one reverse Sp1 transcription factor recognition site (−263 bp) (Figure S1 in Supporting Information). The sequences of the MREs are similar to the core MRE consensus sequence identified in the promoters of the metallothionein gene, TGCPuCNC, with that of cMRE3 being identical.

Rational design of laccase expression vectors in *A. nidulans*

To assess laccase gene expression with different promoters, we designed and constructed the expression vector with the *pslcc* gene and different auxotroph markers matched with the different host strains. First, we constructed the tool vectors including pYWL14 (with the *gpdA* promoter, *TrpC* terminator and *AppyrG*), pYWL93.4 (with the *gpdA* promoter, *TrpC* terminator and *AfpyroA*) and pYWL94.8 (with the *gpdA* promoter, *TrpC* terminator and *AfriboB*) (Table 1). Then, we cloned the *pslcc* gene expression vectors for *A. nidulans*, including pYWL15 (with the *gpdA* promoter, *TrpC* terminator and *AppyrG*), pYWL17.1 (with the *pslcc* promoter and terminator and *AppyrG*), pYWL53.1 (with the *aflR* promoter, *pslcc* terminator and *AppyrG*), pYWL55.3 (with the *aflR* promoter, *pslcc* terminator and *AfriboB*), pYWL97.2 (with the *gpdA* promoter, *TrpC* terminator and *AppyrG*), pYWL99.2 (with the *pslcc* promoter and terminator and *AfpriboB*) and pYWL100.1 (with the *pslcc* promoter and terminator and *AfpyroA*) (Table 1). Finally, the *pslcc* expression cassette with its own promoter and terminator was cloned into the vector containing the *AMA1* (autonomous maintenance in *Aspergillus*) sequence (Alek-

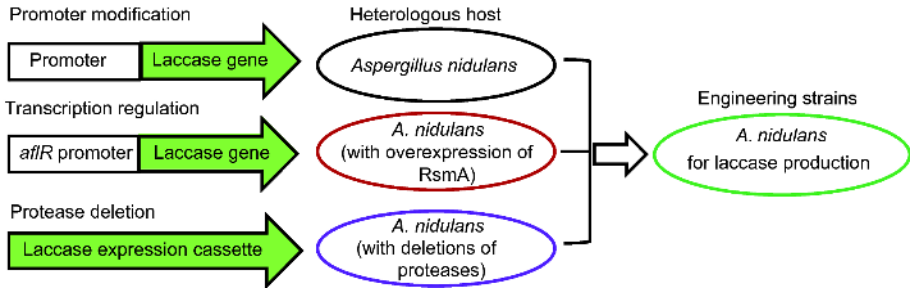

Figure 1 (Color online) Strategy for laccase heterologous expression in *A. nidulans*.

Table 1 Plasmids used in this study[a)]

Plasmid name	Description	References
pJMP9.1	*gpdA(p)::A. para pyrG*, *Amp*	(Baccile et al., 2016)
pRG-AMA1-*Not* I	*pyrG*, *AMA1*, *Amp*	(Aleksenko and Clutterbuck, 1997)
pWY25.16	*A. fumigatus pyroA::gpdA* in pGEMT easy vector	(Yin et al., 2012)
pYH-WA-pyrG-KI	*URA3*, *WA flanking*, *AfpyrG*, *Amp*	(Yin et al., 2012)
pYWB2	*URA3*, *WA flanking*, *AfriboB2*, *Amp*	(Zhang et al., 2017)
pYWL14	*gpdA(p)*, *TrpC(t)*, *AppyrG*, *Amp*	This study
pYWL15	*pslcc(p)::pslcc*, *AppyrG*, *Amp*	This study
pYWL16	*pslcc(p)::pslcc* in pRG-*AMA1-Not* I	This study
pYWL17.1	*pslcc(p)::pslcc*, *AppyrG*, *Amp*	This study
pYWL53.1	*aflR(p)::pslcc*, *AppyrG*, *Amp*	This study
pYWL55.3	*aflR(p)::pslcc*, *AfriboB2*, *Amp*	This study
pYWL90.2	*mnn9 deletion cassette*, *AfpyroA* in pEASY-Blunt	This study
pYWL91.4	*dppV deletion cassette*, *AfpyroA* in pEASY-Blunt	This study
pYWL92.6	*pepA deletion cassette*, *AfriboB2* in pEASY-Blunt	This study
pYWL93.4	*gpdA(p)*, *TrpC(t)*, *AfpyroA* in pWY25.16	This study
pYWL94.8	*gpdA(p)*, *TrpC(t)*, *AfriboB2* in pWY25.16	This study
pYWL96.1	*dppV* deletion cassette, *AppyrG* in pEASY-Blunt	This study
pYWL97.2	*gpdA(p)::pslcc*, *AfriboB2*, *Amp*	This study
pYWL99.2	*pslcc(p)::pslcc*, *AfriboB2*, *Amp*	This study
pYWL100.1	*gpdA(p)::pslcc*, *AfpyroA*, *Amp*	This study

a) pXX, plasmid.

senko and Clutterbuck, 1997) to obtain plasmid pYWL16 (Table 1).

