Two pal genes of Pleurotus ostreatus participate in primordium formation and heat stress response

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Abstract

Background

Phenylalanine ammonia-lyase (PAL, EC 4.3.1.24) is the first key enzyme in the phenylpropanoid pathway. The pal gene is widely studied in plants and participates in plant growth, development and defense systems. However, the biological function of pal in P. ostreatus development and abiotic stress has not been reported.

Results

In this study, we cloned and characterized the pal1 (2232 bp) and pal2 (2244 bp) from basidiomycete Pleurotus ostreatus CCMSSC 00389. The pal1 and pal2 genes are interrupted by 6 and 10 introns and encode proteins of 743 and 747 amino acids, respectively. Furthermore, prokaryotic expression experiments showed that PAL enzymes catalyzed the conversion of L-phenylalanine to trans-cinnamic acid. The function of pal1 and pal2 was determined by constructing overexpression (OE) and RNA interference (RNAi) strains. The results showed that the two pal genes had similar expression patterns during different developmental stages. The expression of pal genes was higher in the reproductive growth stage than in the vegetative growth stage. And the overexpression of pal1 and pal2 resulted in the formation of primordia earlier. The results of heat stress assays showed that the RNAi-pal1 strains had enhanced mycelial tolerance to high temperature, while the RNAi-pal2 strains had enhanced mycelial resistance to H2O2.

Conclusions

These results indicate that two pal genes may play a similar role in the development of P. ostreatus fruiting bodies, but may alleviate stress through different regulatory pathways under heat stress.

Background
*Pleurotus ostreatus* is one of the most widely cultivated mushroom species globally [1], being an edible mushroom with high nutritional and medicinal value. The mechanism of fruiting body development of edible fungi has been a popular topic in recent years, with numerous studies having been conducted on fruiting body development and the associated signaling pathways. A number of functional genes, such as nicotinamide adenine dinucleotide phosphate oxidase [2], cytochrome P450 gene [3], superoxide dismutase [4], multicopper oxidases [5] and catalase [6], have been identified and characterized with respect to mushroom development. In addition, the AMP signaling pathway has been reported in mushrooms [7]. The development of mushrooms is a complex process, that is regulated - by gene products and by environmental factors. In China, because *P. ostreatus* is mostly primarily cultivated in horticultural facilities, its cultivation is greatly affected by seasonal temperature changes, especially the high temperature that occurs in summer. A number of studies have shown that high temperatures can affect mycelial growth and fruiting body development [8], and can even lead to spawn burning [9] and *Trichoderma* contamination [10]. In addition, previous studies on the heat stress response of *P. ostreatus* have investigated programmed cell death [11], the role of catalase in fruiting body development and heat stress [6], and the effect of trehalose on mycelial damage mitigation [12]. Recently, Zou et al. studied proteome changes in *P. ostreatus* mycelia during heat stress and recovery and identified 204 proteins exhibiting altered expression during heat stress or the recovery phase, among which the expression of *pal* was found to be altered under stress and in the recovery phase [13].

PAL is the first enzyme in the phenylpropanoid pathway and catalyzes the conversion of L-phenylalanine to trans-cinnamic acid by nonoxidative deamination [14-16]. Furthermore, PAL is the first key enzyme in the phenylpropanol pathway, affecting the formation of a
series of structural and defensive phenolic compounds, such as lignin, phenolic acid and hydroxybenzoic acid, flavonoids and stilbene [17]. The pal gene is widely studied in plants and participates in plant growth, development and defense systems [18, 19], including lignin synthesis in cell walls, nutrient transport, and the regulation of seed color [20]. In addition, plants can induce PAL enzymes under abiotic stresses (ultraviolet-B (UV-B) light, high and low temperature, injury, salt, etc.), leading to the accumulation of phenolic compounds such as flavonoids and phenolic acids [21]. Under salt stress, the antioxidant capacity of plants has been shown to be enhanced by increasing PAL activity [22]. Under UV-B stress, the roots and leaves of soybeans increased their salicylic acid (SA) content by increasing PAL activity and subsequently became stress resistant [23]. The pal gene has also been studied in mushrooms in recent years, such as in Flammulina velutipes, where the pal gene was cloned and characterized. The different expression patterns of the F. velutipes pal gene and its activity in different organs of the mushroom indicated that pal is associated with mushroom growth [24]. In Tricholoma matsutake, transcriptome analysis revealed a pattern of pal gene expression that was dependent on the developmental stage, suggesting that pal has many physiological functions in this mushroom [25]. In several basidiomycete fungi, a metabolic pathway for the metabolism of phenylalanine via cinnamic, benzoic, p-hydroxybenzoic, and protocatechuic acids has been reported that is similar to that observed in plants [26]. However, the biological function of pal in P. ostreatus development and abiotic stress has not been reported.

At present, many studies have used molecular and genetic methods to silence the pal gene and study its biological function in plant growth, development and environmental stress [20, 27]. In recent years, RNAi and OE technologies have been widely used to study of gene function in P. ostreatus. For example, the overexpression of a methionine sulfoxide reductase A gene enhances stress tolerance in P. ostreatus [28], which provides
a more effective method for studying the function of genes in *P. ostreatus*. In this study, we searched and cloned the *pal* genes from the *P. ostreatus* genome. On the basis of describing their characteristics, we studied the role of *pal* genes in fruiting body development and heat stress using RNAi and OE technologies.

