Progress and Research Needs of Plant Biomass Degradation by Basidiomycete Fungi

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Abstract Basidiomycete fungi are efficient organisms for conversion and degradation of plant biomass. This is due to combination of their extracellular enzymes and chemical reactions targeted to plant cell wall degradation. Wood- and litter-decomposing white rot fungi have unique ability to degrade and even mineralize all polymeric components of plant cell walls, including aromatic lignin, which makes them promising candidates for biotechnological applications using plant biomass as feedstock.

Rapidly increasing whole genome sequence data has revealed the content of plant biomass modification related genes in different basidiomycete species. Comparative genome analyses have enlightened evolutionary events that have led to development of different fungal plant cell wall decay strategies, which are reflected in nuances detected between basidiomycete rot types and lifestyles in nature. However, basidiomycete genomes harbour a large number of genes encoding proteins with unknown functions, which remains to be characterized to fully understand the degradation process. In addition, fungal aromatic metabolism of plant biomass derived compounds has gained relatively little attention, although aromatic metabolic enzymes specifically acting on lignin structures would provide interesting options for biotechnological use. Still, low production levels of basidiomycete enzymes in commonly used ascomycete or bacterial host organisms often hamper their use in biotechnological applications. Another aspect that is in its infancy in basidiomycetes and restricts the use of their full potential is understanding of the regulatory systems driving the production of plant biomass degrading enzymes. In this chapter, recent developments in basidiomycete research with respect to plant biomass conversion will be discussed.

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1. Introduction

Wood and lignified annual plant biomasses such as cereal straws are usually called lignocellulose because they are mainly composed of the three natural polymers, i.e. the polysaccharides cellulose and hemicelluloses, and aromatic lignin. These biomasses are renewable, and huge amounts of lignocellulose are annually synthesized and degraded in nature. Degradation of plant biomass is in a central position in the global carbon cycle, because most renewable carbon is either in lignin or in plant cell wall polysaccharides (Kirk 1983). Especially in forest ecosystems, basidiomycete saprotrophic wood decaying and litter-decomposing fungi have an essential role in this degradation process.

The use of plant biomass resources, such as residues from agriculture and forestry, energy crops and municipal waste streams, as feedstock for production of biofuels, biochemicals and biomaterials is interesting due to their abundance and relatively low cost. In addition, they do not compete with food production (Tolbert et al. 2014). As basidiomycete fungi are efficient plant biomass degrading organisms and produce versatile sets of enzymes targeted on depolymerisation of all the polymeric components of lignocellulose, they are promising candidates for various biotechnological applications.

For a long time, the studies on plant biomass degrading basidiomycetes were focused on lignin degradation, especially on the so-called white rot fungi that can efficiently degrade and even mineralize the recalcitrant lignin polymers. In relation to this, many research needs and future perspectives in basidiomycete research with respect to lignin degradation were recognized about 20 years ago (Hatakka 2001). At that time, it was considered important to study (i) taxonomically or functionally different fungi, (ii) involvement of accessory factors, i.e. small molecular weight effectors and mediators, (iii) isoenzymes or isoforms expressed under natural conditions, (iv) enzymes or factors involved in the degradation of macromolecular native or isolated lignins, (v) the relative importance of individual ligninolytic enzymes in the whole degradation process, and (vi) *in vitro* degradation of polymeric lignin using mixtures of enzymes, small molecules, and different conditions. The same items are valid and remain as major challenges even now. However, the role of basidiomycete fungi as sources of other biotechnologically interesting enzymes has also been recognized, especially after the rapid progress in the genomics and post-genomics studies of lignocellulose degrading basidiomycetes in the 2010's. Figure 1 shows the strong increase in the number of the scientific articles related to plant biomass degradation by basidiomycete fungi during the last decade.

In this chapter, we emphasize the recent developments and needs on basidiomycete research, to fully exploit the biotechnological potential of these fungi and their enzymes in terms of plant biomass utilization.

2. Plant cell wall polymers

Cellulose is the most abundant biopolymer on Earth (Klemm et al. 2005). It is the main constituent of wood, and approximately 40% of the dry weight of most wood species is cellulose (Sjöström 1993). Cellulose is a homopolysaccharide composed of β-D-glucopyranoside units, which are linearly linked together by β-1,4-glycosidic bonds. Cellulose can be crystalline, para-crystalline and even amorphous, depending on the tissue in native plant, or the way that cellulose is isolated (Andersson et al. 2004, Ding and Himmel 2009, Karimi and Taherzadeh 2016), and its degree of polymerization may vary between 1000 (e.g. in wheat straw) up to 4000-5000 (in hardwood and softwood) (Hallac and Ragauskas 2011). Fibre aggregation found in isolated celluloses and caused by sample processing does not necessarily represent the native cellulose structure, and the detailed molecular structure of plant cell wall cellulose remains unknown (Ding and Himmel 2009).

Hemicelluloses in wood consist of relatively short, mainly branched heteropolymers of glucose, xylose, galactose, mannose and arabinose as well as uronic acids of glucose, galactose and 4-O-methylglucose linked by β-1,3-, β-1,6- and β-1,4-glycosidic bonds. Galacto(gluco)mannans are the principal hemicelluloses in softwoods (approx. 20%), whereas their xylan content is lower (5-10%). Depending on hardwood species, the xylan content varies within the limits of 15-30% of the dry wood. Acetyl groups are present as substituents particularly in the glucomannans of gymnosperms and the xylans of angiosperms (Sjöström 1993). Other hemicelluloses are xyloglucan (β-1,4-linked D-glucose), found mainly in the primary walls; β-glucan (β-1,3;1,4-linked D-glucose); and mannan (β-1,4-linked D-mannose) (Harris and Stone 2009). Xylan, xyloglucan and mannan backbones are decorated with branched monomers and short oligomers consisting of D-galactose, D-xylose, L-arabinose, L-fucose, D-glucuronic acid, acetate, ferulic acid and *p*-coumaric acid that are cleaved by various debranching enzymes. Hemicelluloses are reported to be linked to lignin through cinnamate acid ester linkages, to cellulose through interchain hydrogen bonding, and to other hemicelluloses *via* covalent and hydrogen bonds (Decker et al. 2009).

Lignin is a phenylpropanoid structural polymer of vascular plants, which gives the plants rigidity and binds plant cell walls together (Sarkanen and Ludwig 1971). It is a key structural component in water transportation (Tolbert et al. 2014) and protects plant tissues from invasion of pathogenic microorganisms (Eriksson et al. 1990). Lignin in plant cell walls is intimately mixed with carbohydrate components. It also forms lignin-carbohydrate complexes (LCC), thereby increasing the recalcitrance of the biomass (Tolbert et al. 2014). Therefore, when lignocellulosic biomasses are used in enzymatic biotechnological processes, they must be pretreated either physically, chemically or biologically before enzymatic depolymerisation of the plant cell wall polysaccharides to e.g. monosaccharides for the production of bioethanol. The main aim of the pretreatment is the removal or partial or total degradation lignin, and therefore there has been an increasing interest in the use of the white rot fungi or their lignin degrading enzymes as a part of pretreatment, studied already by Hatakka (Hatakka 1983). This approach has received a considerable interest and has been reviewed frequently during recent years (e.g. Johnson and Elander 2009; Salvachua et al. 2011; Shirkavand et al. 2016; Sindhu et al. 2016).

Lignin is one of the most abundant biopolymers in the world, and comprises about 25% of the dry weight of cellular carbon stored in the biosphere (Zeikus 1981). To avoid confusion, it is important to understand the basic terminology and follow definitions, when describing lignin and aromatic substrates. Lignins can be defined as outlined by Zeikus (1981): natural lignins, which are water insoluble polymers arising from an enzyme-initiated dehydrogenative polymerization of coumaryl, coniferyl or sinapyl alcohol in plant cell walls; industrial lignins, which are chemically or physically modified, largely aromatic residues from pulp and paper, and bioethanol producing industrial processes utilizing wood, straw or other lignocellulose; and model lignins, which include water-soluble aromatic compounds that are synthesized from cinnamyl alcohol derivatives and contain an intermonomer linkage(s) present in natural lignin.

