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EXPERIMENTAL ARTICLES

Activity and Expression of Laccase, Tyrosinase, Glucanase, and Chitinase Genes during Morphogenesis of *Lentinus edodes*

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Abstract—Activation of expression of the *lcc4* and *tir* genes encoding laccase and tyrosinase was observed during transition of the xylotrophic basidiomycete *Lentinus edodes* from the vegetative to the generative growth stages. This was especially pronounced in the brown mycelial mat (the stage preceding formation of the fruiting bodies). Development of this structure was shown to be associated with a sharp increase in laccase and tyrosinase activities, as well as with rearrangements in the phenol oxidase complex. Formation of the tissues with thickened cell walls was associated with enhanced expression of the *chi* and *exg1* genes encoding chitinase and glucanase, respectively. Exogenous treatment of the vegetative mycelium with a laccase preparation from the brown mycelial mat promoted formation of this morphological structure. Activation of the *lcc4, tir, chi*, and *exg1* genes may be used as a marker of readiness to fruition in xylotrophic fungi.

Keywords: basidiomycete morphogenesis, brown mycelial mat, *Lentinus edodes,* gene expression, protein markers

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The basidiomycete *Lentinus edodes* (the shiitake mushroom) is of interest due to its nutritive value and palatability of the fruiting body, as well as the presence of a unique complex of biologically active and medicinal compounds [1, 2]. Shiitake is a wood-destroying xylotroph and can be cultivated on wastes of timber and the woodworking industry as substrates [3].

Despite its obvious timeliness and practical importance, the problem of optimization of artificial growth of valuable edible and medicinal basidiomycetes has not been completely solved. One of the major reasons lies in the specific features of the macromycete life cycle and lack of knowledge on molecular and physiological aspects of their morphogenesis.

Studies aimed at investigation of the genes and proteins associated with the regulation of growth and morphogenesis in basidiomycetes are scarce. Phenol oxidases (laccases and tyrosinases), which xylotrophs require for wood decomposition and nutrient release, have been hypothesized to participate in fungal morphogenesis [4]. Formation of fruiting bodies was proposed to be associated with laccase-catalyzed synthesis of extracellular pigments, which are involved in binding the cell wall components of mycelial hyphae [5]. Unlike the revertants with the normal enzymatic activity, laccase-negative mutants of *Pleurotus florida* did not form fruiting bodies [6]. In *Agaricus bisporus* laccase activity depended on the growth phase, decreasing drastically after formation of the fruiting bodies [7]. While the physiological functions of tyrosinase remain unclear, available data indicate a protective role of this enzyme associated with its role in the synthesis of melanin pigments, which contribute considerably to resistance of fungi to pathogens and environmental impacts, especially during basidiome formation [8].

The results of some works indicate an important role of glucanases in fungal growth due to limitation on hydrolysis and continuous reconstruction of the cell wall components involved in extension and fusion of the hyphae [9, 10], as well as of chitinases, which are required for apical growth, cell division, spore germination, and mycelial branching [11, 12]. The patterns of expression of the genes involved in cell differentiation of the fungal mycelium may probably act as markers of specific stages of morphogenesis.

In the process of development, *L. edodes* forms well-differentiated morphological structures: vegetative non-pigmented mycelium, brown mycelial mat (BMM), primordia, and fruiting bodies [13]. The results of our previous studies demonstrated considerable changes in the intracellular polypeptide composition depending on the stage of morphogenesis [14]. The most pronounced differences were found in the tissues of the BMM, the structure development of which is a necessary prerequisite for the formation of proper shiitake fruiting bodies.

The goal of the present work was to study the transcriptional activity of laccase, tyrosinase, glucanase,

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and chitinase genes and to detect the changes in the phenol oxidizing complex and ultrastructural morphology of the fungal hyphae in the course of cell differentiation in *L. edodes*.

MATERIALS AND METHODS

Organisms and cultivation conditions. We used three strains of the xylotrophic basidiomycete *Lentinus edodes* (Berk.) Sing [*Lentinula edodes* (Berk.) Pegler] (shiitake mushroom): strains F-249 and 2-T from the Collection of Higher Basidial Fungi, Department of Mycology and Algology, Lomonosov Moscow State University, as well as strain 363 obtained from the Collection of Pileate Fungi of the Kholodny Institute of Botany, National Academy of Sciences of Ukraine. Cultures of the fungi were grown on beer-wort agar (4^B) for 14 days at 26°C and stored at 4°C. Fourteen-day cultures grown on the same medium at 26°C were used as the inoculum.

The basidiomycetes were grown using an intensive technique, which is the closest to natural growth on a wooden substrate under laboratory conditions [13]. Non-pigmented mycelium, BMM, primordia, and fruiting bodies of *L. edodes* were investigated.

Isolation of RNA and synthesis of cDNA using the reverse transcription reaction. Tissues of the above-listed morphostructures of the three *L. edodes* strains were separately collected, immediately frozen, and homogenized by grinding in a porcelain mortar with liquid nitrogen. Isolation of total RNA from fungal cells was performed using the RNeasy Plant Mini Kit (Qiagen, United States) according to the manufacturer's recommendations. RNA concentration was determined on a NanoDrop ND-1000 (NanoDrop Technologies, United States) spectrophotometer. Quality of the isolated RNA was assessed by electrophoretic separation patterns in 1% agarose gel after staining with ethidium bromide [15].

