TUM School of Life Sciences

Genetic and regulatory investigation of fungal brown rot decay and its response to wood acetylation

Martina Petra Kölle

Vollständiger Abdruck der von der TUM School of Life Sciences der Technischen Universität München zur Erlangung des akademischen Grades einer

Doktorin der Naturwissenschaften (Dr. rer. nat.)

genehmigten Dissertation.

Vorsitzender: Prof. Dr. Dr. h.c. Hans Pretzsch

Prüfende der Dissertation: 1. Prof. Dr. Johan Philipp Benz

2. Prof. Dr. Klaus Richter

Die Dissertation wurde am 07.05.2021 bei der Technischen Universität München eingereicht und durch die TUM School of Life Sciences am 28.10.2021 angenommen.

"Success isn't about how your life looks to others. It's about how it feels to you. We realized that being successful isn't about being impressive, it's about being inspired."

Michelle Obama

Acknowledgements

When I decided to take the step to write a doctoral thesis, I couldn´t even imagine what this experience would bring to my life, including both professional and personal growth. A wide range of people have contributed to this growth, which I want to mention here.

It was Annica Pilgård who set the ball running, giving me the opportunity to write my thesis within her project. The spirit she brings to a room, was the same spirit that amazed me from the very first time I entered the Wood Research Institute to apply for a job as a student associate. She never got tired of sparking joy, and being supportive, which I am very grateful for. She shared my despair, when we had to overthrow initial project plans and built me up when I thought that I couldn´t continue.

I am also very grateful for having invaluable guidance by Philipp Benz as my supervisor, who tickled out the precision and love for details that was needed to create publications that I now can be really proud of. Thank you for having an open door and ear, as well as advise whenever I needed it.

Furthermore, I want to thank Klaus Richter for always checking up on me, also when his time schedule was working to capacity, it was a great time and experience to work at the Wood Research Institute of Munich. Special thanks to Lisa for mentoring me during this time, always having advice when I needed it and always encouraging me to stick to it. Also, thanks to Maria, Rebecka and Nils for giving me advise, supporting me with databases, excel sheet madness and when I lost vista.

I want to thank my colleagues Anja, Michael, Raphaela, Gabi and Pia for dispelling black thoughts and doubts during sometimes endless coffee breaks and lunch breaks with a lot of laughing, scientific talks and nonsense, as for example the foundation of a goodies-container-

police. I will also never forget the long nights of the museums with you, which have been a great experience.

One of the most important experiences in my life was probably my time in Norway. Never have I ever thought to lose my heart for a country as I did for Norway. This is also thanks to the amazing people I met during the project meetings in Oslo. I want to thank the people from NIBIO for being amazing colleagues during my stay in Oslo, with special thanks to Gry, for being the best supervisor I could imagine for my time in Norway. And thank you Greeley, for being such an awesome first dinner partner in Oslo, a funny and inspiring person, my roomey for 10 weeks and now an appreciated friend.

I am very grateful to have so many great friends, who always kept my back. I feel more than honoured to gather a great group of ladies around me, every single of them being strong, confident, encouraging and amazing – thank you Julia, Martina, Mel, Nane and Domo (and some more) – for countless talks and shared glasses of wine and for keeping me sane. Thank you, Mo, for being such a loyal friend, and for having my back, when I didn´t have it.

I feel very gifted and am very grateful for having the most amazing family I could imagine. Thank you for never stopping believing in me, for always being there and giving your support. Thank you for being a port for shelter and safety, whenever I need it. Thank you for the endless love that I receive every day. Without you this wouldn´t have been possible. Thank you, Steffi, not only for being the best sister on earth, but also for being my best friend and therapist. Thank you, Clemens, for being a loyal and humorous friend since the first day at university.

Thank, you Alex, for accompanying me during the last one and a half years, for always cheering me up, for the love and the laughter you give me every day. Thank you for always being there for me and for being this overwhelmingly supportive and funny human being!

Summary

Wood is an attractive building material with a wide range of positive aspects, as for example being a renewable resource, its biodegradability, carbon storage capacity and its´ mechanical properties. Wood used in outdoor constructions is exposed to several types of decay, necessitating protection. As an alternative to copper-based preservatives, which have been partly banned or limited, wood modification has been shown to be effective against rots. One modification method that has been shown to delay fungal decay is acetylation. Even though several theories exist on how acetylation inhibits fungal growth, the exact mode of action remains unclear. Moreover, a detailed understanding of the molecular processes during brown rot decay is mandatory to improve protection methods against this type of degradation.

Brown rot fungi are known to be the most destructive group of decay fungi. It has been shown that brown rot fungi degrade wood in a two-step process, in which an initial oxidative degradation phase using low molecular weight agents is superseded by enzymatic hydrolysis of lignocellulose. *Rhodonia placenta* and *Gloeophyllum trabeum* are used in standardised decay tests in both Europe and North America. However, for *R. placenta* two different strains are employed (FPRL280 and MAD-698, respectively) for which differences in colonisation-rate, mass loss, as well as gene expression have been observed, limiting the international comparability of results.

The objective of this thesis was to provide new insights into the biochemical mechanisms involved in brown rot decay of the above-mentioned species and emphasis on the effects of wood acetylation. These would be beneficial for biotechnological applications as well as the development of novel wood preservation methods.

Initially, the genome of *R. placenta* FPRL280 was sequenced and compared to the known genome of MAD-698 to elucidate the inherent divergence between the two strains and identify the molecular basis for their differing phenotypes. On a next step, a comparative analysis of the decay strategies and degradative capacities of *R. placenta* and *G. trabeum* on untreated and acetylated wood (15% WPG) was performed to study the impact of wood acetylation on the behaviour of the three brown rot strains. To this end, transcriptomic data were compiled by RNA-seq from both the oxidative and enzymatic degradation phase. Additionally, using quantitative real time polymerase chain reaction, the impact of an acetylation gradient (10, 15

and 20%) was investigated in more detail by focusing on ten genes that are likely involved in the initial oxidative degradation of *R. placenta.*

Significant phenotypic and genomic differences were found between FPRL280 and MAD-698. Sequencing of the genome revealed an identity of 98.4%. Specific analysis of the carbohydrate-active enzymes (CAZymes) identified differences in many families related to plant biomass degradation, including SNPs, indels, gaps or insertions within structural domains. Four genes from MAD-698 belonging to the AA3_2 family could not be found in FPRL280 at all.

In degradation tests, all tested strains revealed clear regulatory differences between the two decay stages. Gene compositions of genes being active during oxidative and enzymatic decay varied not only between *R. placenta* and *G. trabeum* species but also between the two strains of the same species. Wood acetylation led to a generally delayed decay process, displaying signs of gene expression attenuation, particularly of genes involved in cell wall degradation. By hierarchical clustering, several transcription factors could be localised in vicinity to CAZy genes within gene clusters that were heavily affected by acetylation.

Furthermore, genes involved in initial brown rot decay were found to be upregulated group-wise with increasing acetylation level by *R. placenta*.

Overall, the results of this thesis help to explain the phenotypic differences observed between both strains of *R. placenta*, since the identified gene variants could interfere with enzymatic performance, substrate binding ability or protein folding. The results furthermore suggest that regulatory circuits evolve rapidly and are probably the major cause behind the different decay strategies as observed even between the two *R. placenta* strains. These findings underline that studies using different strains of *R. placenta* should only be compared with care, considering their different aggressiveness and decay capacity. Furthermore, describing brown rot as a two-step mechanism seems to be a little bit too simplistic, considering that multiple groups of genes work together synergistically and in potentially individual regulatory circuits. Seeing that all three strains used different molecular strategies for degradation as well as when coping with the wood acetylation highlights the complexity of the brown rot degradation system. Additionally, a high number of genes of all three strains is still uncharacterised, warranting further annotation efforts. Identifying these genes can not only help in decay detection and identification of the fungus *in situ*, but might also be interesting for other fields, such as biopulping.

Zusammenfassung

Holz ist ein beliebtes Baumaterial mit einem breiten Spektrum positiver Eigenschaften, wie zum Beispiel Erneuerbarkeit und biologische Abbaubarkeit. Holz, welches im Außenbereich verbaut wurde, muss allerdings gerade deshalb geschützt werden. Als Alternative zu kupferbasierten Holzschutzmitteln, welche aufgrund ihres Gefahrenpotentials für Gesundheit und Umwelt teilweise verboten wurden, hat sich Holzmodifizierung, wie zum Beispiel Acetylierung, bei der Bekämpfung von Fäuleerregern als effektiv erwiesen. Die exakte Wirkungsweise der Acetylierung bleibt, trotz einiger existierender Theorien, weiterhin unklar.

Braunfäulepilze sind für ihr hohes Zerstörungspotenzial bekannt, da sie Holz schneller und effektiver als andere holzzerstörende Organismen abbauen. Braunfäule verursachende Pilze bauen Holz in einem zweistufigen Prozess ab, wobei eine oxidative Initialphase, in welcher niedermolekulare Wirkstoffe zum Einsatz kommen, vor einer enzymatischen Hydrolyse von Lignocellulose stattfindet. In standardisierten Abbautests werden gängige Braunfäulepilze, wie zum Beispiel *Rhodonia placenta* oder *Gloeophyllum trabeum* verwendet. In Europa und Amerika werden allerdings, je nach Herkunft, zwei verschiedene Stämme von *R. placenta* (FPRL280 und MAD-698) verwendet, deren Phänotypen sich unterscheiden, was die internationale Vergleichbarkeit der Ergebnisse beeinträchtigt.

Ziel dieser Dissertation ist es, neue Einblicke in die unterliegenden biochemischen Mechanismen während des Braunfäuleabbaus oben genannter Stämme zu geben, mit dem Schwerpunkt des Effekts von Holzacetylierung. Diese Erkenntnisse können für den Einsatz dieser Organismen in der Biotechnologie, aber auch für die Verbesserung und Entwicklung von Holzschutzmethoden von Bedeutung sein.

Zuerst wurde das Genom von *R. placenta* FPRL280 sequenziert und mit dem von MAD-698 abgeglichen, um Unterschiede zwischen den Stämmen festzustellen. Außerdem wurde eine Vergleichsanalyse der Abbaustrategien von *R. placenta* und *G. trabeum* auf unbehandeltem und acetyliertem Holz (15% WPG) durchgeführt, um den Einfluss der Holzmodifizierung auf das Verhalten der Stämme zu untersuchen. Schließlich wurden Transkriptomdaten von sowohl der oxidativen als auch der enzymatischen Abbauphase, mithilfe von RNA-Seq gewonnen. Außerdem wurde der Einfluss von drei verschiedenen Acetylierungsstärken (10, 15 und 20%) auf zehn ausgewählte Gene untersucht, welche eine Rolle während des oxidativen Abbaus von *R. placenta* spielen.

Wesentliche Unterschiede wurden zwischen den Phänotypen und Genotypen der beiden *R. placenta* Stämme gefunden. Die Genome von FPRL280 und MAD-698 stimmten in 98.4% überein. Eine genauere Analyse der kohlenhydrataktiven Enzyme (CAZies) wies Unterschiede in vielen Genfamilien auf, welche mit dem Abbau von pflanzlicher Biomasse in Verbindung stehen, darunter SNPs, Indels, Lücken oder Einschübe innerhalb struktureller Domains. Vier der in MAD-698 vorkommenden Gene der AA3_2 Familie fehlten zudem im Genom von FPRL280.

Die Abbautests zeigten klare regulatorische Unterschiede zwischen den beiden Abbauphasen, sowie allen getesteten Stämmen. Holzacetylierung verhinderte, bzw. verzögerte einen Abbau generell und Teilprozesse des Abbaumechanismus waren abgeschwächt oder gestört. Eine Vielzahl von CAZy Genen gruppierte sich mit Transkriptionsfaktoren und war teils schwer von der Acetylierung betroffen. Eine Steigerung der Acetylierungsintensität zeigte während der Initialphase eine gruppenweise Erhöhung braunfäulerelevanter Gene.

Im Allgemeinen helfen die Ergebnisse des Genomabgleichs die phänotypischen Unterschiede zwischen den beiden *R. placenta* Stämmen zu erklären, da sie Auswirkungen auf das Enzymverhalten, die Substratbindungsfähigkeit oder Proteinfaltung haben können. Die Ergebnisse zeigen, dass sich regulatorische Mechanismen rasant entwickeln können und womöglich die Hauptursache hinter den verschiedenen Abbaustrategien der verschiedenen Stämme darstellen. Dies zeigt außerdem, dass Ergebnisse, welche die Aggressivität und das Abbaupotenzial betreffen, von Studien, welche diese beiden *R. placenta* Stämme verwenden, nur mit äußerster Vorsicht verglichen werden sollten. Den Braunfäuleprozess als einen strikt zweistufigen Prozess zu betrachten, erscheint in Hinsicht auf die Ergebnisse dieser Studie als zu simpel. Vielmehr scheint es sich um ein äußerst dynamisches System zu handeln, in welchem unterschiedliche Enzymgruppen während der Initialphase und der späteren Abbauphase parallel zum Einsatz kommen. Zu sehen, dass alle drei Stämme unterschiedliche Abbaustrategien anwenden, und auch anders mit den durch die Acetylierung bedingten Veränderungen der Wachstumsbedingungen umgehen, hebt die Komplexität des Braunfäule-Abbausystems erneut hervor. Zusätzlich zeigte sich ein Großteil der involvierten Gene als noch nicht annotiert, was sie in den Fokus rückt. Eine vollständige Annotation könnte ein wichtiger Faktor dafür sein, die der Braunfäule unterliegenden Prozesse besser zu verstehen und nicht nur Modifizierungsmethoden zu verbessern, sondern auch zu schnellerer und einfacherer Identifizierung von Schaderregern beitragen oder in anderen Bereichen, wie zum Beispiel der Vorbehandlung von Biomasse zum Einsatz kommen.
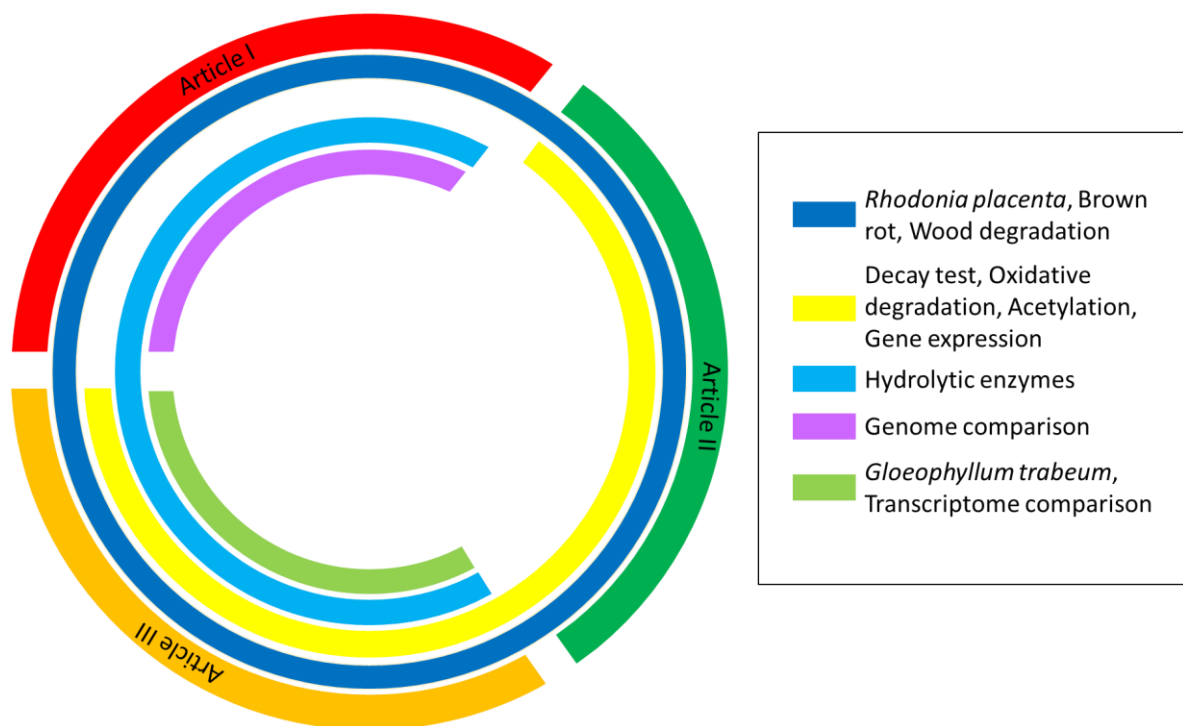
Articles this thesis is based on

Article I: Kölle M, Horta Crivelente MA, Nowrousian M, Ohm R, Benz JP, Pilgård A (2020) Degradative capacity of two strains of *Rhodonia placenta*: from phenotype to genotype. Frontiers in Microbiology, 11 (1338).

Article II: Kölle M, Ringman R, Pilgård A (2019) Initial *Rhodonia placenta* gene expression in acetylated wood: group-wise upregulation of non-enzymatic oxidative wood degradation genes depending on the treatment level. Forests 10 (1117).

Article III: Kölle M, Horta Crivelente MA, Benz JP, Pilgård A (2021) Comparative transcriptomics during brown rot decay in three fungi reveals strain-specific degradative strategies and responses to wood acetylation. Frontiers in Fungal Biology, 2 (701579).

An overview on how the three publications are connected is given in Figure 1.

Figure 1 Schematic map on how the three publications thematically cluster together.

Table of contents

Index of figures

Abbreviations

AA	Auxiliary activities
AlOx	Alcohol oxidase
BRS	Brown rot specific
CAZy	Carbohydrate-active enzyme
CBM	Carbohydrate-binding module
CCA	Chromated copper arsenate
cDNA	Complementary deoxyribonucleic acid
CE	Carbohydrate esterase
CMF	Chelator mediated Fenton reaction
CML	Middle lamella
Cro	Copper radical oxidase
CyOx	Oxaloacetate dehydrogenase
DMDHEU	1,3-Dimethylol-4,5-dihydroxyethyleneurea
DNA	Deoxyribonucleic acid
DP	Degree of polymerisation
EMC	Equilibrium moisture content
Enz	Enzymatic
EXN	Expansin
FSP	Fibre saturation point
GH	Glycoside hydrolase
GlyD	Glyoxylate dehydrogenase
GMC	Glucose-methanol-choline (oxidoreductase)
GOx	Glucose oxidase
GT	Glycosyl transferase
Lac	Laccase
LF-NMR	Low-field nuclear magnetic resonance
LPMO	Lytic polysaccharide monooxygenase
MC	Moisture content

NGS	Next-generation sequencing
mRNA	Messenger ribonucleic acid
OahA	Oxaloacetate acetylhydrolase
OH-group	Hydroxyl group
Ox	Oxidative
OxaD	Oxaloacetate dehydrogenase
P	Primary layer
PCR	Polymerase chain reaction
PL	Polysaccharide lyase
PQT	Putative quinate transporter
QRD	Quinone reductase
RH	Relative humidity
RNA	Ribonucleic acid
ROS	Reactive oxidative species
S	Secondary layer
SCA	Sorbitol and citric acid-modified wood
TCA	Tricarboxylic acid
WPG	Weight percentage gain

1 Introduction and State-of-the-Art

One of the most important lignocellulosic materials is wood. Wood is an attractive building material since it is renewable, biodegradable and it stores carbon (Rowell, 2005; Huß and Krötsch, 2013). Most of the carbon that is photosynthetically fixed by land plants is directed into lignocellulose, consisting of cellulose, hemicelluloses, pectins and lignin (Wagenführ and Scholz, 2012; Cragg et al., 2015). Furthermore, its mechanical properties qualify wood to serve as an alternative to concrete and steel (Bergman et al., 2014; Jakes, J.E. et al., 2016). The use of wood as construction material has the potential to store significant amounts of atmospheric carbon over a long timeframe (Hill and Norton, 2014) and that this compensates for the emissions caused during production processes (Hill and Dibdiakova, 2016). These positive aspects make wood an advantageous material compared to other building materials. In addition, cascading utilisation of wood, reusing wood multiple times during service life before incineration, is another benefit (Risse et al., 2019). Since the wood demand is being expected to increase in the future (Mantau et al., 2010), using wood over different life cycles, including reuse and recycling is a promising way to supply the demand, to decrease environmental impact, to increase resource efficiency and to exceed carbon storage, contributing to climate change mitigation (Risse et al., 2017). Despite these positive aspects, wood applied in humid conditions is vulnerable to fungal attack (Eaton and Hale, 1993; Ibach and Rowell, 2000). While lignocellulosic material is recalcitrant to enzymatic processing through the paracrystallinity of cellulose, the complexity of the hemicellulose coating of cellulose microfibrils and the interpenetration and encapsulation of polysaccharide components by lignin (Cragg et al., 2015), wood decay fungi are capable of degrading nearly all biochemical forms of carbon from wood, returning CO_2. Around 10% of wood harvested each year is to replace timber decayed by fungi (Morrell, 2005). Softwood is a material that is often used in building constructions in the northern hemisphere and brown rot fungi are the most common and most destructive organisms, attacking softwood products (Eaton and Hale, 1993; Alfredsen et al., 2005). Due to this, brown rot fungi are feared in outdoor wood construction. Their efficient degradative systems raised attention as potential biodegraders of recalcitrant wastes and lignocellulosic biomass into biofuels, biopulping of wood chips, biobleaching of cellulosic pulps and many other applications (Arantes and Goodell, 2014).

1.1 Wood and decay fungi

1.1.1 Wood properties and durability

If not quoted differently the following information is taken from (Wagenführ and Scholz, 2012).

