Section 2
Saprotrophic Fungi

3 Wood Decay

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Introduction

A significant portion of global carbon is sequestered in forest systems. Specialized fungi have evolved to efficiently deconstruct woody plant cell walls. These important decay processes generate litter, soil bound humic substances, or carbon dioxide and water. This chapter reviews the enzymology and molecular genetics of wood decay fungi, most of which are members of the Agaricomycotina subphylum. This chapter emphasizes recent advances derived from a growing number of genome resources but otherwise directs interested readers to previously published reviews for additional information. Along these lines, background on wood cell wall polymer chemistry and the oxidative systems involved in their depolymerization have been extensively reviewed (Eriksson, Blanchette, et al., 1990; Cullen & Kersten, 2004).

Wood Composition and the Challenges Posed as Substrate

Cellulose, a linear polymer of anhydrocellobiose units linked by β-1, 4-glycosidic bonds, constitutes approximately 40 percent of the weight of wood. Through Van der Waals forces and hydrogen bonding, individual cellulose molecules are arrayed into microfibrils, each of which contains approximately 40 cellulose chains. Regions along the cellulose microfibrils are highly ordered and crystalline in diffraction measurements. In the primary cell wall, fibrils appear randomly oriented within a matrix of xyloglucan and pectic substances at the cell surface. In the S2 layer of the secondary wall (the bulk of wood weight) cellulose microfibrils are organized approximately parallel to the cell long axis. The cellulose microfibrils appear embedded in a matrix of hemicelluloses and lignin.

Making up 25 to 30 percent of wood weight, hemicelluloses are linear β-1,4-linked monosaccharide polymers with limited branching consisting of mono-, di-, or trisaccharides. Branches can be sugars, sugar acids and acetylated sugars, and sugar acid esters. In the major hemicellulose of hardwoods,

The Ecological Genomics of Fungi, First Edition. Edited by Francis Martin.

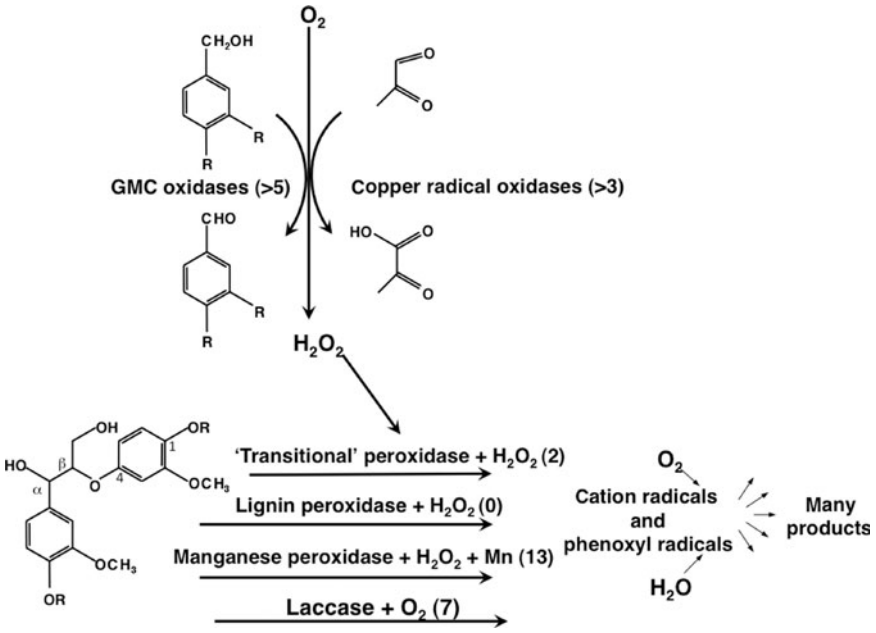

Figure 3.1 Schematic representation of extracellular processes involved in lignin degradation by the white rot fungus *Ceriporiopsis subvermispora*. The model emphasizes the central role of peroxide. For each enzyme class, the predicted gene number is shown in parentheses (Fernandez-Fueyo, Ruiz-Dueñas, et al., 2012).

O-acetyl-4-O-methylglucuronoxylan, approximately 7 of 10 xylosyl residues are acetylated, and about every tenth contains α-glucoronic acid. The same basic structure occurs in conifers, but without acetyl groups, with more glucuronic acid residues, and with α-arabinose residues on about every eighth xylosylresidue.Amajorhemicelluloseofconifers,O-acetylgalactoglucomannan, contains galactose-to-glucose-to-mannose at a ratio of approximately 1:1:3. Lignin is covalently bonded via infrequent linkages to the hemicelluloses.

The third major component of wood, lignin, is comprised of carbon-carbon and ether bonds between phenylpropanoid residues. Formed through free radical-induced polymerization of monolignols *p*-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol, the structures are often referred to as *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) subunits, respectively (Ralph, Lundquist, et al., 2004). Proportions vary between species. Softwoods generally tend toward G-lignins with little or no H and S units, whereas hardwoods contain varying ratios of G/S lignins. Often referred to as β-O-4 linkage, the ether substructure shown (Fig. 3.1) is representative of the major linkage (about 90 percent) in lignins. A consequence of such ether bonds is that degradation involves oxidative mechanisms, as opposed to hydrolytic mechanisms.

