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Cloning, expression and phylogenetic analysis of a divergent laccase multigene family in *Auricularia auricula-judae*

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ABSTRACT

Laccases (*p*-diphenol: oxygen oxidoreductase; EC 1.10.3.2) are multi-copper oxidases encoded by gene family in white rot fungi. *Auricularia auricula-judae* is one kind of white rot fungi with a soft, jelly-like texture and an ear-like shape. In the present study, seven laccase genes containing the signature sequences L1–L4 were isolated from *A. auricula-judae* strain Au916 on the basis of the mycelium-derived transcriptome. In the basidiomycetes, the predicted substrate binding loops of the *A. auricula-judae* laccases were found to be uncommon. Phylogenetic analysis showed that the laccases of the *Auricularia* were nested into the ascomycete laccases, indicating that the laccase genes from *Auricularia* are distinctly different in function from other basidiomycetes. Among the seven laccases, the intron positions and cluster distributions in the NJ tree varied from each other and the expression patterns of seven genes estimated by qRT-PCR were also discrepant. The *lcc3* gene was highly expressed not only in the free-living mycelium but also in substrate mycelium, furthermore, the *lcc5* gene was mostly expressed during the fruiting body formation and maturation indicating that *lcc5* might play a major role during the sexual reproduction stage.

1. Introduction

Laccase (*p*-diphenol: oxygen oxidoreductase; EC 1.10.3.2) is a blue multi-copper oxidase that catalyzes the oxidation of phenols, aromatic amines, and other aromatic compounds concomitantly with the reduction of molecular oxygen to water (Thurston, 1994). Laccases contain two copper centers which are responsible for the electron transfer during redox reactions. The copper centers are usually differentiated as mononuclear center (T1) with one type-1 Cu being responsible for the blue color, and the trinuclear cluster (T2/T3) consisting of one type-2 Cu and two coupled type-3 Cu (Messerschmidt and Huber, 1990; Hoegger et al., 2004). In all laccases, the copper-binding residues, ten histidines and one cysteine, are conserved as copper ligands, among which two histidines and one cysteine serve as ligands for type-1 Cu and the rest eight histidines for type-2 and type-3 Cu. These conserved residues are spread over four conserved amino acid regions, which are designated as signature sequences L1–L4 that can be used to identify the laccases (Kumar et al., 2003).

The laccase was first identified in the varnish tree *Rhus vernicifera* in 1883 (Yoshida, 1883), and since then, it has been detected in higher plants, some insects, a few bacteria, and fungi (Mayer and Staples, 2002). Most of the known laccases are of fungal origin, in particular from the lignin-degrading white rot fungi (Saito et al., 2003). In the white rot fungi, the laccases are usually encoded by a gene family, as described in *Trametes villosa* (Yaver and Golightly, 1996; Yaver et al., 1996), *Agaricus bisporus* (Perry et al., 1993; Billette et al., 2011), *Pleurotus sajor-caju* (Soden and Dobson, 2001), *Polyporus brumalis* (Ryu et al., 2008), *Pleurotus ostreatus* (Castanera et al., 2012) and *Laccaria bicolor* (Courty et al., 2009). The largest laccase family is from *Coprinopsis cinerea* that comprises up to 17 non-allelic laccase genes (Kilaru et al., 2006).

Besides lignin degradation, the fungal laccases have been linked to vegetative growth (Ohga et al., 1999; Ohga and Royse, 2001), fruiting body formation (Kües and Liu, 2000; Chen et al., 2004), sporulation (Mayer and Staples, 2002), and pigment production (Langfelder et al., 2003; Nagai et al., 2003). It is noteworthy that the members of the laccase gene family showed differential expression patterns, suggesting that they play different roles during the life cycle of fungi (Hoegger et al., 2006). For instance, in *L. bicolor*, among the 11 laccase genes, *lcc3* and *lcc8* are very abundant in ectomycorrhiza, *lcc7* in fruiting bodies, and *lcc9* and *lcc10* in the free-living mycelium growth (Courty et al., 2009). In *P. ostreatus*, among the produced laccases, *lacc10* plays a major role in

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vegetative growth and *lacc2*, an additional physiological role during fructification (Pezzella et al., 2013).

Laccase genes are usually cloned from a cDNA or genomic library using degenerate primers designed according to the highly conserved laccase copper-binding regions (Soden and Dobson, 2001; Hoegger et al., 2004). In recent years, the newly sequenced genomes of fungi have rapidly increased the number of laccases, such as in *C. cinerea*, *L. bicolor* and *P. ostreatus*. Lately, transcriptome, the complete set of transcripts from a sample in a specific developmental stage or physiological condition provided by RNA-Seq, has been used to reveal the molecular constituents and the expression levels of each transcript (Wang et al., 2009). Consequently, one or more laccase genes of a target organism could be predicted by the transcriptome.

Although the phylogeny of laccase genes does not strictly follow the species phylogeny, the fungal laccases can be divided into basidiomycetous and ascomycetous clades in phylogenetic analyses (Hoegger et al., 2006). Nevertheless, *Auricularia delicata*, one kind of basidiomycetes, is classified into the ascomycetous clade for its laccase genes were nested within the ascomycete laccases (Floudas et al., 2012). *Auricularia auricula-judae* [(Bull.) Quél.], together with *A. delicata*, belongs to genus *Auricularia* of Incertae sedis under Agaricomycetes (Kirk et al., 2008). It is one of the most cultivated mushrooms in China due to its high nutritive, economic and medicinal values. To the best of our knowledge, no report has been published about laccases in *A. auricula-judae*.

