



A comparative genomic analysis of the oxidative enzymes potentially involved in lignin degradation by *Agaricus bisporus*



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ARTICLE INFO

Article history:

Available online 10 April 2013

Keywords:

Agaricus bisporus

Oxidase

Peroxidase

Cytochrome P450 monooxygenase

Comparative genomics

Litter-rot fungi

ABSTRACT

The oxidative enzymatic machinery for degradation of organic substrates in *Agaricus bisporus* (Ab) is at the core of the carbon recycling mechanisms in this fungus. To date, 156 genes have been tentatively identified as part of this oxidative enzymatic machinery, which includes 26 peroxidase encoding genes, nine copper radical oxidase [including three putative glyoxal oxidase-encoding genes (GLXs)], 12 laccases sensu stricto and 109 cytochrome P450 monooxygenases. Comparative analyses of these enzymes in Ab with those of the white-rot fungus, *Phanerochaete chrysosporium*, the brown-rot fungus, *Postia placenta*, the coprophilic litter fungus, *Coprinopsis cinerea* and the ectomycorrhizal fungus, *Laccaria bicolor*, revealed enzyme diversity consistent with adaptation to substrates rich in humic substances and partially degraded plant material. For instance, relative to wood decay fungi, Ab cytochrome P450 genes were less numerous (109 gene models), distributed among distinctive families, and lacked extensive duplication and clustering. Viewed together with P450 transcript accumulation patterns in three tested growth conditions, these observations were consistent with the unique Ab lifestyle. Based on tandem gene arrangements, a certain degree of gene duplication seems to have occurred in this fungus in the copper radical oxidase (CRO) and the laccase gene families. In Ab, high transcript levels and regulation of the heme-thiolate peroxidases, two manganese peroxidases and the three GLX-like genes are likely in response to complex natural substrates, including lignocellulose and its derivatives, thereby suggesting an important role in lignin degradation. On the other hand, the expression patterns of the related CROs suggest a developmental role in this fungus. Based on these observations, a brief comparative genomic overview of the Ab oxidative enzyme machinery is presented.

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1. Introduction

Lignocellulose, the most abundant biopolymer on earth, and its degradation is an essential component of the global carbon cycle. This substrate is composed of lignin, hemicellulose and cellulose and is degraded in nature by fungi, most efficiently by basidiomycetous fungi. Based on patterns of decay, these fungi are often classified as white-rots, brown-rots or litter-rots (Eriksson, 1990; Floudas et al., 2012; Kirk and Farrell, 1987; Martinez et al., 2005). The white-rot fungi have the enzymatic machinery required to completely breakdown all plant cell wall components including cellulose, hemicelluloses and the more recalcitrant lignin. Brown-rot fungi possess the capacity for rapid and efficient cellulose depoly-

merization through the action of highly reactive hydroxyl radicals, while leaving lignin as a substantially modified residue (Martinez et al., 2011; Yelle et al., 2008). In contrast to these wood decay fungi, litter decomposers (litter-rots) of the Agaricomycotina degrade cellulose via hydrolytic mechanisms. The ability to remove or modify lignin has been found in some litter decomposing basidiomycete fungi; although genome-based evidence is still lacking (Steffen et al., 2000). This variation in decay patterns is likely due to the distinctive nature of the oxidative systems present in these fungi.

The oxidative machinery generally attributed to ligninolytic fungi includes peroxidases, glyoxal oxidases (GLX), copper radical oxidases (CRO), laccases and cytochrome P450 monooxygenases (P450s). The peroxidases, belonging to the class II type, are heme-containing proteins and are typically secreted enzymes. They include lignin peroxidases (LiPs), manganese peroxidases (MnPs), and versatile peroxidases (VPs) (Hatakka, 1994; Hofrichter et al., 2010). Heme-thiolate peroxidases (HTPs) are a different class of peroxidase enzymes, which catalyze the transfer of peroxide-oxygen to substrate molecules (Hofrichter et al., 2010). Another

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class of heme-peroxidases, dye-decolorizing peroxidases (DyPs, Kim and Shoda, 1999; Zubieta et al., 2007), are capable of oxidizing various anthraquinone derivatives and phenolic dyes (Sugano et al., 2009).