Natural lignins can be divided into three major groups: guaiacyl lignins found in conifers but also in some other plants, guaiacyl-syringyl lignins, found in angiosperms, and guaiacyl-syringyl-*p*-hydroxyphenyl lignins, found in grasses (*Graminae*). Because natural lignins are highly polydisperse materials, their molecular weight is difficult to determine. The non-uniformity of the chain lengths of lignin prevents the characterization of a specific molecular weight. Thus, it is necessary to characterize lignin in terms of average molecular weight, using e.g. number average molecular weight (Mn) and weight average molecular weight (Mw) (Tolbert et al. 2014).

Due to the complex and irregular structure of the lignin polymer, isolation of native lignin is difficult (Buswell and Odier 1987; Brunow 2001) and therefore many industrial

lignins, such as alkali lignins, are highly modified preparations. In a recent review by Tolbert et al. (2014), different methods to isolate lignin from native biomass are summarized, including the determination of its average molecular weight. The most common isolation methods are milled wood lignin (MWL), cellulolytic enzyme lignin (CEL), and enzymatic mild acidolysis lignin (EMAL) methods.

Lignin model compounds, e.g. dimeric β-O-4 model compounds and synthetic lignin (dehydrogenation polymer, DHP), are commonly used in microbiological studies (Kirk and Farrell 1987). However, when small molecular weight aromatic compounds are used as substrates, they do not have the polymeric nature of lignin, which is essential characteristic of natural lignins.

3. Life styles of plant biomass converting basidiomycetes

Plant biomass degrading basidiomycete fungi are traditionally categorized based on the visually observed wood-decay pattern (Hildén and Mäkelä 2018). White rot fungi are able to degrade all the components of the plant cell wall. The decayed wood is characteristically white including fiber-like cellulose enriched material, whereas brown rot fungi mainly degrade wood polysaccharides and only modify lignin, which results in dry, brown residual wood. Based on their genome content, fungal species which show intermediate characteristics to white and brown rot have been suggested to be named as grey rot (Riley et al. 2014; Floudas et al. 2015; Nagy et al. 2016)

White rot causing fungal species in the subphylum Agaricomycotina have a special role in plant biomass degradation since they can efficiently degrade all components of plant cell walls and even mineralize the most recalcitrant natural polymer, lignin (Kirk 1983; Eriksson et al. 1990; Hatakka 2001). While the white rot fungi usually belong to basidiomycetes, some ascomycete fungi such as those belonging to Xylariaceae can mineralize lignin to some extent causing greyish sponge-like soft rot (Blanchette 1995; Liers et al. 2006). Basidiomycete white rot species colonize the wood cell lumen where the hypha can enter from cell to cell *via* bordered pits or through the cell walls (Blanchette 1995; Kuhad et al. 1997). White rot fungi can be divided into simultaneous and selective lignin degraders. In simultaneous degradation, lignin and wood cell wall polysaccharides are concomitantly depolymerized, whereas in selective degradation, lignin and hemicelluloses are degraded leaving cellulose almost intact (Eriksson et al. 1990; Kuhad et al. 1997, Hakala et al. 2004). Selective degradation is usually limited to the initial stages of decay (Adaskaveg et al. 1995). However, selective lignin degradation has been suggested to significantly differ between mono- and dikaryotic

white rot fungal strains (Marinović et al. 2018b). Litter-decomposing basidiomycete fungi are also able to cause white rot type decay. The growth and degradation capacity of these species is usually restricted to the soil environment (Steffen et al. 2000). The common feature of all white rot species is the ability to secrete oxidative lignin-modifying enzymes such as lignin-modifying heme peroxidases, laccases and H_2O_2-generating enzymes (Kirk and Farrell 1987; Hatakka 2001; Hammel and Cullen 2008).

Brown rot fungi mainly depolymerize cellulose and hemicelluloses in wood, but lignin modification occurs only at some extent, mostly by demethoxylation (Eriksson et al. 1990; Akhtar et al. 1997; Hammel 1997). The decayed wood remains brown and breaks down into cubical cracks. The main decay mechanism of brown rot fungi is non-enzymatic attack using highly reactive oxygen species (ROS), especially hydroxyl radicals ($HO^{\cdot}$) produced by Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO^- + HO^{\cdot}$) (Jensen Jr. et al. 2001). ROS enable loosening of wood structure by depolymerizing lignin, which is followed by partial repolymerization and enzymatic polysaccharide degradation in wood cell lumen (Yelle et al. 2008; Goodell et al. 2017).

Recently, the traditional classification to white rot or brown rot species has been challenged by species that produce white rot like appearance of wood, but lack key gene families for lignin degradation (Riley et al. 2014; Floudas et al. 2015). Their ability to degrade wood is limited, and in that regard they resemble ascomycete soft rot causing fungi (e.g. *Trichoderma* and *Xylaria* species), which are able to degrade wood polysaccharides in moist or aquatic conditions. Particularly, a key enzyme family involved in lignin degradation, class II lignin-modifying heme peroxidases, is missing in their genomes (Nagy et al. 2016).

It is noteworthy that ectomycorrhizal fungi possess genes encoding plant cell wall degrading enzymes, but their ability to decompose lignocellulose is lower than in most other wood-decaying basidiomycetes. Degradation mechanism of some ectomycorrhizal species resembles that of brown rot type of decay (e.g. *Paxillus*), while others use a white rot type mechanism (e.g. *Cortinarius*) (Kohler et al. 2015; Lindahl and Tunlid 2015). In addition to mycorrhizal species, plant pathogenic basidiomycetes have also been reported to be able to degrade lignocellulose, but less efficiently than wood decaying species (Rytioja et al. 2014; Kohler et al. 2015). Therefore, we focus on wood and litter-degrading basidiomycetes in this chapter.

3. Plant biomass modifying enzymes of basidiomycetes

Due to the structural complexity of plant biomass, multiple carbohydrate-active enzymes (CAZymes) and oxidoreductases are needed to degrade the plant polysaccharides and lignin. CAZy database (www.cazy.org) consists of thousands of enzymes involved in the modification of plant biomass components (Lombard et al. 2014). Plant polysaccharides degrading hydrolytic enzymes include a variety of activities that are grouped in glycoside hydrolase (GH) families. Many of these enzymes harbour a cellulose binding module(s) (CBM), which binds to the substrate and promotes enzyme catalysis (Várnai et al. 2014). Most of the oxidoreductases acting on lignin as well as lytic monosaccharide monooxygenases (LPMOs) that oxidatively cleave various polysaccharides are classified to the family of Auxiliary Activities (AA) in the CAZy database.

3.1 Carbohydrate modifying enzymes

So-called classical cellulases, namely endoglucanases, exoglucanases (cellobiohydrolase; CBH) and β-glucosidases, and oxidative enzymes, LPMOs and cellobiose dehydrogenases (CDHs) (Rytioja et al. 2014), are involved in cellulose degradation. Endoglucanases, which belong to several GH families (GH5, 7, 12, 45) hydrolyse non-crystalline regions of cellulose by opening and cleaving internal glycosidic bonds at random positions. They generate free cellulose chain ends for processively acting CBHs. CBH I (GH7) acts on non-reducing and CBH II (GH6) on reducing end of cellulose chain resulting in the release of oligosaccharides. In addition to these hydrolytic cellulases, oxidative LPMOs (AA9-11, AA14,15) together with CDH (AA3_1) act on crystalline cellulose regions providing new access points for endoglucanases and CBHs (Bissaro et al. 2018). β-Glucosidases (GH 1, 3) finalize the concerted action of cellulases by hydrolysing the released oligosaccharides to glucose molecules (Shewale 1982, Rytioja et al. 2014).