Fermentative optimization of laccase gene expression

Transformants of *pslcc(p)*::*pslcc* (TYWL33.9) were cultivated in LMM medium for optimizing the fermentation of laccase production in *A. nidulans*. The fermentation parameters, including culture temperature (28°C, 32°C and 37°C), inoculation number of spores (10^4 spores, 10^5 spores and 10^6 spores/100 mL LMM media) and Cu^{2+} concentration (0.1, 0.2 and 0.5 mmol L^{-1}) as an inducer were assessed for the induction of laccase expression. The results showed that inoculation amount is very important for laccase production. There was no difference in laccase activity with the inoculation of 10^4 spores at different temperatures and Cu^{2+} concentrations (Figure 2A). The conditions for laccase expression was the highest at both 32°C with 10^5 spores (Figure 2B) and at 28°C with 10^6 spores/100 mL (Figure 2C). The Cu^{2+} concentration at 0.1 mmol L^{-1} greatly improved laccase expression, whereas laccase expression was decreased with the addition of 0.5 mmol L^{-1} Cu^{2+}. Therefore, the optimized conditions for laccase production are 10^6 spores per inoculation per 100 mL LMM medium at 32°C culture with shaking at 220 r min^{-1} and the addition of 0.1 mmol L^{-1} Cu^{2+}. The laccase activity was detected in five days.

Assessment of laccase expression with different promoters

Transformants of *pslcc*-AMA1 (TYWL16.2), *pslcc(p)::pslcc* (TYWL33.9) and *gpdA(p)::pslcc* (TYWL61.1) were verified by PCR (Figure S2A in Supporting Information) and culti-

vated in LMM media to assess the effect of different promoters on laccase production in *A. nidulans*. Laccase activity indicated that laccase production (approximately 37×10^{-3} U mL^{-1}) in the strain carrying *pslcc(p)::pslcc* was notably higher than both the *pslcc*-AMA1 and *gpdA(p)::pslcc* strains (approximately 20×10^{-3} U mL^{-1}), whereas there was no difference between strains carrying *pslcc*-AMA1 and *gpdA(p)::pslcc* (Figure 3A). Concomitantly, the expression of the *pslcc* gene was detected by qPCR and showed significant upregulation of laccase in strains carrying both *pslcc*-AMA1 and *gpdA(p)::pslcc* compared to controls (Figure 3B). The results showed that the *gpdA* promoter and AMA1 sequence effectively improved the transcription of the *pslcc* gene but they do not work well at the protein level.

RsmA overexpression effectively improves laccase production in *A. nidulans*

The previously identified transcription factor RsmA greatly increases SM production by binding to the *aflR* promoter region in *A. nidulans* (Yin et al., 2012). To evaluate the effect of RsmA overexpression on laccase production by *A. nidulans*, we constructed the *OE::rsmA* strain TYWL13.2 (Figure 4 and Table 2). The verification of *OE::rsmA* mutant in *A. nidulans* LO4389 was performed by diagnostic PCR (Figure S3 in Supporting Information). The schematic illustration of the RsmA overexpressing strain was shown in Figure 4. Transformants of the *OE::rsmA* host carrying *aflR (p)::pslcc* (TYWL23.19) and the LO4389 strain carrying *aflR(p)::pslcc* (TYWL69.6) were verified by diagnostic PCR (Figure S2 in Supporting Information). They were cultivated in LMM media to assess the effect on laccase production in *A. nidulans*. The laccase activity assay indicated that laccase production (approximately 60×10^{-3} U mL^{-1}) in the *OE::rsmA* strain carrying *aflR(p)::pslcc* was 15 times higher than the control (approximately 4×10^{-3} U mL^{-1}) (Figure 3C). The qPCR showed that the transcriptional level of the *pslcc* gene in the *OE::rsmA* background was higher than that in LO4389 (Figure 3D).

Protease deletions effectively improve laccase production in *A. nidulans*

By analyzing two protease genes and one mannosyltransferase, including *dppV*, *pepA* and *mnn9*, respectively, in the *A. niger* in NCBI database, we identified *dppV* (AN2572.2), *mnn9* (AN7672.2) and *pepA* (AN6888.2) in *A. nidulans* (Table S1 in Supporting Information). To study the effects of these enzymes on laccase production, we constructed their deletion mutants. The schematic illustration for protease deletions was shown in Figure 4. The mutants with a single protease deletion (*ΔdppV*, *Δmnn9* and *ΔpepA*) and double protease deletions (*ΔdppVΔmnn9*, *ΔdppVΔpepA* and *Δmnn9ΔpepA*) in *A. nidulans* LO4389 were obtained by using the corresponding deletion cassette (Table 2). The verification of transformants was performed by diagnostic PCR (Figure S4 in Supporting Information). The growth was not affected for mutants with a single protease deletion and with two protease deletions of *ΔdppVΔpepA* (TYWL63.4). However, the double mutants of *ΔdppVΔmnn9* (TYWL65.1) and *Δmnn9ΔpepA* (TYWL60.5) grew very slowly compared to controls (Figure 4C).