1.1.1.1 Wood anatomy

The microscopic structure of the evolutionary older softwoods differs from the one of hardwoods. Softwoods are built by only two cell types: tracheids and parenchyma cells. The structure of hardwoods is more complex compared to softwoods. It mainly contains pores, libriform fibres and parenchyma cells as well as more seldomly tracheids. Wood cells consist of several layers, mainly consisting of cellulose, hemicellulose and lignin. Long cellulose chains form fibrils with partly crystalline structure that are responsible for the longitudinal strength of wood. These fibrils are embedded in a lignin matrix, giving compressive strength. Hemicellulose links cellulose fibrils with the lignin matrix. The layers are divided into compound middle lamella (CML), the primary layer (P) and the secondary layer (S), which is further segmented into three sublayers (S1, S2 and S3) (Figure 2) (Kühnen and Wagenführ, 2002). The middle lamella connects the different cells and is mainly containing pectin and lignin. The primary layer is very thin with diffused fibril orientation, low amounts of cellulose fibrils and high contents of lignin. The secondary layer shows a parallel fibril orientation with high amounts of cellulose fibrils and low lignin content. The S1-layer is also called a transitional layer between the P- and the S2-layer.
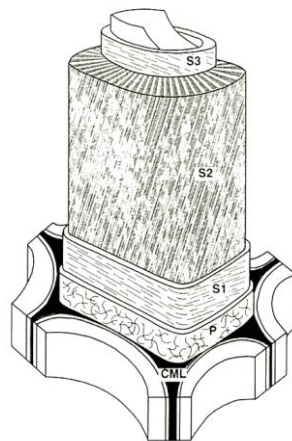
Figure 2 Cell wall structure according to Sell/Zimmermann (Kühnen and Wagenführ 2002); CML: compound middle lamella, P: primary layer, S1: outer secondary layer, S2: central secondary layer, S3: inner secondary layer.

The main part of the S-layer is formed by the S2 layer, containing the largest amount of lignin (due to its thickness). The S3 layer is forming the border to the lumen and is highly resistant against for example solvents and brown rots. The secondary cell wall is chemically consisting of 35% semicrystalline cellulose microfibrils embedded in a matrix of hemicelluloses (30%), amorphous cellulose (20%) and lignin (15%) (Rowell, 2005).

1.1.1.2 Chemical composition

Chemically the wood cell wall consists of cellulose (43-46%), hemicellulose (27-37%, also called polyoses) and lignin (20-27%). Cellulose and hemicellulose, combined in the term of holocellulose are hydrophilic polysaccharides, while lignin is hydrophobic. The close network of polysaccharides and lignin leads to the often-used term of wood as lignocellulose. Extractives, also called secondary metabolites are a group of cell wall chemicals that can form 0.5 – 10% of the cell wall. They can act as hydrophobing agent, supporting lignin, protecting against microorganisms. Further constituents of the wood are minerals and water.

Cellulose, the most abundant biopolymer on earth is built by β-1,4-linked D-glucose molecules. The degree of depolymerisation (DP) is an important parameter for cellulose and in wood, values around 7000 can be isolated. The higher the DP of a material, the greater longitudinal strength levels can be expected. Other important parameters influencing longitudinal strength are hydrogen bridge linkages and microfibrils. The smallest repeating unit is the cellobiose-unit, forming a glucan-chain. The combination of many of these glucan-chains is forming the elementary and micro-fibrils, which are important for longitudinal strength of wood. Two crystalline structures of cellulose (I_α and I_β) can be formed during this process (also called polymorphism). I_β is the mainly occurring cellulose form in wood, I_α can also occur in softwoods. The degree of crystallinity (in natural celluloses between 50 and 95%) is an important characteristic value for cellulose. Hemicelluloses are not crystalline as cellulose, but amorphous and able to absorb water resulting in swelling of the wood and its viscoelasticity. Hemicelluloses in wood can roughly be separated into xylans and glucomannans. Glucomannans are also supporting longitudinal strength, more than xylans. Hard- and softwoods contain different rates of xylans and glucomannans. Hemicelluloses are formed around the cellulose fibrils, lignin around the hemicelluloses. Lignin is the second most abundant biopolymer on earth. It is amorphous, polymeric and differentially distributed in softwoods (27%) and hardwoods (21%). It is classified into G-, GS- and HGS-lignin, depending on the main lignin component. Softwoods predominantly consist of G-lignin, while in

hardwoods GS-lignin prevails. Lignin is closely connected to the hemicellulose and therefore hard to remove, which makes the depolymerisation of lignocellulosic materials challenging in the industry.

Extractives are divided into primary and secondary ingredients. Primary extractives are physiologically active metabolic intermediates for example fat, sugar and starch. They serve the photosynthesis, respiration and the synthesis of substances forming supportive structures. Secondary extractives are released during the formation of heartwood and serve as natural protection against microorganisms and therefore extractive-content is influencing the natural durability of wood (Rowell, 2005).

One other important constituent, influencing several wood properties for example mechanical, thermal and dimensional stability, is water (Glass and Zelinka, 2010; Brischke and Alfredsen, 2020).

1.1.1.3 Role of water in wood

The moisture content of wood is calculated by dividing the wood mass with water by oven-dried wood mass (Glass and Zelinka, 2010). The surrounding temperature, as well as relative humidity (RH), influence the moisture content (MC) of wood (Jakes, J. et al., 2016). Storing wood under constant RH and temperature leads to a constant MC, called the equilibrium moisture content (EMC). The EMC depends on the surrounding circumstances and the wood species. During the wood drying process, there will be a point where the moisture content will transition from saturated to unsaturated. This point was firstly defined in 1906 as fibre saturation point (FSP), describing the state when lumens no longer carry liquid water, cell walls begin to dry and strength increases (Tiemann et al., 1906). Engelund *et al.* (2013) suggested considering fibre saturation not as a state at a certain moisture content, but as a gradual transition between the state that newly absorbed water molecules break intra- and intermolecular H-bonds in the wood cell polymers (MC below 30%) and the state that newly absorbed water molecules are gathered in the cell wall without breaking these bonds (MC between 30-40%). The water, which is inside the wood until the FSP is called bound water, interacting with the wood polymers, mainly forming hydrogen bonds with the hydroxyl groups (OH groups) of the wood constituents (Engelund et al., 2013). The most so-called sorption sites (Simpson, 1980) are found in hemicelluloses (Christensen and Kelsey, 1959). In hemicelluloses, twice the amount of accessible OH groups are found, compared to lignin and four times higher amounts compared to cellulose fibrils (Thybring, 2017). Hydroxyl groups that are bound in and between cellulose

chains, constituting the microfibril, are inaccessible, while hydroxyl groups on the surface of these microfibrils are accessible sorption sites (Engelund et al., 2013). Therefore, the term "fibre saturation" might be misleading, since not all fibres are saturated, but the cell wall is. The term "cell wall saturation" was therefore suggested as more descriptive (Brischke and Alfredsen, 2020), a state that can only be reached theoretically. New experimental techniques revealed that the cell walls of specimens were not saturated until the whole wood specimens were saturated with water (Fredriksson and Thybring, 2019). This means that there is no state where the cell walls are swollen to their maximum with simultaneously capillary water being absent (Brischke and Alfredsen, 2020). Water that is absorbed above the FSP through capillary condensation, starts accumulating in the lumina and pits and is called free water (Hoffmeyer et al., 2011; Engelund et al., 2013). Below the FSP moisture transport is mainly proceeding via diffusion, which is also important for chemical transport through the wood cell wall. It has been suggested, that electrical conduction in wood might be explainable by a percolating network and that a continuous path of Type II water exists below the FSP (Zelinka et al., 2008). The formation of a continuous cell wall water network might enable ion transport in wood (Thybring, 2017).

1.1.1.4   *Durability and wood degradation*

Water is influencing the durability of wood and wooden products, since for example fungi need water and oxygen to degrade wood. Moisture contents between 23% and 25% and below have reportedly been protecting wood from being degraded (Cardias Williams and Hale, 2003; Hill and Ormondroyd, 2004; Thybring, 2013). New techniques have shown that some brown rot species (*Serpula lacrymans* and *Gloeophyllum trabeum*) are able to diffuse reagents at significantly lower moisture contents than fungi are traditionally thought to be able to degrade wood (Meyer-Veltrup and Brischke, 2015; Zelinka et al., 2015; Kirker et al., 2017). Fungi need water to establish the initial oxidative system and later to enable the diffusion of polysaccharides, solubilised during decay back to the fungal hyphae (Goodell et al., 1997). The moisture optimum for decay fungi lays in the range between 50 and 100% (Zabel and Morrell, 2020). Decayed samples showed an MC high above the FSP, indicating that the hyphae itself further supports the transportation of ions and water (Brischke and Alfredsen, 2020). A review by Jakes *et al.* (2019) brought out the theory that not only aqueous pathways are used for diffusion processes. They concluded, that diffusion could also be possible through solid polymers and that both amorphous polysaccharides as well as lignin might have moisture-

induced glass transitions that allow them to transport ions (Jakes et al., 2019). How these conclusions affect fungal growth is not quite clear, but hypotheses and arising questions were stated by Brischke and Alfredsen (2020). Wood can also be attacked by bacteria. The two main forms of bacteria, degrading lignified wood, are tunneling and erosion bacteria, but there are also other bacteria known to attack wood (Daniel, 2014). The degradation pressure through fungi is much higher in middle Europe, compared to insects and marine borers or bacteria. Fungal hyphae are small enough to penetrate into the cell lumen of wood cells, hyphae of decay and stain fungi are also able to grow through and penetrate pit membranes (Figure 3) (Goodell, 2020).
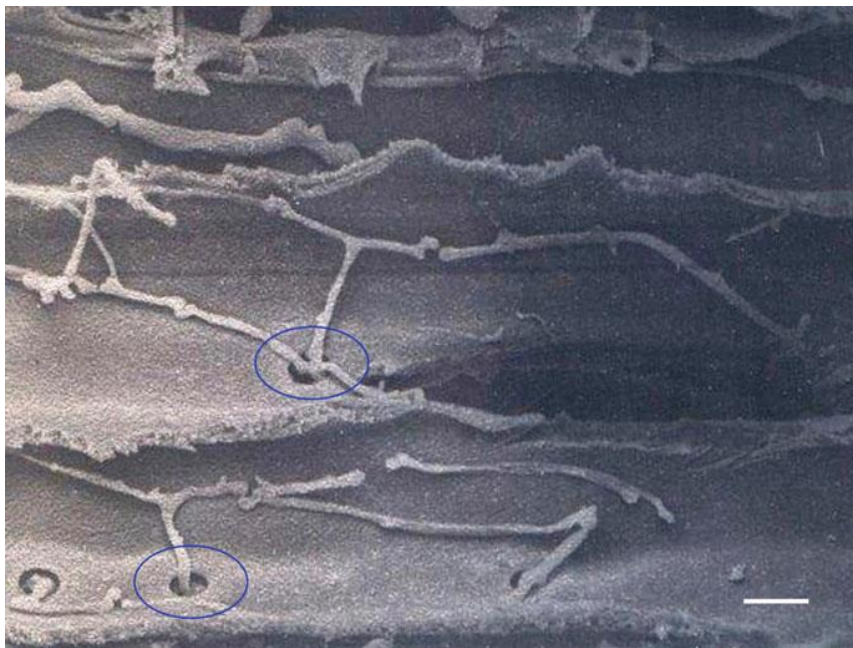
Figure 3 Hyphae of wood decaying fungi, growing through the tracheid cell walls of softwood. The blue circles mark the bordered pits of wood, where decay fungi preferably penetrate through during early stages of wood degradation to grown from one wood cell to another (SEM image, scale = 10 µm). (Image taken from Goodell 2020).

Brown rot decay, for example, can lead to severe strength loss within a short time period and further when no mass loss can be detected yet (Rowell, 2005). Wood decaying fungi are divided into three groups: soft rot, white rot and brown rot. Soft rot mainly occurs under excessive moisture conditions and is mainly caused by *Ascomycota*. Furthermore, soft rot has been observed to be one of the major forms of attack in wood, treated with preservatives (Daniel, 2014). Wood without soil contact is mainly deconstructed by the more competitive *Basidiomycota*, causing white and brown rot. The natural durability of wood and wooden products needs to be tested before commercial use. Durability against white and brown rot is

tested with the EN 350-1 or CEN/TS 15083 in a laboratory test. *R. placenta* is an often-used brown rot fungus in this test in Europe and North America. However, two different strains are employed (FPRL280 and MAD-698, respectively) for which differences in colonisation-rate, mass loss, as well as in gene expression have been observed, limiting the comparability of results. So far, results obtained with either the European or American strain have been seen as equivalent. Differences in either the genome or in regulatory mechanisms however could cause varying decay capacities and aggressiveness, which is why it is important to reveal how strong these differences are. The genome of the European *R. placenta* strain has so far not been sequenced, so it cannot be used as a reference for European studies.

1.1.2 Wood decay

Basidiomycota represent 32% of the described fungi in the world (Kirk et al., 2008), belonging to the group of filamentous fungi and can be further divided into two groups, white rot and brown rot fungi (Blanchette, 1991; Daniel, 1994; Coelho et al., 2017) (Figure 4). This grouping might however be too simplistic since decay mechanisms are much more diverse than assumed (Riley et al., 2014). White rot is evolutionary older than brown rot, which evolved several times independently from different types of white rot ancestors (Floudas et al., 2012). Morphologically, brown rot and white rot fungi share some similarities in decay, for example that they both secrete non-enzymatic agents, supporting the degradation by hydrolytic enzymes (Daniel, 2014). Furthermore, lignin demethoxylation occurs in both white and brown rot fungi, although their lignin metabolism differs substantially (Vanden Wymelenberg et al., 2010). Brown rot fungi were found to have reduced key enzymes for lignin and cellulose degradation, which they seem to have lost over time (Arantes et al., 2012). A study comparing extracellular proteins found 79 and 67 proteins in the extracellular fluids of the cultures of the white rot fungus *Phanerochaete chrysosporium* and the brown rot fungus *Rhodonia (Postia) placenta* respectively (Vanden Wymelenberg et al., 2010). Whereas *P. chrysosporium* expressed a high number of extracellular glycosyl hydrolases, *R. placenta* secreted an array of hemicellulases and only few cellulases. While it has been shown that brown rot fungi such as *G. trabeum* and *R. placenta* produce oxalic acid and have high polysaccharide-depolymerisation activity, negligible amounts of oxalic acid have been found in white rot fungi (Espejo and Agosin, 1991). Non-enzymatic processes vary between species of white rot fungi, time or even along a single hypha (Daniel, 2014). The polyphyletic group structure of white and brown rot fungi makes the understanding of their decay mechanisms complex and enhances the likelihood of differences

(Daniel, 2014). Many different theories about the underlying mechanisms have been stated during the last decades, but so far none of these systems has been fully accepted, which makes further research important.
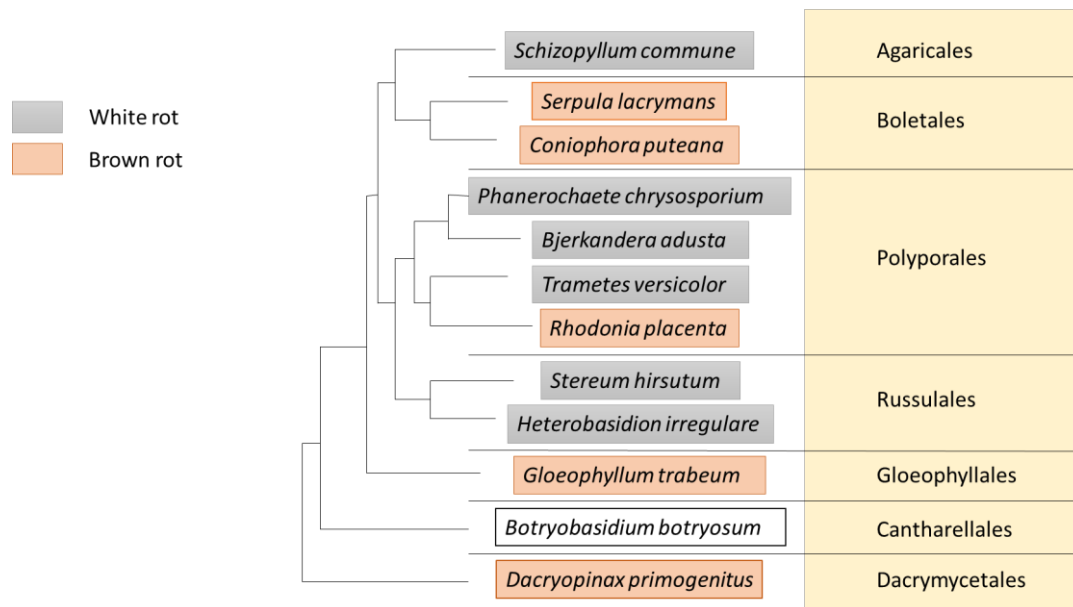
Figure 4 Tree of life of selected white and brown rot fungi based on Coelho *et al.* (2017), including the two brown rot fungi, used in this thesis (*R. placenta*, *G. trabeum*). Grey squares are white rot fungi, orange squares are brown rot fungi. For *B. botryosum*, a clear degradation type is not defined.

1.1.2.1   White rot

White rot, mainly occurring in hardwoods leads to a white colouring of the substrate, precipitated through the depolymerisation of lignin. Wood is colonised via ray canals, providing rapid entrance as well as easily accessible nutrients (Daniel, 2014). Hyphae then grow further through native pits or bore hyphae are developed, penetrating through the cell walls (Daniel, 2014). All polymeric cell wall constituents – cellulose, hemicellulose and lignin – are degraded, either simultaneously or selectively, depending on the fungal species. Wood with progressed white rot becomes fibrous and spongy (Goodell, 2003; Rowell, 2005; Wagenführ and Scholz, 2012). Reduction of lignin leads to the typical bleached appearance of the wood and further, strength properties are reduced substantially (Rowell, 2005). White rots use hydrolases to

degrade cellulose gradually and lignin is mineralised completely by lignin peroxidases, manganese peroxidases or versatile oxidases (Kirk and Farrell, 1987; Martinez, 2002; Hammel and Cullen, 2008). In addition, the genome of *Ph. chrysosporium* showed cellobiohydrolases, capable of degrading crystalline cellulose (Riley et al., 2014). The specificity of enzymes, discovered in white rot fungi so far raised the biotechnological potential of this type of decay (Daniel, 2014). A range of endoglucanases, cellobiohydrolases and β-glucosidases is produced by white rot fungi, in order to degrade cellulose (Daniel, 2014). Degradation of lignin is, unlike cellulose, somehow restricted and limited to a number of extracellular oxidative enzymes primarily peroxidases and laccases, together with associated enzymes and cofactors (Hammel and Cullen, 2008; Daniel, 2014).

1.1.2.2   *Brown rot*

Worldwide, more species of white rot fungi are known. Hence, 80% of the wood decay fungi found in wooden constructions are brown rot fungi (Liese, 1970; Alfredsen et al., 2005; Martin, 2007; Schmidt, 2007; Eastwood, 2014). Brown rot fungi prefer degrading softwoods, making them the main recyclers of lignocellulose in coniferous forests of the Northern Hemisphere. Furthermore, decay associated with brown rot is reportedly the most destructive type (Zabel and Morrell, 1992; Goodell, 2003; Vanden Wymelenberg et al., 2010), since it rapidly leads to significant strength loss (Curling et al., 2002; Filley et al., 2002). Both modulus of elasticity and modulus of rupture could be reduced by 70%, when mass loss has only received 10% (Wilcox, 1978), without the wood appearing to be degraded (Goodell, 2020). Brown rot fungi colonise wood by passing through the cell lumen into the parenchyma cells, where they penetrate the pit membranes. After reaching the lumens of the tracheid cells they penetrate the wood cell walls via bore holes (Goodell, 2003). Other than white rot, brown rot fungi are lacking lignin degrading peroxidases and cellobiohydrolases are either absent or missing a carbohydrate binding module 1 (CBM1) (Riley et al., 2014). Infected wood appears brownish with cubic cracks, since the white coloured cellulose and hemicellulose is degraded (Figure 5). The dark lignin that is highly modified, remains (Arantes et al., 2011; Yelle et al., 2011; Wagenführ and Scholz, 2012). Since this thesis is concentrating on brown rot degradation mechanisms, this subject is described in more detail in section 1.2. Nevertheless, there is still too little known about the exact mechanisms that play a role during brown rot decay. To understand how brown rot exactly proceeds it is important to add further detailed knowledge on the single processes during initial and enzymatic decay.

Figure 5 Brown rotted wood, showing the typical brown colour and the cubic cracks. Image: M. Risse, Holzforschung München, TUM

1.1.3 Brown rot fungi used in this thesis

Studies on brown rot have mainly been made using the organism *Gloeophyllum trabeum* (Pers.) *Murill*. The results have to some degree been verified in *Rhodonia placenta* (Fr.) *Niemelä, K. H. Larss. & Schigel*, *Coniophora puteana* (Schumach.) *P. Karst.*, and *Serpula lacrymans* (Wulfen) *J. Schröt. Gloeophyllum trabeum* and *Rhodonia placenta* (previously *Postia placenta*) are indeed distantly related, but represent independent origins of brown rot (Hibbett and Donoghue, 2001). Comparisons of decay mechanisms between these two species have to consider that their decay mechanisms are in common with white and brown rot species or have evolved convergently (Martinez et al., 2009).

1.1.3.1 Rhodonia placenta

Rhodonia placenta, which is the current name of this brown rot species according to Niemelä *et al.* (2005) has previously been known as *Postia placenta*, *Poria placenta* or many more other synonyms (Niemelä et al., 2005). It belongs to the kingdom of the fungi, the division of *Basidiomycota*, the class of the *Agaricomycetes*, the order of *Polyporales* and the family of *Fomitopsidaceae* (Schmidt, 2006). *R. placenta* and the white rot fungus *Ph. chrysosporium* are closely related, both belonging to the Phlebia clade (Binder et al., 2005; Hibbett et al., 2007). The American strain of *R. placenta* (MAD-698) was sequenced and analysed in 2009 (Martinez et al., 2009). A monokaryotic version of this strain (MAD-SB12) was then sequenced and published in 2017 (Gaskell et al., 2017). Analysis of the draft genome of *R. placenta* revealed the absence of conventional cellulase genes and most class II peroxidases as well as the presence of a wide range of genes likely involved in the generation of extracellular reactive oxygen species (Martinez et al., 2009). *R. placenta* has been used in a broad range of studies, investigating decay mechanisms of brown rot degradation (Martinez et al., 2009; Alfredsen and Fossdal, 2010; Vanden Wymelenberg et al., 2010; Ryu et al., 2011; Schmöllerl et al., 2011; Pilgård et al., 2012; Ringman et al., 2016; Beck et al., 2018). In this study, two strains of *R. placenta* were used, the American strain (MAD-698) and the European strain (FPRL280).