To obtain cDNA, 1.5 μg total RNA pretreated with 0.1 U/μL DNase (Fermentas, Lithuania) at 37°C for 30 min was used. Reverse transcription was performed in a reaction mixture of the total volume of 25 μL containing 100 μM oligo-dT18 primers, 300 μM dNTPs, and 100 U M-MuLV reverse transcriptase (Fermentas) in a relevant buffer. At the first stage, RNA solution was heated at 70°C for 5 min in the presence of oligonucleotide primers (oligo-dT18) and then cooled on ice. At the second stage, the remaining components were added and the mixture was incubated at 37°C in a DNA Engine thermocycler (Bio-Rad, United States) for 60 min. The reaction was terminated by heating of the mixture to 70°C for 10 min; 2 μL of thus obtained cDNA was used in PCR.

Amplification of *L. edodes* DNA fragments by real-time PCR (RT PCR). To amplify *L. edodes* DNA fragments (cDNA), we constructed the primers complementary to fragments of the genes *lcc4, tir, chi, exg1*, and *gpd* encoding laccase, tyrosinase, chitinase, glucanase, and glyceraldehyde 3-phosphate dehydrogenase, respectively. The latter gene was used as a reference one.

Primers and TaqMan oligonucleotide fluorescent probes were constructed using the Vector NTI version 9 software package using the nucleotide sequences of *L. edodes* [16]. Since the information on the shiitake genome sequence in available databases is very limited, we compensated for the lack of data by building consensus sequences of the coding regions of the genes using the databases on other basidiomycete species. The primers and fluorescent probes were synthesized by NPO Syntol (Moscow, Russia).

The list of the constructed primers and TaqMan fluorescent probes is presented in the table.

PCR was performed in a reaction mixture of the total volume of 25 μL containing buffer (67 mM Tris-HCl, pH 8.8, 17 mM $(NH_4)_2SO_4$, 2.5 mM $MgCl_2$, and 0.1% Tween 20), 100 μM of each dNTP, 2 μL cDNA, 0.1 μM of each primer, and 0.04 U TaqMan probe.

Temperature and duration of the reaction stages (10 s at 94°C and 1 min at 60°C) and dynamics of fluorescence were controlled with a CFX96 (Bio-Rad) thermal cycler equipped with an optical module. The duration of the procedure was 40 cycles. The content of cDNA corresponding to transcripts of each of the genes under study was determined using the calibration curves built on the basis of reactions performed with four tenfold dilutions of cDNA. To evaluate the efficiency of the reactions and to build the calibration curves, the CFX Manager Software package (Bio-Rad) was used. The absence of considerable amounts of genomic DNA was confirmed by PCR with the samples that were not subjected to the reverse transcription stage of sample preparation. The relative level of expression was determined by comparing the amount of cDNA of a target gene (*lcc4, tir, chi,* or *exg1*) and the reference gene (*gpd*).

Isolation and purification of the enzymes. To prepare protein extracts, the mycelium or individual morphostructures of *L. edodes* were separated from the cultivation medium, ground mechanically at 18°C with quartz sand in a porcelain mortar to break the cell walls, extracted with 2 mL 20 mM Na-K-phosphate buffer (pH 6.0) per 20 mg wet mycelium for 2 h, centrifuged at 12000 *g* for 15 min, and filtered. The supernatant was salted out with ammonium sulfate to the final concentration of 85% until saturation on an ice bath and centrifuged. The pellet was resuspended in the minimal volume of the buffer.

To separate and purify the enzymes, protein fractions were desalted on a Sephadex G-25 column, then applied to a Sephadex G-75 (Sigma-Aldrich, Sweden) column equilibrated in 20 mM Tris-HCl buffer (pH 7.5). The proteins were eluted at the flow rate of 0.5 mL/min with the same buffer. Further separation of the protein fractions was performed by ion exchange HPLC on a TSK Bioassist Q column equil-

List of constructed primers and TaqMan fluorescent probes

Primers and probes	5'–3' Sequence
gpd F	gACgCACTgACAATCTgACTg
gpd R	gATgCgAgAgTTCAggTTTTAC
gpd Z	(FAM-C)CCTgTCCgAg(T-BHQ-1)CCTTTCCgTgATAC
exg1 F	CggggTAAgTTCTCCTCCAAg
exg1 R	gAACgAgTAggACgAACCATg
exg1 Z	(FAM-C)TgggCg(T BHQ-1)TTgAAgTTggTCCg
chi F	CTTCgggAgCCTATgTAACTgTg
chi R	CCAggCTTTCCAATgATTACTgC
chi Z	(FAM-C)ACTACC(T-BHQ-1)gCgACgggCTCC
lcc4 F	CAggCACCCCAgCAAATgACC
lcc4 R	CggCACgCTgggAgACACAAAg
lcc4 Z	(FAM-C)gTAACCA(T-BHQ-1)CgCACCATCATCATCgggg
tir F	GCCCAGGTTGATCGTCTGCTTTC
tir R	CTGCCAATGTGACCGATACGTCC
tir Z	(FAM-C)ATGGACTA(T-BHQ-1)CCCTCCCGACACTGTAGTTGGAAAG

ibrated in 20 mM Tris-HCl buffer (pH 7.5). The proteins were eluted with a continuous gradient of 0.01 to 1 M NaCl. Protein fractions were detected using an Uvicord S-II (LKB, Sweden) equipment at λ = 280 nm. The fractions exhibiting phenol oxidase activity were dialyzed against water and used in subsequent experiments.