These mutants were used as receptor hosts for the transformation of *pslcc(p)::pslcc* expression vectors. Transformants with different hosts, including the control (TYWL33.9), *Δmnn9* (TYWL64.9), *ΔdppV* (TYWL70.2), *ΔpepA* (TYWL62.4), *ΔdppVΔpepA* (TYWL68.5), *Δmnn9ΔpepA* (TYWL66.16) and *ΔdppVΔmnn9* (TYWL71.2), were verified by diagnostic PCR (Figure S2C in Supporting Information). Then, the transformants were cultivated in LMM media to assess laccase production. The result indicated that laccase production in the *ΔdppVΔpepA* host was the highest followed by the *Δmnn9ΔpepA* host. The highest laccase activity was 8- to 13-fold higher than the control. The laccase activity in the *Δmnn9* host, *ΔdppV* host, *ΔpepA* host and *ΔdppVΔmnn9* host was lower than the control (Figure 3E). The qPCR analysis showed that the expression level of the *pslcc* gene at the transcriptional level was highest in the *Δmnn9* host, which was followed by the *ΔdppVΔpepA* host (Figure 3F).

DISCUSSION

Laccases are eco-friendly enzymes that have generated enormous biotechnological interest as versatile biocatalysts. Laccases are distributed mostly in white-rot fungi. However, the application of laccases is limited due to the slow growth of laccase producing strains and their low laccase expression levels. To overcome these obstacles, several fungi have been used for the heterologous production of laccase. For example, the laccase gene (*Lcs-1*) from *Ceriporiopsis subvermispora* was expressed in *A. nidulans* and *A. niger*. Laccase production in *A. nidulans* had a similar molecular mass to the native system (Larrondo et al., 2003). In this study, we performed the heterologous expression of the laccase gene *pslcc* using *A. nidulans* LO4389 as the host, which abolished production of the toxin sterigmatocystin (Ahuja et al., 2012). Because of the importance of the promoter in the heterologous expression host, we choose *gpdA* as the promoter, which has been used for the expression of heterologous extracellular proteins (Mayfield et al., 1994). Production of the laccase gene from *Pycnoporus cinnabarinus* was improved by an 80-fold increase in *A. niger* with the *gpdA* promoter (Record et al., 2002). Some promoter sequences from basidiomycetes, such as the *Coprinopsis ci-*

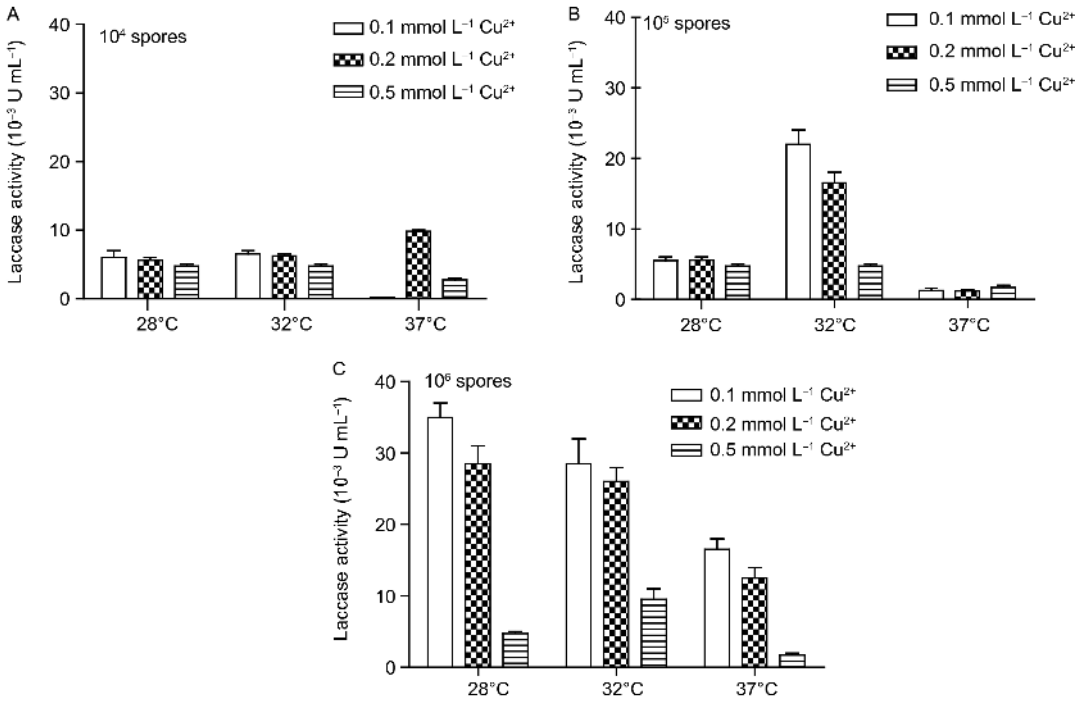

Figure 2 Fermentative optimization for laccase production in the heterologous host of *A. nidulans*. A, Laccase activity in the mutant of 10^4 spores/100 mL at 28°C, 32°C and 37°C with 0.1, 0.2, 0.5 mmol L^{-1} $CuSO_4$ and control, respectively. B, Laccase activity in the mutant of 10^5 spores/100 mL at 28°C, 32°C and 37°C with 0.1, 0.2, 0.5 mmol L^{-1} $CuSO_4$ and control, respectively. C, Laccase activity in the mutant of 10^6 spores/100 mL at 28°C, 32°C and 37°C with 0.1, 0.2, 0.5 mmol L^{-1} $CuSO_4$ and control. Values are means of three biological replicates for each culture. Error bars represent the standard deviations. Data were analyzed using the GraphPad Instate software package, version 5.01 (GraphPad Software) according to the Tukey-Kramer multiple comparison test at $P \leq 0.05$.