**Results**

**Cloning and bioinformatics analysis of *pal***

Two *pal* genes were identified in the *P. ostreatus* genome and were named *pal1* and *pal2*, the full-length cDNA sequences which are 2232 and 2244 bp, respectively. DNA sequence analysis showed that 7 exons are interrupted by 6 introns in *pal1*, while 11 exons are interrupted by 10 introns in *pal2* (Fig. 2B). The two sequences were deposited in GenBank with the accession numbers MK207023 and MK207024, respectively.

To understand the phylogenetic relationship between the PAL proteins and other fungal PALs, phylogenetic analysis was performed. Phylogenetic analysis of 19 PAL sequences showed that PAL can be divided into two distinct branches (Fig. 2A). The phylogenetic tree showed that PAL1 and PAL2 have higher similarities to the protein sequences of other mushrooms or fungi than to each other. The cladogram revealed the variation in the PAL protein sequence among fungi.

The *pal1* and *pal2* sequences were bioinformatically analyzed to determine their physicochemical properties and possible structure. The *pal1* gene encodes a putative 743-amino-acid polypeptide with an approximate molecular weight and calculated pI of 79.845 kDa and 5.28, respectively. The *pal2* gene encodes a putative 747-amino-acid polypeptide of 79.946 kDa with a predicted isoelectric point of 6.11 [29]. An online analysis revealed a Pfam lyase aromatic domain in both *pal1* and *pal2*, whereas only *pal2* was observed to have a SCOP d1qj5a_domain. The gene models for *pal1* and *pal2* from different organisms are
shown in Fig. 3A, which primarily describes the amino acid identities and similarities among \textit{pal} genes in different organisms. The \textit{pal} motif is labeled with a red box, and the conserved active-site motif (Ala-Ser-Gly) and specific amino acids are also shown in Fig. 3A. The conserved active-site motif is labeled with circles under the specific amino acids, which can be converted into an MIO (4-methylidene-imidazole-5-one) prosthetic group (Fig. 2C). The other active-site residues are labeled with red circles in Fig. 3A. The 3-D structure of PAL (Fig. 2C) showed that it is composed of an MIO domain, a core domain and an inserted shielding domain [30]. Thus, the PAL amino sequences are highly conserved with other characterized PAL proteins in fungi.

To investigate the activity of the PAL proteins \textit{in vitro}, the transformant strains (\textit{E. coli}) were induced to express the PAL protein by the addition of IPTG. Subsequently, the proteins were purified by nickel column affinity chromatography, and analyzed by SDS-PAGE. The results showed that the purified proteins (PAL1 and PAL2) had molecular weights of approximately 75 kDa (Fig. 3B), which is consistent with predictions. The activity of the purified enzymes was determined by spectrophotometry (Fig. 3C). The results showed that the activity of PAL1 (24.469 ± 2.296 u/mgprotein) was significantly lower than that of PAL2 (43.387 ± 2.551 u/mgprotein).

\textbf{Expression of \textit{pal1} and \textit{pal2} during different P. ostreatus developmental stages}

To investigate the expression patterns of \textit{pal1} and \textit{pal2} during \textit{P. ostreatus} development, the expression of these genes during different developmental stages and different parts of the fruiting bodies of the WT strain were assessed (Fig. 4). The results showed that compared with that in mycelia, the expression of \textit{pal1} was significantly upregulated in primordia (3.5-fold), fruiting bodies (19.3-fold) and spores (11.8-fold) (Fig. 4A). In
addition, the expression of *pal2* was upregulated significantly and continuously during *P. ostreatus* development stages and was higher than that of mycelia in primordia (7-fold), fruiting bodies (15.2-fold), and spores (68-fold) (Fig. 4B). Fig. 4C and D shows *pal1* and *pal2* gene expression in different parts of the *P. ostreatus* fruiting body. The results showed that the expression of *pal1* and *pal2* in different parts of fruiting bodies had the same trend, with the highest expression observed in the gills.

**Expression of *pal1* and *pal2* and damage to mycelia under heat stress**

As shown in Fig. 5A, the mycelial growth was slightly affected at 32 °C, severely inhibited at 36 °C and completely abrogated at 40 °C. The expression of *pal1* increased significantly with increasing temperature, while *pal2* expression was first downregulated and then upregulated through a small series of changes. According to the results, 40 °C was selected as the stress temperature for further study. The H$_2$O$_2$ and MDA content, which are two indicators of oxidative damage, increased with the duration of processing, especially when the stress time exceeded 24 h. The results showed that oxidative damage occurred in mycelia under heat stress (Fig. 5D, E). Fig. 5F and G show the changes in the mycelial total respiration rate and relative ion leakage under different temperature stresses. The results showed that with increasing heat stress time, the total respiration rate of mycelia increased temporarily and then decreased rapidly. At the same time, the relative ion leakage increased significantly with increasing stress time, indicating that the degree of mycelial damage increased.