The heterogeneous composition of hemicelluloses requires a diverse set of enzymes for degradation of the backbones and branches depending on each hemicellulose types (Shallom and Shoham 2003; Rytioja et al. 2014). The two main groups, which participate in hemicellulose degradation are xylanolytic and mannanolytic enzymes. The backbone of xylan is hydrolysed by endoxylanases (GH10, 11) to shorter oligosaccharides. In addition, xylobiohydrolase (GH30) is able to cleave xylan backbone to xylobiose units. Exo-acting β-xylosidases (GH3, 43) release monomeric xylose residues from the ends of xylan chain. Xyloglucan has the same backbone as cellulose, but with xylose-based side chains. Xyloglucan backbone is hydrolysed by xyloglucanases (GH74), endoglucanases, CBHs and β-glucosidases. The mannan

backbone is cleaved by endo-mannanases (GH5, 26) into manno-oligosaccharides, which are further hydrolyzed by β-mannosidases (GH2) into mannoses. In addition, β-glucosidases act on the mannan backbone.

In addition to the backbone degrading enzymes, a variety of debranching enzymes are needed in hydrolysis of xylan and mannan (Rytioja et al. 2014). As their side chains include monomers and oligomers which can contain acetate, arabinose, fucose, glucuronic acid, galactose or ferulic acid residues, they are hydrolyzed by different sets of debranching enzymes depending on the type of hemicellulose. Side chains of xylan are cleaved e.g. by acetyl xylan esterases (CE1, 5), arabinoxylan arabinofuranohydrolase (GH62) and α-glucuronidases (GH67, 115), whereas mannan debranching enzymes include α-galactosidases (GH27, 36) and galactomannan acetyl esterase (not included in CAZy database). Debranching enzymes for xyloglucan are α-fucosidases (GH29, 95), α-arabinofuranosidases (GH51, 54) and α-xylosidase (GH31). Feruloyl (EC 3.1.1.73) and glucuronoyl esterases (CE15) catalyze the cleavage of ester bonds between plant cell wall polysaccharides and lignin (Dilokpimol et al. 2016; Dilokpimol et al. 2018).

3.2 Lignin modifying enzymes

Class II heme containing peroxidases and laccases are considered as classical lignin modifying enzymes. In addition, assisting enzymes such as H_2O_2-producing enzymes participate in lignin degradation (Lundell et al. 2014). They are classified as auxiliary activities (AA) in the CAZy database (Lombard et al. 2014).

Manganese peroxidases (MnP), lignin peroxidases (LiP) and versatile peroxidases (VP) are unspecific lignin modifying heme peroxidases, which are exclusively secreted by the white rot species. All lignin-modifying peroxidases belong to the AA2 family. For all heme peroxidases, H_2O_2 or organic peroxides act as the primary oxidant. Two electrons derived from substrate molecules gradually reduce the enzyme back to the resting stage with concomitant release of two water molecules (Dunford 1991; Gold et al. 2000; Hammel and Cullen 2008). As a result, organic radicals are formed, which rapidly undergo subsequent chemical reactions leading to formation of oxidized or coupled (oligomeric) end-products.

MnP catalyzes the oxidation of Mn(II) to Mn(III) in the presence of H_2O_2. Highly reactive Mn(III) is stabilized in chelated form by organic acids. Chelated Mn(III) acts as a low molecular weight diffusible redox mediator, which is able to penetrate into

wood cell wall. This yields spontaneous break down of unstable organic radicals from phenolic lignin structures, carboxylic acids, and unsaturated lipids (Wariishi et al. 1992; Blanchette et al. 1997; Gold et al. 2000; Hofrichter 2002). Nonphenolic structures of lignin cannot be directly oxidized by MnP, but MnP-mediated lipid peroxidation reactions enable the slow degradation of nonphenolic structures (Kapich et al. 1999a; Kapich et al. 1999b). In addition, significant cell-free mineralization of ^{14}C-labelled synthetic and natural lignins by MnP has been reported (Hofrichter et al. 1999a; Hofrichter et al. 1999b) further highlighting the importance of MnPs in lignin degradation.

LiP catalyzes the oxidation of variety of substrates, such as phenolic and nonphenolic aromatic compounds, including the substructures of lignin (Kirk and Farrell 1987; Hatakka and Hammel 2010). The catalytically active tryptophan residue on the enzyme surface participates in the so-called long-range electron transfer from lignin or other bulky recalcitrant substrate that cannot directly interact with heme in the active centre of LiP (Doyle et al. 1998). Typically, the oxidation reactions of LiP result with formation of organic radicals leading to C_α-C_β cleavage, aromatic ring cleavage and demeth(ox)ylation (Kirk and Farrell 1987; Lundell et al. 1993).

VP combines the structural and catalytic properties of MnP and LiP (Ruiz-Dueñas and Martínez 2009). Therefore, they are capable of degrading a wide range of substrates. Similarly to MnP, VP is able to efficiently oxidize Mn(II), and also nonphenolic structures in the absence of Mn(II) ions by using the similar approach as LiP (Perez-Boada et al. 2005).

A turning point in lignin biodegradation studies was the discovery “ligninase” in 1983 in the model white rot basidiomycete fungus *Phanerochaete chrysosporium*. Kirk (1983) evaluated developments before that, and some advances since 1983 were listed by Hatakka (2001). The discoveries include finding and characterization of ligninolytic peroxidases, LiP and MnP, in 1983-1984, one-electron oxidation mechanism and cation radical formation by LiP in 1985, and the concept of "enzymatic combustion" (Kirk and Farrell 1987). Some pioneering molecular biological studies (for references, see Hatakka 2001) until 2000 include the first cloning and sequencing of *lip* and *mnp* gene, heterologous expression of laccase and peroxidases, homologous expression of peroxidases, and 3D structures of LiP, MnP and laccases (in 1993-1998), and detailed characterization of VP. The need to study more than one model fungus was obvious in the 1990’s, when lignin biodegradation had almost exclusively been studied by the canonized white rot model fungus *P. chrysosporium*. Later, the profiles of ligninolytic enzymes produced by different white rot fungi were found to show considerable variations (Hatakka 1994).

In addition to family AA2 heme peroxidases, dye-decolorizing peroxidases (DyP) and members of heme-thiolate peroxidase (HTP) superfamily are suggested to participate in plant biomass degradation. DyPs have been shown to degrade wheat straw, non-phenolic β-O-4 lignin model compounds and substituted phenols (Liers et al. 2010; Linde et al. 2015). HTPs include chloroperoxidases, unspecific peroxygenases (UPOs) and intracellular cytochrome P450 monooxygenases. UPOs are able to catalyse a variety of oxidation and oxyfunctionalization reactions with H_2O_2 (Hofrichter et al. 2015). The UPO catalyzed reactions include aromatic oxygenations, alkyl hydroxylations and oxidations of different aromatic and aliphatic compounds. UPOs are also able to cleave dimeric nonphenolic β-O-4 lignin model compounds suggesting that UPOs may have a role in the degradation of lignin derived methoxylated compounds (Kinne et al. 2011). The physiological function of DyPs and HTPs and their natural substrates remains unclear (Hofrichter et al. 2015; Linde et al. 2015).

Laccases (family AA1_1) belong to the multicopper oxidase family and they catalyse one-electron oxidations of wide range of substrates with the concomitant reduction of O_2 to water via a radical-catalysed reaction (Thurston 1994; Munk et al. 2015). First the substrate is oxidized at the mononuclear copper center T1 followed by intramolecular electron transfer into the trinuclear copper center T2/T3 in the laccase active site (Solomon et al. 2008). Laccases are able to directly oxidise a variety of phenolic compounds, e.g. methoxy-substituted phenols, polyphenols, aromatic amines and benzenethiols (Thurston 1994; Call and Mücke 1997). The laccase-mediator system was discovered in late 1990's (Call and Mücke 1997) showing that in the presence of low molecular weight compounds that act as redox mediators, the substrate range of laccases can be indirectly expanded to non-phenolic compounds (Bourbonnais and Paice 1990). As non-phenolic aromatic structures comprise ca. 80% of lignin, laccase-mediator systems play an essential role in lignin degradation. Laccases are applied in many industrial applications such as in bleaching and removal of lignin from wood and non-wood fibres. In addition, they are the most popular enzymes studied for the modification of various industrial lignins.