Protein composition was studied by denaturing PAGE in a 7.5% gel [17].

Protein concentration was determined according to Bradford [18].

To visualize the proteins with laccase activity, the gels were specifically stained in the reaction mixture of the following compositions: 1% acetic acid; 0.2% *o*-dianisidine (Sigma, United States); and 50 mM sodium tartrate buffer (pH 4.5) [19]. To stain the gel for tyrosinase activity, 2 mM L-dihydroxyphenylalanine (L-DOPA, Serva, Germany) and 50 mM Tris-HCl buffer (pH 7.5) were used [20].

Phenol oxidase activity determination. Activity of the enzymes was determined on a Specord M40 spectrophotometer (Carl Zeiss, Germany) in quartz cuvettes with 1-cm optical path length at 18°C.

Laccase activity was determined by the rate of oxidation of 0.2 mM 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonate) (ABTS, Sigma, United States) in 50 mM Na-tartrate buffer (pH 4.5) to a stable cation radical measured as an increase in absorption at the wavelength of 436 nm (ε_{436} = 29300 M^{-1} cm^{-1}) [21]. Tyrosinase activity was determined by the rate of oxidation of 2 mM L-DOPA in 50 mM Tris-HCl buffer (pH 7.5) to DOPA-quinone through measuring the increase in absorption at 475 nm (ε_{475} = 3700 M^{-1} cm^{-1}) [20]. Time course of the reaction was 5 min. The amount of enzyme catalyzing the formation of 1 μmol of the product per min was considered an activity unit and expressed in μmol/min/mg protein.

Sample preparation for electron microscopy. For further investigation of ultrathin sections, fungal hyphae were fixed according to the following technique. Mycelial hyphae and morphostructures of *L. edodes* were collected by centrifugation at 13000 *g* for 20 min at room temperature. The material was transferred into 2 mL polypropylene tubes and fixed with 0.5% glutaraldehyde solution for 3 h. Further fixation was performed in a 2.5% glutaraldehyde solution in 0.1 M phosphate buffer (pH 7.2) for 12 h. The material was then treated with 1% OsO_4 solution in the same buffer supplemented with 34 mg/mL sucrose for 4 h.

The samples were dehydrated in a series of alcohol solutions of increasing concentrations and in absolute acetone. Then, the samples were transferred into propylene oxide for 45 min, mixtures of EPON epoxy resin and propylene oxide in 1 : 2, 1 : 1, and 2 : 1 ratios (24 h each), and pure resin. The resin was polymerized at 37, 45, and 57°C (24 h each). Ultrathin sections were prepared on an LKB-III (LKB, Sweden) microtome, mounted on grids, and stained with 2% uranyl acetate and lead citrate solution [22]. Analysis of the ultrastructure of fungal cells at various stages of morphogenesis of *L. edodes* was performed on a Libra 120 (Carl Zeiss, Germany) transmission electron microscope at 120 keV.

Statistical processing of the results. The experiments were performed in three replicates of three independent experiments. Statistical treatment of the data was performed using the standard mathematical approaches (mean square root deviation calculation

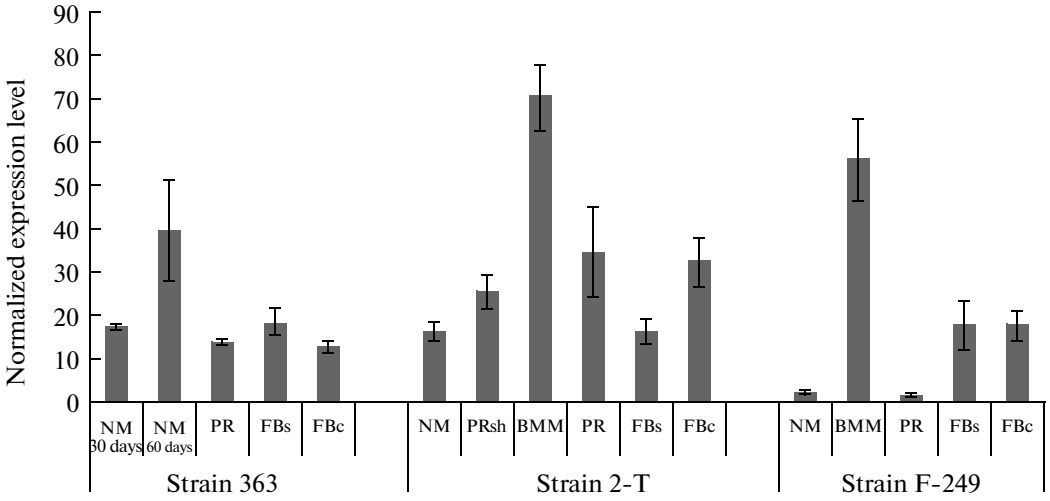

Fig. 1. The level of expression of the laccase gene (*lcc4*) in *L. edodes* strains at various morphogenesis stages. The data are normalized by the transcriptional activity of the *gpd* gene. NM, non-pigmented mycelium (30 and 60 days of cultivation); BMM, brown mycelial mat; PR, primordial; PRsh, shapeless primordia; FBs, fruiting body (stipe); and FBc, fruiting body (cap).

and comparison of the means using Student's *t*-test) using Microsoft Excel 2000 software.