Lignin polymers are stereoirregular and insoluble, properties that present significant experimental challenges. Model compounds, particularly those mimicking non-phenolic interunit linkages (*see* Fig. 3.1), have proven especially useful for characterizing high-oxidation potential enzymes. However, it should be noted that Cα-Cβ cleavage of such compounds, by itself, does not address the question of lignin depolymerization. Use of commercially available lignin as a substrate can be similarly misleading because the product typically contains various contaminants, and the lignin is partially depolymerized and modified (e.g., sulfonated). Compelling evidence for lignin degradation is gained from direct measurements of lignin mineralization in wood or metabolism of synthetic lignins. No microbe has been convincingly shown to use lignin as sole carbon source.

General Characteristics of Wood Decay Fungi

The principal wood-decay fungi lie within the Agaricomycetes (Basidiomycota), although representatives from other groups of Basidiomycota and Ascomycota have been documented (Eriksson, Blanchette, et al., 1990). Two broad categories of wood decay are recognized; white rot and brown rot. Phylogenetic and comparative genomic studies support the view that white rot is plesiomorphic in Agaricomycetes and that brown rot has evolved repeatedly (Hibbett & Donoghue, 2001; Floudas, Binder, et al., 2012). As of this date, genome analyses of 17 wood decay fungi have been published (Table 3.1).

Only white rot Basidiomycetes have been convincingly shown to efficiently mineralize lignin. This unique ability to completely degrade lignin is generally viewed as a strategy to gain access to carbohydrate polymers of plant cell walls for use as carbon and energy sources. Decay patterns may differ substantially among white rot species and strains (Eriksson, Blanchette, et al., 1990; Blanchette, 1991; Daniel, 1994), and two gross morphologies are recognized: simultaneous decay of cellulose, hemicelluloses, and lignin; and selective delignification, in which lignin and hemicelluloses are removed more rapidly than the cellulose. During simultaneous decay, erosion troughs appear beneath hyphae, the cell walls become gradually thinner, and holes appear between cells as decay advances. In contrast, cell walls retain their morphology during selective ligninolysis. Simultaneous and selective white rot fungi are exemplified by *Phanerochaete chrysosporium* and *Ceriporiopsis subvermispora*, respectively.

Brown rot fungi modify but do not remove bulk lignin. Instead, the lignin remains as a polymeric residue following removal of cellulose and hemicellulose (Blanchette, 1995; Worrall, Anagnost, et al., 1997; Niemenmaa, Uusi-Rauva, et al., 2007; Yelle, Ralph, et al., 2008). Brown rot residues resist further decay and contribute to the carbon pool in humic soils. Early in decay,

Table 3.1 Wood decay fungi with published genomes; taxonomic order, number of genes, and secreted proteins.[a]

White rot	Order	LiP	MnP	VP[b]	Lac	CROs	GLX	CDH	ALE	P450	GH61	GH6	GH7
Phach	Polyporales	10	5	0	0	6	1	1	2	149	15*	1*	6*
Cersu	Polyporales	0	13*	2	7*	3	0	1	0	222	9	1*	3*
Dicsq	Polyporales	0	9	3	11*	5*	5	1*	3*	187	15*	1*	4*
Trave	Polyporales	10	13*	2*	7*	5*	5*	1*	1*	190	18*	1*	4*
Stehi	Russulales	0	5	0	15	5*	3	1*	2*	215	16*	1*	3*
Hetan[c]	Russulales	0	8	0	13	5	0	1	1	144	10	1	1
Aurde	Auriculariales	0	5	0	7	7*	2	1	2	249	19*	2*	6*
Fomme	Hymenochaetales	0	16*	0	10*	4	0	1	1	130	13	2	2
Punst	Corticales	0	10*	0	12*	6*	3*	1*	2	144	14*	1*	5*
Schco[c]	Agaricales	0	0	0	2	2	0	1	2	115	22	1	2
Brown rot													
Pospl	Polyporales	0	0	0	3	3*	0	0	0	254	2	0	0
Fompi	Polyporales	0	0	0	5	*	0	0	1	190	4	0	0
Wolco	Polyporales	0	0	0	5*	4*	0	0	0	206	2	0	0
Conpu	Boletales	0	0	0	6	6	0	1*	1*	238	10	2	2
Serla[c]	Boletales	0	0	0	4	3	0	2	2	164	5	1	0
Glotr	Gloeophyllales	0	0	0	4	2*	0	1	2*	130	4*	0	0
Dacsp	Dacrymycetales	0	0	0	0	3	0	0	0	126	0	0	0

Aurde, *Auricularia delicata*; CDH, cellobiose dehydrogenase; Cersu, *Ceriporiopsis subvermispora*; Conpu, *Coniophora puteana*; CROs, copper radical oxidases; Dacsp, *Dacryopinax* sp; Dicsq, *Dichomitus squalens*; Fomme, *Fomotiporia mediterranea*; Fompi, *Fomitopsis pinicola*; Glotr, *Gloeophyllum trabeum*; GLX, Glyoxal oxidase; Hetan, *Heterobasidion annosum*; Lac, laccase; LiP, lignin peroxidase; MnP, manganese peroxidase; P450, cytochrome P450 and GH61, GH6, GH7, members of the Glycoside Hydrolase families 61, 6, and 7, respectively; Phach, *Phanerochaete chrysosporium*; Pospl, *Postia placenta*; Punst. *Punctularia strigo-zonata*; Schco, *Schizophyllum commune*; Serla, *Serpula lacrymans*; Stehi, *Stereum hirsutum*; Trave, *Trametes versicolor*; VP, versatile peroxidase; Wolco, *Wolfiporia cocos*.

