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Research paper

The *Trametes hirsuta* 072 laccase multigene family: Genes identification and transcriptional analysis under copper ions induction

Daria V. Vasina [a, *], Orkhan N. Mustafaev [b], Konstantin V. Moiseenko [a], Natalia S. Sadovskaya [b], Olga A. Glazunova [a], Alexander A. Tyurin [b], Tatiana V. Fedorova [a], Andrey R. Pavlov [a], Tatiana V. Tyazhelova [a], Irina V. Goldenkova-Pavlova [b], Olga V. Koroleva [a]

[a] A.N. Bach Institute of Biochemistry, Russian Academy of Sciences, Leninsky prospekt, 33, build. 2, Moscow 119071, Russia
[b] Institute of Plant Physiology, Russian Academy of Sciences, st. Botanicheskaya, 35, Moscow 127276, Russia

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ABSTRACT

Laccases, blue copper-containing oxidases, can play an important role in a variety of natural processes. The majority of fungal laccases are encoded by multigene families that express closely related proteins with distinct functions. Currently, only the properties of major gene products of the fungal laccase families have been described. Our study is focused on identification and characterization of laccase genes, which are transcribed in basidiomycete *Trametes hirsuta 072*, an efficient lignin degrader, in a liquid medium, both without and with induction of laccase transcription by copper ions. We carried out production of cDNA libraries from total fungal RNA, followed by suppression subtractive hybridization and mirror orientation selection procedures, and then used Next Generation Sequencing to identify low abundance and differentially expressed laccase transcripts. This approach resulted in description of five laccase genes of the fungal family, which, according to the phylogenetic analysis, belong to distinct clusters within the *Trametes* genus. Further analysis established similarity of physical, chemical, and catalytic properties between laccases inside each cluster. Structural modeling suggested importance of the sequence differences in the clusters for laccase substrate specificity and catalytic efficiency. The implications of the laccase variations for the fungal physiology are discussed.

1. Introduction

Scientific saga of laccase enzymes began in 1883 with the first laccase discovered in the Chinese lacquer tree *Rhus vernicifera*, and since then it has been found in all domains of life: higher plants, some insects, a few bacteria, and fungi [1,2]. Most of the known and well-studied laccases are of fungal origin, in particular from the lignin-degrading white rot fungi [3].

Laccases participate in various physiological processes, such as: lignin modification, vegetative growth, fruiting body formation, sporulation, detoxification, synthesis of humic substances, pigment production, etc. [3,4]. It is assumed that the physiological function of the enzymes is determined by the structure of their catalytic active center, which is common for fungal laccases. It includes copper centers of three types, which are responsible for the electron transfer during redox reactions [5]. The copper centers are usually determined as a mononuclear center (T1) containing one Cu of type-1 responsible for the blue color, and the tri-nuclear cluster (T2/T3) consisting of one Cu of type-2 and two coupled Cu of type-3. In all laccases, the copper-binding residues are strictly conserved: Cu of type-1 is chelated by two histidines and one cysteine, and Cu of type-2 and type-3 use another eight histidines. These conserved residues are spread over four conserved amino acid regions, which were defined as signature sequences L1–L4 that can be used to identify the laccases [6].

Abbreviations: SSH, suppression subtractive hybridization; MOS, mirror orientation selection; RACE-PCR, rapid amplification of cDNA ends; GOI, gene of interest; RQ, relative quantities; PTR, partial transcripts; qPCR, quantitative RT-PCR; ABTS, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid); 2,6-DMP, 2,6-dimethoxyphenol; PCBs, polychlorinated biphenyls; PAHs, polycyclic aromatic hydrocarbons.

* Corresponding author.
E-mail address: d.v.vasina@gmail.com (D.V. Vasina).

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The genes encoding fungal laccases are commonly form multigene families [4,5]. The term "multigene families" is broadly used to describe groups of genes from the same organism that encode proteins with sequence similarity either over their full lengths or limited to a specific domain. To date, such laccase multigene families have been identified in many fungi; *e.g.*, *Coprinopsis cinerea* has up to 17 genes [7]. The importance of finding the multigene families is clear, since the proteins of a family may perform a range of related functions. However, traditional methods for identification of such families may present certain difficulties, as genes have identical or nearly identical sequences. On the other hand, genes with extensive divergence derived from a common ancestral gene could be described as unrelated. Moreover each isozyme can be presented by several isoforms, largely, due to various post-translational modifications (especially, glycosylation) [8], which can make it almost impossible to identify a complete family of specific proteins without modern genetic approaches.

Previously laccase genes were identified in cDNA or genomic libraries using degenerate primers designed according to the highly conserved laccase copper-binding regions and then cloned. However, these methods are not always successful [9]. Recently, advances in DNA sequencing techniques suggested new approaches for identification of laccase multigene families.

The newly sequenced fungal genomes [4,10–12,91] have demonstrated an increased number of laccase multigene family members. On the other hand, genomic studies do not provide information about expression level of individual genes. Application of RNA-Seq analysis of transcriptome allows to reach this goal. For example transcriptome sequencing has been used for laccase multigene family in *Auricularia auricula-judae* to reveal the molecular constituents and the expression levels of each transcript from a sample in a specific developmental stage or physiological condition [13].