RESULTS AND DISCUSSION

Specific features of morphogenesis in *L. edodes* strains with normal and deficient development. Three *L. edodes* strains were chosen as the objects of study: F-249, 2-T, and 363. F-249 is a reference strain; in the process of morphogenesis it forms the morphostructures typical of practically all shiitake strains: vegetative non-pigmented mycelium, BMM, primordia, and fruiting bodies. In strain 2-T, the normal fructification program is impaired. After substrate colonization and mycelium tightening, the fungus does not form the BMM, but rather shoots out hypertrophied shapeless primordia and forms untypical fruiting bodies without differentiation into cap and stipe [13]. After a while, the nonpigmented mycelium becomes covered with a network of tightly intertwined thick-wall melanized hyphae; BMM is formed, followed by formation of normal primordia and fruiting bodies. Shiitake mushrooms were found to form mature fruiting bodies only after BMM formation. This structure is typical of practically all *L. edodes* strains. Some shiitake strains not forming the BMM were also incapable of fructification [13]. Yet, there is an exception to the rule, i.e. strain 363, which can form normal primordia and fully featured fruiting bodies without the preliminary BMM formation on the non-pigmented mycelium.

Based on this, it seemed reasonable to find out how the genes of the enzymes (laccases, tyrosinases, chitinases, and glucanases) supposedly involved in morphogenesis were expressed at each stage of morphogenesis in the strains with normal and impaired development cycles.

Specific features of laccase gene (*lcc4*) expression. Transcripts of the genes under study (*lcc4, tir, chi*, and *exg1*) were detected at all stages of *L. edodes* morphogenesis, although the level of their expression depended considerably on the stage of development and was particularly different in the BMM, where expression was the highest. At initial stages of development, when vegetative hyphae grew actively, divided, and colonized the substrate, rather high expression of laccase genes was observed (Fig. 1). *L. edodes* belongs to white rot fungi capable of decomposing all components of wood and utilization of the products of decomposition of phenolic compounds as nutrients. This is the very reason why xylotrophs require active enzymes, phenol oxidases. However, upon BMM formation, when the fungus initiated its preparation to fructification, expression of the *lcc4* gene encoding laccase increased sharply (20-fold) in strain F-249, unlike strain 2-T, in which the level of *lcc4* expression was the same at the stage of non-pigmented mycelium and upon formation of shapeless primordia. The absence of activation of laccase gene expression was probably one of the reasons why fully featured fruiting bodies failed to form in these cases. Subsequently (during BMM formation), a 3.5-fold increase in expression of the *lcc4* gene was observed in this strain. In primordia and fruiting bodies of all strains, a decline in laccase activity compared to the BMM was noted. The highest expression of *lcc4* in strain 363 occurred at the stage of non-pigmented dense mycelium, immediately before basidiome formation.

Changes in laccase activity and rearrangement of the enzyme complex at various stages of morphogenesis in *L. edodes* strains. PCR analysis results were confirmed by biochemical tests. Using native electrophoresis and specific phenol oxidase staining we found that the fungus produced a complex of laccases

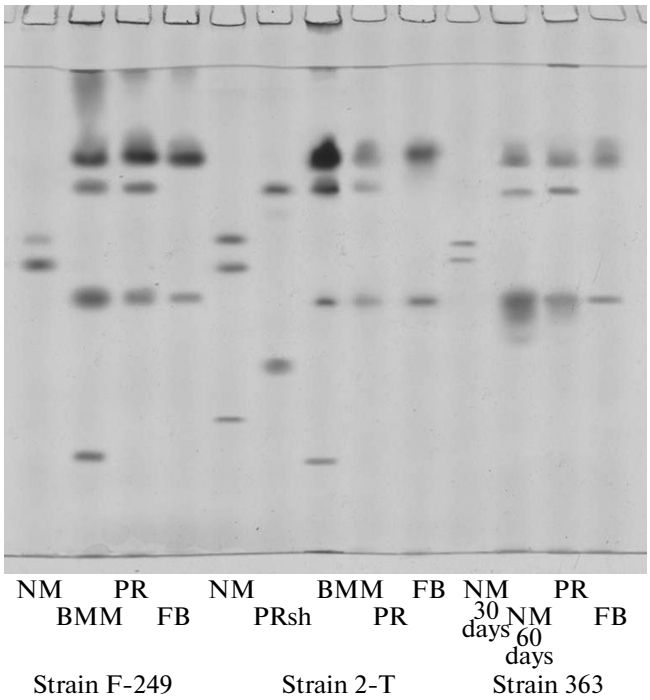

Fig. 2. Native electrophoresis and specific staining of laccases in *L. edodes* strains at various morphogenesis stages. See Figure 1 caption designations.

throughout its life cycle (Fig. 2). Differences in the composition of the phenol oxidase complex were observed between different stages of development in various strains; the dynamics of enzymatic activity also differed considerably. The first laccase activity peak occurred in the period of active substrate colonization; then, the activity decreased (Fig. 3). With mycelium pigmentation, enzymatic activity resumed, and the second peak occurred in the period of BMM formation. This agrees with some literature data, which evidence a link between the increase in laccase activity of some fungi and melanin synthesis and pigment formation in the structures which were denser than the simple mycelial aggregate [5, 6]. In strain 363, the stage of dense non-pigmented mycelium prior to formation of primordia was characterized by certain similarity with the BMM stage in F-249 by the molecular weight and activity of laccases. Strain 2-T was characterized by low laccase activity in both white mycelium and shapeless primordia. Apparently, formation of a fruiting body is accompanied by the activity of phenol oxidases, is associated with the formation of extracellular pigments, and occurs in parallel with oxidative polymerization of cell wall components, which promotes intercellular junction.