[a]*NanoLC-MS/MS unambiguously identified at least one protein in media containing ground aspen as sole carbon source. See supplemental files published for *Postia placenta* (Martinez, Challacombe, et al., 2009), *Ceriporiopsis subvermispora* and *Phanerochaete chrysosporium* (Fernandez-Fueyo, Ruiz-Dueñas, et al., 2012), and refer to Floudas, Binder, et al., 2012 for others.

[b]VPs include two "transitional" peroxidases recently characterized in *C. subvermispora* (Fernandez-Fueyo, Ruiz-Dueñas, et al., 2012).

[c]Secretome data derived for media containing ball-milled aspen not available.

cellulose is depolymerized, a process that leads to rapid loss of wood strength (Kleman-Leyer & Kirk, 1992). This unusual depolymerization is markedly different from the more gradual cellulose degradation attributed to conventional hydrolytic enzymes.

Most species of wood-decaying Agaricomycetes exhibit characteristic substrate preference for either conifers (gymnosperms) or hardwoods (angiosperms). Some species are more or less restricted to one or a few wood species, and others appear to be true generalists. Typically, brown rot species are associated with conifer decay, but some have been isolated from hardwoods (Gilbertson, 1981; Hibbett & Donoghue, 2001). Certain wood decay Agaricomycetes can attack living trees (e.g., *Heterobasidion annosum*) or colonize freshly cut sapwood (e.g., *Phlebiopsis gigantea*), whereas many decay only dead trees (Blanchette, 1991).

Microscopic analysis of selective delignification (white rot) and cellulose depolymerization (brown rot) during incipient decay argue against direct interactions between enzymes and their polymeric substrates. Simply put, enzymes are too large to penetrate sound, intact wood. Blanchette, Krueger, et al. (1997) demonstrated this limited accessibility by showing that during *C. subvermispora* decay of pine, the walls only gradually became permeable to insulin (5.7 kDa), and then to myoglobin (17.6 kDa), but not to ovalbumin (44.3 kDa), even in relatively advanced stages of decay. Lignin-depolymerizing enzymes and many cellulases are in the same size range as ovalbumin, and it is generally thought therefore that small molecular weight, oxidizing species are generated and that these diffuse into the walls. The remainder of this chapter describes mechanisms of lignocellulose degradation with particular emphasis on insight gained from the genomes of wood decay fungi.

Mechanisms of Wood Decay

Peroxidases

Lignin peroxidase (LiP) catalyzes Cα-Cβ cleavage of propyl side chains of lignin and lignin models (*see* Fig. 3.1), hydroxylation of benzylic methylene groups, oxidation of benzyl alcohols to the corresponding aldehydes or ketones, phenol oxidation, and aromatic cleavage of nonphenolic lignin model compounds. A wide array of oxidations, all dependent on H_2O_2, has been demonstrated. In a mechanism described as "enzymatic combustion" (Kirk & Farrell, 1987), LiP oxidizes aromatic compounds by a single electron and the resulting aryl cation radicals undergo spontaneous reactions that yield many different products dependent on substrate structure (*see* Fig. 3.1). The biochemistry of peroxidases in ligninolysis has been reviewed (Hammel & Cullen, 2008).

Ten LiP-encoding genes have been identified in the white rot fungi *P. chrysosporium* and *Trametes versicolor* (*see* Table 3.1). Transcript levels of *P. chrysosporium* LiP are substantially altered by culture conditions (Stewart & Cullen, 1999). In soil cultures, transcript patterns are modulated in response to specific pollutants, for example, anthracene versus pentachlorophenol (Cullen, 2002).

More recent secretome and transcriptome studies revealed complex patterns of LiP gene expression in defined media (Vanden Wymelenberg, Sabat, et al., 2005; Vanden Wymelenberg, Sabat, et al., 2006; Vanden Wymelenberg, Gaskell, et al., 2009) and in more complex lignocellulose-containing media such as red oak (Sato, Feltus, et al., 2009) and ball milled aspen (BMA) suspended in basal medium (Vanden Wymelenberg, Gaskell, et al., 2010). Systematic studies of the *Phanerochaete carnosa* transcriptome show that wood species significantly impacts LiP transcript levels (Macdonald, Doering, et al., 2011; Macdonald & Master, 2012; MacDonald, Suzuki, et al., 2012).

Beyond the LiPs, evidence strongly supports a role for manganese peroxidase (MnP) involvement in lignin degradation by white rot fungi (Hammel & Cullen, 2008). Discovered in *P. chrysosporium* cultures, MnP oxidizes Mn^{2+} to Mn^{3+} using H_2O_2 as oxidant. Organic acids, such as oxalic acid, stimulate MnP through stabilization of Mn^{3+} and form diffusible oxidizing chelates. The interactions between oxalate and Mn as they relate to MnP activity and to peroxide generation in *C. subvermispora* cultures have been investigated (Urzua, Kersten, et al., 1998). MnPs lack sufficient oxidative potential to cleave the major non-phenolic units of lignin but can oxidize phenolic structures. Scission of non-phenolic structures within lignin may be mediated by lipid peroxidation mechanisms (Cullen & Kersten, 2004; Hammel & Cullen, 2008). This view is supported by recent analysis of the *C. subvermispora* genome (Fernandez-Fueyo, Ruiz-Dueñas, et al., 2012).

