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MOLECULAR GENETICS

Range of Gene Candidates Involved in Biosynthesis of Laccase from Basidiomycete *Trametes hirsuta*

D. V. Vasina[a], D. S. Loginov[a], O. N. Mustafaev[b], I. V. Goldenkova-Pavlova[b], and O. V. Koroleva[a]

[a] *Bach Institute of Biochemistry, Russian Academy of Sciences, Moscow, 119071 Russia*
e-mail: d.v.vasina@gmail.com
[b] *Timiryazev Institute of Plant Physiology, Russian Academy of Sciences, Moscow, 127276 Russia*

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Abstract—A comparative analysis of transcripts from the basidiomycota *T. hirsuta* grown with and without an inducer of the laccase biosynthesis was carried out. Methods of subtraction hybridization and massive parallel sequencing were used for this purpose. Unique transcripts encoded by genes that have a relatively high level of expression and belong to different gene ontology categories were identified. Also, a large number of transcripts were found to encode for predicted proteins, as well as noncoding transcripts. The latter may represent regulatory RNA molecules. Transcripts that increase their abundance when the laccase synthesis is induced are selected as gene-candidates involved in the laccase biosynthetic pathway.

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INTRODUCTION

Basidiomycetes, an infectious agent that causes white rot, has a unique enzyme complex that can efficiently degrade and mineralize lignin [1]. Currently, it is thought that laccase, which belongs to the family of copper-containing oxidases, is one of the key enzymes of this complex (EC 1.10.3.2). The enzyme catalyzes the reduction of molecular oxygen to water and the concurrent oxidation of an electron donor without an intermediate step of the peroxid formation [2]. Although laccases have been studied for more than 100 years, their biosynthetic mechanism is still of considerable interest. There are many papers on how nitrogen and carbon sources, as well as different inducers, affect the laccase biosynthesis [3–5]. A variety of organic substances, mainly of phenolic and aromatic origin, as well as inorganic compounds, e.g., copper sulfate, are used as inducers. However, in most studies on laccase synthesis, the principle aim was to increase enzyme production instead of investigating the biosynthetic process.

Currently, the main tools for studying biosynthesis come from proteomics. These include 2D electrophoresis, mass spectrometry, and others. This approach allows one to identify individual proteins, which makes it possible to suggest their involvement in a variety of biochemical processes [6, 7]. Nevertheless, in the case of basidiomycota, the proteomic methods are difficult to apply as far as there are no annotated genomes available, which makes it difficulty to identify proteins. One alternative may be an approach based on the comparative analysis of transcripts that originated from fungi grown with or without the inducer, which helps in the identification of differentially expressed genes [8, 9]. The induction of the laccase biosynthesis with copper sulfate is a convenient model for this purpose.

The aim of this work was to find suggested gene-candidates involved in the laccase biosynthesis in the basidiomycota *Trametes hirsuta* using the comparative analysis of transcriptomes and current proteomic methods.

MATERIALS AND METHODS

Fungus. The basidiomycota *Coriolus hirsutus* (*Trametes hirsuta* (Wulfen) Pilat, 1939), from the collection of the Komarov Botanical Institute, Russian Academy of Sciences (LEBIN), was kindly presented by Dr. N.V. Psurtseva.

Fungus growing. The laccase producer *T. hirsuta* was grown on GLP medium as described previously [10]. The medium included 10.0 g/L glucose, 3.0 g/L peptone, 0.6 g/L KH_2PO_4, 0.001 g/L $ZnSO_4\square \cdot 7H_2O$, 0.4 g/L K_2HPO_4, 0.0005 g/L $FeSO_4\square \cdot 7H_2O$, 0.05 g/L $MnSO_4$, and 0.5 g/L $MgSO_4\square \cdot 7H_2O$. For induction, the cultivation medium was supplied with 0.25 g/L $CuSO_4$. The fungal mycelium was collected after 3 days of growth.

Measurement of laccase activity. The enzymatic activity was measured spectrophotometrically using the syringaldazine as a substrate (Sigma, $\varepsilon_{520} = 65000\ M^{-1}\ cm^{-1}$) solution buffered with 0.1 M Na-acetate (pH 4.5) [11]. The amount of enzyme needed to oxidize 1 μM of substrate in 1 mL of reaction mixture for 1 min was assumed to be the unit of the activity.

Isolation of total RNA. Total RNA was purified from the mycelium samples with the use of the QIAamp RNA kit (QIAGEN, United States).