1.1.3.2 Gloeophyllum trabeum

Gloeophyllum trabeum (Pers.) *Murril*, also belongs to the kingdom of the fungi, division of *Basidiomycota* and the class *Agaricomycetes*. Further, it belongs to the order of *Gloeophyllales*, the family *Gloeophyllaceae* and the genus *Gloeophyllum* (Schmidt, 2006). Many studies regarding brown rot degradation systems have been using *G. trabeum*, making it a suitable species for comparing processes, active during lignocellulose degradation (Goodell et al., 1997; Kerem et al., 1999; Jensen et al., 2001; Cohen et al., 2002; Hammel et al., 2002; Jensen et al., 2002; Arantes and Milagres, 2006; Suzuki et al., 2006; Zhuang et al., 2015; Presley and Schilling, 2017; Zelinka et al., 2021).

1.2 Brown rot degradation process

The exact mechanisms that lay behind brown rot degradation are still not clear. Therefore, many statements regarding brown rot degradation still remain based on assumptions or only partly proven theories. Since saccharification enzymes of the fungi are too large to diffuse into the

wood cell walls (Cowling, 1961), low molecular weight components, able to diffuse into the cell, pretreat the cell wall. These agents are able to diffuse through the nanopores and establish an oxidative system involving reactive oxygen species (Goodell et al., 1997; Cohen et al., 2002; Hammel et al., 2002). Thus, it has been shown that the degradation mechanism of brown rot fungi is a two-stepped process, which works very efficiently. During non-enzymatic oxidative degradation, several premises must be abided, since oxidants/reductants should a) be generated remote to fungal hyphae, protecting it from attack, b) be sufficiently stable and only react when they are inside the wood cell wall and c) have a self-regenerating system (Daniel, 2014). Non-enzymatic oxidative reagents are secreted by the fungus, which diffuses into the wood cell wall, where they depolymerise cellulose and hemicellulose (Curling et al., 2002; Filley et al., 2002; Martinez et al., 2005; Niemenmaa et al., 2007; Arantes and Goodell, 2014). The initial step is followed by the secretion of hydrolysing enzymes (Baldrian and Valášková, 2008; Arantes et al., 2012; Arantes and Goodell, 2014). The currently most accepted theory is that solubilised sugars and oligosaccharides diffuse out of the cell wall and saccharification occurs only in the cell lumen (Goodell et al., 2017). The two steps were also found to be spatially separated (Zhang et al., 2016). A schematic map on the processes proceeding during both the oxidative and the enzymatic degradation of brown rot is imaged in Figure 6. Explanations of the details regarding the two degradation steps can be found in the following sections.
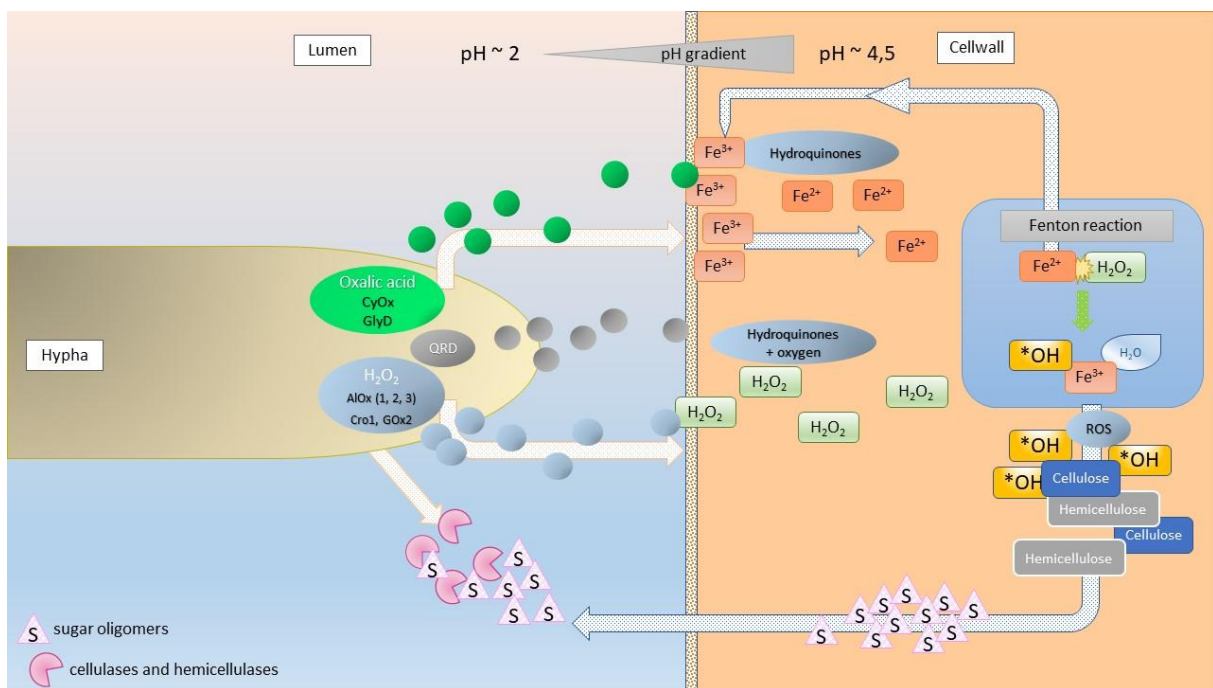
Figure 6 Schematic figure of the processes during initial and progressed brown rot decay, including genes that were used in this study (Article II).

1.2.1 Non-enzymatic oxidative degradation

The details regarding the non-enzymatic oxidative degradation phase are still not fully understood. The current theory is that brown rot fungi secrete oxalic acid, produced via the tricarboxylic acid (TCA) cycle (Munir et al., 2001b), which diffuses into the lumen, where it chelates Fe^{3+} (Goodell et al., 1997; Eastwood et al., 2011; Arantes et al., 2012; Zhu et al., 2016). Hydrogen peroxide (H_2O_2), also capable of reducing Fe^{3+} is believed to be produced through the reaction between hydroquinones and oxygen within the cell wall. Furthermore, Fe^{3+} that is bound to the wood cell wall is suggested to be reduced to Fe^{2+} by hydroquinones (Paszczynski et al., 1999; Jensen et al., 2001). Hydroxyl radicals ($^\bullet OH$) are then extracellularly formed through the chelator-mediated Fenton (CMF) reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^-OH + {}^\bullet OH$) between hydrogen peroxide and Fe^{2+}, which polymerise cellulose and hemicellulose and modify lignin (Fenton, 1894; Goodell et al., 1997; Baldrian and Valášková, 2008; Arantes et al., 2012; Arantes and Goodell, 2014). It has further been shown that phenolic compounds (e.g. 2,5-dimethoxy-1,4-benzoquinone and 4,5-dimethoxy-1,2-benzoquinone) and biomimetic fungal phenolic compounds are also able to chelate ferric iron (Fe^{3+}) and are sources of electrons, promoting CMF reactions (Enoki et al., 1997; Goodell et al., 1997; Kerem et al., 1999; Paszczynski et al., 1999). For the fungi, the location where the CMF takes place is of importance, since the highly reactive $^\bullet OH$ radicals that have an extremely short half-life (Arantes and Goodell, 2014) would otherwise damage the fungal hyphae. Furthermore, the OH radical formation needs to be close to the target holocellulose or lignin molecules (Arantes and Goodell, 2014). Therefore, the CMF has to progress within the wood cell wall. A pH gradient is potentially generated by the fungi themselves to enable reactants (e.g. Fe-oxalate complexes) to diffuse into the wood cell wall (Goodell et al., 1997; Arantes and Goodell, 2014). In 1997, it was found that oxalate decarboxylase may be involved in active pH regulation, which may function as a safety mechanism for the fungal hyphae (Micales, 1997). It has, however, been shown that pH regulation is not the only strategy to protect fungal hyphae from oxidative damage. Instead, it seems that brown rot fungi use avoidance (controlling of reactive oxygen species potential), suppression (antioxidants) and tolerance (of early-secreted CAZy genes) to be able to establish their wood decay strategy (Castaño et al., 2018). The oxalate concentration and the pH have further been shown to have influence on the reactivity of Fe^{3+} and oxalate (Hyde and Wood, 1995). At a low pH and high oxalic acid concentrations, ferric iron that is present in the wood is readily released by oxalic acid (Arantes et al., 2009). Fe-oxalate complexes formed through this reaction diffuse into the cell wall where the oxalate

concentration is lower and the pH is higher (around 4.5). This condition causes the Fe^{3+} to be released, being either transferred to the wood or being reduced, initiating the CMF (Arantes et al., 2012). During brown rot degradation, lignin is extensively demethylated (Filley et al., 2002; Yelle et al., 2008; Martínez et al., 2011), which occurs simultaneously to holocellulose loss (Filley et al., 2002). This further enhances the iron-reducing capability in brown rot degraded wood (Goodell et al., 2006). It has also been suggested that Fe^{3+}-reductants could be part of a self-regenerating system (Hammel et al., 2002) since multiple moles of Fe^{3+} can be reduced by a single mole of fungal or biomimetic reductants (Goodell et al., 1997; Goodell et al., 2002; Arantes and Milagres, 2006). Chemical transport via diffusion is used by the fungi to be able to establish the CMF within the wood cell wall, therefore moisture is a limiting factor regarding decay initiation and progress (Goodell et al., 1997; Goodell et al., 2017).

1.2.2 Lignin modification

As mentioned above, lignin is heavily modified during brown rot decay. Brown rot fungi such as *R. placenta* lack class II peroxidases (lignin and manganese peroxidases) (Martinez et al., 2009). However, Goodell *et al.* (1997) suggested that lignin is depolymerised during CMF degradation by brown rot fungi. The observed modification of lignin moieties (Filley et al., 2002; Yelle et al., 2008) might be crucial for brown rot decay (Arantes and Goodell, 2014).

1.2.3 Enzymatic degradation

Sugars, solubilised during oxidative decay, can diffuse through the cell wall into the lumen (Martinez et al., 2005; Goodell et al., 2017). These sugars are suggested to trigger the transition from oxidative to enzymatic degradation (Zhang et al., 2016), which might not be the only explanation. During enzymatic degradation of cellulose four main classes of enzymes are involved: endoglucanases, cellobiohydrolases, β-glucosidases and lytic polysaccharide monooxygenases (LPMOs). The enzymes form glucose in a synergistic process (Goodell, 2003). Endoglucanases randomly cut the cellulose chains, providing chain ends as binding sites for cellobiohydrolases. These produce cellobiose, which then gets hydrolysed by β-glucosidases to glucose (Aro et al., 2005). Additionally, LPMOs are able to oxidise crystalline cellulose surfaces leading to chain breaks in this polymer, which make it accessible for the hydrolytic enzymes. These polysaccharides are further degraded to mono- and disaccharides which the fungus is able to metabolise (Baldrian and Valášková, 2008; Arantes et al., 2012). Other important enzymes during brown rot decay are hemicellulases assisted by carbohydrate

esterases. Hemicelluloses that are expressed by brown rot fungi are for example xylanases, arabinanases, mannanases and glucuronidases (Floudas et al., 2012). Studies on brown rot genomes have revealed that the number of enzymes used during enzymatic decay have been reduced compared to several white rot fungi and that a more efficient initial degradation phase precedes enzymatic actions (Eastwood et al., 2011; Floudas et al., 2012; Riley et al., 2014).

1.3   Genes involved in brown rot degradation

1.3.1   Lignocellulose- and carbohydrate-active enzymes in fungi

The ability to deconstruct lignocellulose is the fundamental process in nature to ensure the carbon cycle. The basis of the principle has multiple origins and evolved in diverse branches (Cragg et al., 2015). There are many different ways for lignocellulose to be deconstructed by organisms, for example via oxidative attack and hemicellulases, improving the access for depolymerising enzymes (Cragg et al., 2015). The deconstruction of biomass is achieved under a wide range of environmental conditions, e.g. pH, temperature and pressure, explaining the diversity of organisms that are involved (Cragg et al., 2015). However, the number of organisms that can degrade lignin is limited, primary degraders are white rot *Basidiomycota* and some ligninolytic bacteria by secreting oxidative enzymes such as peroxidases and laccases (Bugg et al., 2011; Pollegioni et al., 2015). Brown rot fungi do not have lignin degrading enzymes but depolymerise lignin via reactive oxidative species (ROS), leading to the release of cellulosic components (Arantes and Goodell, 2014). A variety of different carbohydrate-active enzymes (CAZy), complementarily and synergistically acting together as a cocktail to enzymatically degrade cellulose and hemicellulose (Payne et al., 2015). While white rot attacks primarily enzymatically, brown rot fungi lost a wide range of cellulolytic enzymes (Floudas et al., 2012). This cellulolytic enzyme machinery has been substituted by generating an alternative efficient mechanism for the depolymerisation of biomass, the CMF (Eastwood et al., 2011; Arantes and Goodell, 2014). The Carbohydrate-Active Enzymes (CAZy) database was launched in 1999 and is the common resource for information on the sequence, structure and molecular mechanism of CAZymes (Lombard et al., 2013). The enzymes are classified into families (Cantarel et al., 2008), which are: Glycoside hydrolases (GH), glycosyl transferases (GT), carbohydrate esterases (CE), auxiliary activities (AA) and polysaccharide lyases (PL). Some

enzymes are multimodular, meaning they have several domains belonging to different families, as well as associated carbohydrate-binding modules (CBMs).

1.3.1.1 *Glycoside hydrolases (GHs)*

The largest class in the CAZy database is the GH class with 168 families (December 2020). They are the primary enzymes that act on the glycosidic linkages of cellulose and hemicellulose, supported by polysaccharide esterases that remove methyl and acetyl groups as well as phenolic esters (van den Brink and de Vries, 2011). GHs that depolymerise cellulose can be either exo- or endo-acting. The cellobiose producing cellobiohydrolases belong to the group of exo-acting cellulases of which some only seem to evolve in a small number of organisms, for example, in filamentous cellulolytic fungi, enzymes of the GH families 6 and 7 (Payne et al., 2015). The cellobiose is then further hydrolysed to glucose by β-glucosidases. As mentioned above, brown rot fungi possess fewer GHs than white rot fungi, but seem to compensate for this by secreting higher amounts of their remaining GHs (Presley et al., 2018). Processive cellobiohydrolases are generally missing in brown rot fungi, which is why they rely more on endoglucanases, which putatively break down cellulose randomly (Eriksson et al., 1990; Vanden Wymelenberg et al., 2010). These putative endo-acting cellulases belong to the CAZy families GH5 and GH12 (Ryu et al., 2011; Floudas et al., 2012; Lombard et al., 2013). Co-operating the cleaving of hemicelluloses are hemicellulases, found in several GH families (Ryu et al., 2011; Brigham et al., 2018). Hemicellulose degrading enzymes are therefore frequent constituents of wood-degrading organisms (Riley et al., 2014). GHs attack polysaccharides in a relatively inefficient way, since the glycosidic bonds are often inaccessible to the active site of the suiting enzymes. To enhance efficiency many of the GHs are modular, containing catalytic modules appended to one or more carbohydrate binding modules (Boraston et al., 2004).

1.3.1.2 *Auxiliary activity enzymes (AAs)*

Besides the previously mentioned CAZy families, wood degrading fungi encode a large set of auxiliary activity redox enzymes that are evidently or potentially involved in lignocellulose depolymerisation (Floudas et al., 2012; Levasseur et al., 2013; Riley et al., 2014). Members of the families CBM33 and GH61 have been observed to be, in fact, lytic polysaccharide mono-oxygenases (LPMOs). LPMOs and lignin fragments are likely acting in concert, which is why lignin degradation enzymes were combined with LPMOs to a new CAZy class, the "auxiliary activities" (AAs) (Levasseur et al., 2013). To this date, the CAZy class of the AAs contains 16

families. Lignin degrading enzymes of the families AA1 and 2 (laccases and class II peroxidases) are only used by white rot fungi. Flavoenzymes, belonging to the CAZy family AA3 are also known as GMC oxidoreductases and oxidise several sugars and alcohols by either reducing O_2 to H_2O_2 or phenolic compounds. Also likely involved in the production of extracellular H_2O_2 are copper radical oxidases of the family AA5, known to be expressed during wood decay by brown and white rot fungi (Kersten and Cullen, 2014). Cellulose degrading fungal LPMOs are meanwhile classified into the families AA9-16, enzymes and are capable of acting on crystalline cellulose (Beeson et al., 2015) and hemicelluloses (Agger et al., 2014). They also demonstrated non-enzymatic deconstruction of cellulose, including Fenton chemistry, depending on iron, which was found in brown rot degrading fungi (Arantes and Goodell, 2014).

1.3.1.3 Carbohydrate esterases (CEs)

Carbohydrate esterases (CEs) are enzymes that catalyse the O- or N-deacylation to remove the ester decorations from carbohydrates (Cantarel et al., 2008). This facilitates GHs to get access to efficiently depolymerise the polysaccharides (Biely, 2012). The CAZy class of CEs currently contains 18 families, the CE10 family was however withdrawn in 2002, since the majority of the members were acting on non-carbohydrate substrates. Most CE families include members that catalyse the removal of the acetylated moieties of polysaccharides, accelerating their degradation (Nakamura et al., 2017). Most CEs can be divided into the categories of hemicellulose deacetylating (CE1-7 and CE16) and pectin deacetylating (CE8 and CE12) enzymes (Sista Kameshwar and Qin, 2018). CEs have considerable potential in several branches of biotechnology (Nakamura et al., 2017).

1.3.1.4 Polysaccharide lyases (PLs)

Polysaccharide lyases (PLs) generate an unsaturated hexenuronic acid residue and a new reducing end by the cleavage of uronic acid-containing polysaccharide chains via a β-elimination mechanism (Lombard et al., 2010). At this time, 40 families are classified as PLs.

1.3.1.5 Glycosyl transferases (GTs)

Glycosyl transferases (GTs) are involved in the biosynthesis of disaccharides, oligosaccharides and polysaccharides (Sinnott, 1990). They are, among others, necessary for the biosynthesis of

the fungal cell wall (Klutts et al., 2006) and represent one to two per cent of the gene products in eukaryotic organisms (Lairson et al., 2008). GTs help with the formation of glycosidic bonds by catalysing the transfer of sugar moieties from donor molecules to specific acceptors (Campbell et al., 1997; Coutinho et al., 2003). According to the stereochemistry of the substrates and the reaction products, GTs can be classified as inverting or retaining enzymes (Sinnott, 1990) and to this time 111 families are classified as GTs.

1.3.1.6   *Carbohydrate-binding modules (CBMs)*

Carbohydrate-binding modules (CBMs) are contiguous amino acid sequences within a CAZy gene with mainly carbohydrate-binding activity. Previously, CBMs were classified as cellulose-binding domains (Gilkes et al., 1988; Tomme et al., 1988), excluding other modules found in CAZy genes that bind carbohydrates. Therefore, the CBMs were newly classified into 88 families, based on their amino acid similarity (Boraston et al., 2004; Lombard et al., 2014). The CBMs display variation in ligand specificity, for example, crystalline and non-crystalline cellulose, chitin, xylan among others (Boraston et al., 2004). This makes their utilisation interesting for numerous biotechnological applications (Tomme et al., 1998). There are three types of CBMs, one binding crystalline ligands (Type A), while the other two types (B and C) bind internal or terminal regions of polysaccharides (Boraston et al., 2004; Chalak et al., 2019). Besides targeting the enzymes to their substrates to promote catalysis (Hervé et al., 2010; Couturier et al., 2011), they can sometimes also adjust enzyme specificity (Cuskin et al., 2012). CBMs that are appended to GHs have been studied intensely (Gilbert et al., 2013). More recent studies have found that CBMs can also play an important role in driving the enzymes of the class of auxiliary activities (lytic polysaccharide monooxygenases) to the substrate, resulting in the release of more soluble sugars (Riley et al., 2014; Chalak et al., 2019).

1.3.2   Genes involved in brown rot decay

Overall, the two strains of *R. placenta* (FPRL280 and MAD-698) encode 317 (to this time) carbohydrate-active enzymes (CAZy) and *G. trabeum* encodes 352 CAZy genes.