nerea tub1 gene, *Agaricus bisporus gpdII* gene, *Lentinus edodes priA* gene and *Schizophyllum commune SC3* gene, were reported to achieve laccase heterologous expression. Laccase production with these promoters was clearly different (Kilaru et al., 2006). This indicated that the promoter used for protein expression is dependent on the conditions. This is consistent with our finding that using the original promoter for laccase expression is effective compared to using *gpdA* promoter. It may be that several putative MREs in the promoter regions of the *pslcc* gene could improve the expression of the laccase gene with the addition of the relevant elements, such as Cu^{2+} (Pezzella et al., 2013). Furthermore, the *AMA1* sequence was incorporated into the plasmid, and the use of this sequence was previously shown to upregulate production of protein in *A. nidulans* (Aleksenko and Clutterbuck, 1997). However, the *AMA1* sequence does not work well for laccase production under our conditions.

RsmA is a putative YAP-like bZIP protein identified in a multicopy suppressor screen for the restoration of sterigmatocystin (Staaden et al., 2010), which greatly increases SM production by binding to two sites in the *A. nidulans aflR* promoter region (Yin et al., 2012). *OE::rsmA* resulted in a 100-fold increase in sterigmatocystin (Yin et al., 2013b). Here, we used the *aflR* promoter for laccase expression under control of RsmA in *A. nidulans*. When the laccase gene *pslcc* was integrated into the *OE::rsmA* host, laccase production was increased by 15-fold compared to the control (Figure 3C). This indicated that the RsmA regulatory mechanism could be used for the improvement of protein expression. The disruption of proteases can improve the yields of heterologous proteins in filamentous fungi. DppV, Mnn9 and PepA were reported to improve heterologous protein production (Beauvais et al., 1997; Mander et al., 2006; van den Hombergh et al., 1997; Wang et al., 2008). Therefore, single or double deletions of *dppV*, *pepA* and *mnn9* genes were performed in *A. nidulans*. The results showed that laccase activities were increased approximately 8- and 13-fold in the double deletion hosts of *ΔmnnΔpepA* and *ΔdppVΔpepA* than in the control strains, respectively. These results showed that disruption of proteolytic enzymes and mannosyltransferase contributes to the secretion of heterologous proteins in *A. nidulans*. Therefore, multiple factors should be considered for laccase production for industries in the future.

MATERIALS AND METHODS

Strains and cultural conditions

The fungal strains used in this study were indicated in Table 2. *Pycnoporus sanguineus* mk528 for cloning laccase gene *pslcc* was cultivated on potato dextrose agar (PDA) medium