Involvement of laccases in the process of morphogenesis of the *L. edodes* basidiomycete. It should be noted that laccases isolated and purified at the BMM stage of the F-249 strain upon application onto the surface of growing non-pigmented mycelium initiated the formation of a mycelial mat in all shiitake strains under study. The transition from one stage to another is probably regulated by the presence or change in the activity of laccases or of the products formed by these enzymes and acting as specific marker molecules.

Changes in the expression of *tir* gene, activity, and enzymatic composition of tyrosinases depending on the developmental stage of *L. edodes* strains. Activity of tyrosinase increased together with mycelium pigmentation and exhibited maxima at the stages of BMM and fruiting bodies, the highly melanized structures of the fungus (Fig. 4). At these stages, new forms of tyrosinases not detected in other morphological structures were also revealed. The results of electrophoresis followed by specific staining with the L-DOPA substrate for strain F-249 are presented in Fig. 5. In the case of BMM and fruiting body, there were considerable differences in the composition of the enzymatic complex compared to other stages of development. In strains 2-T and 363, the number of tyrosinase forms at various stages of morphogenesis was similar to that in F-249 strain. Shapeless primordia of strain 2-T and dense white mycelium of strain 363 had a single tyrosinase each. This agrees with PCR analysis data: the level of *tir* gene expression was the highest at the most pigmented stages of the fungus development, brown mat and fruiting body (Fig. 6).

This is easily explained since tyrosinase is involved in the synthesis of melanin pigments, which play an important role in resistance of the fungus to environmental impacts. Increased concentration of the pigments is noted in dense structures, which can carry out

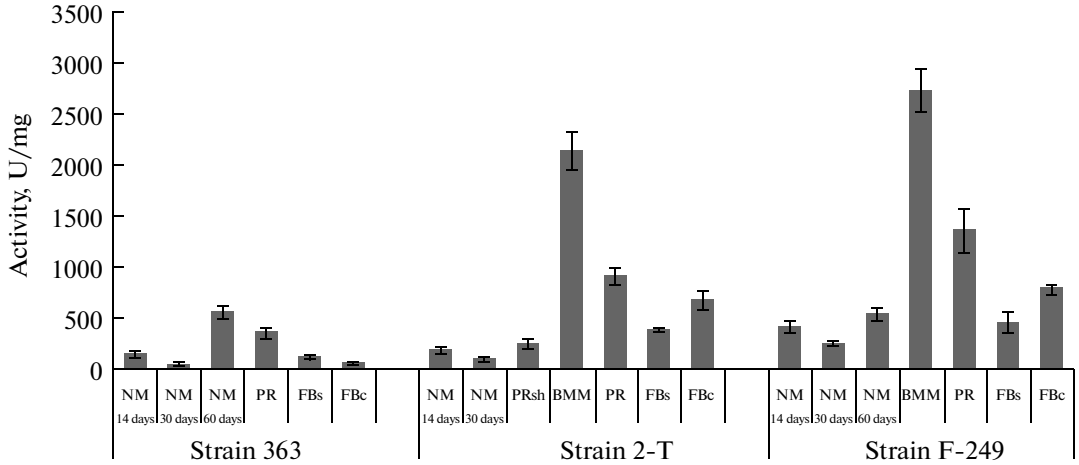

Fig. 3. Dynamics of laccase activity in *L. edodes* strains at various morphogenesis stages. See Figure 1 caption for designations.

a protective function in the beginning of fruiting body formation under unfavorable environmental conditions. Melanin formation is supposed to provide for resistance to UV and visible light. The presence of melanin also produces a strong effect on the stability of the chitin–glucan–melanin complex to the effect of chitinase and 1,3-β-glucanase. The pigments may be organized in individual granules on the outer surface of the hyphae or inside the cell wall forming a complex with chitin, which results in an outer barrier exhibiting high stability to the enzymes of pathogens [12, 23].

Therefore, transition from the vegetative stage of development to basidiome formation is accompanied by an increase in laccase and tyrosinase activity, as well as by a rearrangement of the oxidase complex, which is associated with the diverse processes these enzymes are involved in. Phenol oxidases catalyze synthesis of melanins, which perform a protective function and are also required for fastening of mycelial cell wall components during formation of the morphological structures.

Expression of glucanase and chitinase genes (*chi* and *exg1*) necessary for cell wall rearrangement in the course of morphogenesis. Chitin-containing organisms produce chitinases necessary for morphogenesis of the cell wall, which is one of the most important structures determining the direction of morphogenesis [11, 24]. A cell wall is a complex, biochemically and morphologically dynamic structure; changes in its composition accompany cell differentiation and are of a certain functional importance. During the periods of vegetative growth and space colonization, it promotes cell protection from unfavorable conditions and pathogens, while in idiophase it is involved in formation of morphological structures and basidiomes [12, 25]. The cell wall of basidiomycetes has a high content of chitin forming complexes with β-glucans [11, 12]. For example, in basidiomes of fungi of the genus *Agaricus*, chitin fibrils embedded in the β-glucan matrix make up over 80% of the cell wall [26]. Study of the biosynthesis of various components of the fungal cell wall proves that chitin, glucans, and glycoproteins are tightly bound by covalent bonds, and the interactions between them are dynamic processes [27].