**Generation of *pal* OE and RNAi strains**

Gene transformation with a gene knockout vector is a useful approach to explore the function of genes in fungi [24]. To study the roles of *pal1* and *pal2* in *P. ostreatus*, two RNAi-*pal* silencing vectors and two OE-*pal* OE vectors were constructed containing the hyg
gene as a selectable marker (Fig. 6). The efficiency of RNAi and OE of the transformants was further confirmed by qPCR analysis. The transcription of pal1 in the OE strains (OE-pal1 7.11-11 and OE-pal1 7.11-9) was approximately 4-fold higher than that of the WT strain, whereas pal1 transcription in the RNAi strains (RNAi-pal1 8.1-26 and RNAi-pal1 8.1-38) decreased by more than 50%. Therefore, these strains were selected for further study (Fig. 6 A). The transcription of pal2 in the OE strains (OE-pal2 7.11-7 and OE-pal2 7.12-11) and RNAi strains (RNAi-pal2 7.18-1 and RNAi-pal2 7.18-19) were significantly different that observed of the WT strain. The pal2 gene expression of the overexpression strains was approximately 3-fold higher than that in the WT strain, while the expression in the RNAi strains decreased to 20% (Fig. 6 B). The PAL enzyme activity in the tested strains was also assessed. The results showed that the PAL activity in the OE-pal1 7.11-11 strain was 1.7-fold greater than that of the WT strain, and the PAL activity in the OE-pal2 7.11-7 was 1.8-fold greater than that of the WT strain (Fig. 6 C, D). In contrast, the PAL enzyme activity slightly decreased in the RNAi strains (Fig. 6 C, D).

**Pal1 and pal2 are involved in primordial formation**

The effects of pal OE and RNAi on mycelial growth are shown in Fig. 7A. The results showed that OE and RNAi of pal1 had no visible phenotypic effects. However, compared with the colony diameter of the WT strain, that of the OE-pal2 strains was slightly larger, while the growth rate of the RNAi strains was significantly lower (Fig. B and C). In mushroom production experiments, we observed that OE-pal strains formed primordia earlier than WT strain, while the RNAi strains exhibited the opposite phenotype (Fig. 7A). Correspondingly, the period of mushroom cultivation was shortened by pal overexpression and prolonged by RNAi. To further explore the biological role of pal in the development of fruiting bodies, the expression of pal1 and pal2 was assessed in the WT, OE and RNAi strains at different developmental stages by qPCR. Fig. 7 D and E shows that the pal gene
expression patterns in the OE-pal and RNAi-pal strains at different developmental stages were similar to those of WT strain. Except for the spores of the RNAi-pal1 strains, the pal gene expression in the other strains during the reproductive growth stage was higher than that observed during the vegetative reproductive stage. In summary, pal1 and pal2 are involved in the formation of P. ostreatus primordia, and the overexpression and interference of the two pal genes have no significant effect on their gene expression patterns.

*Pal1* and *pal2* participate in the regulation of the mycelial response to heat stress

Fig. 8A and C show the growth status and rate of the tested strains. The results showed that the growth rate of the WT strain was seriously affected when the mycelial growth temperature was 32 °C, while that of the pal1 interference strains was significantly increased compared with that of the WT strain. Similar to the WT strain, the growth rate of other tested strains was significantly inhibited, and germination actually stopped at 40 °C. Heat stress can lead to a significant increase in H₂O₂ in mycelia. To investigate whether *pal* genes are involved in the reactive oxygen species (ROS) response, the growth rates of the WT, OE and RNAi strains were evaluated on PDA plates with different concentrations of exogenously added H₂O₂. The resistance of the RNAi-pal2 7.18-1 and RNAi-pal2 7.18-19 strains to H₂O₂ was higher than that of the WT strain, especially when at an H₂O₂ concentration of 10 mM (Fig. 8B). In addition, the resistance of other strains to H₂O₂ did not change significantly under the tested conditions (Fig. 8B and D). In summary, the interference of the pal2 gene reduced the sensitivity of mycelia to H₂O₂.

Discussion

PAL plays an important role in the acquisition of secondary metabolites. The role of PAL in
plants has been extensively studied. However, the biological function of PAL in fungi, which has important research significance, has yet to be fully elucidated. The number and structure of *pal* genes varies greatly in different organisms, and there are several *pal* genes in fungi. In *Rhodosporidium toruloides*, *pal* is encoded by a single gene. In the genomes of *Aspergillus oryzae* RIB 40 and *Aspergillus nidulans* FGSC A4, four and two *pal* genes are encoded, respectively. In this study, two *pal* genes were identified within the *P. ostreatus* genome, which is generally consistent with that observed in other basidiomycetes. For example, two *pal* genes were identified in *Coprinopsis cinerea* and *Schizophyllum commune*. Our phylogenetic tree also supports this result. Po-PAL1 and Po-PAL2 clustered together with PALs from *Coprinopsis cinerea* and *Schizophyllum commune*, respectively. In addition, the *P. ostreatus* *pal1* and *pal2* genes contained 6 and 10 introns, respectively, differing greatly in genetic structure. Previous studies have shown that the number of introns in *Basidiomycota* *pal* genes ranges from 0-13 introns, and our results are consistent with these observations [31]. In phylogenetic trees, the PAL1 and PAL2 sequences were not phylogenetically closely related to each other, suggesting that PAL1 and PAL2 did not undergo a simple gene duplication.