The aims of the current study were to (i) isolate laccase genes from *A. auricula-judae* based on the transcriptome of mycelium, (ii) unravel the expression patterns of different isoenzymes by quantitative reverse transcription PCR, and (iii) infer the phylogenetic relationship between the laccases of *A. auricula-judae* and other fungi.

2. Materials and methods

2.1. Organisms and culture conditions

The strain used in this study was a commercially cultivated strain of *A. auricula-judae*, Au916 (preserved in our laboratory), together with two types of monokaryotic strains PI and PII, which were isolated by protoplast monokaryogenesis from dikaryotic Au916. Both dikaryotic and monokaryotic strains were maintained on solid CYM medium (complete yeast medium: 2 g peptone, 2 g yeast extract, 20 g glucose, 1 g K_2HPO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.46 g KH_2PO_4 and 15 g agar per liter of distilled water) as described by Horgen et al., (1989). *Escherichia coli*, strain DH5α, was used for transformation and subcloning. *E. coli* strains were grown at 37 °C in LB medium for plasmid propagation.

2.2. Sampling

The strain Au916 was incubated at 25 °C in liquid CYM for one week, and then the mycelia were filtered and collected. After 7 days of growth on CYM at 25 °C, the mycelial plugs were inoculated in sterile polypropylene plastic bags (30 cm × 15 cm) containing 360 g sawdust substrate (78% sawdust, 20% wheat bran, 1% $CaCO_3$ and 1% sugar) and 60% water. The inoculated bags were incubated at 25 °C in the dark for 30 days, and then the mycelia with substrate were gathered from three bags and mixed. Subsequently, the substrate with mycelia in the other bags was used as spawns and inoculated in wood-log to obtain primordia and fruiting bodies. Primordia with a diameter of 1–3 mm were selected and blended (pinhead, stage I). Immature fruiting bodies with a diameter of 5–10 mm (stage II), and 10–20 mm (stage III) and the mature fruiting bodies with a diameter of above 20 mm (stage IV) were plucked on the 2nd, 5th, and 15th days after primordia budding, respectively. All the aforementioned samples were immediately frozen by liquid nitrogen and stored at −80 °C.

2.3. Nucleic acids isolation and cDNA preparation

Genomic DNA was extracted from the mycelia using the cetyl trimethylammonium bromide (CTAB) method (Sambrook and Russell, 2001). RNase A (100 mg/mL) was used to degrade the residual RNA. Total RNAs from the samples described above were isolated with TRIzol Reagent (Invitrogen, USA) according to the instruction manual. The integrity of the obtained genomic DNA and total RNA were evaluated via 1.0% (w/v) agarose gel electrophoresis. Nanodrop 2000 (Thermo Scientific, USA) was used to determine the concentration. Total RNA was treated to remove the genomic DNA according to the protocol of Recombinant DNase I (TaKaRa). After DNase I treatment, quality was checked again with the Nanodrop. 1 μg of treated total RNA was used to synthesize the first-strand complementary DNA (cDNA) by M-MLV reverse transcriptase (TaKaRa, Japan) with 1 μM oligo $(dT)_{18}$ primer in a 20 μL reaction system. Genomic DNA and cDNA were stored at −20 °C, and the total RNA was stored at −80 °C.

2.4. Cloning and analysis of laccase genes

According to the annotation in the transcriptome of *A. auricula-judae* Au916 mycelium, 11 putative laccase genes (*plcc1* to *plcc11*) were obtained. The length of the unknown 5′ and 3′ ends for the putative laccase coding regions were speculated using BLASTX on the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov). Subsequently, the primers for amplifying cDNA fragment and 5′ and 3′ ends were designed by using Primer Premier 5.0 software (see supplementary Table S1). Using the first-strand cDNA from dikaryotic mycelia as the template, the 5′-Full RACE Core set, 3′-Full RACE Core Set (TaKaRa) and SMARTer™ RACE (Clontech) cDNA Amplification Kits were used to obtain the complete full-length cDNA according to the manufacturer's instruction. According to the 5′ and 3′ untranslation regions (UTR) of cDNA, primers for DNA sequence amplification were designed. The final concentration of the genomic DNA from dikaryotic mycelia was 50 ng in the 20 μL polymerase chain reaction (PCR) system.

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The putative alleles were identified by PCR, for which the specific primers were designed and the genomic DNA from two types of parental monokaryons were separately used as templates (Table S1). After PCR, the products were sequenced and compared with the DNA sequences of the putative genes to verify the reliability of amplification.

All the PCR products were subjected to the agarose gel electrophoresis. The target PCR fragments were excised from the gel and purified with Gel Purification Kit (BioTeke, Beijing). The purified products were then cloned using the pMD®18-T Vector (TaKaRa, Japan) into *E. coli* DH5α. Finally, 3–5 positive clones of target fragments were sequenced by GenScript Corporation (Nanjing, China).

DNAMAN version 5.2.2 software was used for sequences analysis and assembly. The SoftBerry program (http://linux1.softberry.com) was used to predict the amino acid sequence. The deduced amino acid sequences were aligned with Clustal X 1.83 (Thompson et al., 1997) and then manually adjusted in GeneDoc 2.6 (Nicholas and Nicholas, 1997). Introns were analyzed by aligning the obtained cDNA and DNA sequences and confirmed by identifying the consensus exon/intron splice