Here we found that for all strains the level of the *chi* gene expression was the highest at the stages of BMM and primordia, while the type strain F-249 exhibited higher *chi* expression also at the stage of the fruiting body (Fig. 7). The model of fungal cell wall growth takes into account the importance of lytic enzymes in the maintenance of balance between synthesis and lysis (simultaneous operation of chitin synthases and chitinases) of the cell wall during apical growth of the mycelium [24]. Chitinases are assumed to be involved in the process of division and branching of mycelial hyphae [25]. In basidiomes and dense mycelial formations (mats and sclerotia), there is much more chitin than in a vegetatively growing submerged mycelium, which, judging by the experimental data, leads to increased synthesis of chitinases necessary for the constant rearrangement of the cell wall.

These data were confirmed by electron microscopy. In contrast to thin-walled hyphae of the vegetative mycelium, the cell wall was strongly thickened in the BMM; the electron-dense layer composed mainly of chitin fibrils was very pronounced. At the stage of primordia, there are many supporting hyphae with very thick cell walls reaching 2 μm and possessing a characteristic spotty texture (Fig. 8).

The overwhelming majority of fungi synthesize extracellular and cell wall-associated glucanases. Along with chitin, glucans are the predominant cell wall polysaccharides and, presumably, define the shape and rigidity of the walls of fungal hyphae [9, 10]. Glucanases are involved in continuous rearrangement of glucans of the cell wall in the process of mycelium growth; for example, in the *Coprinus cinereus* basidio-

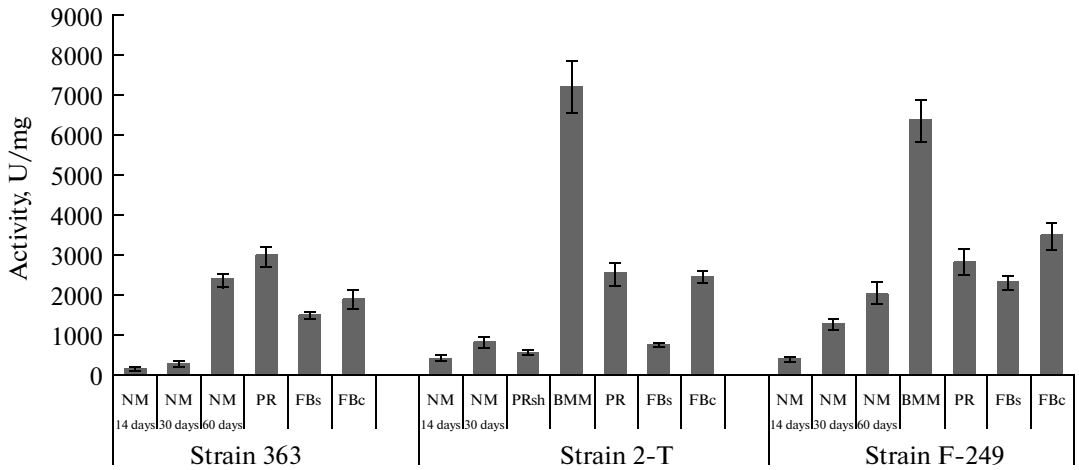

Fig. 4. Dynamics of tyrosinase activity in *L. edodes* strains at various morphogenesis stages. See Figure 1 caption for designations.

mycete endo-β-glucanases play an important role in stretching of the stipe fibers [28].

According to our data, the level of expression of the glucanase gene *exg1*, as well as of the chitinase gene *chi*, was the highest at the stages of BMM, primordia, and fruiting bodies, in contrast to the nonpigmented vegetative mycelium (Fig. 9). The above-listed facts evidence the importance of intracellular glucanases and chitinases in cell rearrangements, particularly in the processes of morphogenesis. These enzymes act directly upon polymers of the cell wall represented by

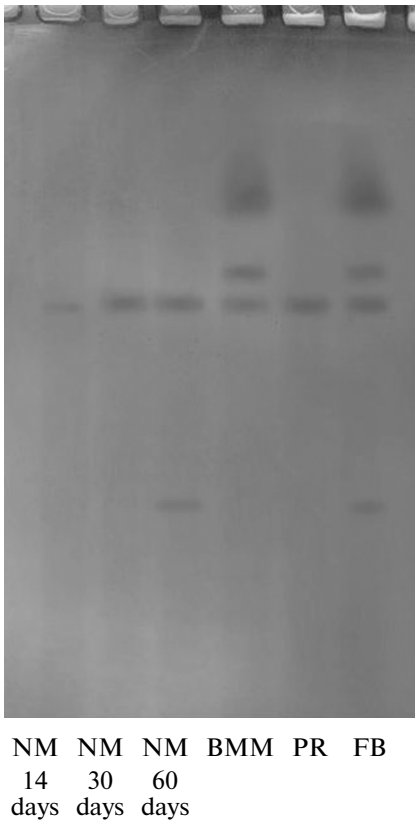

Fig. 5. Native electrophoresis and specific staining of tyrosinases in *L. edodes* F-249 strain at various morphogenesis stages. See Figure 1 caption for designations.