The common feature of these peroxidases is the requirement of H_2O_2 as the electron acceptor. Generation of extracellular H_2O_2 likely involves CROs, of which GLX has been most intensively studied (Hammel et al., 1994; Leuthner et al., 2005; Whittaker et al., 1996). Catalytically distinct from CROs, laccases are also produced by most, but not all, wood decay fungi (Cullen, 1997). Finally, cytochrome P450 enzymes are a superfamily of heme-thiolate proteins that are involved in a wide range of catalytic reactions, most important of which is the hydroxylation of substrates (Anzenbacher and Anzenbacherova, 2001; Ichinose, 2012). These enzymes are widely distributed in both prokaryotic and eukaryotic organisms (more than 11,000 P450 enzymes identified) and have highly diversified during the course of evolution (Nelson, 2009).

Information about the organisms and the mechanisms involved in degradation of lignocellulose has recently grown, especially with the published genome analyses for basidiomycetous fungi, such as *Phanerochaete chrysosporium* (Pc), *Postia placenta* (Pp), *Schizophyllum commune* (Sc), *Serpula lacrymans*, and *Coprinopsis cinerea* (Cc, Eastwood et al., 2011; Martinez et al., 2009, 2004; Ohm et al., 2010; Stajich et al., 2010). Genome analysis of several wood decay fungi was recently reported (Fernandez-Fueyo et al., 2012; Floudas et al., 2012). In contrast to these wood decayers, there is a dearth of similar information regarding those fungi that are involved in metabolizing partially degraded organic and humic substances; thus considered as secondary, litter-decomposing fungi. Augmenting the analysis of *Agaricus bisporus* (Ab) class II peroxidases, HTPs and laccases (Morin et al., 2012), here we present detailed genome-wide analysis of oxidase families, P450s and CROs. Based on comparisons with other sequenced Agaricomycotina and gene expression profiles, a brief overview of the Ab oxidative enzyme machinery is presented.

2. Materials and methods

The oxidase enzymes belonging to the basidiomycete fungus, Ab var. *bisporus* namely LiPs, MnPs, VPs, HTPs, DyPs, GLXs and CROs were identified previously (Morin et al., 2012). The classification of peroxidases has been performed in line with earlier publications (Hofrichter et al., 2010; Liers et al., 2012; Ruiz-Duenas et al., 2009). The mRNA-to-genomic DNA alignment program, Spidey, was used to predict the intron–exon junctions. Transcript levels were determined using microarray data derived from mycelium grown in casing, in compost, and in fruiting bodies. A detailed description of the methods used for growth of the fungus Ab (commercial strain A15), RNA extraction and microarray analyses can be found in the main genome paper (Morin et al., 2012). Cluster analyses presented in this study was generated using the MultiExperiment viewer (MEV) software (Saeed et al., 2003).

A total of 125 predicted cytochrome P450 DNA and protein sequences were downloaded from the Joint Genome Institute's (JGI) website (http://genome.jgi.doe.gov/Agabi_varbisH97_2/Agabi_varbisH97_2.home.html) using the search word “P450”. These sequences were next scanned for two conserved cytochrome P450 protein domains, namely the ERR triad motif (EXXR) and the oxygen binding motif (CXG) to identify a subset of 114 P450 proteins. Of these, five proteins were later identified as non-P450 proteins using BLAST search and hence were not included in the subsequent analyses. The remaining 109 proteins were used for family/clan classification and phylogenetic tree construction as described previously (Doddapaneni et al., 2005). The P450 proteins were assigned family/clan names based on protein homology to other

known fungal P450 proteins. The mRNA-to-genomic DNA alignment program Spidey was used to predict the intron–exon junctions. Comparative analyses was performed for Ab var *bisporus* (H97) strain, Pp, Pc, Cc and *Laccaria bicolor* (Lb) P450 sequences using BLAST and results were compared using custom perl scripts. All publicly accessible fungal genomes, including those mentioned here, are available through the JGI's interactive MycoCosm web portal (<http://genome.jgi.doe.gov/programs/fungi/index.jsf>).