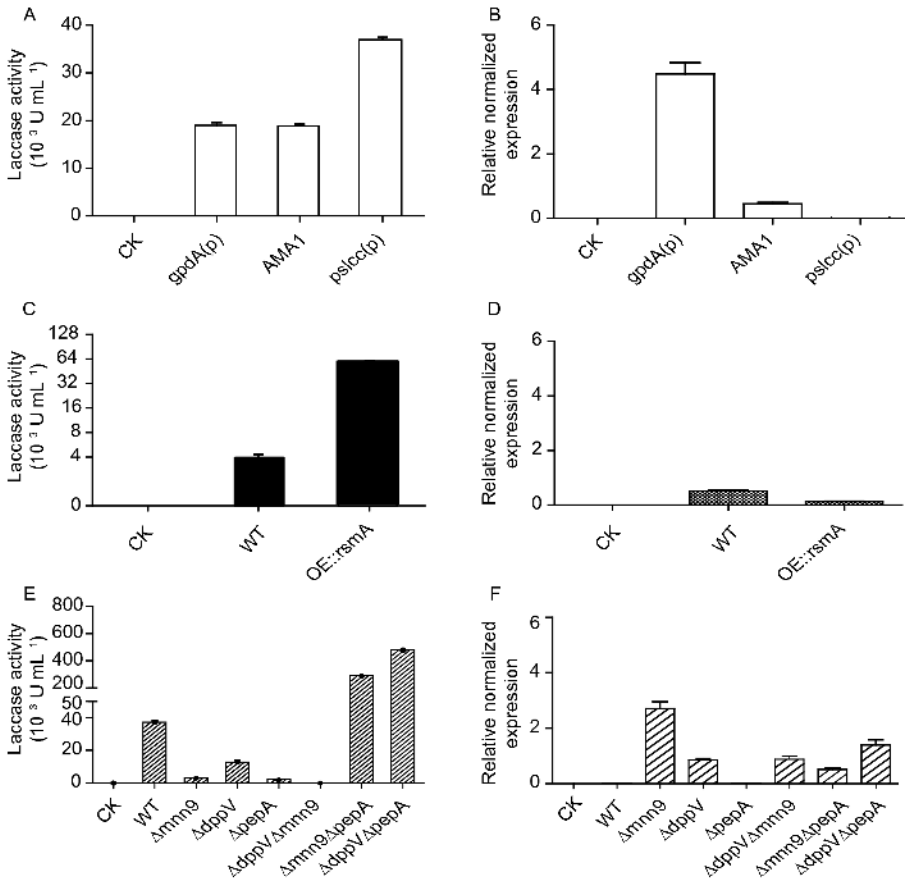

Figure 3 Laccase activity and the qRT-PCR analysis in mutants. A and B, Laccase activity and relative normalized expression of laccase gene in the *A. nidulans* LO4389 with different promoter. CK (LO4389), *gpdA(p)::pslcc* in LO4389 (TYWL61.1), *pslcc-AMA1* in LO4389 (TYWL16.2), *pslcc(p)::pslcc* in LO4389 (TYWL33.9). C and D, Laccase activity and relative normalized expression of laccase gene in the *A. nidulans* LO4389 and *OE::rsmA* host with transcription factor model. CK (LO4389), WT (TYWL69.6, *aflR(p)::pslcc* in LO4389), *OE::rsmA* (TYWL23.19, *aflR(p)::pslcc* in TYWL13.2). E and F, Laccase activity and relative normalized expression of laccase gene in the mutants with protease deletions. CK (LO4389), WT of LO4389 host (TYWL33.9), *Δmnn9* (TYWL64.9, *pslcc(p)::pslcc* in TYWL58.2 host), *ΔdppV* (TYWL70.2, *pslcc(p)::pslcc* in TYWL57.8 host), *ΔpepA* (TYWL62.4, *pslcc(p)::pslcc* in TYWL59.2 host), *ΔdppVΔmnn9* (TYWL71.2, *pslcc(p)::pslcc* in TYWL65.1 host), *ΔmnnΔpepA* (TYWL66.16, *pslcc(p)::pslcc* in TYWL60.5 host), *ΔdppVΔpepA* (TYWL68.5, *pslcc(p)::pslcc* in TYWL63.4 host). A total of three biological replicates were performed. Bars represent standard error of mean.

at 28°C (Zhang et al., 2015). *Aspergillus nidulans* LO4389 was used as the host strain in mutation experiments (Ahuja et al., 2012). *A. nidulans* TWY5.2, RDIT9.32 and RJMP5.32 were used to clone the *rsmA* cassette, the *trapC* terminator and *aflR* promoter, respectively (Yin et al., 2012). All of the *A. nidulans* strains grew on the glucose minimum medium (GMM) with or without appropriate supplementary (0.56 g uracil L^{-1}, 1.26 g uridine L^{-1}, and 0.5 μmol L^{-1} pyridoxine HCl) for sporulation at 37°C for 72 h. *A. nidulans* mutants were cultivated to product laccase in liquid GMM (supplemented with 5 g yeast extracts L^{-1} and $CuSO_4$ mother solution, LMM) for 5 days. *Escherichia coli* DH5α was used routinely for plasmids amplification in LB medium.

Cloning of gene deletion and overexpression cassettes

The plasmids utilized in this work are listed in Table 1. The oligonucleotide sequences for PCR primers are given in Table S2 in Supporting Information. PCR amplification was performed on a T100 Thermal Cycler from Bio-Rad made in Singapore. All PCR steps were performed using an Expand template PCR system according to the corresponding enzyme manufacturer's instructions. PCR reactions were performed with Phusion high-fidelity DNA polymerase (NEW ENGLAND Biolabs, USA). PCR screening for transformants was performed with 2× TSINGKE® Master Mix (TSINGKE, Beijing). The amplified fragment for fungal transformation was purified using the Zymoclean Gel DNA Recovery Kit (D4007, ZYMO RESEARCH, USA). The *rsmA* overexpression cassette (5,670 bp) was amplified with the template from the genomic DNA of *A. nidulans* TWY5.2 strain (Yin et al., 2012), in which the *rsmA* gene promoter sequence (1,079 bp) and partial *rsmA* gene sequence (1,097 bp) are the upstream homologous arm and downstream homologous arm, respectively. The auxotrophic *pyrG* marker cassette and *gpdA* promoter were located in the two homologous arms (Figure 4A).