GMC (glucose-methanol-choline) superfamily oxidoreductases (AA3) and copper radical oxidases (AA5) are essential enzymes in plant biomass degradation as they generate H_2O_2 for peroxidase-catalysed reactions in white rot fungi and for Fenton reactions in brown rot species (Hammel and Cullen 2008). GMC superfamily members include various alcohol and sugar oxidases such as aryl alcohol oxidase (AA3_2), which catalyses the oxidation of lignin-derived compounds, such as phenolic aromatic aldehydes and acids, to their corresponding aldehydes by simultaneously reducing O_2 to H_2O_2 (Hernández-Ortega et al. 2012). Aryl alcohol oxidases are the most common GMC

oxidases in the white rot species. Copper radical oxidases, such as glyoxal oxidase (AA5_1) and galactose oxidase (AA5_2), produce H_2O_2 by catalysing oxidation of simple aldehydes to their corresponding carboxylic acids (Whittaker et al. 1996).

3.3 Intracellular aromatic converting enzymes

Fungal intracellular enzymes involved in conversion of aromatic compounds are mainly cytochrome P450 monooxygenases, glutathione-S-transferases, O-methyl transferases and vanillyl alcohol oxidases.

Basidiomycete genomes are rich in genes encoding putative cytochrome P450s (cytP450s). These enzymes are potentially involved in detoxification of small molecular weight aromatic compounds from lignin degradation process (Mäkelä et al. 2015). In addition, the huge number and sequence-level variation in basidiomycete cytP450s suggests that they play roles also in other metabolic adaptations such as secondary metabolite production and detoxification of xenobiotics (Crešnar and Petrič 2011; Durairaj et al. 2016). While experimental screening is essential to elucidate the catalytic potential of individual cytP450s, a systematic functional characterization has already been conducted e.g. with the cytP450s of the brown rot fungus *Postia placenta* (Ide et al. 2012).

Glutathione-S-transferase (GST; EC 2.5.1.18) superfamily consists of multifunctional enzymes which are involved in metabolic and detoxification pathways. They catalyse the conjugation of the tripeptide glutathione to the compounds containing electrophilic center. In addition, some GSTs are also able to bind non-substrate molecules possessing ligandin function (Lallement et al. 2014). Ligandin properties are known to play an important role in sequestration and transport of toxic compounds inside of cell. Fungal specific GSTs (GSTFuA) have been identified and characterized in white rot species. GSTs include β-etherases, which catalyse the reductive cleavage of β-O-4 bonds. Putative β-etherase encoding genes are widely detected in fungal genomes, however functional β-etherase (GST1) has been only characterized from the white rot basidiomycete *Dichomitus squalens* (Marinović et al. 2018b). GST1 is able to cleave β-O-4 bond in lignin model compound as well as to reduce the size of synthetic lignin (G-DHP) thus suggesting a role in intracellular catabolism of lignin-derived aromatic compounds. GSTFuAs of the other white rot species *P. chrysosporium* have shown ligandin properties towards lignin derived components such as coniferyl and syringaldehyde, vanillin, chloronitrobenzoic acid, hydroxyacetophenone, and catechins (Thuillier et al. 2014).

O-methyl transferase encoding genes are found in the genomes of white rot species. Functionally characterized O-methyl transferase of *P. chrysosporium* converts the aromatic ring linked OH groups at both meta and para positions by utilizing S-adenosyl methionine (Wat and Towers 1975; Coulter et al. 1993; Pham and Kim 2016). They are able to methylate coniferyl alcohol, ferulic acid, vanillic acid and isovanillic acid (Jeffers et al. 1997; Pham and Kim 2016). It is suggested that the biological role of O-methyl transferases in fungi is to remove free-OH phenolic compounds, which inhibit enzymatic activity of LiP, and convert them into non-toxic methylated phenolic ones (Pham et al. 2014; Pham and Kim 2014). Fungal phenol-methylating enzymes may have applications e.g. in the pulp and paper industry. Treatment of mechanical pulp under anaerobic conditions with mycelial extracts of the white rot fungi *Phlebia radiata* and *P. chrysosporium* partially prevents the yellowing of mechanical pulp (Hatakka et al. 1994).

Vanillyl alcohol oxidases (VAOs) convert a wide range of para-substituted phenols producing e.g. vanillin, coniferyl alcohol and chiral aryl alcohols. VAOs are classified into AA4 family in the CAZy database. In the Basidiomycota genomes, only Agaricomycotina contain putative VAO encoding genes (Gygli et al. 2018). However, the only biochemically studied VAO is from an ascomycete species *Penicillium simplicissimum*.

4. Current status of basidiomycete genomics

In 2004, *P. chrysosporium*, the model species for lignin and white rot wood degradation was genome-sequenced as the first species from the phylum Basidiomycota (Martinez et al. 2004). Since that the genomics era has already provided more than 400 basidiomycete genome sequences (JGI MycoCosm, 5th April 2019, https://genome.jgi.doe.gov/programs/fungi/index.jsf), most of which (362) are from species from the subphylum Agaricomycotina (Fig. 2) that includes majority of the plant biomass degrading basidiomycete fungi. At the same time, MycoCosm portal of the Joint Genome Institute (JGI) of the U.S. Department of Energy has become an invaluable fungal genomics resource, which gives access to numerous fungal genome sequences as well as provides tools for analysis of individual genomes and comparative genomics (Grigoriev et al. 2014). After *P. chrysosporium*, the second genome-sequenced wood degrading basidiomycete was the model brown rot species *P. placenta* (*Rhodonia placenta*) (Martinez et al. 2009), and release of its whole genome sequence enabled the first genome level comparative studies between the two wood decay types,

white and brown rot (Vanden Wymelenberg et al. 2010; Vanden Wymelenberg et al. 2011). These pioneering studies were followed by public releases of the genomes of the selectively lignin degrading white rot fungus *Ceriporiopsis subvermispora* (Fernández-Fueyo et al. 2012), the medicinal white rot fungus *Ganoderma lucidum* (Liu et al. 2012) and the litter-decomposing white button mushroom *Agaricus bisporus* (Morin et al. 2012), amongst others. More recently, genome sequencing has expanded to cover multiple strains of the same species, and the white rot fungus *D. squalens* with four available genome sequences from different strains (https://genome.jgi.doe.gov/programs/fungi/index.jsf) provides the best coverage of a filamentous basidiomycete species at the moment. The availability of multiple genome sequences of an individual species will enable e.g. detailed molecular level studies on intraspecies diversity in basidiomycete plant biomass degradation, which is of importance for instance when selecting strains for biotechnology applications.

The first genome sequences of the saprobic basidiomycete fungi supported the biochemically observed differences between white and brown rot wood decay types (Martinez et al. 2004; Martinez et al. 2009). However, extensive genome comparisons of saprobic basidiomycetes (Riley et al. 2014; Floudas et al. 2015) enabled more defined division for different types of fungal wood decay and life-styles based on the content of the CAZyme encoding genes in these species. As already mentioned in the previous section, the classical dichotomous division of the wood rot types was shown to be inadequate to cover the whole spectrum of the degradation strategies of wood degrading basidiomycetes (Riley et al. 2014; Floudas et al. 2015), and thus species that have intermediate decay capabilities were described as species causing uncertain type of rot or grey rot.

The genomes of the saprobic white rot basidiomycetes have been shown to be rich in genes (Riley et al. 2014) encoding enzymes that cover depolymerization of plant cell wall polysaccharides as well as the aromatic lignin polymer (Floudas et al. 2012; Rytioja et al. 2014). The presence of the genes encoding class II fungal heme peroxidases, i.e. MnPs, LiPs or VPs from family AA2, as well the enzymes acting on crystalline cellulose, i.e. GH6 and 7 CBHs, and AA9 LPMOs (Table 1) have been shown to be characteristic for the white rot fungal genomes. Compared to white rot fungi, the lignin-modifying peroxidases encoding genes are absent from brown rot fungal genomes. In addition, the number of cellulase genes is strongly reduced in brown rot genomes, thus highlighting their focus on non-enzymatic strategy for cellulase depolymerization. In contrast, the grey rot species, such as *Botryobasidium botryosum*, *Cylindrobasidium torrendii*, *Jaapia argillacea* and *Schizophyllum commune*, do not possess lignin-modifying peroxidase enzymes, but have diverse enzymes for depolymerization of crystalline cellulose (Riley et al. 2014; Floudas et al. 2015).