3. Results and discussion

3.1. Cytochrome P450 proteins and their redox partners

In fungi, the P450 enzymes play important roles in secondary metabolic pathways. In particular, P450s in basidiomycetous fungi are known to be involved in the breakdown of various xenobiotics and lignin metabolites released by the action of extracellular peroxidases (Ide et al., 2012; Kullman and Matsumura, 1996; Subramanian and Yadav, 2009; Sutherland et al., 1991; Syed et al., 2010).

3.1.1. Gene characteristics, classification, phylogenetic relationships and comparative genomics

The Ab genome has 109 P450 genes varying in length from 0.5 to 4.1 kb with introns in individual genes ranging from two in gw1.10.977.1 (Protein ID 56008) to 23 introns in fgenesh2_pg.6_#_267 (Protein ID 143643). Based on the established criterion for grouping of cytochrome P450 enzymes (>40% sequence similarity being classified as members of a family), the 109 P450 sequences fall under 38 CYP families. Of these, 87 proteins are grouped into 16 families of 2–19 P450s and the remaining 22 are assigned as single member families. Additionally, 105 of these P450 proteins were further classified into 8 fungal clans namely CYP51 (1 protein), CYP52 (6 proteins), CYP53 (3 proteins), CYP61 (1 protein), CYP64 (51 proteins), CYP503 (18 proteins), CYP547 (19 proteins) and CYP614/534 (6 proteins) (Fig. 1).

Genome scaffolds 2 and 12 have the highest concentration of P450s (12 and 14, respectively) while the rest of the P450s are scattered across 17 other scaffolds (Figs. 1 and 2). There are 6 genomic clusters on 6 different scaffolds (Figs. 1 and 2). Each cluster contains 5–7 P450 genes within a 18–58 Kb region. In total, there are 34 clustered genes: scaffold 2 (7 genes spanning 58.5 Kb), scaffold 3 (5 genes spanning 18.2 Kb), scaffold 7 (5 genes, spanning 51.6 Kb), scaffold 12 (6 genes, spanning 29.4 Kb), scaffold 15 (6 genes spanning 45.2 Kb) and scaffold 16 (5 genes spanning 53.6 Kb) (Figs. 1 and 2). With the exception of one gene (GeneMark.9468_g), members of individual clusters belong to the same family. Of the gene clusters only 4 genes on scaffolds 2, 3 genes on scaffold 3 and 2 genes each on the rest of the 4 scaffolds are tandem repeats (total 15 genes). There are two striking examples of gene duplication events (Protein IDs 211278 and 195991, 179058 and 225715), where proteins are not only more than 90% identical at the amino acid level, but their 5' and the 3' UTR sequences (100 bp upstream and downstream) were highly conserved (~90%).

Comparative analyses of P450s in Ab with those of the white-rot, Pc, brown rot, Pp, the other litter fungus, Cc and its closest phylogenetic relative and ectomycorrhizal fungus, Lb revealed enzyme diversity that is indicative of both lifestyle adaptation as well as phylogenetic relevance. The P450omes of Pp and Pc contain 254 genes and 152 genes, respectively, whereas Ab contains only 109 P450 genes (Martinez et al., 2009, 2004).

Ab features a unique protein (ID 143643, Gene model fgenesh2_pg.6_#_267) that is 1021 aa long with 23 introns and is not grouped under the previously known clans in white-rots,

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