Table 1. Primers used in this study

Primer/adapter	Nucleotide sequence
SMART Oligo IIA	5'-AAGCAGTGGTATCAACGCAGAGTACGCrGrGrG-3'
SMART CDS primer IIA	5'-AAGCAGTGGTATCAACGCAGAGTA-d(T)30-3'
SMART PCR primer IIA	5'-AAGCAGTGGTATCAACGCAGAGT-3'
Adapter 1	5'-CTAATACGACTCACTATAGGGCTCGAGCGGCCGCCCGGGCAGGT-3' 3'-GGCCCGTCCA-5'
PCR primer 1	5'-CTAATACGACTCACTATAGGGC-3'
Nested primer 1	5'-TCGAGCGGCCGCCCGGGCAGGT-3'
Adapter 2R	5'-CTAATACGACTCACTATAGGGCAGCGTGGTCGCGGCCGAGGT-3' 3'-GCCGGCTCCA-5'
Nested primer 2R	5'-AGCGTGGTCGCGGCCGAGGT-3'
MOS PCR primer	5'-GGTCGCGGCCGAGGT-3'

cDNA synthesis. cDNA synthesis was accomplished according to the SMART method as described [12]. The first chain synthesis was conducted in the presence of the SMART Oligo II primers, CDS primers (Table 1), and 0.5 μg of total RNA. The reaction was carried out in the 10 μL volume. The second cDNA chain was synthesized using 1 μL of the previous reaction mixture (fivefold dilution), which contained the first cDNA chain and the SMART PCR primers IIA (Table 1). Amplification was accomplished under the following conditions: 95°C for 10 s, 65.5°C for 20 s, and 72°C for 3 min for 21 cycles. Next, cDNA samples were digested with *Rsa*I as recommended by the manufacturer (Promega, United States).

Subtraction of cDNA libraries. For the subtraction of cDNA libraries, suppression subtraction hybridization (SSH) was used in two directions, i.e., Cu+ vs. Cu– and Cu– vs. Cu+, where Cu+ stands for a fungus sample grown in the presence of the inducer (Cu ions), while Cu– refers to a sample grown in the absence of that. For each direction, two control populations were constructed by ligation of cDNA with two different suppressive adapters, Adapter 1 and Adapter 2R (Table 1). The control populations were annealed separately with 30-fold excess of cDNA without adapters (designated as driver cDNA) in a series of DNA denaturation/renaturation. After the first round of hybridization, the two samples were mixed together and hybridized in the same volume. The subtracted cDNA libraries were amplified in two rounds. The primary amplification of each subtracted cDNA (**Cu**+ and **Cu**–) was carried out with the Primer 1 (Table 1) under the following conditions: 95°C for 10 s, 65°C for 30 s, and 72°C for 1.5 min, for 27 cycles. The secondary amplification of each subtracted cDNA sample (**Cu**+ and **Cu**–) was carried out with a primer pair of Nested primer 1 and Nested primer 2R (Table 1) under the following conditions: 95°C for 10 s, 68°C for 30 s, and 72°C for 1.5 min, for 10 cycles. Next, each subtracted cDNA sample was subjected to mirror-oriented selection (MOS) using the MOS PCR primer (Table 1) under the following conditions: 95°C for 10 s, 62°C for 30 s, 72°C for 1.5 min for 22 cycles.

Massive parallel sequencing. Four subtracted cDNA samples (Cu+ and Cu–, both obtained before and after MOS) were sequenced on the same lane of the Illumina HiSeq SR 100 machine according to the manufacturer's instruction.

Transcript assembly. Transcripts were assembled using of Trinity software.

Transcript analysis. Transcripts were analyzed as described previously [13]. We took advantage of the EXPASY bioinformatics portal (http://www.expasy.org) to translate nucleotide sequences in three reading frames in both directions. Furthermore, using the BLASTp search engine (http//blast.ncbi.nlm.nih.gov/Blast.cgi), we searched for gene products in the NCBI databases. The functional Gene Ontology (GO) annotation was carried out using Blast2go software (http://www.blast2go.org/).

RESULTS AND DISCUSSION

As was shown by the preliminary studies, the laccase activity in *T. hirsuta* samples was detectable after 3 days of cultivation, when grown submerged in GLP or GLP+Cu^{2+} media. Notably, when grown in GLP medium supplied with inducer, the laccase activity increased by 25-fold, up to 0.33 ME. Based on these results, we used 3-day-old fungal mycelium to analyze transcripts.