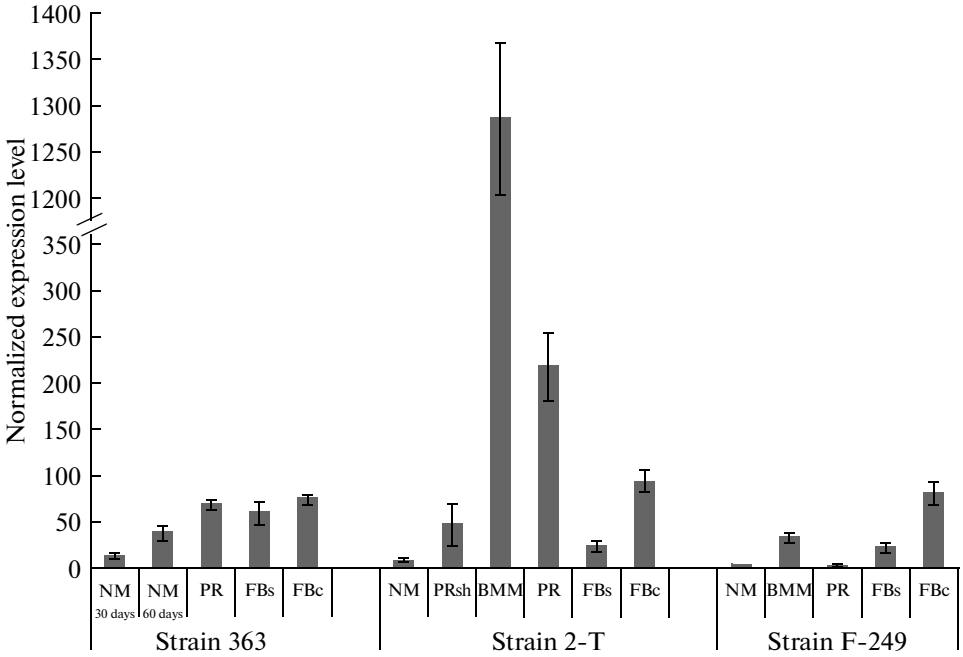

Fig. 6. The level of expression of the tyrosinase gene (*tir*) in *L. edodes* strains at various morphogenesis stages. The data are normalized by the transcriptional activity of the *gpd* gene. See Figure 1 caption for designations.

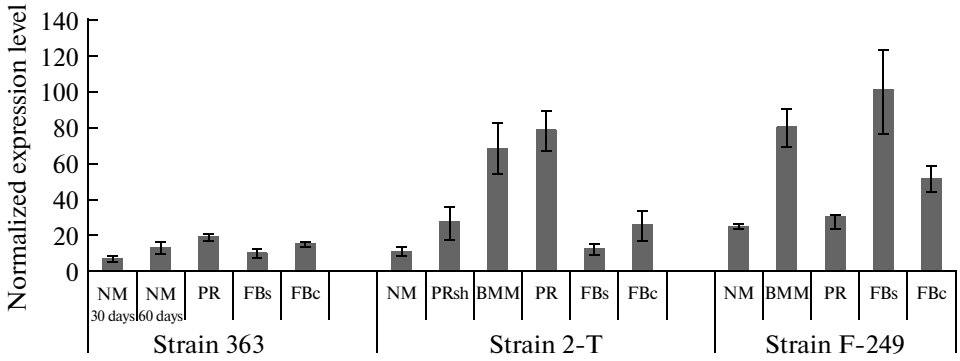

Fig. 7. The level of expression of the chitinase gene (*chi*) in *L. edodes* strains at various morphogenesis stages. The data are normalized by the transcriptional activity of the *gpd* gene. See Figure 1 caption for designations.

β-1,3-glucans linked with chitin, another major component, and can provide for rigidity or flexibility of the cell wall in the processes of apical growth, branching, tightening, stretching, and fusion of fungal hyphae, which are necessary for generation of the macromycete morphological structures.

Therefore, constant presence of laccases, tyrosinases, glucanases, and chitinases and the dynamics of their activity depending on the morphogenesis stage is a specific feature of the fungus and evidences the multifunctionality of these proteins in the process of development. Expression of the *lcc4, tir, chi*, and *exg1* genes was the highest in the BMM. Moreover, active phenol oxidases specific for this morphological structure have been revealed in it and not in the other developmental stages of the fungus. Obviously, the BMM not only protects the fungus, but is also a biochemically active structure. Emergence of certain functional proteins and an increase in their activity prior to the fructification stage evidences their importance for initiation and formation of *L. edodes* basidiome. These results evidence the multifunctional role of phenol oxidases, glucanases, and chitinases in the process of development of a xylotrophic basidiomycete *L. edodes*, indicating the importance of these enzymes not only in substrate colonization, but also in morpho-