Typically, the number of genes encoding lignin-related enzymes is extremely reduced in the genomes of brown rot fungi. For example, most of the brown rot fungi do not encode class II lignin-modifying peroxidases (PODs) (AA2) (Riley et al., 2014). However, they are able to efficiently degrade wood polysaccharides as well as to modify lignin (Eaton and Hale, 1993;

Yelle et al., 2008; Arantes et al., 2012). Furthermore, laccase genes are encoded either very limited in number in *R. placenta* (Martinez et al., 2009; Wei et al., 2010) or completely missing, as in *G. trabeum* (Floudas et al., 2012). As mentioned in the section on brown rot fungal decay, most brown rot fungi (including *R. placenta*) cellobiohydrolases are either absent or lacking a carbohydrate-binding module (Riley et al., 2014). The cellulose attacking cellobiohydrolases belong to the GH families 6 and 7 and are often accompanied by a CBM (mostly CBM1) domain (Lombard et al., 2014). Brown rot fungi rely more on endoglucanases instead (GH5, GH12), which are thought to randomly decompose cellulose (Eriksson et al., 1990; Vanden Wymelenberg et al., 2010; Ryu et al., 2011; Floudas et al., 2012; Lombard et al., 2014). Furthermore, genes of cellulolytic families (GH5, GH12, GH44, GH45) are reduced and cellobiose dehydrogenases (AA3_1) are absent in the majority of brown rot fungi, which does not hinder them to depolymerise polysaccharides from wooden cell walls (Martinez et al., 2009; Eastwood et al., 2011; Floudas et al., 2012; Riley et al., 2014). Hence, brown rot fungi seem to compensate for the lower number of encoded GHs by secreting higher amounts of their remaining GHs (Presley et al., 2018; Zhang et al., 2019). The comparison of the transcriptomes of two white and two brown rot fungi further hypothesised that the combination of reactive oxygen species (ROS) and enhanced expression of the remaining GHs is the reason that degradation by brown rot fungi is not inferior to this of white rot, even though PODs are missing and the set of GHs is smaller (Zhang et al., 2019). Still, the number of genes encoding for cellulose-active enzymes is reduced to a higher extent than the number of genes coding for hemicellulases and pectinases (Martinez et al., 2009; Wei et al., 2010; Riley et al., 2014). The role of single genes or of gene cocktails during brown rot decay are still not fully understood, making further research, elucidating the ongoing mechanisms, important.

1.3.2.1   *Genes involved in the Fenton reaction*

Preceding enzymatic degradation of the wood cell wall, a mechanism based on the Fenton reaction involving chelators is the predominant mode of action of brown rot decay (Goodell et al., 1997; Arantes and Goodell, 2014). Zhang *et al.* (2016) found that 33 genes likely associated with redox processes were upregulated during early stages of decay, and 21 of them may be involved in generating H_2O_2 and Fe^{2+}. Martinez *et al.* (2009) identified a putative quinone reductase in *R. placenta* that is believed to recover iron reductants (hydroquinones) (Paszczynski et al., 1999; Jensen et al., 2002; Qi and Jellison, 2004). It is also putatively mediating the reduction of iron chelators (oxalic acid) (Jensen et al., 2002). *R. placenta* has

only one quinone reductase (QRD Ppl124517), compared with the two in *G. trabeum*, which have been shown to have different functions; one is involved in wood degradation and the other in stress defence (Cohen et al., 2004). *R. placenta* quinone reductase belongs to the CAZy family AA6 and is likely involved in oxidative processes and the formation of intracellular enzymes for fungal protection. It was suggested that Ppl44553 is a quinate transporter (PQT), belonging to the major facilitator superfamily domain (Mueckler et al., 1985). This domain includes sugar transporters, which bind and transport a variety of carbohydrates, organic alcohols, and acids. A major part of this family catalyses sugar transport (Yan, 2015). The exact function of the *R. placenta* PQT remains unclear. Four putative laccases have been found in *R. placenta* (Martinez et al., 2009). Laccases appear to play an important role in the oxidation of methoxyhydroquinones into semiquinones that reduce Fe^{2+} (Wardman, 1989). Further, they are believed to be effective producers of H_2O_2 (Wei et al., 2010). Extracellular H_2O_2 has also been suggested to be produced by copper radical oxidases (Cro), gluco-oligosaccharide oxidases, and glucose-methanol-choline (GMC) oxidoreductases, including alcohol oxidases (AlOx) and glucose oxidases (GOx) (Martinez et al., 2009; Floudas et al., 2012; Levasseur et al., 2013). Alcohol oxidase is upregulated in the presence of cellulose and wood (Martinez et al., 2009; Vanden Wymelenberg et al., 2010). *R. placenta* has three genes encoding alcohol oxidases, belonging to the CAZy family AA3, AlOx1 Ppl44331, AlOx2 Ppl129158, and AlOx3 Ppl118723. Copper radical oxidase (Cro1 Ppl56703) belongs to the CAZy family AA5 and is, together with glucose oxidase 2 (GOx2 Ppl108489), involved in the H_2O_2 production (Zhang et al., 2016).

1.3.2.2 *Genes coding for enzymes of the oxalate cycle*

Brown-rot fungi accumulate significant amounts of oxalic acid, which has been shown to be involved in their wood decaying system (Takao, 1965). Oxaloacetate dehydrogenase (CyOx Ppl112832) is presumed to be involved in oxalic acid synthesis, which is important for sequestering and reducing Fe^{3+} to Fe^{2+} (Beck et al., 2018). Glyoxylate dehydroxygenase (GlyD Ppl121561) is likely involved in oxalic acid synthesis, by catalysing the production of oxalate through oxidation of glyoxalate (Akamatsu and Shimada, 1994). It is also involved in the glyoxylate cycle (Munir et al., 2001a). In previous studies (Tokimatsu et al., 1998; Munir et al., 2001a), a GlyD that catalyses dehydrogenation of glyoxylate to oxalate in the presence of cytochrome c was purified. Munir *et al.* (2001) also found that GlyD exhibited a strong correlation with the biosynthesis of oxalic acid and fungal growth.

1.3.2.3 Genes coding for lignocellulose-active enzymes

Following the "pretreatment" by the CMF degradation, enzymatic degradation changes the wood cell wall in composition (Fackler et al., 2010). Several studies on fungal genomes throughout the last years have revealed that lignocellulose-active enzymes are significantly reduced in number in brown rot fungi compared to white rot (Eastwood et al., 2011; Floudas et al., 2012; Riley et al., 2014; Nagy et al., 2015; Zhang et al., 2016; Presley et al., 2018). In a study on brown rot fungi, using a wafer method to separate initial from later decay, it was shown that brown rot fungi involve a set of differentially regulated oxidoreductases accompanied by high expression of pectinase (Zhang et al., 2016). This may loosen lignocellulose as a "pretreatment" to enhance the accessibility for hydrolytic enzymes (Xiao and Anderson, 2013). GHs were increased in samples of later decay by *R. placenta* and *G. trabeum*, when growing on aspen wafers (Presley et al., 2018). Most enzymes secreted by brown rot fungi, acting on cellulose are endoglucanases belonging to the GH5 and GH12 family and LPMOs of the AA9 family (Floudas et al., 2012; Lombard et al., 2014). *R. placenta* showed a significant increase of endoglucanase activity in enzymatic samples, while *G. trabeum* increased xylanase activity in the same decay phase (Presley et al., 2018). This indicates that different brown rot species may prefer different carbohydrates, perhaps leading to chemical variability in the decay substrate among species (Presley et al., 2018). It has been suggested that early-stage pectinolysis might be necessary to convert wood lignocellulose enzymatically without harsh pretreatments (Presley et al., 2018). Hemicellulases are also important during brown rot decay, assisted by carbohydrate esterases (Floudas et al., 2012; Riley et al., 2014). During early stages of brown rot decay, CEs are required for hemicellulose removal, for example, to remove acetyl groups from xylan in hardwoods (Cowling, 1961; Puls, 1997). An increased set of genes attributed to the CEs were found in brown rot secretomes during initial brown rot decay (Presley et al., 2018).

1.3.3 Transcription factors in filamentous fungi

Transcription factors (TFs) form the last link between signal flow and gene expression of target genes and are important for signal transduction pathways (Shelest, 2008). The regulatory capacity of an organism depends on the variety of its TFs and around 80 families have been revealed by whole-genome annotation of more than 200 fungal species, of which three families are fungal-specific (Shelest, 2008, 2017).

1.3.3.1 Fungal specific transcription factors

The largest class of fungal-specific domains are the $Zn(II)_2$-Cys_6, are interacting with DNA as monomers or as homo- or heterodimers (Shelest, 2008). The zinc-cluster superfamily is among others regulating cellular processes that are crucial for the survival of the fungus: sugar and amino acid metabolism, cell cycle, stress response and many more (MacPherson et al., 2006). The zinc cluster superfamily is multifunctional, while the other two fungal-specific superfamilies (DNA-binding domain of MBP1 and copper-regulated zinc domain) are more restricted in their functions (Shelest, 2008). The second largest TF class after the zinc cluster is the "fungal-specific transcription factor domain" (Shelest, 2008). TFs containing this domain, are involved in sugar metabolism, amino acid metabolism, gluconeogenesis and respiration, fatty acid catabolism and so on (Shelest, 2008). Cellular differentiation in Ascomycetes is regulated by a conserved ASPES domain, which can be modelled as a basic bHLH-like structure (Shelest, 2008). These proteins might be involved in both, activation and repression of gene expression (Doedt et al., 2004).

1.3.3.2 Other transcription factors

Functionally highly conserved are the bZIP proteins, belonging to one of five domain families that are found in eukaryotes and viruses, but not in bacteria (Shelest, 2008). Interestingly, some TFs of fungal and metazoan genomes possess two or more different DNA-binding specificities (Shelest, 2017).

1.4 Laboratory tools

Traditionally wood degradation was investigated by screening alterations in the wood itself using, for example, microscopy with less focus on the microorganism responsible for the damage (Schmidt, 2006; Gelhaye and Morel, 2009). Different molecular tools, capable of detecting and identifying decay fungi have been developed during the last decades, for example genetic, transcriptomic and proteomic tools (Gelhaye and Morel, 2009).

The polymerase chain reaction (PCR) has been shown to be a suiting method for investigating DNA of Basidiomycetes in wood (Pilgård et al., 2011).

1.4.1 Next-generation sequencing (NGS)

Functional genomics is used to get information on the functions of genes and other sequences in the genome (Schmidt and Hildebrandt, 2017). In the advancement of molecular biology, DNA sequencing technologies have played an important role (Gilbert, 1981) and are a fast-developing area in biomedical research (Zhou et al., 2010). There are several sequencing technologies available, one of them being the next-generation sequencing (NGS) where nucleotides are sequenced faster and cheaper than during Sanger sequencing (Park and Kim, 2016). NGS technologies have extended to an impressive array of applications and are varying according to the experimental purpose (Zhou et al., 2010). A new genome of an unknown species can be built by using de novo sequencing with assembly, where fragmented reads of DNA are put together by aligning regions with overlap, building a genome sequence (Baker, 2012). Genetic variation between different genomes can be detected by mapping the genome to an existing reference, identifying single nucleotide polymorphisms (SNPs), structural variations, changes in copy number and so on (Ng and Kirkness, 2010). A partly sequencing of mRNA is often sufficient to get information on gene expression and to compare the set of transcribed genes to a certain time point in a certain condition (Schmidt and Hildebrandt, 2017). For analyses of differential gene expression, complementary DNA has to be synthesised from RNA and the transcriptome has to be sequenced (Wang et al., 2009; Park and Kim, 2016). The transcriptome reflects the complete set of transcripts in a cell, as well as their quantity and is important for interpreting functional elements of the genome (Wang et al., 2009).

1.5 Wood protection

Wood protection is necessary to prolong the service life of wood and wooden products made of nondurable wood. Thereby it is important to change the living preconditions of the fungi in such a way, that growing on wood is completely inhibited, or at least aggravated (Schmidt, 2006). There are mechanical ways, preventing fungal attacks, starting with the right choice of the suitable wood species and its application through to design issues, chemical preservation and modification (Goodell, 2003; Schmidt, 2006). Modifications are gaining importance since they have reduced environmental impact and they have been shown to influence weathering behaviour of wood substantially (Temiz et al., 2007).

1.5.1   Wood preservatives

The application of softwoods in construction in northern countries enhances the need of finding ways to protect timber products. Copper-based preservatives were traditionally used to treat wood. However, restrictions and regulations regarding the use of these preservatives have been issued after concerns on ecology and human health of these products arose (Hill, 2006; Temiz et al., 2007; Gress et al., 2015). Chromated copper arsenate (CCA) has therefore been banned from residential use and for wood in contact with water (Hingston et al., 2001; Lebow, 2004; Townsend et al., 2005; Hill, 2006). Newer preservation methods for wood in residential use are for example alkaline copper quaternary, borates, copper azole, copper naphthenate, with alkaline copper quaternary currently being the most widely used preservative for residential application (EPA, epa.gov).

1.5.2   Wood modification

An alternative, non-biocidal approach is wood modification which enhances the properties of wood physically, biologically or chemically. By definition, modified wood should be non-toxic itself and not release any toxic substances during or at the end of service life following disposal or recycling (Hill, 2006). Two types of modification are differentiated, active and passive modification. During active modification reagents react with and bind to the hydroxyl groups in the cell wall polymers, leading to an alteration of the wood properties (Hill, 2006). The majority of wood modification systems work as shown in Figure 7 (Jones and Sandberg, 2020). These are for example the commercially used furfurylation and acetylation. Another modification method is the impregnation with dimethylol-dihydroxyethyleneurea (DMDHEU). Passive modifications include for example thermal wood treatment. The commercial production of modified wood is led by thermally modified wood with 1,110,000 m^3/year, followed by acetylated wood (120,000 m^3/year) and furfurylated wood (45,000 m^3/year). Other processes, predominantly based on resin-based systems provide 330,000 m^3/year (Jones and Sandberg 2020). In this thesis only acetylation is included for analysis, thus furfurylation and thermal modification are only treated briefly.
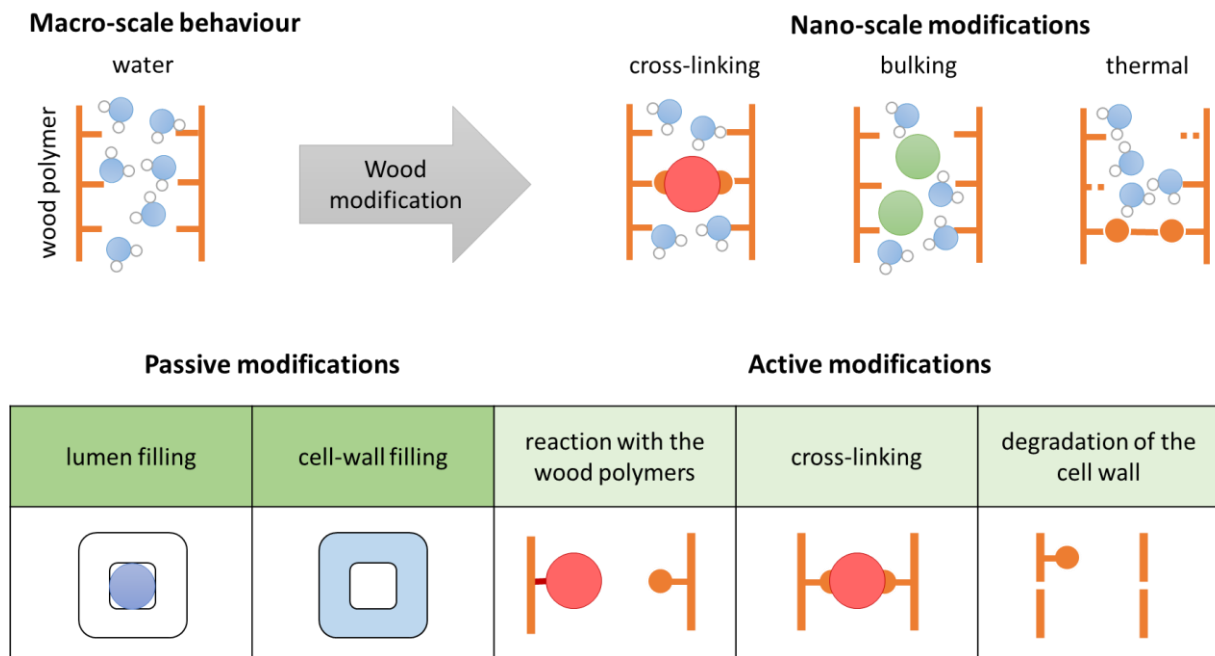
Figure 7 Schematic diagram illustrating the effect of modification (according to Jones & Sandberg 2020).

1.5.2.1 *Thermal modification*

Heat treatment without the usage of chemical additives and with a limited supply of oxygen to prevent oxidative combustion, is a generally accepted and commercialised procedure for improving some characteristics of wood (Jones et al., 2019). Wood is thermally modified via mild pyrolysis (Tjeerdsma et al., 1998; Welzbacher, 2007; Windeisen et al., 2009; Pfriem et al., 2010) applying temperatures between 180 °C and 260 °C for several hours (Militz, 2002; Hill, 2006). Heat treatment leads to auto condensation of lignin and degradation of hemicellulose. Lignin further builds up crosslinking with polysaccharides (Tjeerdsma et al., 1998; Hakkou et al., 2006; Mohareb et al., 2012). During thermal modification the wood gets discoloured and partly degraded while enhancing dimensional stability and biological resistance (Tjeerdsma et al., 1998). Furthermore, hygroscopicity and abrasion resistance is reduced (Hill, 2006).

1.5.2.2 *Furfurylation*

During furfurylation, wood products are impregnated with furfuryl alcohol which polymerises inside the wood cell wall (Schneider, 1995; Lande et al., 2008; Bryne and Wålinder, 2010; Thygesen et al., 2010). This process is followed by a heat-curing step and drying including recycling of the chemicals used. The heating results in a hard and resistant product (Jones and Sandberg, 2020). It has been shown that with increasing weight percentage gain (WPG) and

EMC, also decay resistance increases (Lande et al., 2004; Esteves et al., 2010). One possible effect of treatment with furfuryl alcohol on wood, leading to enhanced decay resistance, is bulking.

1.5.2.3   Acetylation

Acetylation is widely studied and a commonly used chemical wood modification (Rowell, 2006). During acetylation hydroxyl groups of the wood cell wall, polymers react with acetic anhydride, forming stable, covalently-bonded ester groups while acetic acid remains as byproduct (Figure 8) (Rowell, 2006). One hydroxyl group reacts with one acetyl group without polymerisation. Thus, the weight gain can directly be recalculated into units of hydroxyl groups blocked (Rowell and Bongers, 2015).
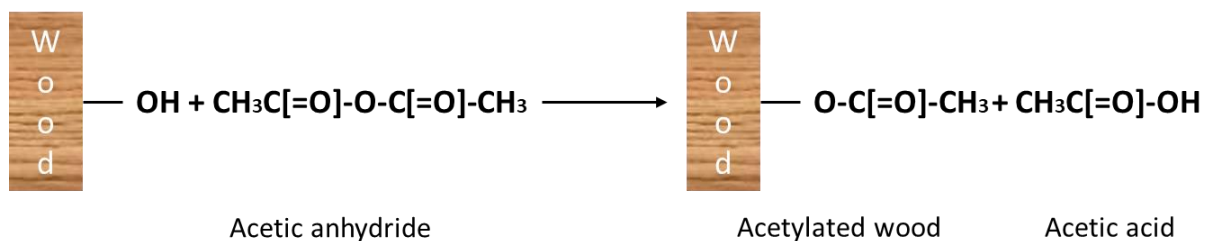
Figure 8 Chemical reactions during the acetylation process (according to Rowell & Bongers 2015).

Since this reaction is limited by temperature, higher temperatures are applied to enable better diffusion of the reactants. Other factors, influencing the acetylation reaction are for example dimensions, density and moisture content of the sample, the wood species, application of catalysts and/or swelling agents and time of incubation (Hill, 2006). The acetylation process is starting in the S2 layer, moving further to the middle lamella. At a WPG between 16 and 19%, 89% of the lignin and 25% of holocellulose hydroxyls are substituted (Rowell et al., 1994). As for furfurylated wood, the WPG of the wood after acetylation characterises the degree of acetylation. The WPG of different wood species increases with the time of acetylation (Beck et al., 2018).

1.5.3 Mode of action of wood acetylation

1.5.3.1 Most accepted theory

Early research concentrated on the enzymatic degradation stage of brown rot decay, for example, the theory that enzymes are not capable of recognising the substrate, due to the modification and therefore the fungi are not able to degrade it (Rowell, 2005). These and other theories were reviewed and some were proven to be wrong (Ringman et al., 2014a). More recent research highlighted the importance of the CMF degradation as being the crucial point, when investigating the inhibition of fungal growth by wood modification (Goodell et al., 1997; Arantes et al., 2012). Several theories on how acetylation inhibits fungal growth have been discussed throughout the years and different hypotheses have been stated. Numerous studies have shown that acetylation is excluding water due to the inhibition of water sorption by directly substituting OH groups with acetyl groups in the wood cell wall (Larsson Brelid et al., 2000; Rowell, 2005; Hill, 2006). This reduces the hygroscopicity and leads to volumetric swelling further leading to a bulking effect (Papadopoulos and Hill, 2003; Engelund et al., 2013; Popescu et al., 2014; Beck et al., 2017). The limitation of cell wall water through acetylation might disrupt a continuous network, mandatory for the transportation of agents needed for the degradation as well as solvents solubilised by the degradation process (Zelinka et al., 2008; Zelinka et al., 2015; Zelinka et al., 2016; Thybring, 2017). This theory was further supported by two other studies, observing a decreased diffusion of K^+ ions with increasing level of acetylation (Hunt et al., 2018) and one characterising moisture in acetylated wood, using low-field nuclear magnetic resonance relaxometry (LF-NMR) (Beck et al., 2017). The interrupted chemical transport would disable a Fenton reaction inside the wood cell wall (Goodell et al., 1997; Arantes et al., 2012). Ringman *et al.* (2014) reviewed the most popular theories, concluding that moisture exclusion, caused by reductions in the wood cell wall void volume is the most reasonable cause for delay of fungal decay. A lower EMC, caused by modification was further endorsed to be the main effect against decay fungi (Ringman et al., 2019). A WPG of 10% does only lead to limited decay inhibition, whereas a WPG of 20% significantly increases decay resistance in acetylated wood (Stamm and Baechler, 1960; Ibach and Rowell, 2000; Larsson Brelid et al., 2000; Hill, 2006; Hill, 2009). It has been shown that modification is not lethal for brown rot fungi, meaning that colonisation of the wood is still possible (Pilgård et al., 2010; Schmöllerl et al., 2011; Pilgård et al., 2012). Hence, the exact mechanisms of how

and to what extent acetylation is inhibiting or delaying fungal growth are lacking information and need to be further investigated.