MnP genes are widely distributed among white rot fungi but are absent from brown rot genomes (see Table 3.1). Manganese concentration dramatically affects transcriptional regulation and may also influence MnP secretion, at least in *C. subvermispora* cultures (Mancilla, Canessa, et al., 2010). Putative metal response elements (MREs) have been implicated in the regulation of *P. chrysosporium mnps* but not that of *T. versicolor mnp2* (Cullen & Kersten, 2004). In nutrient limited medium, transcripts and peptides corresponding to *P. chrysosporium mnp1* accumulated, whereas *mnp2* transcripts were upregulated in nitrogen-starved cultures not in carbon-starved cultures (Ravalason, Jan, et al., 2008; Vanden Wymelenberg, Gaskell, et al., 2009). Differentially regulated transcription of *mnps* has also been observed in more complex substrates (Janse, Gaskell, et al., 1998; Stuardo, Vasquez, et al., 2004), and depletion of the polycyclic aromatic hydrocarbon (PAH) fluorine roughly correlates with transcript levels of

mnp1, *mnp2*, and *mnp3* (Bogan, Schoenike, et al., 1996a). The latter observation supports lipid peroxidation mechanisms in *P. chrysosporium* (Watanabe, Tsuda, et al., 2010), as does the simultaneous upregulation of MnPs and putative lipid biosynthesis genes in *C. subvermispora* (Fernandez-Fueyo, Ruiz-Dueñas, et al., 2012).

Versatile peroxidases (VPs) oxidize Mn(II) as well as non-phenolic substrates (e.g., veratryl alcohol) in the absence of manganese (Mester & Field, 1998; Camarero, Sarkar, et al., 1999). These enzymes feature Mn-binding residues and a conserved Trp required for electron transfer. VP-encoding genes have not been observed in any brown rot fungi, but white rot species *Dichomitus squalens*, and *T. versicolor* feature three and two genes, respectively (*see* Table 3.1). Transcriptome studies have not yet been reported for these fungi, but VP-derived peptides have been identified in *T. versicolor* cultured on BMA medium (Floudas, Binder, et al., 2012).

Certain sequence deviations resist simple classification, such as LiP, MnP, or VP. Two *C. subvermispora* proteins were classified as LiP and VP genes based on homology modeling and conservation of catalytic residues. Predictably, the corresponding proteins were capable of oxidizing non-phenolic model compounds, but the putative VP was unable to oxidize Mn, and both enzymes exhibited catalytic properties intermediate between conventional LiPs and MnP (Fernandez-Fueyo, Ruiz-Dueñas, et al., 2012).

Possibly involved in ligninolysis, heme thiolate peroxidases (HTPs) and dye decolorization peroxidases (DyPs) (Hofrichter, Ullrich, et al., 2010) have received increasing attention. HTPs include chloroperoxidases and peroxygenases that can catalyze a variety of reactions such as oxidations of various aliphatic and aromatic compounds (Ullrich & Hofrichter, 2005; Gutierrez, Babot, et al., 2011). Recent studies have attributed high redox potentials for HTP from the white rot fungus *Auricularia auricula-judae* (Liers, Bobeth, et al., 2010). Multiple HTP-encoding genes occur in all wood decay genomes and peptides corresponding to *A. auricularia*, *Fomitopsis pini*, and *Dacryopinax* sp. have been identified in media containing BMA (*see* Table 3.1). Three predicted *P. chrysosporium* HTP genes exhibited differential regulation in response to substrate composition (Vanden Wymelenberg, Gaskell, et al., 2011).

DyP genes are irregularly distributed among genomes. Excluding *Gloeophyllum trabeum*, none were detected in brown rot genomes. Analysis of the white rot genomes showed HTP genes absent from *P. chrysosporium* and *C. subvermispora*, whereas *T. versicolor* and *D. squalens* featured two and one gene, respectively. Analysis of BMA culture filtrates suggest that a *D. squalens* DyP and a *T. versicolor* DyP protein are especially abundant, constituting 1.3 percent and 2.2 percent of the total spectra, respectively (Fernandez-Fueyo, Ruiz-Dueñas, et al., 2012).

Laccases

Phenolics, aromatic amines, and other electron-rich substrates are oxidized by laccases, members of the multicopper oxidase family (Hoegger, Kilaru, et al., 2006). The one-electron oxidation of the phenolic units in lignin generates phenoxy radicals that may lead to aryl-Cα cleavage (Kawai, Umezawa, et al., 1988), but the dominant non-phenolic substructures do not serve as substrates. These are only oxidized in the presence of auxiliary substrates, such as ABTS (2,2'-azino-bis-3-ethylthiazoline-6-sulfonate). In this context, the white rot fungi *Pycnoporus cinnabarinus* and *T. versicolor* produce small molecular weight compounds that may act as mediators for oxidation of non-phenolic lignin substructures (Eggert, Temp, et al., 1996; Johannes & Majcherczyk, 2000). Most white rot fungi secrete multiple laccase isozymes. In contrast, *P. chrysosporium* produces none indicating that the enzyme is not uniformly required for lignin degradation. Laccases have been reviewed (Giardina, Faraco, et al., 2010).