While the overall CAZy gene content can be considered largely comparable between fungal species representing a certain wood rot type, variable numbers of genes encoding enzymes with certain (putative) activity are present in these species (Table 1). For example, several white rot genomes are particularly rich in laccase encoding genes, whereas *P. chrysosporium*, *Phanerochaete carnosa*, *Phlebiopsis gigantea* and *Bjerkandera adusta* lack *sensu stricto* laccases, thus showing that these enzymes are not required for efficient lignin degradation. This is further confirmed by the presence of laccase genes in almost all studied brown rot fungi as well as in the species with intermediate wood decay abilities. White rot fungi also have differences in their set of lignin-modifying heme peroxidases. MnPs are the most common ligninolytic peroxidases, while LiP and VP encoding genes are relatively rare in the white rot fungal genomes (Table 1). Interestingly, a high number of LiP encoding genes, form 10 to 12, are present in *P. chrysosporium*, *Trametes versicolor* and *B. adusta*. This redundancy of certain plant cell wall degradation related CAZy genes is not yet understood, but it may endow basidiomycetes with e.g. adaptability in changing environmental conditions.

In addition to cataloguing the gene content related to plant biomass degradation, the comparative genomics studies on different basidiomycete fungi have shed light on evolutionary patterns leading to the currently existing plant cell wall degradation approaches among basidiomycetes (Floudas et al. 2012; Floudas et al. 2015; Nagy et al. 2016). First, the brown rot fungi were shown to be evolved several times from an ancestor white rot species (Floudas et al. 2012), and more recently, comparative genomics of early-diverging mushroom forming fungi suggested that most of the white rot fungal lignin-degrading oxidative enzymes have appeared after the origin of the first white rot species (Nagy et al. 2016).

Although the intracellular aromatic metabolic enzymes and their encoding genes of wood-rotting basidiomycetes have gained relatively little attention (Mäkelä et al. 2015), their genomes have been shown to be particularly rich in genes encoding putative cytP450s. For example, the white rot fungi *P. chrysosporium* (Doddapaneni et al. 2005) and *D. squalens* (Morel et al. 2013) possess 149 and 200 cytP450 genes, respectively, while even a higher number, 236, is present in the genome of the brown rot fungus *P. placenta* (Martinez et al. 2009). So far, the most significant expansion of cytP450s is reported for the white rot fungus *P. carnosa* with 266 candidate cytP450s (Suzuki et al. 2012). This enrichment could be advantageous for growth on substrates that have high content of lignin and extractives, such as coniferous heartwood (Jurak et al. 2018). Brown rot fungi are primarily found associated with conifers, although there are also some brown rot species that attack hardwoods (Eriksson et al. 1990).

It should be noted that approximately one-half of the proteins predicted to be encoded by the basidiomycete genome sequences do not have a known or putative function (Riley et al. 2014). Conceivably, part of these candidate enzymes plays a role in lignocellulose conversion. For example, the phylogenomic analysis by Nagy et al. (2017) revealed 73 gene families without any known domains in the Pfam protein family database (El-Gebali et al. 2019) and 49 gene families that have domains of unknown function (DUF), which were predicted to participate in white rot fungal wood decay. Functional characterization of the unknown proteins is undoubtedly one of the future challenges in basidiomycete research.

The development of genome annotation methods has produced improved fungal genome sequences, e.g. with respect to the number and quality of predicted gene models (Ohm et al. 2014). While recently the gold-standard genome of the filamentous ascomycete fungus *Aspergillus niger* strain NRRL3 was published (Aguilar-Pontes et al. 2018), most of the fungal genomes are still incompletely sequenced and assembled, containing gaps and insufficient gene annotations. In addition to the risk of missing genes due to gaps in the genome, lack of high-quality genome sequences crucially complicates the analysis of omics data as well as genetic studies, by hampering targeted gene deletions and modifications. Recently approved genome sequencing project at DOE-JGI (https://jgi.doe.gov/csp-2019-finishing-genomes/) aims to result in approximately 25 full fungal genomes with telomere-to-telomere validated assemblies of all chromosomes and manually verified gene models. These high-quality genome sequences, including well-studied basidiomycete species for wood degradation – the white rot fungus *D. squalens* and the brown-rot fungus *P. placenta* – will provide better references for gene prediction and functional annotation in other basidiomycete species. These gold-standard genomes will therefore improve the quality of other genome sequences and provide a better understanding of abilities and differences between the fungal species.

5. Post-genomic analyses of basidiomycetes

Shortly after the availability of the fungal genome sequences, the high-throughput functional genomics studies, i.e. transcriptomics and proteomics, of basidiomycete fungi have increased exponentially. These omics studies have contributed to harnessing the full potential of basidiomycetes in plant biomass based biotechnological applications by facilitating the exploration of key genes and enzymes that are involved in plant cell wall degradation, and validating functions of these enzymes. However,

global metabolomics studies are still in their infancy in basidiomycetes, mostly due to lack of reference databases for fungal metabolites. Further development of analytical methods and bioinformatics tools will be needed to enable systems biology level studies on plant biomass modification by basidiomycetes.

So far, transcriptome and proteome analyses have been performed for several individual plant biomass degrading basidiomycete species, but more extensive studies have focused on relatively few model species. These include e.g. the white rot fungi *P. chrysosporium*, *P. carnosa* and *D. squalens*, and the brown rot fungus *P. placenta* (Vanden Wymelenberg et al. 2009; Vanden Wymelenberg et al. 2010; MacDonald et al. 2011; Vanden Wymelenberg et al. 2011; Rytioja et al. 2017; Casado López et al. 2018; Daly et al. 2018; Jurak et al. 2018). The basidiomycete omics studies have been conducted from variable culture conditions including liquid, semi-solid and solid cultivations. These cultivations have contained various types of plant biomass with different chemical composition as a substrate covering both non-wood and wood biomass (Vanden Wymelenberg et al. 2011; Miyauchi et al. 2017; Rytioja et al. 2017) as well as plant biomass derived mono-, oligo- and polymeric compounds (Vanden Wymelenberg et al. 2009; Casado López et al. 2018). In addition, the used biomass substrates have had highly different physical properties varying from fine-powdered plant biomass to wood sawdust and more intact pieces of wood such as sticks and wafers (Rytioja et al. 2017; Daly et al. 2018; Jurak et al. 2018; Marinović et al. 2018a; Presley et al. 2018). Initially, the omics studies focused on early stages of basidiomycete growth and degradation, and usually included sampling of an individual time point after few days of cultivation (Vanden Wymelenberg et al. 2009; Fernández-Fueyo et al. 2012). Later on, the omics analyses have also been performed from prolonged cultivations with several sampling points to capture the temporal dynamics of the plant biomass conversion and to predict the most critical genes and enzymes at early and later stages of the degradation process (Kuuskeri et al. 2016; Marinović et al. 2018a; Jurak et al. 2018).

In line with the content of the white rot genomes, the functional genomics studies have indicated a central role for class II lignin modifying peroxidases and H_2O_2-producing enzymes, together with GHs and LPMOs for white rot decay. Expectedly, the variations detected in CAZy gene expression and protein production by different white rot fungal species have been larger than those observed in the corresponding genome content. In the molecular level studies, several white rot species have shown preference for lignin degradation during early stages of growth (Patyshakuliyeva et al. 2015; Kuuskeri et al. 2016; Rytioja et al. 2017; Marinović et al. 2018a), most probably as a prerequisite for getting access to plant biomass carbohydrates to warrant further growth and propagation. So far, no conclusive results with respect to the dogma of the selective

lignin degradation have been obtained from the (post)genomics studies of white rot fungi (Fernández-Fueyo et al. 2012; Marinović et al. 2018a). The mechanism behind selective ligninolysis has been suggested to possibly involve MnPs as well as desaturases that are putatively involved in the cleavage of dominant non-phenolic structures of lignin through lipid peroxidation (Fernández-Fueyo et al. 2012). In addition, lower expression and production of cellulolytic genes and enzymes, respectively, were implicated when the selective lignin degrader *C. subvermispora* was compared to the non-selective *P. chrysosporium* (Fernández-Fueyo et al. 2012).