1.5.3.2 Effect of acetylation on gene expression

Genes that are involved in the CMF degradation have been found to be upregulated in modified samples compared to untreated samples (Alfredsen and Fossdal, 2010; Schmöllerl et al., 2011; Pilgård et al., 2012; Zhang et al., 2016), which might be a reaction to the acetylation but also a stress response.

2 Positioning of this thesis within the scientific field

2.1 Gaps of knowledge

Within the global carbon cycle, brown rot fungi belong to the most important groups of biomass degraders. Enhancing the understanding for the wood degradation mechanisms used by these fungi will help to understand fundamental processes in nature. Since fungi are also of importance in lignocellulose processing, improved knowledge of the brown rot mechanisms will promote the development of novel wood preservation methods. When performing degradation and durability tests with brown rot fungi, *R. placenta* is an often-used species. In Europe and America, however, different strains are employed: FPRL280 and MAD-698, respectively. So far, results obtained from these studies have been seen as equivalent. Due to differences in phenotypes, suspicion arose that the strains have evolutionarily separated, but genomic data of the European strain, enabling genomic comparison, were absent. Differences in either the genome or in regulatory mechanisms could cause varying decay capacities and aggressiveness, which is why it is important to reveal how strong these differences are. If the differences between two strains of one species are big enough, results from studies using different strains could no longer be comparable, since statements referring to the aggressiveness and harming potential for wooden products of one strain might not be transferable to the other.

Brown rot fungi evolved several times independently from white rot fungi, explaining their versatile mechanisms of polysaccharide degradation. Many theories on the different underlaying mechanisms in brown rot fungi have been stated so far, but none of these has been fully accepted, highlighting the importance of further research in this field. Many studies that investigate brown rot mechanisms use only single brown rot species or do not differentiate between the degradation phases, but drawing conclusions from these does not capture the overall complexity of brown rot degradation systems.

Especially the initial decay phase seems to be crucial, when wood protection methods are considered, which is why an improved understanding of processes during oxidative decay is of importance. The roles of single genes in the degradation machinery, working in synergy with others, is still not fully understood, warranting more detailed research approaches.

Wood modification is an effective method to protect wooden products in outdoor constructions from fungal attack. Even though wood acetylation has proven to inhibit or at least delay fungal

decay to some extent, the exact mechanism, how it helps to counteract fungal attack is still not fully understood. Several studies have addressed this problem, but so far none have used a sample design that separates the hyphal front from later decay on acetylated samples. Investigating gene regulation of brown rot fungi during initial degradation could reveal coping mechanisms to overcome the modification as well as differing strategies between strains, to changes in growth conditions.

2.2 Objectives and Aims

In light of the above-mentioned gaps of knowledge, the overall aim of this thesis was to provide new insights into the biochemical mechanisms involved in brown rot decay as well as the effects of acetylation on the gene regulation. These would be beneficial for fungal biotechnological applications as well as the development of novel wood preservation methods.

To this end, a number of individual objectives were targeted:

- Identifying genetic and regulatory differences between the American and the European strains of *R. placenta*, MAD-698 and FPRL280, respectively, that can explain the observed phenotypic differences in appearance and decay behaviour. Derive recommendations on the comparability of studies investigating brown rot decay using the *R. placenta* strains FPRL280 and MAD-698.

- Provide a reference genome for further studies using the European strain of *R. placenta* FPRL280.

- Elucidate the differential gene expression in oxidative and enzymatic degradation phase, not only between two strains of *R. placenta*, but also between two brown rot species, *R. placenta* and *G. trabeum* when growing on untreated wood.

- Study the effects of wood acetylation on the overall gene expression behaviour, with a focus on brown rot-related genes, to elucidate coping mechanisms in the three brown rot strains.

- Compile a list of genes that are specifically important in brown rot decay to be used in future studies.

2.3 Approach

To enable both, the comparison of genetic data and the elucidation of the genetic impact on the phenotype, the genome of the European strain of *R. placenta*, FPRL280, was sequenced and compared to the American strain MAD-698. Additionally, phenotypes were tested as well as the strain´s ability to fuse (**Article I**).

To gain information of how brown rot fungi react to acetylation, gene expression of ten brown rot-specific genes during the initial degradation phase of *R. placenta* (FPRL280) was tested on samples, acetylated to three different levels (10, 15 and 20%) by q-PCR (**Article II**).

The comparison of the two degradation phases (oxidative and enzymatic), observed in brown rot fungi in two strains of *R. placenta*, as well as in another distantly related brown rot species, *G. trabeum*, was performed, by sequencing the transcriptomes of all three strains growing on wood wafers of Scots pine. Differences between the two phases as well as between decay strategies between the strains were investigated (**Article III**).

To get a broader view, transcriptomic data of *R. placenta* and *G. trabeum* growing on untreated wood and wood acetylated to an intermediate level (15%) were additionally investigated by RNA-seq (**Article III**).

A more detailed analysis of genes with probably important roles during both, initial and progressed decay as well as in coping with changed growth conditions through the acetylation was performed to identify genes of interest and possible candidates to be used as biomarkers (**Article III**).

3 Materials and Methods

3.1 Wood samples

3.1.1 Wafer design

Since softwood is often used in outdoor construction in the northern hemisphere and the substrate that is preferably degraded by brown rot species, wood of the domestic Scots pine (*Pinus sylvestris L.*) was used in this study. Further, pine sapwood is due to its anatomy a suitable wood species to be modified by acetylation. In accordance to the method introduced by Schilling and Zhang in 2013 (Schilling et al., 2013), wood boards from *Pinus sylvestris* sapwood were cut into wafers (80 x 18 x 2.5 mm^3), with the largest area being the cross section. This method enables the spatial separation of the initial, non-enzymatic oxidative and the enzymatic degradation phase (Zhang et al., 2016) and it has been shown, that mycelium on the outer parts of the samples is growing as fast as in the inner parts of the sample (Schilling et al., 2013). The samples were dried (103 °C for 24 h) and their dry weights were measured.

3.1.2 Acetylation of the samples

A standard protocol for the acetylation of wood samples in the laboratory was optimised for the impregnation of the samples with the wafer design (Hill, 2009). The setup used for the acetylation can be seen in Figure 9. Dried samples (50 for 15% and 20% acetylated samples and 25 for 10% acetylated samples) were put into a 1 L reaction vessel (B) and vacuum was attached for 30 minutes. Then, 50 mL (25 for 10% acetylation) of acetic anhydride were injected, followed by the same amount of pyridine. A vacuum was attached for another minute, followed by an incubation of the samples for 3 h at room temperature. Following this, the reaction vessel was lowered into an 80 °C water bath for 15 (10% acetylation), 30 (15% acetylation) or 60 (20% acetylation) minutes. The reaction was interrupted by washing the samples twice with ice-cold acetone and twice with an acetone-water mixture with a ratio of 1:1. All samples were rinsed in distilled water several times for 3 days, vacuum-impregnated with water, to remove all accessible chemicals and then again oven dried and weighed. Before the decay test all samples were packed and autoclaved.
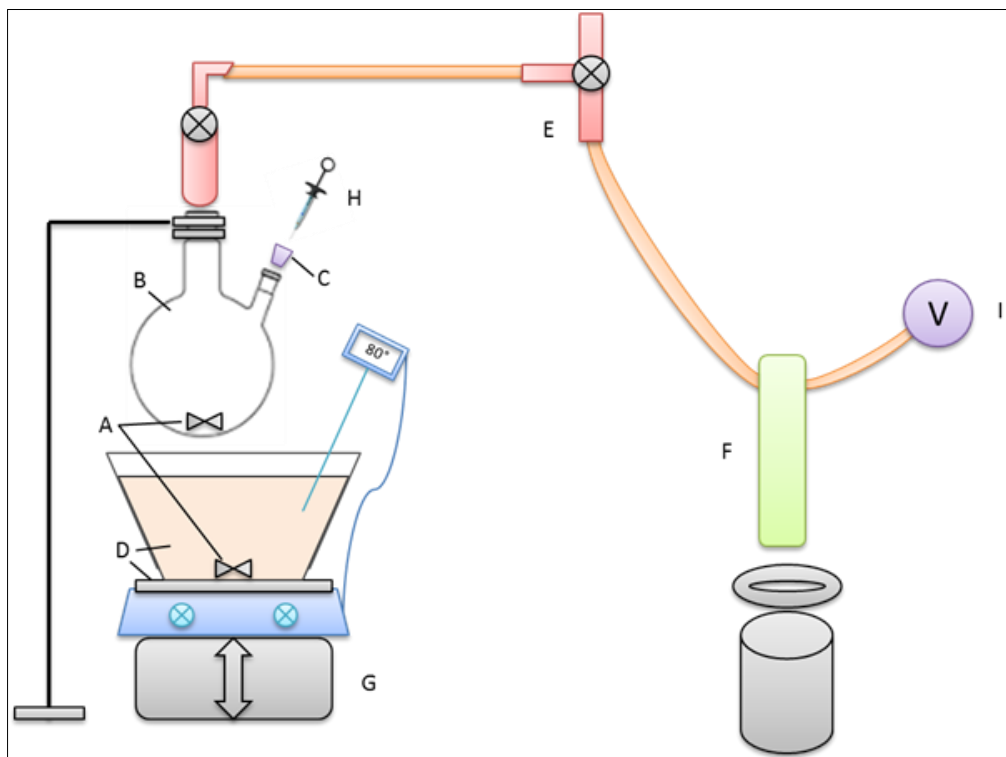
Figure 9 Schematic setup of the acetylation in the laboratory. **A** = stirring bars; **B** = customised two-neck round-bottom flask; **C** = rubber plug; **D** = water bath with stirring bar, hot plate and adjustable contact thermometer; **E** = t-valve for pressure balance; **F** = c ondensation trap in Dewar flask (inhibits the permeation of reaction vapor into the vacuum pump); **G** = lifting table; **H** = injection syringe; **I** = vacuum pump.

3.1.3   Decay test

Details on the setup of the decay tests are provided in Article II and III. The fungi that were used were either *Rhodonia placenta* (strain FPRL280), *Rhodonia placenta* (strain MAD-698) and *Gloeophyllum trabeum* (strain BAM 115). For the degradation test of Article I, 16 glasses of *R. placenta* (FPRL280) were prepared for each treatment (n = 16) and for the degradation test of Article II, samples from 15% acetylation and untreated samples of the previous test were used for FPRL280. Three glasses of untreated and 15% acetylated samples were prepared for *R. placenta* (MAD-698) and *G. trabeum*. An illustration of the setup can be seen in Figure 10. Again, the jars were stored in the climate chamber until the wafers were ¾ overgrown with mycelium. A 5 mm section containing the hyphal front, representing the oxidative decay, was taken, the rest of the mycelia containing samples was taken for enzymatic analysis. A 5 mm section has been shown to represent a 48 h window, since the expected growth rate is 2.5 mm/day, during which the non-enzymatic degradation phase has been shown to take place (Zhang et al., 2016). These growth rates were reproduced using our samples. Three samples

from each weck jar were pooled to one biological replicate.
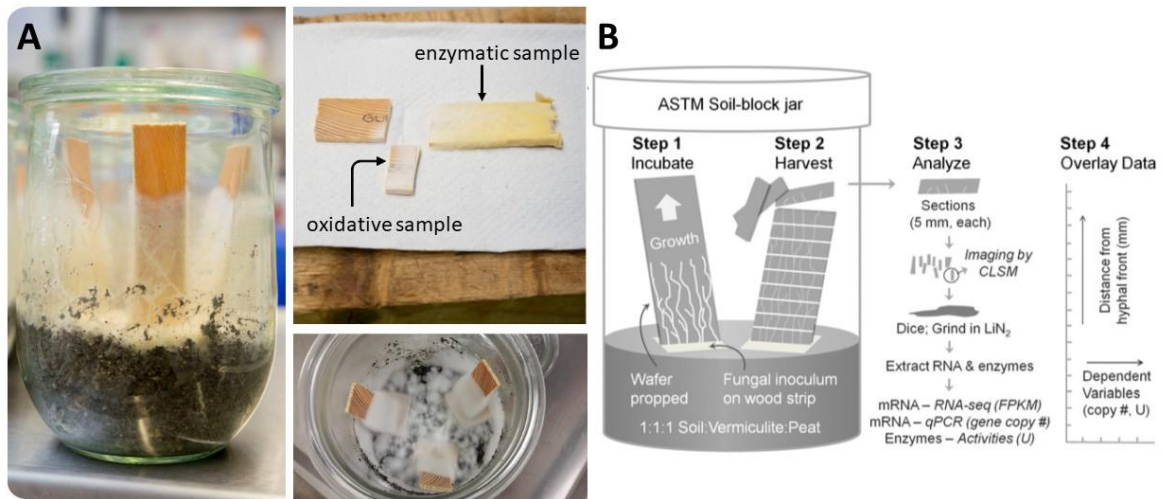
Figure 10 Setup of the wafer degradation test. **A**: The overgrown wafers in the weck jar on the growth medium as well as how the samples were later harvested and cut into oxidative and enzymatic samples. **B**: Schematic figure on how the test was set up by Zhang *et al.* (2016).

3.2 Gene expression

RNA was extracted from the previously milled 5 mm sections for Article II and for both, the 5 mm section as well as the rest of the mycelium containing sample, for Article III. Details on the device and protocol that were used are provided in Article II and III. RNA of the samples used for Article II was converted to cDNA and gene expression of ten genes, likely involved in early brown rot decay were analysed using qPCR (see Article II). The expression levels for each treatment (untreated (UT), acetylated (AC10, AC15 and AC20)) were compared.

3.3 Phenotype test

The tests on the phenotypes of the two *R. placenta* strains FPRL280 and MAD-698 were done for Article I, containing tests on the growth rates, mass loss (Figure 11) as well as a fusion test (Figure 12). These tests were used to compare the strains and genomic data were screened for differences in important genes, potentially explaining the phenotypic differences.
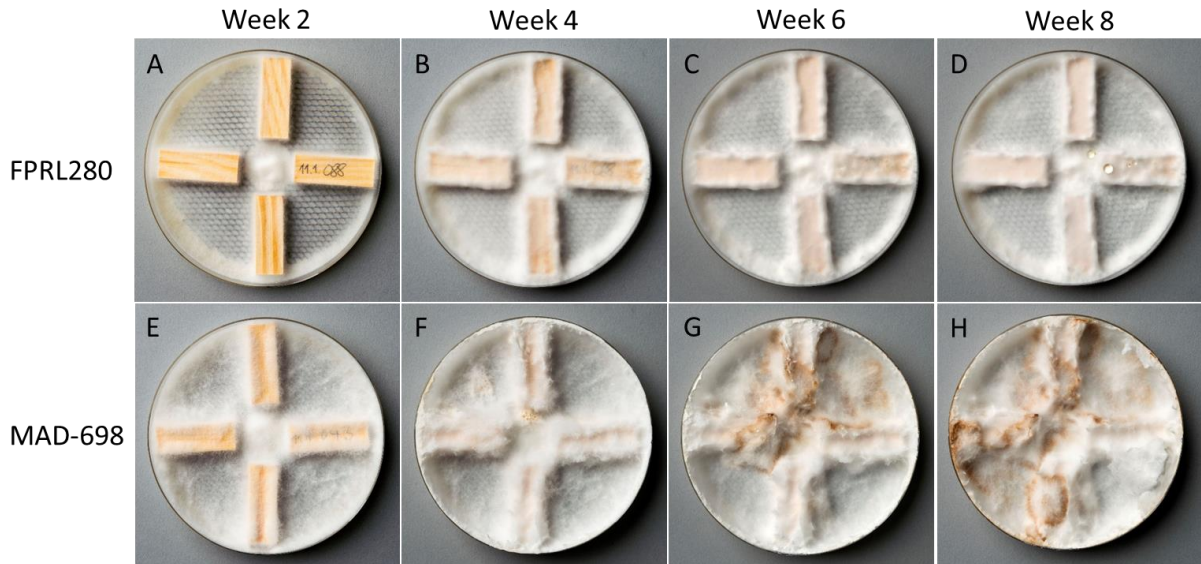
Figure 11 Wood degradation test to observe appearance of both strains (FPRL280 and MAD-698) when growing on miniblock wood samples. **A-D** show growth of FPRL280 after two to eight weeks. **E-H** show growth of MAD-698 after two to eight weeks.
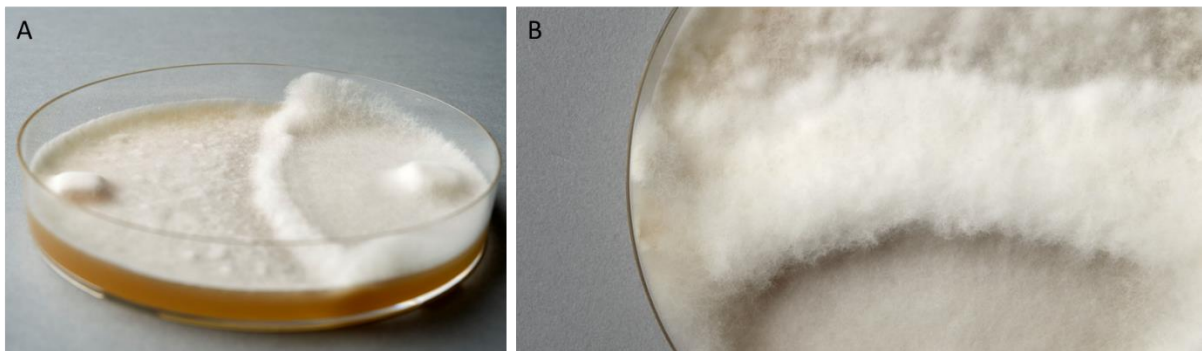
Figure 12 Fusion of the two *Rhodonia placenta* strains growing on one plate: **A**: FPRL280 (left side) and MAD-698 (right side), two weeks after incubation and **B**: to a later time point.

3.4 Sequencing

The dikaryotic strain of *R. placenta* (MAD-698) has already been sequenced in 2009 (Martinez et al., 2009) as well as the corresponding monokaryotic strain (MAD-SB12) in 2017 (Gaskell et al., 2017). The genome of the European strain FPRL280 has not been sequenced yet, which has been done for this study. Details on the sequencing, mapping, de novo assembly, as well as on the annotation are provided in Article I. Further, a phylogenetic tree was created to determine degrees of relationships between the strains and closely related other brown and white rot fungi.

3.5 Transcriptome analysis

To further support our findings on differences between the two strains of *R. placenta*, transcriptomic data were generated and analysed. Information on the proceedings of the sequencing and the analysis are provided in Article III. The aim of the analysis of transcriptomic data was also to further elucidate strategies of brown rot fungi during initial and enzymatic decay. Therefore, a degradation test on untreated and 15% acetylated wood wafers was performed (as mentioned in 5.1.3), including the three brown rot fungal strains of *R. placenta* (FPRL280), *R. placenta* (MAD-698) and *G. trabeum* (BAM 115). To compare the oxidative non-enzymatic phase with the enzymatic decay phase, both, the 5 mm section, containing the hyphal front and the remaining sample with mycelium were taken for analysis. Three jars containing three samples each were prepared for each condition and each strain, resulting in 36 glasses. The three samples of each glass were pooled to one biological replicate and later the transcriptomic data of all three technical replicates were combined.

4 Results

4.1 Article I

Article I: Kölle M, Horta Crivelente MA, Nowrousian M, Ohm R, Benz JP, Pilgård A (2020) Degradative capacity of two strains of *Rhodonia placenta*: from phenotype to genotype. Frontiers in Microbiology, 11 (1338).

4.1.1 Summary

Brown rot causes the main damage to wood in outdoor construction and is therefore feared. To better understand the mechanisms during brown rot decay, intense research has been done in this field during the last decades. *Rhodonia placenta* is a commonly used brown rot species to investigate mechanisms used by brown rot fungi to degrade wood. Phenotypic differences between the American (MAD-698) and the European (FRPL280) strain have been observed previously. This raised the question, if the spatial separation of the two strains has led to such genetical differences that their decay behaviour might be different enough to directly compare them as one species.

The monokaryotic (MAD-698) and dikaryotic (MAD-SB12) strain of the American *R. placenta* have already been sequenced in 2009 (Martinez et al.) and 2017 (Gaskell et al.). The aim of this study was to sequence the European strain FPRL280 ad to compare its genome to the published MAD-SB12 genome, to figure out differences that could explain the phenotypic observations. Furthermore, this genome could be used as reference for further downstream analyses as well as for a better understanding of the species lineage.

Besides the sequencing of the FPRL280 genome, phenotypes of MAD and FPRL280 have been compared investigating growth speed, appearance, mass loss and the ability of fusion. The whole genome was mapped against the MAD-SB12 genome and a phylogenetic analysis was performed.

The results confirmed phenotypic differences in decay initiation and degradative capacity and showed that the two strains are no longer able to fuse. The genomic analysis showed an overall identity of 98.4% with SNPs in roughly every 60th basepair. Phylogenetic clustering showed that the two strains are closely related. Several variants could be detected in carbohydrate-active

enzymes (CAZymes) as well as four genes that are completely missing in the FPRL280 genome.

Mutations in domains of important genes (for example regulatory genes) could be the explanation for phenotypic differences, but also regulatory mechanisms are likely having a powerful impact. Small changes in the genome could affect the degradation behaviour of fungal strains, highlighting the importance of a united strain selection for standardised decay tests. This study is further useful for the understanding of brown rot degradation mechanisms and the development of this important brown rot species.

4.1.2 Contributions of the authors

The research was designed by Annica Pilgård, Martina Kölle, J. Philipp Benz and Maria Augusta Crivelente Horta. Martina Kölle and Maria Augusta Crivelente Horta performed the analyses, together with Minou Nowrousian, who did the sequencing and primary analyses and Robin Ohm, who generated the phylogenetic tree. Martina Kölle, Annica Pilgård, J. Philipp Benz and Maria Augusta Crivelente Horta co-wrote the manuscript with support of Minou Nowrousian and Robin Ohm. The results were interpreted by the help of all authors, as well as final approve and editing of the manuscript.

