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SUMMARY OF DOCTORAL THESIS

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Title: Evolution of multigene families for lignin degradation in *Pholiota microspora*

（*Pholiota microspora*でのリグニン分解のための多重遺伝子ファミリーの進化）

Lignin is a complex aromatic polymers, amorphous and important in the formation of higher plant cell walls. It enables trees to grow taller and compete for sunshine. Wood and other vascular tissues generally are 20-30% lignin and forming a matrix that surrounds the orderly cellulose microfibrils. Lignin physically protects most of the world's cellulose and hemicelluloses from enzymatic hydrolysis. In which fungi play the major role in lignin biodegradation. The white rot fungi capable of extensive aerobic lignin biodegradation. This property is based on the white rot fungi capability to produce one or more extracellular lignin modifying enzyme (LME). The white rot fungi produce extracellular enzymes that break down the woody cell wall. The lignin modifying enzymes, such as manganese peroxidase (MnP), lignin peroxidase (LiP) and laccase (Lcc) are directly involved in the degradation of lignin in their natural lignocellulosic substrates. Some white rot fungi produce all three LMEs while others produce only one or two of them.

The wood-rotting basidiomycete *Pholiota microspora* (also known as *P. nameko* or "nameko," the common name in Japanese). Nameko mushroom is one of the most popular edible mushroom and is widely cultivated in Japan. It is sold in local markets for food ingredient in Japan and is widely used as material in fungal research. In order to understand the lignin degradation system in *P. microspora*, we identify lignin degrading genes that belong to LME. Initially, the deduced amino acid sequence of known MnP, LiP and laccases of different basidiomycetes such as *Phanerochaete chrysosporium* and *Trametes versicolor* from public databases were subjected to BLASTP searches of the *P. microspora* genome. *LiP* gene was not detected by this inquiry. It suggests that *LiP* absence in *P. microspora*, also, Xu and colleague (2013) reported that the enzyme activity of LiP was not detected in their experiment. Therefore, the result obtained, *P. microspora* provides five *MnP* and nine laccase genes in their genome.

Laccase is belong to phenol oxidases (PO) that are enzymes containing copper atoms in the catalytic centre and are usually called multicopper oxidases. The catalytic activity of these enzymes is oxidation of diphenols to the corresponding quinones. Not only laccase is PO, but also includes tyrosinases. It is obvious that the laccases are very ancient enzymes from an evolutionary of view. However, fungal laccase were discussed on the role in lignin degradation. Laccases can degrade lignin in the absence of LiP and MnP. But laccase

performed browning process in *Lentinula edodes*. Moreover, laccase activity is strongly regulated and promoted fruiting body development in *Agaricus bisporus* and *Schizophyllum commune*.

To estimate physiological roles of MnP and laccase multigene families in *P. microspora* which were five and nine genes, respectively. We conducted quantitative RT-PCR (qRT-PCR) technique to study the transcriptional level of genes in *P. microspora*. Firstly, to understand differential gene expression in different stages during mushroom development. Total RNA were extracted and quantified from mycelia grown in sawdust substrate, primordia and fruiting bodies. Secondly, to understand the substrate specificity for each gene. *P. microspora* was grown in M4 liquid medium containing various aromatic compounds, which were lignin related compounds. Total RNA were extracted and quantified from mycelia to investigate transcriptional level of MnP, laccase and related genes. Thirdly, nucleotide and deduced amino acid sequences were analysis. Number and position of introns were inspected. Phylogenetic trees were generated and analyzed of evolutionary relationship across basidiomycetous fungi.

The results obtained, firstly, to analyse lignin degrading genes. Five *MnP* genes were identified. Nucleotide and amino acid sequences were analyzed intron-exon position and phylogenetic relationship, respectively. *PnMnP5*, *3, 2* and *4* were clustered tightly, but *PnMnP1* was clustered relatively far from *MnP5*. Moreover, qRT-PCR unveiled that *PnMnP5* gene only that was strongly transcribed, 15-fold higher expression than other *MnPs* in M4 liquid medium. While transcription of *PnMnP5* in sawdust medium was 100 times higher than in M4 liquid medium. Therefore, the results indicate that *PnMnP5* plays a major role in the ligninolytic peroxidase reaction during mycelial growth in *P. microspora*. Based on a comparison of the position of introns, the phylogenetic relationships among *PnMnPs* and the predominant expression of *PnMnP5*, we believe that all *PnMnPs* are of the same origin and that they were amplified by duplication events in the ancient *P. microspora* genome.

Secondly, to estimate the physiological role of phenol oxidase. We analyzed nucleotide sequences of phenol oxidase genes; nine laccases and a tyrosinase. The expression of *Lcc1* to *Lcc9* and *Tyr* genes in *P. microspora* was examined by qRT-PCR. We quantified transcripts of these ten genes in mycelia, primordia, and fruiting bodies grown on sawdust substrate and in mycelia grown in M4 liquid medium supplemented with aromatic compounds. All *Lcc* genes were expressed at a very low level in mycelia grown on sawdust medium, but *Lcc1* was transcribed at a level 8-fold higher in M4 liquid medium when supplemented with 3 mM veratryl alcohol. On the other hand, *Lcc9* and tyrosinase were highly expressed in primordia and fruiting bodies. These results suggest that the content of melanin and related pigments in the fruiting body might be determined by complementary activity of two types of phenol oxidase, such as *Lcc* and *Tyr*, in *P. microspora*.

Final conclusion, we investigated the possible physiological role of manganese peroxidase and phenol oxidase expression in *P. microspora* at the transcriptional level. Manganese peroxidase is required for lignin degradation in mycelia during growth on sawdust medium, but phenol oxidase including laccase and tyrosinse are required for related pigment synthesis in the fruiting body of *P. microspora*.