Transcriptome and proteome analyses have clearly shown that the ability of the lignocellulose degrading basidiomycete fungi to convert plant biomass is not restricted to substrates they encounter in their natural habitats. For example, the white rot fungus *D. squalens* shows nearly as good molecular response to non-wood as to woody biomass (Rytioja et al. 2017). Furthermore, *D. squalens* is able to partially adjust the enzyme sets is produces to match the composition of the wood substrate it grows on (Daly et al. 2018). This suggests the existence of fine-tuned regulatory mechanisms behind white rot plant biomass and wood cell wall degradation.

Although the brown rot wood decay mechanism is not fully understood yet, functional genomics studies have supported the important role of diffusible ROS and Fenton chemistry type redox system in non-enzymatic depolymerization of carbohydrates (Vanden Wymelenberg et al. 2010; Vanden Wymelenberg et al. 2011; Gaskell et al. 2016). Recent transcriptome analyses have indicated that variable degradation patterns exist among brown rot species. While *P. placenta* has suggested to initiate lignocellulose degradation by chemical attack that is followed by the action of its relatively narrow set of cellulolytic and hemicellulolytic GHs (Zhang et al. 2016), this pattern was not observed for *Fomitopsis pinicola* that is a common brown rot species in boreal and temperate forests in the Northern Hemisphere (Wu et al. 2018). In addition, *Wolfiporia cocos* and *P. placenta* that both are members of the clade Antrodia, were detected to express and upregulate a diverge set of genes putatively related to redox cycling (Gaskell et al. 2016). This may indicate that even the closely related brown rot species have differences in their approaches for lignocellulose conversion. Transcriptome and proteome data from various species combined with detailed metabolomics analyses will be needed to further clarify the brown rot mechanisms of wood decay.

Typically, brown rot fungi strongly decrease the methoxyl content of lignin in wood (Eriksson et al. 1990), and therefore, the demeth(ox)ylation mechanisms of these fungi are of great interest, and most probably a high number of cytP450s reflects these activities. Variability among different brown rot species is also expected in the

regulation and extent of demeth(ox)ylation. When demethoxylation reactions in the cultures of the brown rot fungi *Gloeophyllum trabeum* and *P. placenta* were studied by determining the evolution of $^{14}CO_2$ from a non-phenolic β–O–4 lignin model dimer, [$O^{14}CH_3$]-labelled at position 4 in the A ring, and from [$O^{14}CH_3$]-labelled vanillic acid, the results indicated that these common brown rot species possess different mechanisms for demeth(ox)ylation of lignin model compounds (Niemenmaa et al. 2008).

In line with the basidiomycete genome content, the putative cytP450s encoding genes have been shown to be abundantly expressed and upregulated during fungal growth on lignocellulosic substrates. For example, approximately 25% of the cytP450s of the white rot fungus *P. carnosa* were highly expressed when the fungus was cultivated on aspen and spruce as carbon sources (Jurak et al. 2018). In several white and brown rot species, including *D. squalens* (Daly et al. 2018), *Pycnoporus coccineus* (Couturier et al. 2015), *P. placenta* (Vanden Wymelenberg et al. 2011) and *W. cocos* (Gaskell et al. 2016), cytP450s have been detected to be upregulated on coniferous softwood compared to deciduous hardwood as a substrate. This could possibly reflect the role of these monooxygenases in detoxification of wood extractives, the composition of which differ between softwoods and hardwoods.

Genes encoding putative non-catalytic proteins such as expansin-like proteins (or "loosenins") and hydrophobins, have also been reported to be co-expressed with CAZymes in the lignocellulose cultures of the white rot species (Couturier et al. 2015; Kuuskeri et al. 2016; Rytioja et al. 2017; Jurak et al. 2018). However, exact roles of these proteins are not known. In plants, expansins facilitate loosening of cell walls during growth (Marowa et al. 2016). It may be possible that the fungal expansin-like proteins have a similar function during degradation of plant cell walls, thus increasing accessibility of CAZymes to lignocellulose polymers, as implicated by an expansin-like protein from *B. adusta* with cellulose disrupting activity (Quiroz-Castañeda et al. 2011). The surface-active hydrophobin proteins have been speculated to facilitate hyphal attachment to lignocellulose substrate (Linder et al. 2005), which could be advantageous for fungal colonization of biomass.

Genes encoding proteins with unknown function form a major portion of basidiomycete genomes. Transcripts of these genes or their protein products have repeatedly been observed in functional genomic studies of different wood rotting fungi addressing lignocellulose conversion, thus supporting that a portion of this genomic "dark matter" contributes to plant cell wall depolymerization. In addition to upregulation and/or high expression levels of unknown protein encoding genes (Vanden Wymelenberg et al. 2010; Korripally et al. 2015; Gaskell et al. 2016; Jurak et al. 2018), their co-expression with annotated CAZy genes in wood cultivations has been reported (Jurak et al. 2018).

Markedly, 146 genes representing 50% of the differentially expressed genes between pine and spruce cultures of *F. pinicola* encoded proteins with unknown functions (Wu et al. 2018). Determining functions of these proteins will be essential for understanding the basidiomycete strategies for plant biomass degradation (Couturier et al. 2018).

The recent transcriptomic and exoproteomic analyses on wood degrading basidiomycetes have indicated that cultivation conditions and level of substrate processing cause larger differences in gene expression and protein production profiles than the use of different wood species as a substrate. This is seen when submerged cultures have been compared to solid state cultures, and when the use of powdered wood has been compared to wood sticks and wafers (Rytioja et al. 2017; Daly et al. 2018; Wu et al. 2018). This suggests that as the cultivation setups used for omics analyses as well as the experimental platforms used are heterogeneous, comparison of the datasets is challenging and therefore care should be taken when drawing conclusions from the results originating from highly divergent experimental conditions. However, the first larger scale meta-analysis on plant biomass degradation related basidiomycete transcriptome data was recently conducted, which involved 10 fungal species and 22 individual datasets (Peng et al. 2018). This comparative analysis defined a core set of commonly upregulated CAZyme encoding genes in these species, thus suggesting a key role for the corresponding enzymes in plant biomass degradation.

6. Challenges in basidiomycete research

As already briefly mentioned in the previous sections, there are several hurdles to overcome in order to harness the full biotechnological potential of plant biomass converting basidiomycete fungi. In this section, we present the current knowledge on molecular level regulation of plant biomass related enzyme production in basidiomycetes and in relation to this, discuss the limitations of genetic manipulation of basidiomycetes as well as recent developments in genome editing systems.

6.1 Regulation of plant biomass conversion related enzyme production

The regulatory mechanisms behind production of plant biomass degrading enzymes in basidiomycete fungi are poorly understood. In ascomycetes on the other hand, many transcription factors (TFs) that regulate expression of CAZyme encoding genes have been characterized (Benocci et al. 2017). These TFs respond to the environmental

signals (e.g. varying carbon and nitrogen source, pH, light and temperature), and bind to the conserved promoter elements in their target genes thus activating and/or repressing the expression of these genes. Several characterized TFs are sugar-specific, which means that they are activated only in the presence of a certain plant polysaccharide-derived inducer, such as mono- and disaccharides or their metabolites (Kowalczyk et al. 2014). Identification of sugar-specific TFs and their inducing compounds in filamentous ascomycete cell factories, such as *A. niger* and *Trichoderma reesei*, was a key step to knowledge-driven engineering of strains with improved plant biomass degradation capabilities (reviewed in Alazi and Ram 2018). Therefore, a better understanding of the regulatory mechanisms that govern expression of polysaccharide and lignin-degrading genes in basidiomycete fungi is crucial to fully exploit their biotechnological potential in future.