PAL proteins participate in the growth and development of plants, playing different roles in different species. In *Arabidopsis*, Antje et al. reported that *pal1* and *pal2* mutants had no obvious morphological phenotype, but rather became sterile [20]. Junli et al. showed that three independent *pal1* and *pal2* double mutants generated yellow seeds due to the lack of condensed tannin pigments in the seed coat [32]. In fungi, many *pal* genes have been cloned, but little research has been performed to elucidate their biological function. In this study, the results showed that the gene expression patterns of *pal1* and *pal2* during different developmental stages were essentially the same, with *pal1* and *pal2* expression increasing during the transformation from the vegetative to the reproductive growth
stages. The gene expression of *pal1* and *pal2* was also consistent in different parts of the fruiting body. At the same time, the overexpression of *pal1* and *pal2* resulted in the formation of primordia earlier, suggesting that these genes may play a similar role in the development of fruiting bodies. In *Flammulina velutipes*, the expression of *pal* in the stipe increased significantly, suggesting that *pal* may be involved in stipe elongation [33]. We observed that *pal* gene expression in stipe was significantly lower than that observed in fruiting the body, possibly because the long stipe is not a beneficial trait during *P. ostreatus* development. The transcription *pal* in the cap was significantly higher than that in the stipe. Considering that phenolic compounds in plants are synthesized through activity of *pal* in the phenylpropanoid pathway, *pal* expression in the *P. ostreatus* cap may also be involved in the production of phenolic compounds, which may improve the antioxidant capacity of mushrooms. In this study, different levels of *pal1* and *pal2* transcription were observed in spores. Previous studies have shown that the transcription of *tmpal2* is the highest in the gill in *Tricholoma matsutake* [34]. Our results also indicate that *pal2* transcription in spores is significantly higher than that of *pal1*, which may indicate that *pal2* may play a major role in spore-related progress.

In this study, *pal1* gene expression increased significantly after heat stress, but the RNAi-*pal1* strains showed a significant growth advantage over the WT strain at 32 °C. The OE-*pal2* and RNAi-*pal2* strains showed no significant difference at 32 °C, but showed obvious resistance to exogenous H₂O₂. Previous biochemical studies using isotope feeding demonstrated that a number of plants can synthesize SA from cinnamate, synthesized by PAL from phenylalanine [35, 36]. Furthermore, some studies have shown that the accumulation of SA can promote the production of H₂O₂, which leads to the production of ROS and cell death [37, 38]. Thus, we speculated that the increase in *pal1* gene expression may lead to ROS generation by regulating SA production. In plants, oxidative
stress is produced as a secondary stress during the heat stress response, which results in the abundant production of ROS [39]. ROS poses a serious threat to cell function by damaging lipids and proteins [40]. ROS are a series of oxygen-containing compounds, such as superoxide anion (O$_2^-$), H$_2$O$_2$, and hydroxyl radicals (·OH) [41]. We speculate that pal2 interference may reduce the sensitivity of mycelia to H$_2$O$_2$, while pal1 interference may reduce the sensitivity of mycelia to other types of ROS. Thus, pal1 and pal2 may respond to stress by regulating different pathways. The results showed that pal may have a negative regulatory effect on the response of P. ostreatus to heat stress. Similar reports have been published in plants. In Arabidopsis thaliana, pal1 and pal2 RNAi strains were more sensitive to UV-B radiation but were more resistant to drought stress [42]. In Brachypodium, no significant difference in UV-B radiation and drought resistance was observed between RNAi-pal and WT plants [43].

Conclusions

In summary, in this study, two pal genes were cloned and the structural characteristics of the encoded proteins was studied. Through a qPCR analysis, we observed that the gene expression patterns of pal1 and pal2 were essentially the same during P. ostreatus different developmental stages. In addition, this study confirmed that pal overexpression could promote the formation of primordia. These results indicate that pal genes are involved in the development of P. ostreatus fruiting bodies. In addition, this study assessed the role of pal in heat stress, providing a basis for exploring the role of the phenylpropanoid pathway in the development and stress response of P. ostreatus.

Methods

Strains, plasmids and media

The dikaryotic P. ostreatus strain CCMSSC00389 from the Center for Mushroom Spawn
Standards and Control of China was used in this study as a parent strain for OE and RNAi experiments. The wild-type (WT), OE and RNAi strains were maintained on potato dextrose agar (PDA) at 4 °C. For the selection and maintenance of transformants, complete media (CM) was supplemented with 90 μg/mL hygromycin (hyg) (Invitrogen, U.S.A.).