4.2 Article II

Article II: Kölle M, Ringman R, Pilgård A (2019) Initial *Rhodonia placenta* gene expression in acetylated wood: group-wise upregulation of non-enzymatic oxidative wood degradation genes depending on the treatment level. Forests 10 (1117).

4.2.1 Summary

Wood modification is a promising alternative to traditionally used wood preservatives to protect wooden products against fungal decay. During modification the wood properties are changed to such extent that fungal growth is inhibited or at least delayed. One possible modification method is acetylation. Hence, the exact mechanisms behind the decay resistance of acetylated wood are not known. The most likely theory is that chemical transport of degradation agents is inhibited by an interrupted water-network within the wood cell wall, due to moisture exclusion.

Brown rot fungi use a two-stepped degradation process to degrade wood in a very efficient way, leading to massive strength loss within a short period of time. The currently most likely mode of action during brown rot is that the fungi secrete oxalic acid, which diffuses into the lumen, where it functions as a chelator to sequester Fe^{3+}, which is then reduced to Fe^{2+} by hydroquinones. Additionally, hydroquinones are believed to react with oxygen to produce H_2O_2, which further reacts with the Fe^{2+} to hydroxyl radicals, capable of polymerising cellulose and hemicellulose and modify lignin. The sugars, solubilised during this process, can diffuse through the cell wall into the lumen, where they become accessible to cellulases and hemicellulases.

It has been shown that these two degradation steps are spatially segregated, which is why a sample design was developed to be able to investigate the two phases separately. In this study the wafer design was applied to samples of untreated and to three different levels acetylated (10%, 15% and 20%) Scots pine (*Pinus sylvestris*). The samples were inoculated with the brown rot fungus *Rhodonia placenta*. The upper sample part, containing the hyphal front that represents the non-enzymatic oxidative brown rot decay was taken as samples. Gene expression of ten genes, potentially involved in initial brown rot decay was investigated. It was expected that the fungus increases the expression of certain genes as a reaction to the acetylation.

The results showed decreased mass loss in to 10% acetylated samples and almost no mass loss in 15% and 20% acetylated samples, while untreated samples showed 10% mass loss after the

same time. Gene expression data showed a group-wise upregulation of six of the chosen genes with increasing level of acetylation, with most genes being upregulated in 20% acetylated samples. The other four genes were downregulated on acetylated samples compared to untreated samples.

The group-wise upregulation of first, genes involved in H_2O_2 production and then genes involved in oxalic acid production leads to the assumption that the fungus is trying to increase the output of the Fenton reaction step by step with increasing pressure through the acetylation. The results further indicated that not all genes that were thought to play a role during initial brown rot decay are involved as much as expected or probably have not the role they were thought to have. Another interesting observation was a relatively strong reaction of the fungus in 10% acetylated samples, even though growth and degradation were not inhibited. The findings suggest that there must be other mechanisms inducing the transition from oxidative to enzymatic degradation in addition to the cellobiose switch, which was proposed by earlier research.

4.2.2 Contributions of the authors

The research design and methodology were developed by Martina Kölle, Annica Pilgård and Rebecka Ringman. The decay test as well as the measurements and result visualisations were performed by Martina Kölle. The original draft was written by Martina Kölle, Annica Pilgård and Rebecka Ringman and all authors contributed to reviewing and editing the final version.

4.3 Article III

Article III: Kölle M, Horta Crivelente MA, Benz JP, Pilgård A (2021) Comparative transcriptomics during brown rot decay in three fungi reveals strain-specific degradative strategies and responses to wood acetylation. Frontiers in Fungal Biology, 2 (701579).

4.3.1 Summary

Understanding the mechanisms of the two-stepped degradation of brown rot fungi is important to improve existing and to find new ways to protect wooden products. A new sample design, enabling the individual investigation of both decay phases was firstly applied on acetylated samples and three strains of commonly used brown rot fungi (*Rhodonia placenta* MAD-698, and FPRL280; *Gloeophyllum trabeum*).

The aim was to figure out differences in fungal behaviour by investigating the transcriptomes of the fungi when growing on untreated and samples, acetylated to 15% - as well as the two decay phases, the non-enzymatic oxidative and the enzymatic decay. Further we wanted to create a list with genes that possibly have an important role during initial and later brown rot decay.

The results showed that the strains follow different strategies, not only those from different species, but also the closely related strains of *R. placenta*. The differences between the two decay phases, as well as the strains were mainly caused by one gene, a well-known endoglucanase (Cel12A), being expressed to high extent during enzymatic decay. Differences in gene expression could also be observed between untreated and acetylated samples, indicating that the conventional processes are somehow delayed or dysfunctional, caused by the treatment. The upregulation of genes, related to sugar metabolism could be explained by a scouting reaction, induced by the fungi to counteract starvation.

Differences in gene expression could probably evolve in a relatively short period of time and by small regulatory changes. Identifying those genes could not only help with fast decay recognition but also to find enzymes that are outstanding in degradation of lignocellulosic material for other fields, for example biopulping.

4.3.2 Contributions of the authors

The research was initiated and designed by Annica Pilgård, Martina Kölle, J. Philipp Benz and Maria Augusta Crivelente Horta. The decay test and the extractions were performed by Martina Kölle, the sequencing was done by the Chair of Animal Breeding (TUM). Subsequent analyses were performed by Martina Kölle and Maria Augusta Crivelente Horta. Martina Kölle wrote the manuscript with support of Annica Pilgård, Maria Augusta Crivelente Horta and J. Philipp Benz. All authors read and approved the final manuscript.

5 General discussion

5.1 Differences in decay strategies between different brown rot strains

One of the main aims of this study was to figure out, if observed phenotypical differences between two commonly used strains of *R. placenta* (MAD-698 and FPRL280) (Thaler et al., 2012) could be explained by either, differences on a genetic base or at the regulatory level. To answer this question, the genome of FPRL280 was sequenced and mapped to the previously published monokaryotic genome of MAD-SB12 (Gaskell et al., 2017) to identify mutations between the sequences and to provide a reference sequence for follow-up research (Article I). Another aim was to reveal differences in decay strategies between different strains of brown rot fungi and between two brown rot species. Therefore, the transcriptomes of *R. placenta* (strains: FPRL280 and MAD-698) and *G. trabeum*, growing on wood wafers, were sequenced (Article III). The data were analysed to verify genomic differences as well as to investigate expression levels of genes during initial and progressed brown rot decay.

Several phenotypic and genomic differences were found between the two strains of *R. placenta* (Article I). Even though the overall similarity was 98.4% and some missing genes and mutations in CAZy genes were found, the results indicate that most decisive differences between the two strains most likely are to be found in regulatory mechanisms. Transcriptomic data revealed similar expression profiles when comparing the two strains of *R. placenta* with *G. trabeum*, but detailed analysis showed that the two strains of *R. placenta* might already have developed different decay strategies as well (Article III).

During the last decades many studies on differential behaviour of decay fungi (brown and white rot) included the investigation of fungal genomes and secretomes (Martinez et al., 2009; Wei et al., 2010; Ryu et al., 2011; Floudas et al., 2012; Riley et al., 2014). While some studies concentrated on a smaller number of genes (Schmöllerl et al., 2011; Pilgård et al., 2012; Alfredsen et al., 2016; Beck et al., 2018; Ringman et al., 2018; Ringman et al., 2020), improved techniques enabled faster and more reliable data generation on whole transcriptome basis (Zhang et al., 2016; Presley and Schilling, 2017; Castaño et al., 2018; Presley et al., 2018; Zhang et al., 2019; Bari et al., 2021). Furthermore, older studies on brown rot degradation were not working with methods to segregate initial and progressed decay, which made it difficult to properly catch the exact phases. This changed with the introduction of the wafer method by Schilling *et al.* (2013), where the fungi grow longitudinally upwards a wood wafer. As already

explained in the introduction and the method section, this sample design enables to separate the hyphal front from the rest of the sample. The sample part that contains the hyphal front represents early decay and the lower sample parts later decay stages. In this study the wafer method was used, enabling the investigation of the individual decay phases by qPCR (Article II) and transcriptome sequencing (Article III). Phenotypical analyses were performed to see previously observed differences on a different sample design as well as to underpin genotypical findings. General information on the sequencing data can be found in Article I.

The results showed that MAD-698 started its growth earlier and produced more mycelium. Hence, FPRL280 caused more mass loss after a shorter period of time (Article I). This indicates that FPRL280 invests more energy in launching the decay machinery rather than into the vegetative hyphal growth as MAD-698 does, or recognises the substrate earlier than MAD-698. These findings are further supported by the upregulation of more BRS "Ox" genes during early brown rot decay by FPRL280 compared to MAD-698 (Article III).

Mass loss data from samples with more progressed decay however revealed heavier degradation rates caused by MAD-698 which might be explained by some genetic differences as for example four missing AA3_2 genes in FPRL280. This CAZy family further contained the highest number of variances (Article I). Members of the AA3 family do not directly act on polymeric constituents of lignocellulosic material, but support degradation by reducing low-molecular weight components, with one of the main products being H_2O_2. Changes in AA3_2 genes could affect this support substantially, resulting in a less effective Fenton reaction. Brown rot fungi have most likely a fine-tuned complex network of gene products working together to deliver radicals via the Fenton reaction and small changes in these genes could probably lead to measurable effects. Another group with many mutations was the group of GHs, particularly in the endoglucanases, which are responsible for the depolymerisation of the structural carbohydrates in the wood cell wall (Ryu et al., 2011; Floudas et al., 2012; Lombard et al., 2014) (Article I). Other important genes with mutations were for example hemicellulases and a member of the CE15 family. Hemicellulases help to break down complex hemicelluloses (Ryu et al., 2011; Brigham et al., 2018) and carbohydrate esterases are required for hemicellulose removal during early stages of brown rot decay (Cowling, 1961; Puls, 1997). A broad spectrum of CEs was found in secretomes of brown rot fungi during early stages of decay (Presley et al., 2018).

The differences in decay capacity could also be explained by regulatory factors, as a proportionally large part of the variances found in FPRL280 was located in regulatory genes (Article I). MAD-698, for example used a more diverse gene cocktail during enzymatic degradation than FPRL280, probably suggesting a more variable way of attacking wood and therefore perhaps explaining the higher decay rate (Article III). Furthermore, it has been shown that brown rot fungi use different carbon sources and therefore different enzyme sets are used to depolymerise the polysaccharides (Presley et al., 2018). The differences in gene sets could therefore also be an indication that the preferred carbon source has already changed to a certain level. Additionally, MAD-698 upregulated a high number of BRS "Ox" genes during enzymatic decay which might further fuel the degradation process. Expression levels of the smaller gene set of FPRL280 were comparably higher during both, oxidative and enzymatic decay (Article III). This probably shows a compensatory function for both, a less diverse set of genes and probably a lowered effectiveness of the genes, further underlining the decreased degradative capacity of FPRL280.

The test to grow both strains on the same plate showed that they are not able to fuse and they form a border between them (Article I). In nature, it is usual that several different species inhabit the same piece of wood, resulting in competition on nutrients and growth space. One possible response to this competition is the expression of cell-wall degrading enzymes, degrading chitin and β- glucans which the competitors cell walls are composed of, serving as nutrient source (Boddy and Hiscox, 2016). The competition between *R. placenta* and *G. trabeum* by growing them on the same wood wafer, was previously investigated (Presley et al., 2020). Searching for genes involved in competitor defence on the same sample where both *R. placenta* strains grow together might be a possibility to further underpin the theory that MAD-698 and FPRL280 have evolved differently, which should be considered when comparing them. Another possibility might be to investigate if the strains already prefer different carbon sources. Genomic research and industrial application become interesting when considering the differences of the two strains, not only caused by mutations in important genes, but also regulatory mechanisms which likely have a powerful impact.

However, when bringing in a second brown rot fungus, *Gloeophyllum trabeum*, the close relation between the two *R. placenta* strains was clearly seen. Even though all strains expressed similar numbers of genes throughout both decay stages and on both, untreated and treated samples, the expression profiles differed significantly (Article III). The expression profiles of all differentially expressed genes in FPRL280 differed significantly from those in MAD-698

and *G. trabeum*, mostly caused by uncharacterised genes. Therefore, further research on the functions of these genes is of great interest and importance for a better understanding of the different decay strategies. FPRL280 seemed to put much more effort into oxidative degradation than MAD-698 and *G. trabeum*, indicating that the efficiency is not as high as in the other two strains (Article III). Previous work suggested that a shift in brown rot evolution on the one hand involved shifts in gene portfolios and on the other hand might also involve shifts in energy investment strategies (Zhang et al., 2019). For several brown rot related genes, as for example endoglucanase, mannanase, glucosidase and xylosidase, remarkably higher activity was observed in *R. placenta* compared to *G. trabeum*, independent of the decay phase the fungi were in (Presley et al., 2018). This could explain the differing intensities of gene expression between the two *R. placenta* strains, further underpinning their evolutionary distance. Comparing only accumulated TPMs of differentially expressed genes created the impression that MAD-698 and *G. trabeum* follow more similar strategies than MAD-698 and FPRL280 (Article III).

It has been hypothesised that *G. trabeum* is a more aggressive degrader than *R. placenta* (Fackler et al., 2010), even though it has been shown that *R. placenta* produces more oxidoreductases during early decay, compared to enzymatic decay. In another study it has been shown that *G. trabeum* accumulates lower amounts of oxalic acid (Wei et al., 2010), which indicates that *G. trabeum* uses a different strategy during decay than *R. placenta*. In *G. trabeum* a higher rate of oxidureductases was found to be expressed during enzymatic decay, potentially fuelling the degradation and explaining the more aggressive decay (Presley et al., 2018). This probably also explains the differences in aggressiveness between FPRL280 and MAD-698 (Article I), since the latter uses a wider range of BRS "Ox" genes during enzymatic decay (Article III). Furthermore, *R. placenta* and *G. trabeum* showed a higher activity of different GHs during progressed decay, which lead to the assumption that different brown rot species may have different carbohydrate preferences (Presley et al., 2018). This could also imply that the two *R. placenta* strains already developed slightly different preferences as well, as mentioned above.

5.2 Relevance for decay tests

Both strains of *R. placenta* are used in standardised decay tests of wood products (AWPA, 2016). Regarding their differences in decay capacity and degradation strategies, the results of a study using one of the two strains are not directly transferable to the behaviour of the other

strain (Article I, Article III). Modification could be more effective for one strain, or enzymes could be used for the depolymerisation of different polysaccharides in the application in biofuels.

5.3 The effects of acetylation

Another aim was to figure out effects of acetylation on gene expression and probable adaption of the fungi to changed growth conditions due to the modification. To reach this aim, the wafer method was applied to modified samples, to be able to separate initial and progressed decay. In one part of this study, wafers were acetylated to three different levels of WPG (10, 15 and 20%) and gene expression of a small number of genes (10) was investigated by qPCR (Article II). Furthermore, the transcriptomes of *R. placenta* (FPRL280 and MAD-698) and *G. trabeum* when growing on untreated and 15% acetylated wood wafers (*Pinus sylvestris*), were sequenced and gene expression, as well as differential expression were analysed (Article III). The non-enzymatic oxidative degradation phase was represented by the first 5 mm containing the hyphal front, while the remaining part of the sample represented the enzymatic degradation phase (more detailed explanations are provided in the methods section).

Understanding the impact of acetylation on fungal growth is mandatory for improving modification methods for a better wood protection. This problem was addressed from different points of view during the last decades. Acetylation, or the mode of action of acetylation can for example be observed by understanding what happens in the wood itself, which has been intensely studied. Decreased diffusion and a therefore interrupted moisture network seems to be a promising pitch to explain growth inhibition (Hill, 2009; Thygesen et al., 2010; Thybring, 2013; Ringman et al., 2014a; Zelinka et al., 2016; Beck et al., 2017; Hunt et al., 2018; Ringman et al., 2019; Jones and Sandberg, 2020). Another possibility is to observe how fungal behaviour is altered when growing on acetylated wood, meaning how fungi react to the acetylation, which has been subject of several studies in the past (Schmöllerl et al., 2011; Pilgård et al., 2012; Alfredsen and Pilgård, 2014; Ringman et al., 2014b; Alfredsen et al., 2015; Alfredsen et al., 2016; Ringman et al., 2017; Beck et al., 2018). So far, the wafer method has not been applied to modified wood, enabling the sophisticated contemplation of the individual decay phases. To the best of my knowledge, no previous research has been made using this method on acetylated wood.

In this study, acetylated samples showed lower rates of mass loss with increasing WPG compared to untreated samples (Article II), while the growth rates were similar on all treatments. It would have been interesting to test if the mass of produced mycelium differed between the treatments. With this information, it would have been possible to gain information on the fitness of the fungi. Testing different levels of acetylation showed a group-wise upregulation of genes that are likely involved in initial brown rot with increasing acetylation level compared with untreated samples (Article II). Higher overall expression rates of differentially expressed genes were observed in acetylated samples during initial brown rot decay in all three strains compared to untreated samples, indicating that the strains enhance their effort of enzyme production to overcome the modification (Article III). MAD-698 seemed to upregulate more genes related to oxalate production than FPRL280 in acetylated samples during oxidative decay, indicating that the two strains react differently to modification. In *G. trabeum* the reaction to the acetylation was more clearly shown than in *R. placenta*. In *G. trabeum*, in the AC/Enz samples, many genes from the AA family clustered together with TFs, showing that a proper enzymatic decay was not yet established. In addition to this, it indicates that the fungus was also heavily struggling to overcome the modification. Furthermore, it might also be possible that the acetylation changes the pH in such way that the fungal enzymes are no longer in their functional optimum. This might also explain why enzymes are still expressed, but mass loss is not increased significantly on acetylated samples compared to untreated samples.

The currently most accepted theory on how acetylation hinders fungal degradation is that diffusion is inhibited in acetylated wood (Ringman et al., 2014a). This was further studied by Hosseinpourpia and Mai (2016), who investigated iron diffusion in acetylated wood. Their findings suggest that diffusion is inhibited under saturated conditions (Hosseinpourpia and Mai, 2016b). Zelinka *et al.* (2020) noted that these observations are not conclusive, since the lower iron rates in acetylated wood could be the result of either slower diffusion into the wood or a lower binding capacity for iron. Furthermore, they showed that diffusion rates were not dependent on the level of acetylation and they concluded that the CMF reaction might be affected by acetylation through lowering the capacity for iron, required for the Fenton reaction by other means than through iron diffusion (Zelinka et al., 2020). The regulation of genes in modified wood before significant mass loss can be detected seems to be more complex than an overall upregulation of genes related to oxidative decay (Ringman et al., 2020). This shows that there are still many gaps in the understanding of the mechanisms of acetylation, diffusion and

brown rot decay, highlighting its complexity. Further results regarding the impact of acetylation are discussed in more detail in the following paragraphs.

5.4   The complexity of the degradation system of brown rot fungi

Another objective of this study was to give further insight into the complex mechanisms that take part during brown rot decay, in addition to differences between initial oxidative and progressed enzymatic decay. To gain more information about these mechanisms, gene expression data were studied, including hierarchical clustering of all genes that were expressed over a level of 10 TPMs under at least one condition (conditions: untreated/oxidative; untreated/enzymatic; acetylated/oxidative; acetylated/enzymatic) (Article III).

Recent studies revealed that brown rot decay does not "only" contain the bipartite strategy of preceding cell wall treatment via lignocellulolytic oxidative reagents, followed by the secretion of enzymes acting on the thereby released polysaccharides. Instead, brown rot decay seems to be a concatenation of an initial step, driven by reactive oxygen species, but also supported by GHs seemingly tolerating oxidative stress (Castaño et al., 2018). This pattern also has recently been observed in the closely related brown rot fungus *Laetiporus sulphureus* (de Figueiredo et al., 2021). The theory that the CMF degradation is working in concert with enzymatic degradation has been supported by several studies (Goodell et al., 1997; Jensen et al., 2001; Cohen et al., 2002; Hammel et al., 2002; Suzuki et al., 2006; Zhang et al., 2016; Zhu et al., 2020). Furthermore, also the enzymatic phase is not only using genes related to sugar metabolism, but also involves oxidative metabolism (Article III).

Even though the number of genes secreted during degradation by both strains of *R. placenta*, is higher than that of *G. trabeum*, a clear differentiation between oxidative and enzymatic decay could be seen in all three strains (Article III). A similar pattern was seen on acetylated samples, although this has to be interpreted with care. The observation that during the enzymatic phase in acetylated samples (AC/Enz) higher levels of gene expression are reached might not give evidence for a functioning enzymatic degradation. In fact, higher levels in gene expression could be explained by a reaction/adaption of the fungi to still be able to degrade the wood even though it is protected by the acetylation. Noticeably many genes that were upregulated on acetylated samples were related to sugar metabolism (Article III). Some of these GHs clustered

closely together with AAs (AA1_2/AA3_2) especially in *R. placenta*. However, *G. trabeum* mainly clustered GHs together in clusters with genes that were enhanced in acetylated samples. GH families that appeared often in all three strains were GH16, GH31 and GH128.