Excluding *P. chrysosporium*, families of structurally related genes encode laccases in wood decay fungi. This genetic multiplicity appears slightly reduced in brown rot fungi, and *Dacryopinax* sp. contains none (*see* Table 3.1). As a percentage of total mass spectra, a single laccase is the most abundant *T. versicolor* protein observed in BMA culture filtrates (2.8 percent). More modest estimates of laccase abundance were inferred from mass spectrometry analysis of *D. squalens*, *Fomotiporia mediterranea*, *Punctularia strigozonata*, *C. subvermispora*, and *Wolfiporia cocos*. Differential regulation of laccase genes is well established, and a potential ACE response may play a role in the copper induction of *C. subvermispora* laccases and MnP (Alvarez, Canessa, et al., 2009). Laccase regulation has been reviewed (Piscitelli, Giardina, et al., 2011).

Intracellular Enzymes Involved in Ligninolysis

Complete mineralization of many small molecular weight extractives and lignin-derived compounds requires intracellular metabolism. Intracellular systems also generate the secondary metabolites (e.g., veratryl alcohol and quinones) thought to support extracellular metabolism. Examples of important *P. chrysosporium* enzymes include methanol oxidase (Asada, Watanabe, et al., 1995); 1,4-benzoquinone reductase (Brock & Gold, 1996); methyltransferases (Jeffers, McRoberts, et al., 1997); L-phenylalanine ammonia-lyase (Hattori, Nishiyama, et al., 1999); 1,2,4-trihydroxybenzene 1,2-dioxygenase (Rieble, Joshi, et al., 1994); glutathione transferases (Dowd, Buckley, et al., 1997); superoxide dismutase (Ozturk, Bozhaya, et al., 1999); catalase (Kwon & Anderson, 2001); aryl alcohol dehydrogenase (Reiser, Muheim, et al., 1994);

and cytochrome P450s (Kullman & Matsumura, 1997; Yadav & Loper, 2000; Van Hamme, Wong, et al., 2003, Yadav, Soellner, et al., 2003).

Agaricomycotina genomes have revealed impressive genetic diversity and complex organization among cytochrome P450s (*see* Table 3.1). Most of these sequences have be assigned to families and clans but possible relationships to ecological roles have been not been ascertained. Functional characterization of these P450s has been limited, but many are assumed to be involved in the metabolism of aromatic compounds, including lignin breakdown products. Comparisons of aspen- versus pine-grown *P. placenta* showed differential regulation of 14 P450s (Vanden Wymelenberg, Gaskell, et al., 2011), and two *P. chrysosporium* P450s were shown upregulated in ligninolytic cultures (Shary, Kapich, et al., 2008).

Fenton Systems and Iron Homeostasis

Hydroxyl radicals have been repeatedly implicated as diffusible oxidants in brown rot (Xu & Goodell, 2001; Cohen, Jensen, et al., 2002), and to a lesser extent, in white rot. Fenton chemistry ($H_2O_2 + Fe^{2+} + H^+ \rightarrow H_2O + Fe^{3+} + OH$) is often invoked as the underlying system for generating the highly reactive radicals. Mechanisms controlling extracellular Fenton reactions are the subject of considerable debate (Goodell, 2003; Baldrian & Valaskova, 2008), and three overlapping models have been offered. In one case, the importance of cellobiose dehydrogenase (CDH) had been emphasized, but it is now clear that the efficient brown rot fungus *P. placenta* does not produce this enzyme (Martinez, Challacombe, et al., 2009). Another view stresses the role of low molecular weight glycopeptides that catalyze extracellular iron reduction (Tanaka, Yoshida, et al., 2007). The third mechanism involves extracellular quinone redox cycling (Varela & Tien, 2003; Shimokawa, Nakamura, et al., 2004; Suzuki, Hunt, et al., 2006) (Fig. 3.2). The cycle is complicated in oxalate-accumulating fungi such as *P. placenta* (Kaneko, Yoshitake, et al., 2005), because Fe^{3+}-oxalate chelates are poorly reduced by hydroquinones (Jensen, Houtman, et al., 2001). In such cases, laccases may be involved in hydroquinone oxidation (Wei, Houtman, et al., 2009). Irrespective of the precise mechanism(s), hydroxyl radical pretreatment of lignocellulose substrates clearly enhances enzymatic saccharification (Ratto, Ritschkoff, et al., 1997), and it is widely held that brown rot involves sequential oxidation and hydrolysis. Supporting hydroquinone importance in *P. placenta*, genes likely involved in their biosynthesis, transport and reduction are upregulated in a BMA medium relative to glucose-containing medium. Transcripts of *P. placenta* laccases, possibly supporting a redox system via oxidation of hydroquinones (Gomez-Toribio, Garcia-Martin, et al., 2009), also accumulated in BMA medium (Vanden Wymelenberg, Gaskell, et al., 2010).