Agrobacterium tumefaciens (A. tumefaciens) GV3101 (IMCAS, Beijing, China) was grown in Luria-Bertani (LB) medium (Oxoid, England) containing 100 μg/mL kanamycin (kan) (VWR Life Science, U.S.A.) and 50 μg/mL rifampicin (rif) (MP Biomedicals, France) and used to transform P. ostreatus. Escherichia coli (E. coli) DH5α and BL21 (DE3) (Tiangen, Beijing, China) were used for plasmid construction, and grown in LB broth containing kan (50 μg/mL). Restriction endonucleases were purchased from New England Biolabs (NEB), and DNA polymerase, a reverse-transcription kit, and a DNA Gel Extraction kit were purchased from Vazyme (Nanjing, China). Primer synthesis and DNA sequencing were performed by Sangon Biotech (Shanghai, China). The plasmid pCAMIBA 1300 was purchased from YRGen Biotech Company (Changsha, China).

Identification, cloning and sequence analysis of pal genes

The pal gene sequences were obtained from the annotated genome database of P. ostreatus strain PC15 provided by the Joint Genome Institute Website (https://genome.jgi.doe.gov/PleosPC15_2/PleosPC15_2.home.html). Subsequently, the two sequences were used to BLAST against the CCMSSC00389 genome database to identify homologs. The nucleotide sequences were used to design primers (pal1 and pal2 in Table 1) to amplify full-length sequences from CCMSSC00389 complementary DNA (cDNA). Total RNA and DNA were extracted using TRIzol (Omega Bio-Tek, U.S.A.) and cetyltrimethylammonium bromide (CTAB), respectively. The first-strand cDNA was synthesized using a PrimeScript™ RT-PCR kit (Vazyme). The amplified products were
purified and cloned into the vector pGEM-T (Promega, Madison, WI, USA) for sequencing.

All primers used in the experiment are shown in Table 1.

**Bioinformatics analysis of the pal genes**

DNAMAN software was used for multiple sequence alignments. The molecular weights, distribution of amino acids, isoelectric point, and signal peptide of **pal** were predicted using the online ProtParam (http://web.expasy.org/protparam/). The structural domains of the PAL proteins were analyzed online (http://smart.embl-heidelberg.de/). A phylogenetic tree was constructed using the neighbor joining method in MEGA 5.0 based on the PAL nucleotide sequences obtained from GenBank and the maximum composite likelihood model. The three-dimensional (3-D) structure of the PAL proteins was predicted using Modeller.

**Expression and purification of **pal1** and **pal2** in **E. coli**

Expression and purification of **pal1** and **pal2** in **E. coli** were performed as previously described with slight modifications [44]. The PCR product was digested with two restriction enzymes (EcoRI-HF and NotI-HF) and ligated into the vector pET28a (Novagen, Inc., Madison, WI, U.S.A.) that was digested with the same enzymes. The recombinant plasmids that were confirmed by DNA sequencing were named pET28a-**pal1** and pET28a-**pal2** and were subsequently transformed into **E. coli** BL21 (DE3) cells for protein expression. The transformed strains were inoculated into LB medium containing 50 µg/mL kan and incubated at 37 °C with shaking at 180 rpm until reaching an OD600 nm of 0.6-0.8. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to reach a final concentration of 1 mM to induce protein expression, and the culture was incubated overnight at 16 °C with shaking at 180 rpm.

The cultured cells were centrifuged at 4 °C and 5000 rpm for 5 min, washed with PBS buffer, and then suspended in the lysis buffer. After the cells were lysed by
ultrasonication, the enzymes were retained in the supernatant after centrifugation. The supernatant was loaded onto an Ni-NTA column (Qiagen, Duesseldorf, Germany) that was preequilibrated with binding buffer. Subsequently, the column was eluted with binding buffer, washing I buffer, washing II buffer, elution I buffer and elution II buffer. Finally, the fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [45].

OE and RNAi vector construction

The original pCambia1300 vector was modified to harbor the hyg phosphotransferase gene (hyp), which was expressed under the control of the upstream lac promoter [28, 46]. The pal gene OE cassettes were constructed as follows. The P. ostreatus gpd promoter was PCR amplified, after which the pal1 and pal2 cDNA was obtained. The two cassettes were individually cloned into vector to generate the pal gene expression cassette driven by the P. ostreatus gpd promoter (Fig. 1A, B, C). Finally, the vector was introduced into A. tumefaciens GV3101. RNAi-F and a RNAi-R fragments were obtained by PCR, after which the two amplicons were invidious inserted into the vector to construct the interference vectors (Fig. 1D, E, F). Finally, the interference vectors were transferred into P. ostreatus by A. tumefaciens GV3101. The primers used to construct the vectors are shown in Table 1.