It was suggested that brown rot adaption was partly driven by regulatory rewiring, including *cis*-regulatory and *trans*-acting factors (Zhang et al., 2019). Transcription factors (TFs), for example, have been altered to a high extent in Basidiomycetes after their deviation from Ascomycetes (Ohm et al., 2014). TFs are important for signal transduction pathways and they form the last link between signal flow and gene expression of target genes (Shelest, 2008). In *G. trabeum* it was shown quite clearly, that an enzymatic phase has not established yet in acetylated samples (Article III). During enzymatic decay the fungi are expected to secrete larger amounts of GHs. In AC/Enz samples of *G. trabeum*, TFs clustered closely together with several genes belonging to the AA CAZy family. This pattern could not be seen in *R. placenta*. However, studying the behaviour of CAZymes might give similar information. Zhang *et al.* (2019) showed that most CAZymes are expressed with delay and suggested that an earlier expression would not result in the depolymerisation of the cell wall. A maximal effectiveness of these enzymes is ensured after the cell wall has been opened by non-enzymatic CMF action. However, an early secretion of important CAZymes would not be an efficient energy investment (Goodell et al., 1997; Goodell et al., 2017; Zhang et al., 2019). The most important endoglucanases for wood degradation in *R. placenta*, Cel12A and Cel5A, were heavily affected by the acetylation, resulting in significantly lower expression values in acetylated samples (Article III). This indicates that a proper enzymatic decay has also not been established yet on AC/Enz samples by *R. placenta*. Therefore, a direct comparison of the enzymatic phases between the treatments is not recommended. *G. trabeum* was found to probably combine enzymatic and non-enzymatic chemistry during initial brown rot decay (Castaño et al., 2018; Zhu et al., 2020). MAD-698 seemed to include a broad range of genes related to oxidative functions into enzymatic degradation (Article III). Goodell *et al.* (2017, 2020) already suggested that combining enzymatic and non-enzymatic mechanisms under controlled conditions could be the key in modifying fibres or creating platform chemicals. Lignin activation by brown rot fungi was shown to be helpful in several industrial applications (Goodell et al., 2020). The ability to depolymerise cellulose and hemicellulose as a pretreatment of bagasse, wood and lignocellulose substrates, makes brown rot fungi interesting for biorefinery applications (Ray et al., 2010). However, even though brown rot fungi or their enzymes can be used in several biotechnological applications, for example biofuel production

or the detoxification of environmental pollutants (Singh and Singh, 2014) to this moment the processes are not well-engineered enough, as well as prices for input resources are high, not generating economic value (Goodell et al., 2020).

5.5   Important brown rot genes

A further aim of this study was to elaborate a collection of genes that likely play an important role during either initial or progressed brown rot decay (Article III). Therefore, publications on gene expression of relevant brown rot fungi were screened to gain a list of genes possibly involved in brown rot decay (Martinez et al., 2009; Vanden Wymelenberg et al., 2010; Ryu et al., 2011; Zhang et al., 2016; Zhang et al., 2019). Furthermore, differential expression data in this study were used to identify genes that were differentially expressed during either the non-enzymatic oxidative phase or the enzymatic phase of brown rot degradation (Article III). Included were also genes that were noticeably up- or downregulated in acetylated samples. The sorting into oxidative-specific brown rot genes (BRS "Ox") and those specific for the enzymatic phase (BRS "Enz") was done by their putative functions or according to their expression behaviour. Six genes were identified in our study that were upregulated in all three strains during oxidative decay (Article III, Table 1), three of them still being uncharacterised. The fact that they were upregulated in all strains makes them possible candidates for further investigation as well as for biomarkers for initial brown rot decay (Article III).

5.5.1   Oxalate cycle

Extracellular accumulation of oxalate might have an impact on the formation of hydroxyl radicals by regulating the availability of ferric iron (Goodell, 2003). Therefore, oxalate synthesis and decomposition play an important role during early brown rot decay and regulatory mechanisms of the involved genes are of great importance. The results obtained during this thesis showed that *R. placenta* upregulated important genes that are involved in the oxalate synthesis, as for example glyoxylate dehydrogenase (GlyD) and oxaloacetate acetylhydrolase (OahA) during initial decay (Article III). These two genes have been shown to contribute to the majority of oxalate synthesis under different nitrogen conditions in *G. trabeum* (Zhuang et al., 2015). More recently OahA was shown to be upregulated on cellulose but downregulated on cedar medium by *G. trabeum*, while GlyD was upregulated on both, cellulose and cedar medium (Umezawa et al., 2020). These data support the theory that the expression of GlyD is induced when growing on wood (Umezawa et al., 2020). An oxalate decarboxylase (OxaD) did

not show significant regulation patterns in our study for *R. placenta*, to the contrary it was upregulated by *G. trabeum* in enzymatic samples (Article III). This is in accordance with the findings that *G. trabeum* accumulates less oxalate than other brown rot fungi, mainly caused by the presence of OxaD (Connolly and Jellison, 1994; Zhuang et al., 2015). In acetylated samples no significant differences were observed between initial and progressed decay, which could be due to an impaired oxalate cycle, or due to other reasons, discussed below. In *R. placenta*, genes that are potentially involved in the oxalate cycle cluster together with genes that were partly affected by the acetylation (Article III). This supports the "impaired cycle hypothesis" in acetylated samples. This pattern, together with a delayed upregulation of genes related to oxalate production, has been observed before in modified wood (acetylated and furfurylated) (Alfredsen et al., 2016; Beck et al., 2018).

5.5.2 Iron reductants

Together with H_2O_2, reduced iron (Fe^{2+}) is needed to fuel the Fenton reaction (Arantes and Goodell, 2014). Low molecular weight components were, among other enzymes, proposed to be involved in this reduction process (Goodell, 2003; Arantes et al., 2012). Hydroquinones and iron-binding metabolites are capable of penetrating the wood cell wall and to reduce iron during brown rot degradation (Kerem et al., 1999; Arantes and Milagres, 2008). However, polyketide synthases that produce catechol and quinone compounds that could be the source of low molecular weight chelators and hydroquinones (Riley et al., 2014) were not upregulated by *G. trabeum* when growing on cellulose or cedar medium (Umezawa et al., 2020). The results of this study did not show significant regulation for QRD for neither *G. trabeum* nor *R. placenta* (Article III). When comparing different levels of acetylation QRD was only upregulated in samples with high acetylation levels (20%) (Article II), indicating that the *R. placenta* QRD could be involved in stress defence or detoxification of quinones, rather than in the reduction of chelators, which has also been suggested in other studies (Cohen et al., 2004; Pedrini et al., 2015). Interestingly, QRD was heavily affected by acetylation in all three strains in this study (Article III), further questioning its role during brown rot decay.

Umezawa *et al.* (2020) suggested, based on their findings, that there might be other iron chelator biosynthesis pathways involving cytochrome P450 and terpene synthases. Furthermore, glycoproteins, capable of reducing iron were hypothesised to be involved in the extracellular Fenton system (Martinez et al., 2009), which have been found to be upregulated on wood substrate in *G. trabeum* (Umezawa et al., 2020) and *R. placenta* (Vanden Wymelenberg et al.,

2010). This could explain the so far scanty evidence of yet investigated genes (QRD, Lac1) being involved in CMF degradation.

5.5.3   The generation of hydrogen peroxide (H_2O_2)

As mentioned above, H_2O_2 is the second important contributor to a functioning Fenton reaction. Proposed sources of H_2O_2 in brown rot fungi are the reduction of molecular oxygen and oxidation of methanol (Arantes and Goodell, 2014). It is extracellularly generated by members of the GMC oxidoreductase family as for example alcohol oxidases, aryl-alcohol oxidases and glucose oxidases (Martinez et al., 2009). In *G. trabeum*, methanol oxidase was identified to produce hydrogen peroxide (Daniel et al., 2007) and *R. placenta* expressed methanol oxidase when growing on cellulose (Martinez et al., 2009). The role of alcohol oxidase may not only be the generation of H_2O_2 but also the control of the methanol concentration in the fungal system, since it is toxic to the fungi (Arantes and Goodell, 2014). An alcohol oxidase of *G. trabeum* (GLOTRDRAFT_139980) was significantly upregulated on cellulose and cedar medium (Umezawa et al., 2020). The present study showed a group-wise upregulation of genes involved in H_2O_2 production (Cro1, GOx2, AlOx2, AlOx3) with increased acetylation level (Article II), indicating that the fungi first enhance H_2O_2 production before adapting other mechanisms. However, another alcohol oxidase (AlOx1) was downregulated on acetylated samples, indicating that its role is different to the other two alcohol oxidases (Article II). The differential expression analysis (Article III) showed less distinct results for AlOx3 and Cro1, which might be explainable by relatively low overall expression levels. A clear upregulation of GOx2 on acetylated samples was observed in all three strains, highlighting the genes´ importance during initial brown rot decay and its role in adaption to modification, making it also suitable as biomarker (Article III). Other genes from the AA3_2 family are also likely involved in H_2O_2 production, as well as a pyranose 2-oxidase from AA3_4 family (Levasseur et al., 2013). Umezawa *et al.* (2020) found some interesting regulation patterns for some oxidoreductases (AA3_2) when growing *G. trabeum* on cellulose and cedar. Besides Cro1, which was upregulated during initial decay on untreated samples, compared to progressed decay in all three strains in this study, no significant regulation was found for the other mentioned genes (Article III). Furthermore, Cro1 clustered together with genes, little to not affected by acetylation in both strains of *R. placenta* (Article III). Therefore, it seems that Cro1 is an important gene during initial decay, as well as when investigating fungal adaption to

modification in more than one brown rot species. Cro1 might also be a suiting biomarker for both, initial brown rot decay and acetylation focused studies.

5.5.4 Degradation of cellulose and hemicellulose

Rapid and extensive holocellulose degradation is characteristic during early brown rot decay (Cowling, 1961; Eriksson et al., 1990). Hemicellulose is depolymerised through the removal of for example acetyl groups (Puls, 1997). This is done by carbohydrate esterases (CEs). Presley *et al.* (2018) found a wide spectrum of CEs in brown rot secretomes during initial decay. The data obtained from the present study showed stronger upregulation of genes of the CE family in enzymatic samples in all three strains, especially in *R. placenta* (Article III), which is contrary to the findings by Presley *et al.* (2018). Effects of acetylation on CEs did not appear to be strong in differential expression analyses, indicating that acetylation does not affect CEs significantly. However, the results of hierarchical clustering revealed that especially genes of the CAZy family CE16 seemed to be heavily affected by acetylation and some CEs were enhanced in acetylated samples (Article III). This indicates that hemicellulose is not removed to the same extent in acetylated samples as in untreated samples, which could further explain that celluloses are not as accessible to enzymes in acetylated samples. Furthermore, it is also imaginable that the CEs that were upregulated in acetylated sample might contribute to a deacetylation process.

Genes of the CAZy families GH5_5 and GH12 are actively degrading cellulose during brown rot decay (Ryu et al., 2011). Even though white rot fungi evidently encode a larger variety of genes that degrade cellulose (Floudas et al., 2012; Riley et al., 2014), this seemingly does not enhance activities related to endoglucanases compared to brown rot fungi (Presley et al., 2018). Presley *et al.* (2018) conclude that the difference must be driven by abundance and enzyme turnover. Umezawa *et al.* (2020) observed extensive upregulation of GH5_5 and GH12 genes by *G. trabeum*, when growing on cellulose and cedar medium. The upregulation of GH5_5 genes by brown rot fungi when growing on lignocellulose, was also observed in previous studies (Eastwood et al., 2011; Vanden Wymelenberg et al., 2011). Surprisingly, GH12 genes were not upregulated by *R. placenta* when growing on pine cultures, which is contradictory to the findings in the present study. There, Cel5A, Cel5B (GH5_5) and Cel12A (GH12) were the main drivers during enzymatic degradation in all three strains (Article III). The largest difference between the strains, as well as between the different decay stages and/or treatments was precipitated by the well-known endoglucanase Cel12A (Article III). This indicates that regulatory mechanisms of endoglucanases not only compensate the lack of gene diversity

between brown and white rot, but also drive differences between brown rot fungal strains. Especially in FPRL280, expression levels of Cel12A represented a high percentage (15%), considering accumulated TPMs of all differentially expressed genes. In comparison, Cel12A expression levels of MAD-698 represented 8%, which highlights the significance of this gene as well as the differences between the strains, as already mentioned above (Article III). Furthermore, hierarchical clustering revealed that in FPRL280 the endoglucanases Cel5A and Cel12A clustered together with genes that were partly to heavily affected by acetylation, while they were much less affected in MAD-698 (Article III). These findings show that even though FPRL280 produced higher levels of these endoglucanases, it is affected more strongly by modification than MAD-698. Additionally, this might partially help to explain the observation that MAD-698 is more aggressive in decay (Fackler et al., 2010). Rättö *et al.* (1997) found that higher hydrolysis yields were reached by endoglucanases of the brown rot fungus *R. placenta* after pretreatment via Fenton reagents. Furthermore, they found that the residual cellulose that was modified during the oxidative degradation step is more hydrolysable by the brown rot endoglucanases (Rättö et al., 1997). If acetylation hinders or changes the oxidative degradation step, this could also result in a changed modification state of the polysaccharides, leading to less effective mode of action of endoglucanases. It is also possible that acetylation itself changes the outcome of degradation products in a way that they are harder to metabolise by the fungi, contributing to a slower or less effective degradation.

5.5.5   Lytic polysaccharide monooxygenases (LPMOs)

The rapid degradation of carbohydrates while leaving lignin behind made brown rot fungi less interesting for biotechnological applications in the past (Goodell et al., 2020). More recently, some genes, as for example LPMOs were identified to be able to act synergistically with peroxidases (Li et al., 2019; Zhu et al., 2020). The CAZymes of brow rot fungi have been little explored for biorefinery, but recent studies implied that *G. trabeum* LPMOs might be interesting for the usage in chemical feedstocks to pretreat lignocellulose biomass (Ray et al., 2010; Kojima et al., 2016; Goodell et al., 2020; Zhu et al., 2020). However, the results of this study did not show an upregulation of the LPMO enzymes during initial decay by *G. trabeum* compared to enzymatic decay on untreated samples (Article III). A downregulation of one LPMO (GLOTRDRAFT_45893) was seen in acetylated samples during oxidative decay compared to untreated samples (Article III). This might indicate that this LPMO is affected by the acetylation, probably having impact on the solubilisation of aromatic monomers from lignin.

The LPMOs of *R. placenta* are not orthologous to those of *G. trabeum*, but were also not significantly upregulated during initial decay on untreated samples, questioning their importance for initial decay. Furthermore, genes could be upregulated by the fungi even though their functions are suppressed by the acetylation, making it even harder to interpret the results.

5.6 Limitations of this study and critical analysis of the methodology

When comparing publications that consider a small range of genes as well as whole transcriptome studies it is often hard to find comparable data and often the results don´t show the same pattern. It seems that different sample design and only small variations in test conditions could lead to differing results in this sensitive system. Wu *et al.* (2018) found that the brown rot fungus *Fomitopsis pinicola* changes not only gene expression but also RNA editing profiles when growing on different wood species. This shows that even the same strain can develop different strategies under varying conditions (Wu et al., 2018). Hence, statements on fungal behaviour should be treated with care when considering these fast-changing mechanisms. For example, the theory that pectinases are upregulated during early stages of decay (Zhang et al., 2016), was based on only one pectinase, which was, to the opposite, downregulated during early degradation in the results of the present study. This might also be caused by the wood substrate, which was different to the one that Zhang *et al.* (2016) used (Spruce) that that in this study (Scots pine). However, Presley *et al.* (2018) showed high pectinase activity of *R. placenta* during initial brown rot decay compared to sample sections of progressed decay, as well as when compared to *G. trabeum*. Studies using the same methods on a wider range of strains and modification methods could enlarge the pool of comparable data, contributing to a better understanding of the differences in decay strategies between strains. The fast-changing mechanisms, dependent of sample design and condition, underline the complexity of brown rot decay and show that there is still a lot to be done before this complicated network will be understood. To create more comparable data, a large-scale test using exactly the same design and conditions could be applied for several fungal species and conditions in future research. Furthermore, it might be interesting to use different kinds of modification methods.

Additionally, latest research revealed that it might be the composition of genes working in concert that leads to effective brown rot decay (Castaño et al., 2018; Goodell, 2020). This sheds light on the vast number of uncharacterised genes, causing large differences between strains,

conditions and decay phases. The annotation of these genes as well as finding corresponding orthologs in different strains, should be of great interest in future studies.

Furthermore, in this study, the samples (three) from one sample container were pooled, which has presumably a lot of impact on the results and could therefore be another reason that it is hard to compare data from this study to those of others. Pooling of the samples from the sample containers was done to gain more material for RNA-extraction, since it was much material needed for qPCR analyses (Article II) as well as transcriptome sequencing (Article III). Pooling the samples decreases standard deviation and might therefore influence the data. For future studies it might be advisable to use single samples for transcriptome studies.

Regarding the effects of acetylation on fungal growth and the mechanisms used by the fungi to overcome the modification, it might have been more powerful to use samples, acetylated to 20% rather than 15%. It has been shown that modification protects from a WPG of 20% on, which is why a lower modification level might not give the exact mechanisms that hinder the fungi to grow and degrade the substrate. Furthermore, 15% acetylated samples acted out of order for some genes compared to samples, acetylated to 10 and 20% WPG regarding the results on gene expression (Article II). Therefore, the results from 15% samples might be misleading for interpreting adaption mechanisms to modification by the fungi. In this study it was decided to use 15% WPG samples rather than 20% samples, to have the chance to see a clear reaction to the acetylation, while 20% was expected to inhibit growth completely, decreasing the chance of seeing a good reaction.

As discussed earlier the comparability of the oxidative phases of untreated and acetylated samples as well as the enzymatic phases might not be the ideal method, since the fungi are obviously in different stages. It could have been an opportunity to also do a differential expression analysis on the data of the oxidative phase of untreated samples and the enzymatic phase of acetylated samples. Another possibility might have been to take samples from different points of the wafers for a more differential approach, since it is conceivable that the fungi are more situated in an intermediate form of degradation stages in acetylated samples. Even though the wafer method is better separating the two phases, it was shown that the two brown rot phases are overlapping (Zhang et al., 2016) (Figure 1B). This means that a pure separation of the phases is basically not possible, making it even harder to hit the exact spot, where they are most different.

Comparing untreated and acetylated samples during enzymatic decay did not show significant effects (Article III), raising the question if the comparison of UT/Ox and AC/Ox is the optimal way to understand how the fungi are dealing with the modification in this context. It might be better to determine a point where the fungi are in the same degradation stage, which is challenging, since it is not known if this could even be reached. Acetylation could affect the brown rot mechanisms in such way that a direct comparison of "phases" might not be possible. The fungi probably need to use an adjusted system to overcome modifications, meaning that they use a different enzyme cocktail, making it hard to directly compare acetylated samples with untreated samples. For example, more genes could be needed for the deacetylation process. However, the hyphal front of acetylated samples could be the wrong sample part to be directly compared to the respective untreated sample.

The original differentiation of the BRS genes into BRS "Ox" and BRS "Enz" genes should be done with care and probably for each species individually, based on the expression behaviour. Some genes of the AA CAZy family are as well as Glutathione s-transferases, for example, thought to be BRS "Ox" genes but were significantly upregulated during enzymatic brown rot decay.

6 Conclusion and Outlook

The results of this thesis demonstrated that the *R. placenta* strains FPRL280 and MAD-698 differ only slightly in their genomes by expectable SNPs and InDels but nevertheless displayed substantial differentiation related to regulatory mechanisms, which are more likely causative for the observed differences in decay capacity, mass loss and appearance.

The investigation of gene expression in the three different brown rot fungi, *R. placenta* FPRL280 and MAD-698 as well as *G. trabeum*, confirmed the presence of a two-stepped degradation mechanisms in all three strains. The results indicate however that the underlying regulatory switches seem to be dynamic rather than being a rigid two-step system. Furthermore, gene expression patterns revealed varying composition of genes being active during oxidative and enzymatic decay, indicating that both the duration of the early phase as well as the transition time between early and late phases is highly strain-specific. Genes that were expressed during the initial and later decay phases by all three strains could, however, be used as biomarkers.

Acetylation affected gene expression on a genome-wide level, with overall more genes being upregulated in modified samples. However, CAZymes appeared to be attenuated, which is in accordance with a scouting-like response, induced by starvation-stress. This indicates that the fungi are struggling with overcoming the wood modification. The enzyme cocktails used by the strains differed in number and composition, indicating that there is more than just an upregulation of genes involved in oxidative degradation as reaction to acetylation. Nevertheless, glucose oxidase (GOx2) was upregulated by all three strains, making it a promising candidate for the investigation of acetylation effects.

The results suggest a shift in strategies regarding energy investment, not only between the strains of different species, but also between the two strains of *R. placenta*. This clearly shows that a comparison of results using either FPRL280 or MAD-698 should be done with care. For example, declarations on the effectiveness of wood modifications, drawn from only one of the two strains are not directly transferable to the behaviour or harming potential of the other strain.

A small number of genes, as for example the important endoglucanase Cel12A, displayed highly dynamic expression differences between strains, indicating that single genes could have great impact on decay capacity and aggressiveness. This sheds light on the high number of genes still being uncharacterized and the need to improve the genome annotations. The observation that brown rot degradation works with a complex enzyme cocktail to gain access

to nutritional polysaccharides highlights the importance of understanding the composition of regulatory gene groups.

The work performed during this thesis was relevant by providing first access to the genome of the European strain of *R. placenta* (FPRL280), allowing genomic and transcriptomic comparisons of two strains that are commonly used for standardised decay tests in Europe and the US. Furthermore, the wafer method, enabling the separation of initial and progressed brown rot decay, had not been applied to modified samples so far to the best of my knowledge, giving a much more detailed insight into the fungal reaction to chemically treated wood substrates. Additionally, this study provides a list with orthologous genes between two commonly used brown rot species (*R. placenta* and *G. trabeum*) as well as between two *R. placenta* strains, simplifying the comparison of results between different studies in the future for the community.

As mentioned above, the two brown rot stages should not only be looked at as a bipartite mechanism but rather as a synergistically working, interconnected machinery. This should also be considered when thinking about the usage of brown rot specific genes for biorefinery applications. A combination of genes that are active during oxidative and enzymatic brown rot decay could be highly efficient for fiber modification, generation of platform chemicals and therefore for biorefinery applications (Ray et al., 2010; Goodell et al., 2017; Goodell, 2020). The need for brown rot fungi for biomass pretreatment in biorefineries will potentially increase and new markets with products containing lignin residues, as for example lignin-based coatings and polymeric resins, will open (Goodell et al., 2020).