Despite the total predicted number of genes encoding transcriptional regulators in the genomes of basidiomycete fungi is estimated to be between 200 and 800 (Todd et al. 2014; Shelest 2017), identification of TFs specifically involved in plant biomass degradation has proven to be difficult. Similarly to ascomycetes, basidiomycete fungi can regulate expression of sets of CAZyme encoding genes in response to particular plant biomass-related substrate (Peng et al. 2018), which suggests the presence of sugar-specific TFs in both fungal groups. However, low number of orthologs for the ascomycete TF proteins in the available basidiomycete genomes (Todd et al. 2014; Benocci et al. 2017) indicates that the regulatory systems that control expression of CAZy genes in these two phyla evolved independently. This is also reflected in the different general distribution of the TF Pfam families, with the C2H2 and CCHC classes being largely overrepresented in basidiomycetes compared to ascomycetes (Todd et al. 2014). Currently, only two of the sugar-specific TFs characterized in ascomycetes, Cre1 and ACE3, have orthologs in basidiomycete fungi (Benocci et al. 2017). The carbon catabolite repressor Cre1 (called Mig1/CreA/Cre-1 in *Saccharomyces cerevisiae*, *Aspergillu*s species and *Neurospora crassa*, respectively) has been studied in many ascomycetes, in which it downregulates expression of genes encoding enzymes degrading complex carbon sources in the presence of energetically more favourable sugars (e.g. D-glucose, D-xylose). However, the regulatory role of Cre1 seems to be much broader in these fungi as it also controls expression of several TF-encoding genes including *amyR*, *xlnR* and *clrB* in various ascomycetes (Tani et al. 2001; Mach-Aigner et al. 2008; Tamayo et al. 2008; Li et al. 2015), and affects chromatin accessibility of *xyr1* promoter in *T. reesei* (Mello-de-Sousa et al. 2016). The influence of Cre1 modifications on activity and secretion of plant biomass degrading enzymes was recently evaluated in the basidiomycete fungus *P. ostreatus* (Yoav et al. 2018). Deletion and overexpression of *cre1* in this fungus caused carbon source-dependent changes in secreted activity of key CAZymes and indicated presence of a complex regulatory

system in which Cre1 could act (directly or indirectly) as repressor or activator (Yoav et al. 2018). The second orthologous protein, an activator of cellulase expression ACE3, upregulates expression of genes encoding cellulases and partially xylanases in *T. reesei* (Häkkinen et al. 2014), however it has not been studied in any basidiomycete species yet. While the TFs involved in degradation of the same plant polysaccharide substrates in basidiomycetes and ascomycetes evolved in parallel, the induction mechanisms involved in their activation could be similar. An interesting example of such similarity is expression of several cellulolytic genes, which was shown to be upregulated in the presence of disaccharide cellobiose in some *Aspergillus* species (Marui et al. 2002; Kunitake et al. 2013) as well as in the brown rot fungus *P. placenta* (Zhang and Schilling 2017) and white rot fungus *D. squalens* (Casado López et al. 2018).

Additionally, white rot basidiomycetes produce variety of lignin-degrading enzymes, expression of which can be affected by several factors, such as metal ions, nitrogen and cAMP levels (Boominathan and Reddy 1992; Boominathan et al. 1993; Van der Woude et al. 1993; Alvarez et al. 2009; Feldman et al. 2017). Several post-genomics studies showed that genes encoding lignin- and polysaccharide-depolymerizing enzymes in these fungi were often expressed independently from each other and therefore could respond to different environmental inducers (MacDonald et al. 2011; Fernández-Fueyo et al. 2012; Rytioja et al. 2017). This suggests that the development of the lignin-degrading machinery has been accompanied by the development of a separate regulatory system, rather than linking them to the carbohydrate-related regulatory systems. Recently, Nakazawa and colleagues adapted the forward genetic approach to search for novel TF-encoding genes linked to lignin degradation in *P. ostreatus*, leading to identification of several genes, including *pex1* encoding a peroxisome biogenesis factor, *chd1* encoding a putative chromatin modifier, as well as *wtr1* and *gat1* encoding putative DNA-binding TFs (Nakazawa et al. 2017a; Nakazawa et al. 2017b; Nakazawa et al. 2018). Deletion of putative TF encoding gene *wtr1* in *P. ostreatus* abolished expression of *mnp2* gene, and partially reduced its ability to degrade lignin in sawdust medium (Nakazawa et al. 2017b), while deletion of *gat1* caused stronger reduction in degradation of lignin, but not in the polysaccharide-part present in beech wood sawdust medium. Interestingly, Gat1 was initially identified as a TF linked to fruiting body development in *S. commune* (Ohm et al. 2011). In addition to ligninolytic system deficiencies, *gat1* mutation completely abolished fruiting in *P. ostreatus* dikaryon. However, the molecular basis of observed defects remains to be solved. While our knowledge about the regulation of plant biomass degradation in basidiomycete fungi is slowly growing, many pieces of the puzzle are still missing to fully understand this intricate system.

6.2 Transformation systems

Genetic manipulations of fungi are essential to study the function of a certain protein *in vivo* and could facilitate construction of strains with enhanced lignocellulolytic capabilities. However, this has been restricted to a few model basidiomycete species, in which an efficient transformation system has been developed. The outcome of each transformation depends on success of the following steps: engineering of host strains with a desired selective marker, construction of exogenous DNA (linear or circular), removal of host cell wall, and introduction of the exogenous DNA inside the cell and its integration into host genomic DNA or maintenance in an autonomous vector inside the host cell (reviewed in Kim et al. 2015).

One of the limitations in genetic manipulations of basidiomycete fungi is still the lack of versatile molecular tools, such as selection markers and plasmids that can be used in several species. Selection markers are necessary for identification of transformants with foreign DNA. Several synthetic plasmids that carry the drug-resistance genes and incorporate into the genome have been constructed for basidiomycete transformation. The use of antibiotic resistance is advantageous, as it does not require prior engineering of an auxotrophic recipient strain. However, only few antibiotics, i.e. hygromycin, phleomycin, nourseothricin, carboxin and geneticin, are utilized in basidiomycetes, which introduces restrictions in the number of modifications they allow. Additionally, since only small number of basidiomycete promoters are functional across the phylum, construction of many selection markers often requires additional screening for strong constitutive promoters to drive their expression (Kim et al. 2015). The use of selectable auxotrophic markers is difficult in basidiomycete fungi without a well-established transformation system, as it requires an additional engineering step of the host strain. For example, few markers for complementation of *trp1/trp2/trp3*, *ade8* and *pab1* auxotrophy were constructed in the well-studied basidiomycetes *Coprinopsis cinerea*, a model species for regulation of multicellular development and mushroom formation, and *P. chrysosporium* (*leu2, ade1/ade2, ura3/ura5*, *trpC* auxotrophy), a model species for white rot lignin degradation (for references see Gold and Alic 1993; Dörnte and Kües 2016). One way to overcome this limitation is isolation of uridine/uracil auxotrophic strains with spontaneous mutation(s) in pyrimidine biosynthesis pathway genes induced by a combination of UV-irradiation and selection on 5-fluoroorotic acid (5-FOA) as shown for *S. cerevisiae* (Boeke et al. 1984), *A. niger* (van Hartingsveldt et al. 1987), *P. chrysosporium* (Akileswaran et al. 1993) and *P. ostreatus* (Nakazawa et al. 2016), among others. An added advantage of using uridine/uracil auxotrophic marker is that it is counter-selectable, and thus leaves no mark on the genome. Recently, this method was adapted in the white rot fungus *P. ostreatus*, where addition of two

homologous direct repeat sequences on each side of the orotidine-5-phosphate decarboxylase gene (*PopyrG*) allowed marker recycling *via* 5-FOA-induced single crossover between the direct repeats (Nakazawa et al. 2016).