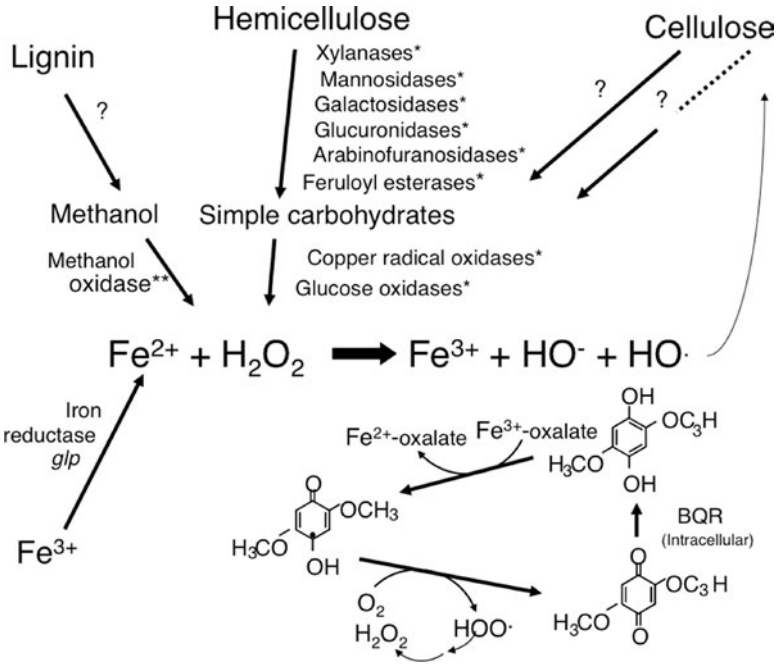

Figure 3.2 Schematic speculation related to systems for generating highly reactive extracellular hydroxyl radical. Enzymes followed by asterisks have been unambiguously identified in culture filtrates of the brown rot fungus, *Postia placenta* (Martinez, Challacombe, et al., 2009). BQR, benzoquinone reductase.

Extracellular Oxidoreductases

Other components commonly ascribed to wood decay systems include extracellular enzymes capable of generating peroxide. Copper radical oxidases and at least four flavin enzymes may be physiologically linked to peroxidases, and possibly to Fenton systems.

Consistent with a close physiological relation, glyoxal oxidase (GLX) is temporally correlated with peroxidases in ligninolytic cultures (Kersten & Kirk, 1987; Kirk & Farrell, 1987; Kersten, 1990), and activity is responsive to peroxidase, peroxidase substrates, and peroxidase products (Kersten, 1990; Kurek & Kersten, 1995). The enzyme will oxidize simple aldehyde, α-hydroxycarbonyl, and α-dicarbonyl compounds; some of which are likely lignin-derived metabolites. Interestingly, such copper radical oxidases (CROs) have two distinct one-electron acceptors, a Cu(II) metal center and an internal Cys-Tyr radical forming a metalloradical complex (Whittaker, 2002).

Initially discovered in *P. chrysosporium* cultures, GLX homologs have been identified in the genomes of most white rot fungi but none of the brown

rot genomes (*see* Table 3.1). Coordinate increases in GLX and peroxidase expression were observed under nutrient starvation (Stewart, Kersten, et al., 1992; Vanden Wymelenberg, Minges, et al., 2006; Vanden Wymelenberg, Gaskell, et al., 2009), in colonized wood (Janse, Gaskell, et al., 1998; Sato, Feltus, et al., 2009), and in soil (Bogan, Schoenike, et al., 1996a, b). Seven structurally related copper radical oxidase genes (*glx*, *cro1-cro6*) have been identified in the *P. chrysosporium* genome, and *cro3*, *cro4*, and *cro5* lie within a LiP gene cluster (Cullen & Kersten, 2004). The clustering of *lip* and *cro* genes may also be related to a physiological connection between peroxidases and these peroxide-generating oxidases. *P. chrysosporium* CRO2 substrate preference differs from that of GLX (Vanden Wymelenberg, Sabat, et al., 2006), and this functional diversity may allow adaptation to shifting substrate accessibility and composition during cell wall degradation.

This view may also explain the absence of a GLX homolog in the selective lignin degrader, *C. subvermispora* (*see* Table 3.1). Perhaps functionally related CROs are better suited for a spectrum of small molecular weight substrates unique to the ligninolytic system of *C. subvermispora*. Supporting this, a *C. subvermispora cro2*-like gene and several MnP genes are upregulated in cultures containing BMA (Fernandez-Fueyo, Ruiz-Dueñas, et al., 2012). Little is known concerning the expression of GLX genes in other fungi, although *T. versicolor* and *P. strigo-zonata* GLX proteins were identified in BMA cultures (Floudas, Binder, et al., 2012).

Extracellular peroxide generation may also be supported by Glucose-Methanol-Choline (GMC) oxidases, a large family of flavin enzymes that includes aryl alcohol oxidase (AAO), methanol oxidase (MOX), and various sugar oxidases (Hernandez-Ortega, Ferreira, et al., 2012). AAOs oxidize benzyl alcohols to aldehydes, transferring the electrons to O_2, producing H_2O_2 (Muheim, Leisola, et al., 1990; Asada, Watanabe, et al., 1995). The AAO genes are widely distributed among wood decay fungi, but at least one white rot fungus, *Auricularia delicata*, and brown rot fungi *Coniophora puteana*, *W. coccos*, and *Dacryopinax* sp. have no detectable AAO gene (Floudas, Binder, et al., 2012). Transcript levels in nutrient-starved medium, Avicel medium, and BMA medium were modest for *C. subvermispora* and *P. chrysosporium* (Vanden Wymelenberg, Gaskell, et al., 2009). AAO-derived peptides have been identified in BMA cultures of *T. versicolor* and *D. squalens* (Floudas, Binder, et al., 2012). Hernandez-Ortega, Ferreira, et al. (2012) provide detailed analyses of 40 AAO genes.