Total RNA was isolated from samples grown both without induction and in the presence of cuprum ions. The quantity and quality of total RNA was confirmed by the electrophoresis in the agarose gel. Figure 1 shows that bands corresponding to ribosomal RNA are

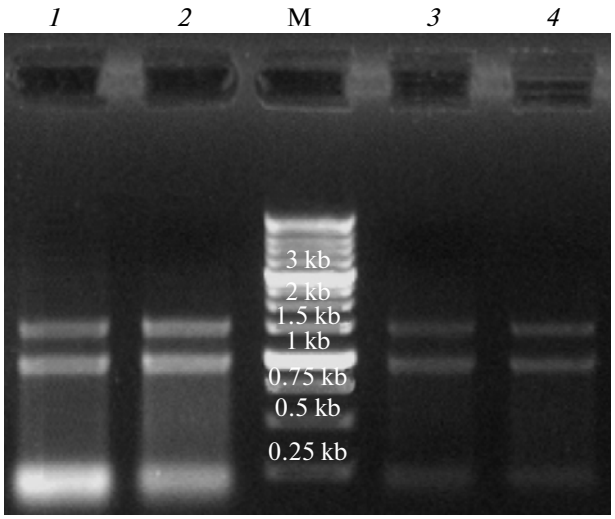

Fig. 1. Electrophoresis of samples of total RNA grown on: (*1*) Cu-supplied media; (*2*) media without Cu; (*3*) Cu-supplied media, after purification; (*4*) media without Cu, after purification. M designates 1-kb ladder.

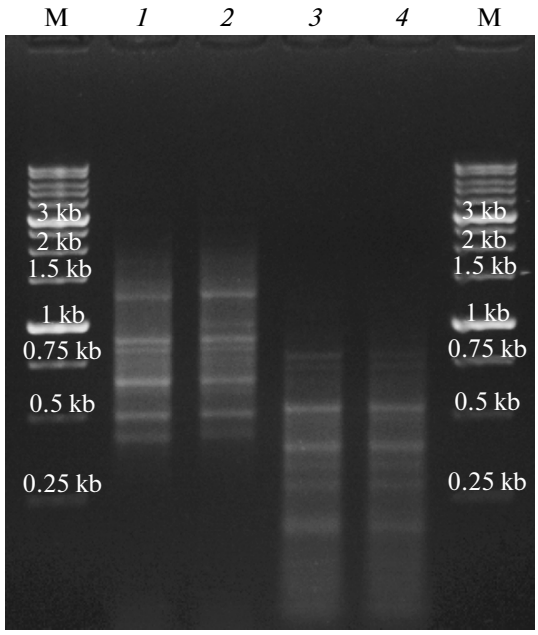

Fig. 2. Electrophoresis of cDNA samples. (*1*) SMART-amplified cDNA, Cu+; (*2*) SMART-amplified cDNA, Cu–; (*3*) *Rsa*I digested cDNA, Cu+; (*4*) *Rsa*I digested cDNA, Cu–. M designates 1-kb ladder.

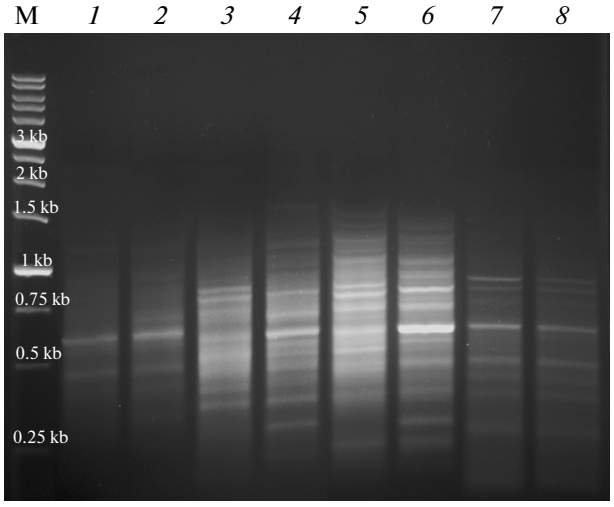

Fig. 3. Electrophoresis of PCR products. (*1*) Primary PCR, Cu+–subtraction; (*2*) primary PCR, Cu– subtraction; (*3*) secondary PCR, Cu+ subtraction; (*4*) secondary PCR, Cu– subtraction; (*5*). MOS PCR, Cu+ subtraction; (*6*) MOS PCR, Cu– subtraction; (*7*) no subtraction, Cu^{+}; (*8*) no subtraction, Cu–. *M* designates 1-kb ladder.

sharp and clearly seen, which makes the RNA samples suitable for the further study.

According to the genome projects database (http://genome.jgi.doe.gov), around 600 fungal genomes and transcriptomes are in progress of sequencing. Amongst them, however, basidiomycota account for less than 60 projects. Moreover, there is no single completed genome which is responsible for the white rot of wood.

Since the basidiomycota genomes are large (from 21 Mb *Pleurotus ostreatus* to 90 Mb *Pluteus cervinus*), and a number of transcripts is potentially high, we used a new approach based on association of subtraction hybridization with subsequent massive parallel cDNA sequencing.