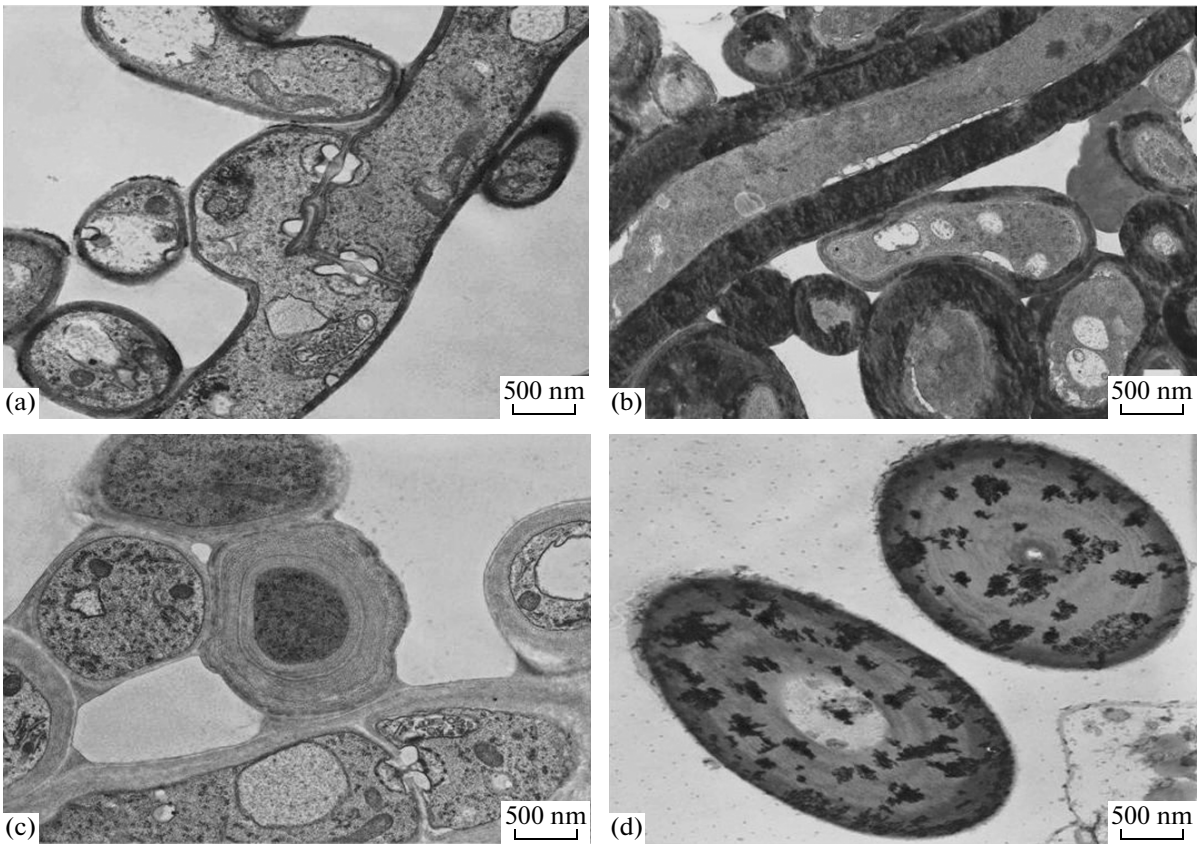

Fig. 8. Transmission electron microscopy of lateral and transverse cross-sections of the mycelial hyphae of *L. edodes* strain F-249. Ultrastructure of the non-pigmented mycelium cells (a); thickened melanized cell walls of the mycelial mat (b); thick-wall supporting hyphae of primordia (c); and thick-wall melanized cells of the fruiting body (stipe) (d). Scale bar is 500 nm.

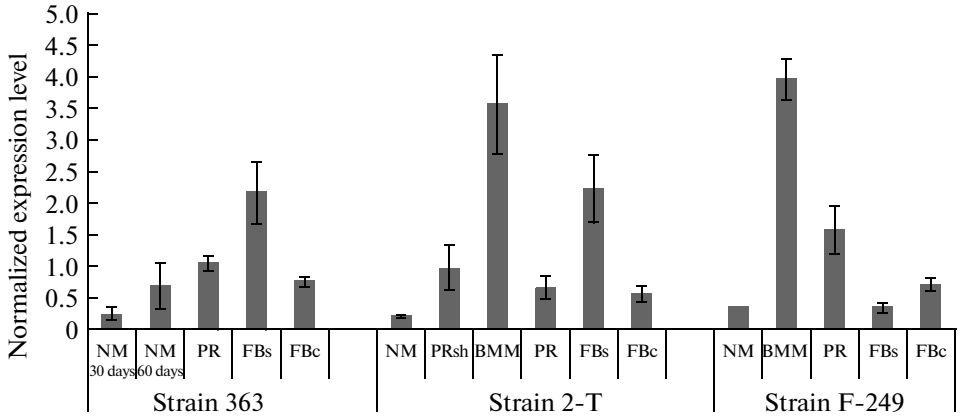

Fig. 9. The level of expression of the glucanase gene (*exg1*) in *L. edodes* strains at various morphogenesis stages. The data are normalized by the transcriptional activity of the *gpd* gene. See Figure 1 caption for designations.

genesis progression from one stage to another, which suggests the hypothesis that these enzymes or their products are involved in regulation of the process of the fungus development. Activation of *lcc4, tir, chi*, and *exg1* gene expression may probably be a marker for the fungus readiness to fructification. The observed positive effect of laccases on acceleration of the fructification process can be used and taken into consideration in the course of development and optimization of cultivation technology of the shiitake mushroom, which is of interest due to its nutritive value and exceptional medicinal properties.

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