Agrobacterium-mediated transformation

P. ostreatus mycelia were inoculated onto PDA plates and cultured at 28 °C until the colony diameter was 5.5-6 cm. Mycelial pellets were cut from the edge of the colony using a cork borer with a 5-mm diameter. Subsequently, 200 pellets were placed into 100 mL of CM liquid medium at 28 °C for 2 days without shaking. A. tumefaciens GV3101 containing the OE-pal or RNAi-pal plasmid was cultivated at 28 °C with shaking at 180 rpm in LB medium with the selective antibiotics (100 μg/mL kan and 50 μg/mL rif) for at least 16 h.
A. tumefaciens cells were collected in sterile tubes (50 mL capacity) by centrifugation at 4500 rpm and 4 °C for 10 min. The bacterial cells were suspended in induction medium (IM, supplemented with 200 μM acetosyringone) and incubated for 5 h (90 rpm, 28 °C) to preinduce A. tumefaciens GV3101 virulence. Then, the A. tumefaciens GV3101 and the mycelia pellets were cocultured at 28 °C for 5 h without shaking. After incubation, the mycelial pellets were dried with filter paper and placed onto IM solid medium at 28 °C for 3 days and then transplanted onto CM medium with selective antibiotics (90 μg/mL hyg and 50 μg/mL cef). Transformants were obtained after 25 days of culturing and were subsequently selected twice for hyg resistance. PCR analysis for the hyg and pal genes was performed using the primers listed in Table 1 [46].

Heat stress treatment

WT, pal-overexpressing (OE-pal 1 7.11-9, OE-pal 1 7.11-11, OE-pal 2 7.11-7 and OE-pal 2 7.12-11) and RNAi transformant strains (RNAi-pal 1 8.1-26, RNAi-pal 1 8.1-38, RNAi-pal 2 7.18-1 and RNAi-pal 2 7.18-19) were used in this study. The strains were cultured on PDA medium incubated at 28 °C for 5 days and then transferred to different temperatures to induce heat stress. To assess the function of pal in the mycelial response to heat stress, the WT, OE-pal and RNAi-pal strains were cultured on PDA plates at different temperatures (28, 32, and 40 °C) for 6 days [6, 47].

Growth susceptibility assay

To assess the susceptibility of the WT, OE-pal and RNAi-pal strains to oxidative stress, mycelial tip pellets with a 5-mm diameter were inoculated onto PDA plates supplemented with 5, 10 and 15 mM H₂O₂. The control group consisted of the nonexogenous addition of H₂O₂. The diameters of the strains were measured after incubation at 28 °C for 7 days [48].

Quantitative real-time PCR (qPCR)
To analyze the expression of *pal* at different developmental stages, samples were collected from the mycelia, primordia, fruiting body and spore stages. The levels of gene-specific mRNA expressed by the WT, OE-*pal* and RNAi-*pal* strains were analyzed using qPCR according to our previous study [6], with the β-actin gene used as a reference. The qPCR amplification procedure was as follows: 95 °C for 3 min, 40 cycles of 95 °C for 3 s, 60 °C for 32 s, and a final extension at 72 °C for 30 s. The relative gene expression was analyzed according to the $2^{-\Delta\Delta CT}$ method.

**Enzymatic activity assay**

The WT, OE-*pal* and RNAi-*pal* strains were cultured on PDA medium incubated at 28 °C for 5 days. Subsequently, the mycelium were quickly scraped, mixed, and frozen in liquid nitrogen for further use. The activity of PAL was determined using a Phenylalanine Ammonia-Lyase Assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer’s instructions.

**Determination of malondialdehyde (MDA) and H$_2$O$_2$**

Intracellular MDA and H$_2$O$_2$ contents were determined using a Malondialdehyde and Hydrogen Peroxide Assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer’s instructions.

**Determination of relative ion leakage and total respiratory rate**

Ten pellet pieces (5 mm) were inoculated into 100 mL of potato dextrose broth medium for 5 days at 28 °C with shaking at 180 rpm. Heat stress was then applied for different times at 40 °C (0, 3, 6, 12, 24, and 48 h). The conductivity of mycelial pellets (C1) was measured by washing electrolytes attached to the surface with deionized water and then putting them into 20 mL of deionized water at 28 °C for 2 h. Then, the sample was autoclaved for 30 min to determine the total conductivity (C2). The relative ion leakage
rate (%) = C1/C2 x 100 [49]. The respiration rate was determined by measuring the production of carbon dioxide with a carbon dioxide meter (MultiRAE IR PGM-54) in sealed containers. The total respiratory rate was measured according to previous studies [9].

Data analysis

GraphPad Prism 6 (GraphPad Software, Inc., San Diego, CA) was used for statistical analyses. The values are reported as the means ± SE and were analyzed by one-way ANOVA, with a P value of <0.05 considered significant.

Abbreviations

PAL, phenylalanine ammonia-lyase; pal, phenylalanine ammonia-lyase gene; P. ostreatus, Pleurotus ostreatus; F. velutipes, Flammulina velutipes; T. matsutake, Tricholoma matsutake; Kan, kanamycin; hyg, hygromycin; A. tumefaciens, Agrobacterium tumefaciens; CM, complete media; PDA, potato dextrose agar; LB, Luria-Bertani; rif, rifampicin. E. coli, Escherichia coli; qPCR, quantitative real-time PCR; MDA, malondialdehyde; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; pl, isoelectric point; cDNA, complementary DNA; CTAB, cetyltrimethylammonium bromide; IM, induction medium; Hyp, hygromycin phosphotransferase gene; MIO, 4-methylideneimidazole-5-one; IPTG, isopropyl-β-D-thiogalactopyranoside; ROS, reactive oxygen species; WT, wild type. OE, overexpression; RNAi, RNA interference. SA, salicylic acid

Declarations

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Availability of data and materials
Data will be shared upon request to the corresponding author huangchenyang@caas.cn

Authors’ contributions
Ludan Hou carried out all experiments, data analysis, and manuscript writing. Lingning Wang collaborated in bioinformatics analysis and in the manuscript writing. Jinxia Zhang and Chenyang Huang participated in the design of the study and in the manuscript writing. Wei Gao and Xiangli Wu collaborated in the manuscript writing.