To identify gene candidates that could be involved in the laccase biosynthetic pathway, total RNA was used to create cDNA libraries. cDNA libraries enriched by full-length sequences were prepared using SMART technology. A primer for cDNA synthesis had a poly(T) sequence with several interspersed C and G nucleotides at its 3'-end. In this case, problems associated with the sequencing of homopolymeric regions using the 454 machine can be avoided. Furthermore, in order to equalize transcript concentrations, cDNA was normalized and depleted. These procedures decreased the highly represented ribosomal RNA and tRNA transcripts. At the same time, the representation of other transcripts was unchanged. This in turn kept the abundance of rare transcripts at the same level. Figure 2 shows the results of the normalization and depletion of the cDNA libraries.

It should be noted that the RNA samples were of high complexity and similarity because they were derived from the same object, basidiomycota, grown under different conditions (cuprum ions present or absent). For this reason, to prepare cDNA, we used an additional MOS procedure. Figure 3 shows a comparison of cDNA samples obtained at different steps of subtraction hybridization. One can see that MOS elevates the abundance of transcripts of low-expressing genes, while the abundance of rRNA and tRNA tran-

Table 2. Classification of transcripts with highest level of expression

GO classifier	Number of transcripts			
	1 (expression >1000)	2 (expression >100)	3 (expression >500)	4 (expression >100)
Hypothetic proteins	9	3	6	4
Ribosomal proteins	2	–	–	–
Cellular respiration	2	1	1	1
Intracellular transport (ER-Golgi)	1	–	2	–
Translation	–	–	3	–
Molecular chaperons	2	2	–	1
Structural and accessory proteins	1	–	1	–
Endocytosis	2	–	1	1
Metabolic pathways (biosynthesis of lipids, proteins and carbohydrates)	1	–	4	–
Proteolysis	2	–	–	–
Oxydases	–	–	1	–
DNA interaction	1	–	–	–
Transcription	–	2	–	–
Stress response	1	1	1	–
Exocytosis	1	–	–	–
Post-translational protein modification	–	–	1	1
Mediator proteins	1	–	1	–
Cell cycle	–	–	1	1
Glycosidases	–	1	–	–
Proteins with unknown functions	1	1	4	1
Noncoding transcripts	8	3	4	5
Total of unique transcripts	35	14	31	15

scripts is decreased. Thus, the procedure leaves all transcripts to be represented.

The next step was massive parallel sequencing of cDNA samples obtained after subtraction hybridization: two samples (**Cu**+ and **Cu**–) without MOS (no MOS variant) and two samples (**Cu**+ and **Cu**–) with additional MOS (MOS variant). This resulted in 98934807 reads (9823569560 nt), including 28248565 (**Cu**+) and 32272465 (**Cu**–) reads for the samples obtained for the no MOS variant, as well as 15968049 (**Cu**+) and 22445728 (**Cu**–) reads for the samples obtained for the MOS variant.

All reads were assembled *de novo* in contigs using the Trinity package. For contig assembly, a set of reads with the average of 180 nt, 30-fold overlap, was used. The assembly resulted in 16088 and 14204 contigs for **Cu**+ and **Cu**– samples, respectively, in the case of the no MOS variant. At the same time, in the case of MOS variant the numbers were 11732 and 11336 contigs for **Cu**+ and **Cu**– samples, respectively.

The results of deep sequencing of the MOS samples led to the identification of 409 unique transcripts that originated from the Cu-induced fungal mycelium, as well as 337 unique transcripts from the noninduced fungus. Moreover, it was found that the levels of expression of 233 transcripts from the Cu-induced samples were elevated more than 100-fold compared to the same transcripts from the noninduced samples. Both the unique and high expression level transcripts. were of considerable interest based on their subsequent functional annotations.

It should be noted that the relative expression level of the unique transcripts fell in a broad range of values. We found also that, in the case of the samples 1 and 3 (Cu+, no MOS variant; Cu+, MOS variant), the expression levels were more than 1000 and 500 relative units, respectively, while the expression levels were slightly more than 100 units in the case of samples 2 and 4 (Cu–, no MOS variant; Cu–, MOS variant).

The further analysis of the highly expressed (100–to 1000-fold) unique DNA sequences was of special interest. The sequences were used in a BLASTp search for known proteins in the NCBI databases (http://blast.ncbi.nlm.nih.gov/Blast.cgi). All identified proteins were divided in groups according to their functional GO annotation. As follows from Table 2, unique, highly expressed transcripts belong to different GO categories, and include many transcripts that encode for predicted proteins, as well as noncoding transcripts. It cannot be excluded that the latter represent regulatory RNA molecules.

Thus, this study resulted in the discovery of unique *T. hirsuta* transcripts with a highly increased level of expression upon laccase induction. It is suggested that the transcripts be transcribed from gene candidates that encode the proteins involved in the laccase biosynthetic pathway. The results represent the basis for the further study of these genes to determine their role in the biosynthesis of laccase from basidiomycota *T. hirsuta*.

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