For creation of the protease deletion strains at the native

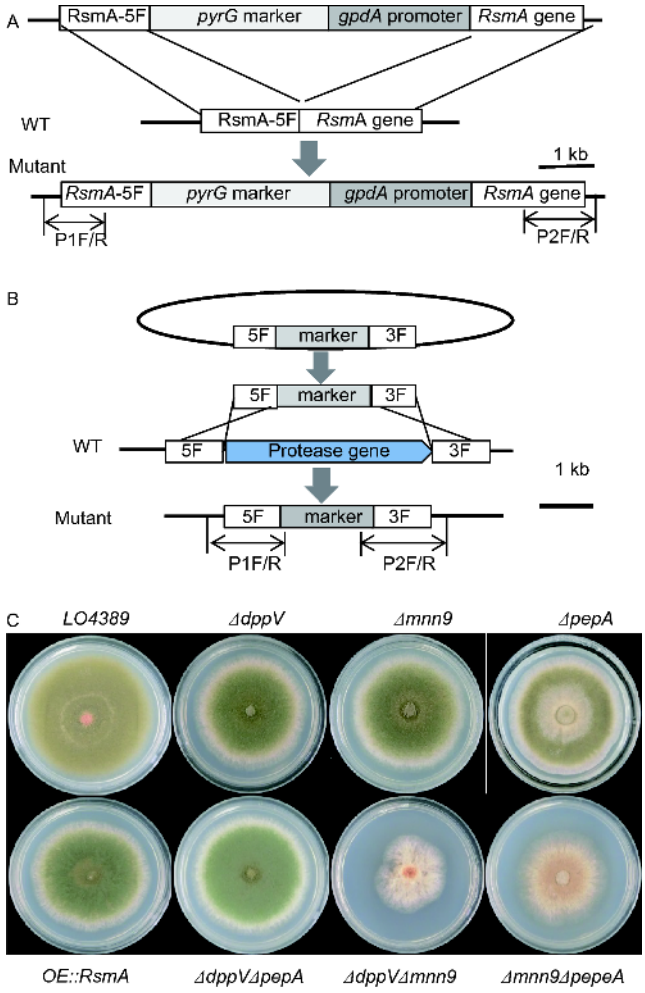

Figure 4 (Color online) Generation of mutants in *A. nidulans*. A, Schematic illustration of overexpression of *rsmA*. B, Schematic illustration for the disruption of protease. C, The colonial morphology of *OE::RsmA* mutants and protease disruption mutants. CK (LO4389), *OE::rsmA* (TYWL13.2), *ΔdppV* (TYWL57.8), *Δmnn9* (TYWL58.2), *ΔpepA* (TYWL59.2), *ΔdppVΔmnn9* (TYWL65.1), *ΔmnnΔpepA* (TYWL60.5), *ΔdppVΔpepA9* (TYWL63.4).

locus, the deletion cassettes were constructed using double-joint PCR procedures (Yu et al., 2004). The *dppV* gene, *mnn9* gene and *pepA* gene in *A. nidulans* were acquired by querying with the separate genes of *A. niger* in the NCBI database. The marker gene *A. fumigatus pyroA* cassette (2,797 bp) was amplified with the plasmid pWY25.16 (Yin et al., 2012). The 1.25 kb fragment upstream and 1.28 kb fragment upstream of *dppV* were amplified from the genomic DNA of *A. nidulans* using the designated primers. The three amplified PCR products were purified with Zymoclean Gel DNA Recovery Kit, quantified, and fused using double joint PCR procedures. The final PCR product was linked to the tool-vector p-Blunt (TRANSGEN BIOTECH, Beijing) to obtain the plasmid pYWL91.4. Using the same strategy as for the creation of the other two gene deletion cassettes, we amplified the marker gene *A. fumigatus ribo* cassette (2,705 bp) with the plasmid pYWB2 (Zhang et al., 2017) and the marker gene *A. fumigatus pyrG* cassette (1,668 bp) with the plasmid pYH-WA-pyrG-KI (Yin et al., 2012). Then, the 1.3 kb fragment upstream and 1.29 kb fragment downstream of *mnn9* and the 1.22 kb fragment upstream and 1.22 kb fragment downstream of *pepA* were amplified from the genomic DNA of *A. nidulans* using the designated primers. We constructed the plasmids of pYWL90.2, pYWL90.2, pYWL92.6 and pYWL96.1 for cloning the deletion cassettes (Table 1). The deletion cassettes of the three protease genes were amplified from separate plasmids using the designated primers.

Construction of *pslcc* gene expression plasmids

To construct the overexpression plasmid, the tryptophan synthase (*TrpC*) terminator sequence was amplified from the genomic DNA of *A. nidulans* RDIT9.32 using the primers TrpC-for and -rev and integrated into plasmid pJMP9.1 to create pYWL14.1 with the *gpdA* promoter and *trpC* terminator using the quick-change strategy (Bok and Keller, 2012). The *pslcc* gene was amplified from the genomic DNA of *P. sanguineus* mk528 using the primers Ps528 lcc-for and -rev and integrated into plasmid pJMP14.1 to create pYWL15 using the abovementioned protocol. The laccase expression cassette, including its own promoter, *pslcc* gene and its own terminator, was amplified from the genomic DNA of *P. sanguineus* mk528 using the primers Ps528PLT-F-*Not* I/-R-*Avr* II or Ps528PLT-for/-rev. The former *pslcc* expression cassette was digested with the restriction enzymes *Not* I and *Avr* II, then the *Not* I-*Avr* II fragment of the cassette was cloned into the *Not* I and *Avr* II sites of pRG-AMA1-*Not* I to create pYWL16. The latter was then integrated into plasmid pYWL14.1 to create pYWL17.1 using the quick-change strategy. To construct the overexpression plasmid with different marker gene cassettes, the plasmid pWL93.4 was constructed by integrating the *trpC* terminator into pWY25.16. Then, plasmids pWL94.8 and pWL95.6 were constructed by replacing the marker gene cassette *AfriboB* (*A. fumigatus* riboflavin biosynthesis gene) and *pyroA* based on pWL93.4. Finally, the overexpression plasmids pWL97.2, pWL99.2 and pWL100 of laccase with the *gpdA* promoter and different marker genes were constructed based on pYWL95.6, pYWL94.8 and pYWL93.4, respectively. To construct the overexpression plasmid with the *aflR* promoter, the *aflR* promoter sequence was amplified from the genomic DNA of *A. nidulans* RJMP1.59 using the primers *aflR*-promoter F and R and integrated into plasmid pYWL17.1 to create pYWL53.1 with the laccase overexpression cassette, including the *aflR* promoter, *pslcc* gene and *trpC* terminator using the quick-change strategy. We amplified the aflR promoter and *pslcc* gene cassette and integrated it into plasmid pYWL94.8 to create pYWL55.3.