Consent for publication
All authors have read and approved the final manuscript and consent to publication.

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Not applicable

Competing interests
The authors declare that they have no competing interests.

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Tables

Table 1 Primers used in this study.

| Primer     | Sequence (5′→ 3′)                                      | Note                                      |
|------------|-------------------------------------------------------|-------------------------------------------|
| Po-gpdfF   | GGTACCTTTATTGGCGGT                                    | Promoter cloning                          |
| Po-gpdfR   | CCAGGTCAGTGAATTTCC                                    |                                           |
| pal1_gF    | ATGACAATCTCTATCCGCAGAC                                | gDNA fragment cloning                     |
| pal1_gR    | TTGAATAAAATCAACGATGATA                                 |                                           |
| pal1_cF    | ATGACAATCTCTATCCGCAGAC                                | cDNA fragment cloning                     |
| pal1_cR    | TTGAATAAAATCAACGATGATA                                 |                                           |
| pal2_gF    | ATGACTATTCTCTCAGGGA                                   | gDNA fragment cloning                     |
| pal2_gR    | CTACGCGAACATCGCTA                                     |                                           |
| pal2_cF    | ATGACTATTCTCTCAGGGA                                   | cDNA fragment cloning                     |
| pal2_cR    | CTACGCGAACATCGCTA                                     |                                           |
| pal1-qF    | CTCCTTCAACATCGCATCTA                                  | qPCR                                      |
| pal1-qR    | CTTAGCCGCGCTATGTTG                                    |                                           |
| pal2-qF    | CAACTGCTGCGTATGTC                                     |                                           |
| pal2-qR    | GATGTAGAGGTATGAGGATT                                  |                                           |
| β-actin-F  | GCGATGAACAATAGCAGGG                                   | Endogenous control                        |
| β-actin-R  | GCTGGTATCCACGAGACAAC                                  |                                           |
| pal1-OE-F  | ttacagtctcaagttATGACAATCTCATCGCAGAC                  | Construction of OE plasmids              |
| pal1-OE-R  | aattctagagggccTTAGAATAATCAACCGATGATA                 |                                           |
pal2-OE-F
tacagctcaagggtagATGACTATTCTCTCA
GGGA

pal2-OE-R
aattctagggcccCTACGCGAACATC
GCTA

pal1-RNAi-F1
actgacctggGATTTGCAACCGTTGTCT
TACG
Construction of RNAi plasmids

pal1-RNAi-R1
gttggagtgcaactccctaCTAAAATCAGAT
GAGGGTTGTAAGCG

pal1-RNAi-F2
tagTGGAGTTGCACTCCAACGTGA

pal1-RNAi-R2
catgccaaattctagggcccGATTTGCAA
CCGTGTCTTTACG

pal2-RNAi-F1
aatttcactgacctggCCACCGACAATCCT
CTCATCG

pal2-RNAi-R1
gcacaaccaaagcaagttaaACTAGAAAATGA
GAATAAGACCTTGCTACC

pal2-RNAi-F2
agTTTACTGCTTGGTTGTGCATTTC

pal2-RNAi-R2
catgccaaattctagggcccCCACCGACA
ATCCCTCTCATCG

hyg F
CGACAGATCCGTCGCGCATCTACTGCTT
ATTTCTT
Detection of transformants

hyg R
TCTCGTGCTTTCAGCTTCGATGTAGG
AGGG

OE_gpd_pal1F
TGCGTGGTAGAAGAATGG

OE_gpd_pal1R
CGATGAAGAAGGTAGAATGC

OE_gpd_pal2F
CGTTTCTCGAGTCTTGTC

OE_gpd_pal2R
TGATAGCGTCTTGCCATC

pal1-PE-F
TCGCGGATCCGAATTCATGCAATCCT
ATCCGCA GAC
Construction of prokaryotic expression plasmid

PAL1-PE-R
GAGTGCGGCCGCTTAGAATAAATCA
ACGATATAGGC

pal2-PE-F
CGATCCGAATTCAGACTATTCTCTA
GGGACCACCG

pal2-PE-R
TGCTCGAGTGCGGCATCGCTACGCA
CATCGCTTACG

Figures
Strategy for the overexpression and RNA interference of pal genes in P. ostreatus.  
(A) The structure of the pal1 OE vector. (B) The structure of the pal2 OE vector. (C) Schematic representation of the OE vector based on the A. tumefaciens right and left borders. On the left side, the HygR cassette is driven by the lac promoter. On the right side, the PAL OE cassette is driven by the P. ostreatus gpd promoter. (D) The structure of the pal1 gene RNAi vector. (E) The structure of the pal2 gene RNAi vector. (F) Schematic representation of the RNAi vector based on the A. tumefaciens right and left borders. On the left side, the HygR cassette is driven by the lac promoter. On the right side, the pal RNAi cassette (pal-RNAiF and pal-RNAiR) is driven by the P. ostreatus gpd promoter.