MOX, highly expressed in the brown rot fungus *G. trabeum* (Daniel, Volc, et al., 2007), may be linked to the demethoxylation of lignin (Niemenmaa, Uusi-Rauva, et al., 2007) and thereby produce H_2O_2. High expression has also been observed in cultures of the white rot fungus *P. chrysosporium* (Vanden Wymelenberg, Gaskell, et al., 2010). In contrast to *P. chrysosporium*, no *D. squalens* and *T. versicolor* MOX proteins were detected by LC-MS/MS in

BMA medium (Floudas, Binder, et al., 2012). The inability to detect soluble MOX protein in filtrates should be cautiously interpreted because cell wall associations are likely (Daniel, Volc, et al., 2007).

Pyranose 2-oxidase genes have been isolated from *T. versicolor* (Nishimura, Okada, et al., 1996), *P. chrysosporium* (de Koker, Mozuch, et al., 2004), and *G. trabeum* (Dietrich & Crooks, 2009), but obvious homologs are lacking from most sequenced genomes. Transcripts of the *P. chrysosporium* gene are upregulated under ligninolytic conditions (de Koker, Mozuch, et al., 2004; Vanden Wymelenberg, Gaskell, et al., 2009), and the protein has been identified in carbon-starved cultures (Vanden Wymelenberg, Gaskell, et al., 2010) and in BMA medium (Vanden Wymelenberg, Gaskell, et al., 2011).

Another oxidoreductase—CDH—oxidizes cellodextrins, mannodextrins, and lactose. Electron acceptors include quinones, phenoxyradicals, and Fe^{3+}. The protein contains a dehydrogenase domain, a heme prosthetic group and a cellulose binding module (Hallberg, Bergfors, et al., 2000). CDH is widely distributed among fungi, including non-wood decay Ascomycotina. The precise role(s) remain uncertain (Zamocky, Ludwig, et al., 2006) but, as mentioned previously, involvement in hydroxyl radical generation has been proposed.

All white rot genomes have a single CDH gene, but the number varies in brown rot genomes, which have none (*P. placenta*, *Fomitopsis pinicola*, *W. cocos*), one (*C. puteana*, *G. trabeum*), or two (*Serpula lacrymans*) copies of the CDH gene. Sequences share a common architecture with separate flavin, heme, and cellulose-binding domains (CBD).

Upregulation of *P. chrysosporium cdh* has been demonstrated by Northern blots in cellulose-containing media, by competitive real time-polymerase chain reaction (RT-PCR) in colonized wood (Cullen & Kersten, 2004) and more recently by microarrays in media containing Avicel or BMA (Vanden Wymelenberg, Gaskell, et al., 2010) and by RNAseq in red oak medium (Sato, Feltus, et al., 2009). The corresponding peptides were identified in media that were nutrient starved (Vanden Wymelenberg, Gaskell, et al., 2009), containing Avicel, or containing complex lignocellulose substrates (Sato, Feltus, et al., 2009; Vanden Wymelenberg, Gaskell, et al., 2011). The wood species influence expression with higher transcript and protein levels in ball milled pine relative to BMA (Vanden Wymelenberg, Gaskell, et al., 2011).

CDH expression is typically coordinate with that of aldose 1-epimerase (ALE) (Vanden Wymelenberg, Sabat, et al., 2005; Sato, Feltus, et al., 2009; Vanden Wymelenberg, Gaskell, et al., 2011). This expression pattern may indicate a physiological relationship through generation of the cellobiose β-anomer, the preferred CDH substrate (Higham, Gordon-Smith, et al., 1994). In this connection, of five recently sequenced wood decay fungi (*T. versicolor*, *D. squalens*, *P. strigoso-zonata*, *Stereum hirsutum*, *C. puteana*), all but *P. strigoso-zonata* simultaneously secreted ALE and CDH in BMA medium. Co-expression of CDH and certain "hydrolases" was also observed. Initially

classified as glycoside hydrolase family 61 (GH61) enzymes, many are now considered copper-dependent monooxygenases (Quinlan, Sweeney, et al., 2011; Westereng, Ishida, et al., 2011). Together, GH61s and CDH boost cellulose depolymerization (Harris, Welner, et al., 2010; Langston, Shaghasi, et al., 2011). In addition to cellulose, secretion of CDH and GH61 were also observed on xylan-containing medium (Hori, Igarashi, et al., 2011). The precise roles(s) and interaction(s) between these genes remain to be clarified.

Glycosyl Hydrolases and Related Carbohydrate Active Enzymes

Employing a battery of hydrolases, cellulose degradation by white rot fungi follows a strategy similar, but not identical, to a diverse array of microbes, particularly the heavily studied industrial ascomycete *Trichoderma reesei*. Key components include exocellobiohydrolase I (CBHI), exocellobiohydrolase II (CBHII), β-1,4-endoglucanase (EG), and β-glucosidase (β-Glu) (Kirk & Cullen, 1998; Baldrian & Valaskova, 2008). Crystalline cellulose is degraded through the synergistic action of the exo- and endo-hydrolases and the resulting oligo- and disaccharides are cleaved to monomers by β-glucosidase. In *T. reesei*, these hydrolases are encoded by relatively few genes principally assigned to glycoside hydrolase families GH6 (CBHII), GH7 (CBHI, EG), GH12 (EG), GH5 (EG), and β-Glu (GH1, GH3). In contrast, six *P. chrysosporium* genes are predicted to encode distinct CBH1 isozymes, and it has been suggested that such diversity reflects subtle functional differences (Munoz, Ubhayasekera, et al., 2001) that allow adaptation to changing environmental conditions during decay.