Table 2 Strains used in this study[a)]

Strains	Characteristics	References
P. sanguineus mk528	*Pycnoporus sanguineus* WT	(Zhang et al., 2015)
TWY5.2	*pyrG89*, *A. fumigatus pyrG::gpdA(p)::rsmA*, *pyroA4*, *ΔnkuA::argB*, *veA*$^{+}$	(Yin et al., 2013b)
A. nidulans RJMP1.59	*pyroA4*$^{-}$, *pyrG89*$^{-}$, *KU*$^{+}$, *veA*$^{+}$	(Soukup et al., 2017)
A. nidulans RDIT9.32	*veA*$^{+}$	(Yin et al., 2012)
A. nidulans LO4389	*ΔAN7804-7825(ST cluster)*, *pyrG89*, *pyroA4*, *riboB2*, *nkuA::argB*, *veA1*	(Ahuja et al., 2012)
TYWL13.2	*pyrG::gpdA(p)::rsmA* in LO4389	This study
TYWL16.2	*pyrG::pslcc(p)::pslcc*, *AMA1* in LO4389	This study
TYWL23.19	*AfpyroA::aflR(p)::pslcc*, *pyrG::gpdA(p)::rsmA* in LO4389	This study
TYWL33.9	*pyrG::pslcc(p)::pslcc* in LO4389	This study
TYWL57.8	*ΔdppV::AfpyroA* in LO4389	This study
TYWL58.2	*Δmnn9::AfpyroA* in LO4389	This study
TYWL59.2	*ΔpepA::AfriboB2* in LO4389	This study
TYWL60.5	*Δmnn9::AfpyroA*, *ΔpepA::AfriboB2* in LO4389	This study
TYWL61.1	*pyrG::gpdA(p)::pslcc* in LO4389	This study
TYWL62.4	*pyrG::pslcc(p)::pslcc*, *ΔpepA::AfriboB2* in LO4389	This study
TYWL63.4	*ΔdppV::pyrG, ΔpepA::AfriboB2* in LO4389	This study
TYWL64.9	*pyrG::pslcc(p)::pslcc*, *Δmnn9::AfpyroA* in LO4389	This study
TYWL65.1	*ΔdppV::pyrG*, *Δmnn9::AfpyroA* in LO4389	This study
TYWL66.16	*pyrG::pslcc(p)::pslcc*, *Δmnn9::AfpyroA*, *ΔpepA::AfriboB2* in LO4389	This study
TYWL68.5	*AfpyroA::pslcc(p)::pslcc*, *ΔdppV::AfpyrG*, *ΔpepA::AfriboB2* in LO4389	This study
TYWL69.6	*pyrG::aflR(p)::pslcc* in LO4389	This study
TYWL70.2	*pyrG::pslcc(p)::pslcc*, *ΔdppV::AfpyroA* in LO4389	This study
TYWL71.2	*AfriboB2::pslcc(p)::pslcc*, *ΔdppV::pyrG*, *Δmnn9::AfpyroA* in LO4389	This study

a) TXX, original transformant.

This construct was confirmed by PCR with primers and then sequenced. Plasmids were isolated by using the Plasmid Mini Kit I (OMEGA, USA). The oligonucleotide sequences for the PCR primers are given in Table S2 in Supporting Information.

Transformation of *A. nidulans*

A. nidulans LO4389 strain was used as the recipient host. The protocols of fungal protoplast preparation and transformation were described previously by Yin and co-worker (Yin et al., 2013a). In *OE::rsmA* experiment manipulation, the *OE::rsmA* fragment was transformed into *A. nidulans* LO4389. The mutants were verified by diagnostic PCR with appropriate primers (Table S2 in Supporting Information), and we acquired the single protease deletion mutant TYWL 13. In the deletion experiment manipulation in *A. nidulans*, the fragments of deletion cassette were amplified from the appropriate plasmids and transformed into *A. nidulans* LO4389 strain, respectively. The disruption mutants were verified by diagnostic PCR with appropriate primers (Table S2 in Supporting Information), and we acquired the single gene deletion mutants of TYWL 57.8, TYWL 58.2 and TYWL 59.2, respectively. Secondly, the fragments of deletion cassette were amplified from the appropriate plasmids and transformed into TYWL 57.8 and TYWL 58.2 strains, and the mutants were verified by diagnostic PCR with appropriate primers. We got the double genes deletion mutants of TYWL 60.5, TYWL63.4 and TYWL 65.1 (Table 2).