Since expression of target genes, such as laccase genes, may have large variations, successful description of a gene family in addition to high quality transcriptomes also requires solving the problem of rare transcripts "loss" in cDNA libraries. In such cases experimental approaches based on DNA hybridization in solution, such as the suppression subtractive hybridization (SSH) and mirror orientation selection (MOS), or their combinations, can increase relative amount of "minor" transcripts in the cDNA libraries [14–17]. Therefore, RNA-Seq analysis of subtractive cDNA libraries, which have unique characteristics, appears to be a better choice, compared to such approaches as microarray and competitive genomic hybridization.

It has been convincingly shown that expression of laccase genes is strongly regulated by metal ions. In particular, copper ions were revealed to regulate genes transcription in *Trametes versicolor*, *Ceriporiopsis subvermispora*, *Pleurotus ostreatus*, *Pleurotus sajor-caju*, and *Trametes pubescens* [18]. Many studies have marked differences in the transcriptional response to copper ions which exhibit different laccase genes within a single species, or gene families [19–22]. The fungus *Trametes hirsuta* potentially produces a wide spectrum of laccase isozymes, including high redox potential ones. However, there were no exact data on the amount of expressed genes, including copper-inducible, as well as regarding the differential gene expression of laccase isozymes and catalytic properties of protein products in *T. hirsuta*.

Hence, we assumed that production of forward and reverse subtracted cDNA libraries of *T. hirsuta* with and without Cu^{2+} ions by SSH in combination with MOS will allow identifying laccases genes, including cooper-inducible ones.

The aims of this study were to (i) identify laccase genes from *T. hirsuta* using a method based on SSH and RNA-Seq, (ii) unravel the expression patterns of different isozymes by quantitative reverse transcription PCR, and (iii) infer the phylogenetic relationship between the laccases of *T. hirsuta* and other fungi of the same genus.

2. Materials and methods

2.1. Fungal strain and culture conditions

Mycelial cultures of Basidiomycete strain *T. hirsuta* 072 used in this study were received from the Collection of the Komarov Botanical Institute, Russian Academy of Sciences (St. Petersburg, Mycobank # 267192). Fungi were stored on wort-agar slants at 4 °C. The static pre-cultivation of mycelium was performed at 26–28 °C in 750-ml Erlenmeyer flasks with 200 ml of GP medium with the following composition (g/liter): glucose, 10.0; peptone, 3.0; KH_2PO_4, 0.6; K_2HPO_4, 0.4; $ZnSO_4 \cdot 7H_2O$, 0.001; $FeSO_4 \cdot 7H_2O$, 0.0005; $MnSO_4$, 0.05; $MgSO_4 \cdot 7H_2O$, 0.5 [23]. For further cultivation, the mycelium was disrupted into small fragments by ceramic beads and inoculated into flasks. The submerged cultivation was performed at 26–28 °C in Erlenmeyer flasks on GP media (Cu-samples) and on GP media supplied with $CuSO_4$ – 0.25 g/l (Cu + samples). The 3-day mycelium was collected for RNA extraction.

2.2. Enzymes activity assays

The laccase activity was measured spectrophotometrically using syringaldazine (Sigma, USA) as a substrate according to Dantán-González et al. [24]. One unit (U) of the enzyme activity was determined as the quantity of laccase that oxidizes 1 μmol of the substrate per minute in 1 ml of the reaction. The optical density was measured using LAMBDA 25 UV/Vis System spectrophotometer (PerkinElmer, USA). All assays were performed in triplicate, and mean values were calculated.

2.3. RNA extraction

Total RNA was extracted from homogenate of fresh cells from 3-day culture with TRIzol Reagent (Life technologies, USA). Total RNA template was further purified using the QIAGEN RNeasy MinElute Cleanup Kit (Qiagen, USA). The integrity and quantity of RNA were determined by spectrophotometer Nanodrop ND-1000 (LabTech International, ES, UK) and by 1.0% agarose gel electrophoresis.

2.4. Subtraction procedure (SSH and MOS)

SSH was performed using a PCR-Select cDNA Subtraction Kit (BD Biosciences Clontech, USA) according to the manufacturer's instructions. Double-stranded cDNA was obtained from the mRNA of the *T. hirsuta* using an SMART PCR cDNA Synthesis Kit (BD Biosciences Clontech, USA) according to the manufacturer's instructions. The cDNA from the tester and driver samples were digested with *Rsa*I. Each cDNA from the tester samples was then separated into two portions, and each ligated with either adaptor 1. (5′-CTAATACGACTCACTATAGGGCTCGAGCGGCCGCCCGGGCAGGT-3′) or adaptor 2R (5′-CTAATACGACTCACTATAGGGCAGCGTGGTC-GCGGCCGAGGT-3′) from Clontech. Subtractions and normalizations were accomplished with a 50-fold excess of driver over tester followed by two rounds of PCR amplification. After checking subtraction efficiency, fresh subtracted PCR products were used for MOS PCR to eliminate false positive clones from SSH libraries following the methods of Rebrikov et al. [14]. Subtraction efficiency was measured by the intensity of the PCR products on agarose gel representing actin and tubulin (S1 Table) before and after the SSH and MOS procedures.