Relationships of fungal PALs, gene structural features and 3-D structural model. (A) A neighbor-joining phylogenetic tree of PAL protein sequences from 11 fungal species. (B) Gene structures of selected pal genes. The exons are represented by red rectangles, and the black lines connecting two exons represent introns. (C) 3-D structural model of the Po-PAL protein. The structure was divided into three parts: the 4-methylideneimidazole-5-one (MIO) domain, the core domain and the inserted shielding domain. The MIO group is highlighted in green.
Figure 3

Partial amino acid sequence alignment and SDS-PAGE results of pal1 and pal2 expressed in E. coli. (A) Partial amino acid sequence alignment of PAL1 and PAL2 of P. ostreatus CCMSSC00389 and other PALs. Positions with identical amino acid residues are indicated by circles below the sequence. (Pleurotus eryngii_AHH55203.1, Schizophyllum commune_XP_003030186.1, Coprinopsis cinerea_XP_001830572.2, Rhodosporidium toruloides_CAA31209.1, Arabidopsis thaliana_NP_181241.1). (B) SDS-PAGE analysis of recombinant PAL protein extracted from E. coli BL21 (DE3) cells. M, protein molecular weight standards; 1, crude lysate of pal1 from E. coli BL21 (DE3) grown at 16 °C for 12 h; 2, crude enzyme of pal1 from E. coli BL21 (DE3)/pSMART-V-PAL induced with IPTG (1 mM) at 16 °C for 12 h; 3, pal1 protein purified with a nickel column; 4, crude lysate of pal2 from E. coli BL21 (DE3) grown at 16 °C for 12 h; 5, crude enzyme of pal2 from E. coli BL21(DE3)/pSMART-V-PAL induced with IPTG (1 mM) at 16 °C for 12 h; and 6, pal2 protein purified with a nickel column. (C) Determination of PAL activity. Three independent biological replicates were performed for all experiments. The values are the means ± SE. Different letters indicate significant differences between the strains (P < 0.05, according to Tukey’s test).
Figure 4

The expression of pal during different developmental stages and different parts of P. ostreatus fruiting bodies. (A) qPCR analysis of pal1 expression in WT strain during different cultivation stages. (B) qPCR analysis of pal2. The relative abundances of the WT transcript levels at different stages were normalized by comparison with that observed in the mycelium stage (relative transcript level = 1). (C) The expression of pal1 in four parts of the P. ostreatus fruiting body. (D) qPCR analysis of pal2 expression. The relative abundances of the transcript levels in the WT strain in the different parts of the fruiting body were normalized by comparison with that observed in the fruiting body (relative transcript level = 1). The values are the means ± SE of three independent experiments. Different letters indicate significant differences between the strains (P < 0.05, according to Tukey’s test).

Figure 5

Effects of heat stress on mycelia. (A) Effects of different temperature stresses on mycelia. (B) Relative expression of pal1. (C) Relative expression of pal2. The relative abundances of transcripts in the mycelia at different temperatures were normalized by comparison with that observed in mycelia at 28 °C (relative transcript level = 1) (D) MDA content. (E) H2O2 content. (F) Total respiratory rate. (G) Relative ion leakage. The mean values and standard deviations of three biological replicates are shown. The error bars with different letters over the columns denote significant differences (P < 0.05, according to Tukey’s test).
Figure 6

Characterization of the pal OE and RNAi strains. (A) qPCR analysis of the expression of pal1 in the tested strains. (B) qPCR analysis of the expression of pal2 in the tested strains. (C, D) Determination of the PAL activity in the tested strains. Three independent biological replicates were performed for all experiments. The values are the means ± SE. Different letters indicate significant differences between the strains (P < 0.05, according to Tukey’s test).

Figure 7

Pal1 and pal2 are involved in the development of P. ostreatus. (A) Developmental stages (mycelia, primordia and fruiting body) in the life cycle of the WT, OE and RNAi strains. (B, C) Colony diameter of the pal mutants. (D, E) qPCR analysis of pal expression in tested strains at different development stages (mycelia, primordia, fruiting body and spores). The relative abundances of the transcript at different stages were normalized by comparison with that observed in mycelia in the WT strain (relative transcript level = 1). The values are the means ± SE of three independent experiments. Different letters indicate significant differences between the strains (P < 0.05, according to Tukey’s test).
Figure 8

Pal1 and pal2 participate in the resistance of mycelia to heat stress. (A) Colony morphology at different temperatures. (B) The effects of exogenous H2O2 at different concentrations on mycelial growth. (C) The growth rate of experimental strains at different temperatures. (D) Effects of different concentrations of exogenous H2O2 on the mycelial growth rate. The values are the means ± SE of three independent experiments. Different letters indicate significant differences between the strains (P < 0.05, according to Tukey’s test).