The aforesaid *A. nidulans* mutants were used as *pslcc* gene heterologous expression recipient hosts. Plasmid pYWL55 containing *pslcc* was transformed into TYWL13 and LO4389 hosts to create the *pslcc* overexpression with *aflR* promoter strain TYWL23.19 and the control strain TYWL69 (Table 2), respectively. Plasmids of pYWL16, pYWL17 and pYWL97 were transformed into LO4389 strain to create the *pslcc* gene overexpression strains TYWL16, TYWL33 and TYWL61, respectively. For *pslcc* gene overexpression in the protease deletion hosts, plasmid pYWL17 was transformed into TYWL57, TYWL58, TYWL59 and TYWL60 strains. Plasmids of pYWL99 and pYWL100 were transformed into TYWL63 and TYWL65 to create the strains TYWL68 and TYWL71, respectively (Table 2). All of the transformants were verified by diagnostic PCR with the appropriate pri-

mers (Table S2 in Supporting Information).

Fermentation of mutants for *pslcc* gene overexpression

A. nidulans mutants were cultivated on a Petri dish containing GMM medium (supplemented with the corresponding nutritional deficiencies) and grew for 6 days at <37°C. For laccase production, the fermentation was performed in liquid GMM (supplemented with 5 g yeast extract L^{-1} and the corresponding nutritional deficiencies, LMM) for 5 days in the dark at 28°C, 32°C and 37°C with 10^4, 10^5 or 10^6 spores in 250 mL baffled Erlenmeyer flasks containing 100 mL medium and shaken at 220 r min^{-1} in a rotary shaker. Different concentrations of $CuSO_4$ (0.1, 0.2 and 0.5 mmol L^{-1}) was designed to the basal medium as the inducer after incubating for 2 days.

Laccase activity analysis

Laccase activity was determined by monitoring the oxidation of ABTS (0.5 mmol L^{-1}, 2,2′-azino-bis-(3-ethylthiazoline-6-sulphonate), Sigma-Aldrich, USA) in sodium-tartrate buffer (50 mmol L^{-1}, pH 4.0) at 420 nm ($\sum_{max}$=3.6×10^4 mol L^{-1} cm^{-1}) and 30°C. All enzymes assays were carried out using a UV array spectrophotometer (Unico, USA). The enzyme activity was expressed in international units (U). One unit of activity leads to the transformation of 1 μmol L^{-1} substrate in one minute (Zhang et al., 2015).

RNA preparation and qRT-PCR of mutants

For analysis of *pslcc* expression in *A. nidulans* mutants, 6×10^6 spores from all the mutants were inoculated into 100 mL LMM and cultivated at 28°C for 5 days with 0.1 mmol L^{-1} Cu^{2+} addition after 2 days. Then, mycelia were harvested and total RNA was extracted using TRIzol® Reagent (Life Technologies, USA). The quality of RNAs was checked in a nucleotide analyzer Quawell Q3000 (Quawell, USA). Single strand cDNAs were synthesized using the Fast Quant RT Kit (Tiangen Biotech, Beijing) according to the manufacturer's protocol. Quantitative real-time PCR (qRT-PCR) was conducted using a CFX96 Real-Time System (Bio Rad, USA). KAPA SYBR FAST qPCR Kit was used for the reactions (2× KAPA SYBR FAST qRCP Master Mix: 10 μL, 10 μmol L^{-1} Forward/Reverse Primer: 0.4 μL, template cDNA: 1 μL, with addition water to 20 μL). Reaction conditions were conducted at 95°C for 3 min followed by 40 cycles of (95°C for 3 s, 60°C for 20 s, 72°C for 20 s) and then were followed by one cycle of 65°C for 5 s and 95°C for holding to calculate the disassociation curves. For transcription assessment, qRT primers were listed and the coding regions of *pslcc* were amplified using designated primer pairs, and expression of ANactin (AN6542) was used to be as the internal control (Table S2 in Supporting Information). Each cDNA sample was performed in triplicate and the average threshold cycle was calculated. Relative expression levels were calculated using the $2^{-\Delta\Delta C_t}$ method.

Statistical analysis

For statistical analyses, values were means of three biological replicates for each culture. Error bars represented the standard deviations. Data were analyzed using the GraphPad Instate software package, version 5.01 (GraphPad software) according to the Tukey-Kramer multiple comparison test at $P \leq 0.05$.

Compliance and ethics *The author(s) declare that they have no conflict of interest.*

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SUPPORTING INFORMATION

Figure S1 Promoter analysis of laccase gene *pslcc* from *P. sanguineus* mk528.

Figure S2 Verifications of laccase gene heterologous expression mutants in *A. nidulans*.

Figure S3 Verification of *OE::rsmA* mutant.

Figure S4 Verifications of protease and mannosyltransferase deletion mutants.

Table S1 Blast analysis of proteases and mannosyltransferases used in this study

Table S2 PCR primer sets used in this study

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