Successful integration of an exogenous DNA in a desired locus in the genome is usually based on homology-directed recombination (HDR). However, HDR is extremely rare in higher fungi, which prefer to repair the double strand DNA breaks *via* non-homologous end joining (NHEJ) resulting in ectopic integrations (for review see Weterings and Chen 2008). Therefore, fungal defense mechanisms itself are a huge problem for targeted genetic modification. In ascomycete model fungus *N. crassa*, increased HDR frequencies were observed after disruption of *ku70/ku80* genes essential for NHEJ pathway (Ninomiya et al. 2004). Shortly after, Δ*ku70* or Δ*ku80* NHEJ-deficient strains were engineered in many other species, including few basidiomycete fungi with well-established transformation systems. For example, construction of recipient strains with improved gene targeting has been successfully performed *via* deletion of *ku80* in *S. commune* (de Jong et al. 2010) and *P. ostreatus* (Salame et al. 2012) or RNAi-mediated silencing of *ku70* and *lig4* in *C. cinerea* (Nakazawa et al. 2011). However, due to low efficiency and the fact that Δ*ku70*/*ku80* are phenotypically indistinguishable from the wild type, this requires screening of multiple colonies to find the correct mutants. Another drawback is potential increased sensitivity of NHEJ-deficient strains to DNA damage and aging.

Another obstacle to overcome when performing genetic manipulations in basidiomycete fungi originates in their distinct life cycle. Basidiomycetes spend most of their life as dikaryotic mycelium (n+n), containing two different nuclei with compatible mating types in every cell. Simultaneous introduction of foreign DNA into two nuclei of a dikaryotic cell is obviously difficult; therefore, the use of monokaryotic cells (basidiospores or young monokaryotic mycelium) seems more advantageous. However, there is still high chance of obtaining heterokaryotic colonies caused by transformation of multi-nucleated protoplasts (da Silva Coelho et al. 2010), fusion of uni-nucleated protoplasts during transformation (Gold et al. 1983) or interleaved growth of transformed protoplasts together with untransformed background. Heterokaryotic colonies will require additional purification, such as re-protoplasting or fruiting and selection of single basidiospores, to obtain mono-nucleated transformants that could be screened for a desired genetic modification.

6.3 Application of CRISPR/Cas9 in basidiomycetes

Development of a powerful genome editing technique CRISPR/Cas9 for several fungal species (Liu et al. 2015; Nødvig et al. 2015; Pohl et al. 2016; Liu et al. 2017 to name a few) has opened new possibilities for precise and efficient gene modifications in transformable basidiomycetes. CRISPR/Cas9 system relies on a synthetic guide RNA (gRNA) to target the Cas9 endonuclease to a specific genomic locus, where it introduces double-strand DNA breaks. The DNA breaks are then repaired by the host's repair mechanisms: NHEJ or, if supplied, with a selectable repair cassette that harbours certain homology, HDR. Successful application of this method in basidiomycete fungi depends on simultaneous delivery of functional gRNA and Cas9 into the nucleus. Functional Cas9 can be achieved by codon optimization of *cas9* from *Streptococcus pyogenes* and its fusion with a strong constitutive promoter and nuclear localization signals as previously shown for *T. reesei* (Liu et al. 2015). In basidiomycete fungi, similarly constructed *cas9* expressing vectors were functional after randomly introduced into the genome in *C. cinerea* (Sugano et al. 2017) and *Ganoderma* sp. (Qin et al. 2017). However, the long-term effects of *cas9* expression in these species are currently unknown. To overcome potential harmful effects originating from integrating of the *cas9* gene into the genome, the plasmids containing self-replicating elements could be used. While certainly beneficial, such plasmids are extremely rare in fungi. In basidiomycete corn pathogen *Ustilago maydis*, a ground-breaking discovery of DNA sequence similar to autonomously replicating sequences (ARS) of *S. cerevisiae* led to construction the first basidiomycete self-replicating plasmid (Tsukuda et al. 1988). In *P. chrysosporium* (Randall et al. 1991) and *P. ostreatus* (Peng et al. 1992), possibly extrachromosomally maintained plasmids were observed after an endogenous replicative sequence recombined *in vivo* with the initial non-replicative vector during transformation, however the exact reason behind such phenomena is not well understood. So far, expression of *cas9* and gRNA from a self-replicating plasmid was only possible in *U. maydis* (Schuster et al. 2016; Schuster et al. 2018) while in *Ustilago trichophora* transient expression was presumably obtained from non-replicative plasmid maintained extrachromosomally (Huck et al. 2018). While application of Cas9 or other programmable nucleases in broader number of basidiomycete species is still a challenge, it seems that a new era of genetic modifications is closer than ever.

7. Concluding remarks and future perspectives

This chapter offered an overview on the current stage and future challenges in research of plant biomass degradation by basidiomycete fungi. Our investigation of the PubMed database (https://www.ncbi.nlm.nih.gov/pubmed) using search words "basidiomycete" or "Basidiomycota" in combinations with "biomass" or "lignin" or "lignocellulose"

returned nearly 3000 publications addressing this topic in 1950-2019. The titles of these articles were then used to generate a word cloud (Fig. 3), which visualizes that the white rot basidiomycetes, especially *Phanerochaete* (438 hits), *Pleurotus* (247) and *Trametes* (158) as well as topics related to production (426) of peroxidases (383), laccases (165) and manganese peroxidases (124) are among the most studied in the field to date. Great progress has been achieved in fungal (post)genomics studies of many taxonomically and functionally different fungi. However, breakthrough or discovery changing paradigm in the understanding how fungi degrade natural lignin has not yet been shown. Only very few researchers have tried to analyse which enzymes are active during natural degradation of wood. A recent metatranscriptomic and metaproteomic study showed that most of the functional oxidoreductase genes were MnPs, while LiPs, VPs or laccases were not detected in field samples of extensively decayed wood (Hori et al. 2018). However, also in this study, the function and significance of the unknown proteins remained unclear. In this respect, the development of proper analytical methods of lignocellulose or lignin to trace the modifications caused by individual enzymes or whole fungal cultures is still a challenge. For example, use of ^{14}C-labelled lignins or lignin model compounds was in a key role when ligninolytic enzymes were first discovered (Kirk and Farrell 1987). Currently very few, if any, research groups use these tedious but unequivocal methods.

At the moment, laccase is the only basidiomycete extracellular oxidoreductase, which can be produced at industrial scale, and therefore it has been the choice in many applications. The lack of efficient recombinant production of lignin-modifying heme peroxidases is still hampering their commercial use. In order to design basidiomycete enzyme mixtures, e.g. causing partial modifications of industrial lignins or small molecular weight aromatic compounds derived from biomass, a more thorough knowledge of the concerted action of enzymes and fungal metabolites would be needed. However, integration of large-scale omics data together with development of powerful genetic tools for gene disruption or suppression for a larger set of basidiomycete fungi will most certainly open up new possibilities to deeply understand the decay mechanisms of plant biomass degradation.

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Figure legends

Figure 1. Number of scientific publications related to plant biomass degradation by basidiomycete fungi per decade. The data was retrieved from PubMed database (5th April 2019) using search words "basidiomycete" or "Basidiomycota" and "biomass" or "lignin" or "lignocellulose". The search resulted in 2837 publications of which 21 have been published in 2019. However, the final number of articles in 2019 is expected to be higher. Number of articles per decade (in parentheses): 1950-1959 (4), 1960-1969 (12), 1970-1979 (8), 1980-1989 (74), 1990-1999 (352), 2000-2009 (874), 2010-2019 (1513).

Figure 2. Number of the sequenced fungal genomes in the phylum Basidiomycota. Distribution of the whole genome sequences between the Basidiomycota classes is shown on the left. The division of the genome sequences in the Agaricomycotina subphyla is depicted on the right. Data was retrieved from JGI MycoCosm (https://genome.jgi.doe.gov/programs/fungi/index.jsf), 5th April 2019.

Figure 3. A word cloud visualizing 30 most studied terms in basidiomycete research related to plant biomass (1950-2019). The PubMed database was searched for all articles

containing search words “basidiomycete” or “Basidiomycota” in combination with “biomass” or “lignin” or “lignocellulose” in any of the fields. The search retrieved 2837 publications (5th April 2019) and their titles were used to generate a word cloud online (https://www.wordclouds.com/). Top 30 terms were visualized and the size of each word corresponds to their relative frequency amongst the titles. The words present in both singular and plural versions were combined (e.g. “enzyme” and “enzymes” were depicted together as “enzyme”) and the words closely related to the search criteria (e.g. “basidiomycete”, “fungus”) were omitted.