Multiple CBHI-encoding genes, and typically 1–2 CBHII genes, have been identified in other white rot fungi, an observation standing in contrast to brown rot fungi, which have few, if any, exocellobiohydrolases (*see* Table 3.1). CBH1 and CBHII proteins of white rot fungi are usually abundant in filtrates of BMA medium (*see* Table 3.1). Thus, the number and expression of white rot cellulases support a conventional hydrolytic attack on cellulose. But in brown rot fungi, the paucity of CBHI and CBHII genes (*see* Table 3.1) points toward the aforementioned oxidative depolymerization of cellulose. However, several putative GH5 β-1,4-endoglucanase genes have been identified in brown rot fungi (Floudas, Binder, et al., 2012). One such putative EG is highly expressed in *P. placenta* cultures containing BMA (Vanden Wymelenberg, Gaskell, et al., 2010), but it seems doubtful that the enzyme could efficiently depolymerize crystalline cellulose in the absence of CBHI or CBHII.

Complete breakdown of wood hemicelluloses requires the combined activities of an array of glycoside hydrolases, carbohydrate esterases (CEs), and polysaccharide lyases (PLs). These include endoxylanase, acetylxylan esterase, α-glucuronidase, β-xylosidase, α-arabinosidase, endomannanase,

α-galactosidase, acetylglucomannan esterase, β-mannosidase, and β-glucosidase (Kirk & Cullen, 1998). As in the case of cellulases, these enzymes are often classified according to carbohydrate active enzyme (CAZy) family (Cantarel, Coutinho, et al., 2009; www.cazy.org/) but, with the possible exception of GH74, CE1, and CE12, the families are dispersed among taxa and show little relationship to ecology and decay patterns (Floudas, Binder, et al., 2012). Relatively little work has focused on the regulation of these genes, but transcript and secretome profiles in colonized aspen versus pine show significant differences for several *P. chrysosporium* genes, including a GH92 α-1,2-mannosidase, a GH27 α-galactosidase, a GH5 1,4 β-mannan endohydrolase, and two carbohydrate esterases (CE15) (Vanden Wymelenberg, Gaskell, et al., 2011). The latter esterases may attack hemicellulose-lignin linkages (Duranova, Spanikova, et al., 2009). The influence of wood species on regulation of these genes has also been shown for *P. carnosa* (Macdonald, Doering, et al., 2011).

Future Prospects

Enumeration and classification of wood decay genes provide considerable insight into mechanisms of carbon cycling by wood decay fungi. Transcriptome and secretome profiles offer additional clues and help focus research on potentially important components. Still, major obstacles remain and, among these, functional analysis of unknown or hypothetical proteins constitutes a major challenge. For perspective, nanoLC-MS/MS (Vanden Wymelenberg, Gaskell, et al., 2009) unambiguously assigned peptides to 55, 32, and 14 genes encoding unknown proteins in lignocellulose-containing cultures of *P. chrysosporium*, *P. placenta*, and *C. subvermispora*, respectively (Vanden Wymelenberg, Gaskell, et al., 2010, Fernandez-Fueyo, Ruiz-Dueñas, et al., 2012). Some of these unknown proteins are particularly intriguing, as is the case for several highly expressed *P. placenta* genes whose transcript levels are differentially regulated in response to wood species (Vanden Wymelenberg, Gaskell, et al., 2011). However, functional analysis has been hindered by the lack of genetic tools and difficulties performing detailed biochemical analysis on purified proteins. In this context, encouraging progress has been made in the development of techniques for targeted RNAi (Salame, Yarden, et al., 2010) and gene replacement (Salame, Knop, et al., 2012) for the white rot fungus *Pleurotus ostreatus*. Such genetic "toolboxes" might be applied to other wood decay fungi.

Another daunting challenge is to attain a deeper understanding of wood decay in more natural substrates including, ultimately, field conditions. Without doubt, white and brown rot fungi interact with bacteria and soft rot fungi during natural decay (Eriksson, Blanchette, et al., 1990), and interactions

with humicolous fungi such as *Agaricus* and *Coprinopsis* at the soil interface likely influence and maintain soil properties. In turn, metabolic activities of these microbes are expected to substantially impact establishment of mycorrhizal systems.

In depth understanding of the structure, physiological activities, and interactions within these complex communities is needed, but here too, research has been stymied by the shortage of experimental tools. Recently however, high throughput metagenomic techniques have been brought to bear (Damon, Lehembre, et al., 2012; de Menezes, Clipson, et al., 2012), and in one case, litter and soil horizons fractions were examined for community composition and for transcript profiles by metagenome and metatranscriptome approaches, respectively (Baldrian, Kolarik, et al., 2012). Focusing on fungi, the genes and transcripts corresponding to CBHIs were also quantified (Baldrian, Kolarik, et al., 2012) and revealed higher numbers and diversity of cellulose decomposers in the litter. Extending such investigations to samples collected over time from multiple ecosystems will identify key species and processes involved in nutrient cycling and forest health.

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