Wood is an increasingly interesting material for construction and other applications in the future and new eco-friendly technologies to prevent fungal degradation, including wood modifications, will be needed. As the knowledge on the exact mode of action during brown rot decay increases incrementally, more specific and cost-effective methods might be found. Some promising research in this field is already in progress, as for example the treatment with citric acid and sorbitol (SCA), which are both low in price and readily available feedstock chemicals (Larnøy et al., 2018; Beck, 2020).

7 References

Agger, J.W., Isaksen, T., Várnai, A., Vidal-Melgosa, S., Willats, W.G., Ludwig, R., Horn, S.J., Eijsink, V.G., Westereng, B., 2014. Discovery of LPMO activity on hemicelluloses shows the importance of oxidative processes in plant cell wall degradation. Proc Natl Acad Sci U S A 111(17), 6287-6292. https://doi.org/10.1073/pnas.1323629111.

Akamatsu, Y., Shimada, M., 1994. Partial purification and characterization of glyoxylate oxidase from the brown-rot basidiomycete Tyromyces palustris. Phytochemistry 37(3), 649-653. https://doi.org/https://doi.org/10.1016/S0031-9422(00)90332-7.

Alfredsen, G., Fossdal, C.G., 2010. Postia placenta gene expression during growth in furfurylated wood. IRG/WP 10-10734. Proceedings, International Research Group on Wood Protection, 9-13.

Alfredsen, G., Pilgård, A., 2014. Postia placenta decay of acetic anhydride modified wood – Effect of leaching. Wood Material Science & Engineering, 8. https://doi.org/10.1080/17480272.2014.887776.

Alfredsen, G., Pilgård, A., Fossdal, C.G., 2016. Characterisation of Postia placenta colonisation during 36 weeks in acetylated southern yellow pine sapwood at three acetylation levels including genomic DNA and gene expression quantification of the fungus. Holzforschung 70(11), 11. https://doi.org/10.1515/hf-2016-0009.

Alfredsen, G., Ringman, R., Pilgård, A., Fossdal, C.G., 2015. New insight regarding mode of action of brown rot decay of modified wood based on DNA and gene expression studies: a review International Wood Products Journal 6(1), 5-7. https://doi.org/10.1179/2042645314Y.0000000085.

Alfredsen, G., Solheim, H., Mohn Jenssen, K., 2005. Evaluation of decaying fungi in Norwegian buildings., Proceedings IRG Annual Meeting, IRG/WP 09-10701. Bangalore, India, p. 11.

Arantes, V., Goodell, B., 2014. Current Understanding of Brown-Rot Fungal Biodegradation Mechanisms: A Review, Deterioration and Protection of Sustainable Biomaterials. American Chemical Society, pp. 3-21. https://doi.org/doi:10.1021/bk-2014-1158.ch001.

Arantes, V., Jellison, J., Goodell, B., 2012. Peculiarities of brown-rot fungi and biochemical Fenton reaction with regard to their potential as a model for bioprocessing biomass. Applied Microbiology and Biotechnology 94(2), 323-338. https://doi.org/10.1007/s00253-012-3954-y.
Arantes, V., Milagres, A., 2008. Relevance of low molecular weight compounds produced by fungi and involved in wood biodegradation. Química Nova 32, 1586-1595.

Arantes, V., Milagres, A.M.F., 2006. Degradation of cellulosic and hemicellulosic substrates using a chelator-mediated Fenton reaction. Journal of Chemical Technology & Biotechnology 81(3), 413-419. https://doi.org/10.1002/jctb.1417.

Arantes, V., Milagres, A.M.F., Filley, T.R., Goodell, B., 2011. Lignocellulosic polysaccharides and lignin degradation by wood decay fungi: the relevance of nonenzymatic Fenton-based

reactions. Journal of Industrial Microbiology & Biotechnology 38(4), 541-555. https://doi.org/10.1007/s10295-010-0798-2.

Arantes, V., Qian, Y., Milagres, A.M.F., Jellison, J., Goodell, B., 2009. Effect of pH and oxalic acid on the reduction of Fe3+ by a biomimetic chelator and on Fe3+ desorption/adsorption onto wood: Implications for brown-rot decay. International Biodeterioration & Biodegradation 63(4), 478-483. https://doi.org/https://doi.org/10.1016/j.ibiod.2009.01.004.

Aro, N., Pakula, T., Penttilä, M., 2005. Transcriptional regulation of plant cell wall degradation by filamentous fungi. FEMS Microbiology Reviews 29(4), 719-739. https://doi.org/10.1016/j.femsre.2004.11.006.

AWPA, A.W.P.A., 2016. E10-16 Laboratory Method for Evaluating the Decay Resistance of Wood-Based Materials Against Pure Basidiomycete Cultures: Soil/Block Test. Birmingham, Alabama, USA.

Baker, M., 2012. De novo genome assembly: What every biologist should know. Nat Methods 9, 333-337. https://doi.org/10.1038/nmeth.1935.

Baldrian, P., Valášková, V., 2008. Degradation of cellulose by basidiomycetous fungi. FEMS Microbiology Reviews 32(3), 501-521. https://doi.org/10.1111/j.1574-6976.2008.00106.x.

Bari, E., Pizzi, A.P., Schmidt, O., Siham, A., Tajick Ghanbary, M., Humar, M., 2021. Differentiation of Fungal Destructive Behaviour of Wood by the White-Rot Fungus Fomes fomentarius by MALDI-TOF Mass Spectrometry. JOURNAL OF RENEWABLE MATERIALS 9, 381-397. https://doi.org/10.32604/jrm.2021.015288.

Beck, G., 2020. Leachability and Decay Resistance of Wood Polyesterified with Sorbitol and Citric Acid. Forests 11(6), 650.

Beck, G., Hegnar, O.A., Fossdal, C.G., Alfredsen, G., 2018. Acetylation of Pinus radiata delays hydrolytic depolymerisation by the brown-rot fungus Rhondonia placenta. International Biodeterioration & Biodegradation 135, 39-52. https://doi.org/10.1016/j.ibiod.2018.09.003.

Beck, G., Thybring, E., Thygesen, L., Hill, C., 2017. Characterization of moisture in acetylated and propionylated radiata pine using low-field nuclear magnetic resonance (LFNMR) relaxometry. Holzforschung. https://doi.org/10.1515/hf-2017-0072.

Beeson, W.T., Vu, V.V., Span, E.A., Phillips, C.M., Marletta, M.A., 2015. Cellulose degradation by polysaccharide monooxygenases. Annu Rev Biochem 84, 923-946. https://doi.org/10.1146/annurev-biochem-060614-034439.

Bergman, R., Puettmann, M., Taylor, A., Skog, K.E., 2014. The Carbon Impacts of Wood Products. Forest Products Journal 64(7-8), 220-231. https://doi.org/10.13073/fpj-d-14-00047.

Biely, P., 2012. Microbial carbohydrate esterases deacetylating plant polysaccharides. Biotechnology Advances 30(6), 1575-1588. https://doi.org/https://doi.org/10.1016/j.biotechadv.2012.04.010.

Binder, M., Hibbett, D., Larsson, K.-H., Larsson, E., Langer, E., Langer, G., 2005. The phylogenetic distribution of resupinate forms across the major clades of mushroom-forming fungi (Homobasidiomycetes). . Systematics and Biodiversity 3, 113-157. https://doi.org/10.1017/S1477200005001623.

Blanchette, R., 1991. Delignification by Wood-Decay Fungi. Annual Review of Phytopathology 29, 18. https://doi.org/10.1146/annurev.py.29.090191.002121.

Boddy, L., Hiscox, J., 2016. Fungal Ecology: Principles and Mechanisms of Colonization and Competition by Saprotrophic Fungi. Microbiol Spectr 4(6). https://doi.org/10.1128/microbiolspec.FUNK-0019-2016.

Boraston, A.B., Bolam, D.N., Gilbert, H.J., Davies, G.J., 2004. Carbohydrate-binding modules: fine-tuning polysaccharide recognition. Biochem J 382(Pt 3), 769-781. https://doi.org/10.1042/BJ20040892.

Brigham, J.S., Adney, W.S., Himmel, M.E., 2018. Hemicellulases: diversity and applications. Routledge.

Brischke, C., Alfredsen, G., 2020. Wood-water relationships and their role for wood susceptibility to fungal decay. Applied Microbiology and Biotechnology 104(9), 3781-3795. https://doi.org/10.1007/s00253-020-10479-1.

Bryne, L.-E., Wålinder, M., 2010. Ageing of modified wood : Part 1: Wetting properties of acetylated, furfurylated, and thermally modified. Holzforschung 64(3), 295-304. https://doi.org/10.1515/HF.2010.040.

Bugg, T.D., Ahmad, M., Hardiman, E.M., Rahmanpour, R., 2011. Pathways for degradation of lignin in bacteria and fungi. Nat Prod Rep 28(12), 1883-1896. https://doi.org/10.1039/c1np00042j.

Campbell, J.A., Davies, G.J., Bulone, V., Henrissat, B., 1997. A classification of nucleotide-diphospho-sugar glycosyltransferases based on amino acid sequence similarities. Biochem J 326 (Pt 3)(Pt 3), 929-939. https://doi.org/10.1042/bj3260929u.

Cantarel, B.L., Coutinho, P.M., Rancurel, C., Bernard, T., Lombard, V., Henrissat, B., 2008. The Carbohydrate-Active EnZymes database (CAZy): an expert resource for Glycogenomics. Nucleic Acids Research 37(suppl_1), D233-D238. https://doi.org/10.1093/nar/gkn663.

Cardias Williams, F., Hale, M.D., 2003. The resistance of wood chemically modified with isocyanates: the role of moisture content in decay suppression. International Biodeterioration & Biodegradation 52(4), 215-221. https://doi.org/https://doi.org/10.1016/S0964-8305(03)00070-2.

Castaño, J.D., Zhang, J., Anderson, C.E., Schilling, J.S., 2018. Oxidative Damage Control during Decay of Wood by Brown Rot Fungus Using Oxygen Radicals. Appl Environ Microbiol 84(22). https://doi.org/10.1128/aem.01937-18.

Chalak, A., Villares, A., Moreau, C., Haon, M., Grisel, S., d'Orlando, A., Herpoël-Gimbert, I., Labourel, A., Cathala, B., Berrin, J.-G., 2019. Influence of the carbohydrate-binding module on

the activity of a fungal AA9 lytic polysaccharide monooxygenase on cellulosic substrates. Biotechnology for Biofuels 12(1), 206. https://doi.org/10.1186/s13068-019-1548-y.

Christensen, G.N., Kelsey, K.E., 1959. The rate of sorption of water vapor by wood. Holz als Roh- und Werkstoff 17, 178-188.

Coelho, M.A., Bakkeren, G., Sun, S., Hood, M.E., Giraud, T., 2017. Fungal Sex: The Basidiomycota, The Fungal Kingdom. pp. 147-175. https://doi.org/https://doi.org/10.1128/9781555819583.ch7.

Cohen, R., Jensen, K.A., Houtman, C.J., Hammel, K.E., 2002. Significant levels of extracellular reactive oxygen species produced by brown rot basidiomycetes on cellulose. FEBS Letters 531(3), 483-488. https://doi.org/doi:10.1016/S0014-5793(02)03589-5.

Cohen, R., Suzuki, M.R., Hammel, K.E., 2004. Differential stress-induced regulation of two quinone reductases in the brown rot basidiomycete Gloeophyllum trabeum. Applied and environmental microbiology 70(1), 324-331. https://doi.org/10.1128/AEM.70.1.324-331.2004.

Connolly, J.H., Jellison, J., 1994. Oxalate production and calcium oxalate accumulation by Gloeophyllum trabeum in buffered cultures, International Research Group on Wood Preservation. Nusa Dua, Bali, Indonesia.

Coutinho, P.M., Deleury, E., Davies, G.J., Henrissat, B., 2003. An Evolving Hierarchical Family Classification for Glycosyltransferases. Journal of Molecular Biology 328(2), 307-317. https://doi.org/https://doi.org/10.1016/S0022-2836(03)00307-3.

Couturier, M., Feliu, J., Haon, M., Navarro, D., Lesage-Meessen, L., Coutinho, P.M., Berrin, J.-G., 2011. A thermostable GH45 endoglucanase from yeast: impact of its atypical multimodularity on activity. Microbial Cell Factories 10(1), 103. https://doi.org/10.1186/1475-2859-10-103.

Cowling, E.B., 1961. Comparative biochemistry of the decay of sweetgum sapwood by white-rot and brown-rot fungi. . US Dept. of Agriculture, Washington, USA.

Cragg, S.M., Beckham, G.T., Bruce, N.C., Bugg, T.D.H., Distel, D.L., Dupree, P., Etxabe, A.G., Goodell, B.S., Jellison, J., McGeehan, J.E., McQueen-Mason, S.J., Schnorr, K., Walton, P.H., Watts, J.E.M., Zimmer, M., 2015. Lignocellulose degradation mechanisms across the Tree of Life. Current Opinion in Chemical Biology 29, 108-119. https://doi.org/https://doi.org/10.1016/j.cbpa.2015.10.018.

Curling, S.F., Clausen, C.A., Winandy, J.E., 2002. Relationships between mechanical properties, weight loss, and chemical composition of wood during incipient brown-rot decay. Forest Products Journal 52(7-8), 34-39.

Cuskin, F., Flint, J.E., Gloster, T.M., Morland, C., Baslé, A., Henrissat, B., Coutinho, P.M., Strazzulli, A., Solovyova, A.S., Davies, G.J., Gilbert, H.J., 2012. How nature can exploit nonspecific catalytic and carbohydrate binding modules to create enzymatic specificity. Proceedings of the National Academy of Sciences 109(51), 20889-20894. https://doi.org/10.1073/pnas.1212034109.

Daniel, G., 1994. Use of electron microscopy for aiding our understanding of wood biodegradation. FEMS Microbiology Reviews 13(2-3), 199-233. https://doi.org/doi:10.1111/j.1574-6976.1994.tb00043.x.

Daniel, G., 2014. Fungal and Bacterial Biodegradation: White Rots, Brown Rots, Soft Rots, and Bacteria, pp. 23-58. https://doi.org/10.1021/bk-2014-1158.

Daniel, G., Volc, J., Filonova, L., Plíhal, O., Kubátová, E., Halada, P., 2007. Characteristics of Gloeophyllum trabeum alcohol oxidase, an extracellular source of H2O2 in brown rot decay of wood. Applied and environmental microbiology 73(19), 6241-6253. https://doi.org/10.1128/AEM.00977-07.

de Figueiredo, F.L., de Oliveira, A.C.P., Terrasan, C.R.F., Gonçalves, T.A., Gerhardt, J.A., Tomazetto, G., Persinoti, G.F., Rubio, M.V., Peña, J.A.T., Araújo, M.F., de Carvalho Silvello, M.A., Franco, T.T., Rabelo, S.C., Goldbeck, R., Squina, F.M., Damasio, A., 2021. Multi-omics analysis provides insights into lignocellulosic biomass degradation by Laetiporus sulphureus ATCC 52600. Biotechnology for Biofuels 14(1), 96. https://doi.org/10.1186/s13068-021-01945-7.

Doedt, T., Krishnamurthy, S., Bockmühl, D.P., Tebarth, B., Stempel, C., Russell, C.L., Brown, A.J., Ernst, J.F., 2004. APSES proteins regulate morphogenesis and metabolism in Candida albicans. Mol Biol Cell 15(7), 3167-3180. https://doi.org/10.1091/mbc.e03-11-0782.

Eastwood, D., 2014. Evolution of Fungal Wood Decay, pp. 93-112. https://doi.org/10.1021/bk-2014-1158.ch005.

Eastwood, D.C., Floudas, D., Binder, M., Majcherczyk, A., Schneider, P., Aerts, A., Asiegbu, F.O., Baker, S.E., Barry, K., Bendiksby, M., Blumentritt, M., Coutinho, P.M., Cullen, D., de Vries, R.P., Gathman, A., Goodell, B., Henrissat, B., Ihrmark, K., Kauserud, H., Kohler, A., LaButti, K., Lapidus, A., Lavin, J.L., Lee, Y.-H., Lindquist, E., Lilly, W., Lucas, S., Morin, E., Murat, C., Oguiza, J.A., Park, J., Pisabarro, A.G., Riley, R., Rosling, A., Salamov, A., Schmidt, O., Schmutz, J., Skrede, I., Stenlid, J., Wiebenga, A., Xie, X., Kües, U., Hibbett, D.S., Hoffmeister, D., Högberg, N., Martin, F., Grigoriev, I.V., Watkinson, S.C., 2011. The Plant Cell Wall–Decomposing Machinery Underlies the Functional Diversity of Forest Fungi. Science 333(6043), 762-765. https://doi.org/10.1126/science.1205411.

Eaton, R., Hale, M., 1993. Decay, pests and protection. Chapman & Hall, Cambridge.

Engelund, E.T., Thygesen, L.G., Svensson, S., Hill, C.A.S., 2013. A critical discussion of the physics of wood–water interactions. Wood Science and Technology 47(1), 141-161. https://doi.org/10.1007/s00226-012-0514-7.

Enoki, A., Itakura, S., Tanaka, H., 1997. The involvement of extracelluar substances for reducing molecular oxygen to hydroxyl radical and ferric iron to ferrous iron in wood degradation by wood decay fungi. Journal of Biotechnology 53(2), 265-272. https://doi.org/https://doi.org/10.1016/S0168-1656(97)01682-9.

Eriksson, K., Blanchette, R.A., Ander, P., 1990. Microbial and Enzymatic Degradation of Wood and Wood Components. Springer, Berlin.

Espejo, E., Agosin, E., 1991. Production and Degradation of Oxalic Acid by Brown Rot Fungi. Applied and environmental microbiology 57, 1980-1986.

Esteves, B., Nunes, L., Pereira, H., 2010. Properties of furfurylated wood (Pinus pinaster). European Journal of Wood and Wood Products 69(4), 521-525. https://doi.org/10.1007/s00107-010-0480-4.

Fackler, K., Stevanic, J.S., Ters, T., Hinterstoisser, B., Schwanninger, M., Salmén, L., 2010. Localisation and characterisation of incipient brown-rot decay within spruce wood cell walls using FT-IR imaging microscopy. Enzyme and Microbial Technology 47(6), 257-267. https://doi.org/https://doi.org/10.1016/j.enzmictec.2010.07.009.

Fenton, H., 1894. Oxidation of tartaric acid in the presence of iron. Journal of the Chemical Society Transactions 65, 12.

Filley, T.R., Cody, G.D., Goodell, B., Jellison, J., Noser, C., Ostrofsky, A., 2002. Lignin demethylation and polysaccharide decomposition in spruce sapwood degraded by brown rot fungi. Organic Geochemistry 33(2), 111-124. https://doi.org/https://doi.org/10.1016/S0146-6380(01)00144-9.

Floudas, D., Binder, M., Riley, R., Barry, K., Blanchette, R.A., Henrissat, B., Martínez, A.T., Otillar, R., Spatafora, J.W., Yadav, J.S., Aerts, A., Benoit, I., Boyd, A., Carlson, A., Copeland, A., Coutinho, P.M., de Vries, R.P., Ferreira, P., Findley, K., Foster, B., Gaskell, J., Glotzer, D., Górecki, P., Heitman, J., Hesse, C., Hori, C., Igarashi, K., Jurgens, J.A., Kallen, N., Kersten, P., Kohler, A., Kües, U., Kumar, T.K.A., Kuo, A., LaButti, K., Larrondo, L.F., Lindquist, E., Ling, A., Lombard, V., Lucas, S., Lundell, T., Martin, R., McLaughlin, D.J., Morgenstern, I., Morin, E., Murat, C., Nagy, L.G., Nolan, M., Ohm, R.A., Patyshakuliyeva, A., Rokas, A., Ruiz-Dueñas, F.J., Sabat, G., Salamov, A., Samejima, M., Schmutz, J., Slot, J.C., St. John, F., Stenlid, J., Sun, H., Sun, S., Syed, K., Tsang, A., Wiebenga, A., Young, D., Pisabarro, A., Eastwood, D.C., Martin, F., Cullen, D., Grigoriev, I.V., Hibbett, D.S., 2012. The Paleozoic Origin of Enzymatic Lignin Decomposition Reconstructed from 31 Fungal Genomes. Science 336(6089), 1715-1719. https://doi.org/10.1126/science.1221748.

Fredriksson, M., Thybring, E.E., 2019. On sorption hysteresis in wood: Separating hysteresis in cell wall water and capillary water in the full moisture range. PLoS One 14(11), e0225111. https://doi.org/10.1371/journal.pone.0225111.

Gaskell, J., Kersten, P., Larrondo, L.F., Canessa, P., Martinez, D., Hibbett, D.S., Schmoll, M., Kubicek, C., P., Martinez, A., T., Yadav, J.S., Master, E., Magnuson, J., Karl, Yaver, D., Berka, R., Lail, K., Chen, C., LaButti, K., Nolan, M., Lipzen, A., Aerts, A., Riley, R., Barry, K., Henrissat, B., Blanchette, R.A., Grigoriev, I., Cullen, D., 2017. Draft genome sequence of a monokaryotic model brown-rot fungus *Postia (Rhodonia) placenta* SB12. Genomics Data 14, 3.

Gelhaye, E., Morel, M., 2009. Molecular Microbiology and Wood Protection, Proceedings IRG Annual Meeting, IRG/WP 09-20416. Beijing, China, p. 13.

Gilbert, H.J., Knox, J.P., Boraston, A.B., 2013. Advances in understanding the molecular basis of plant cell wall polysaccharide recognition by carbohydrate-binding modules. Curr Opin Struct Biol 23(5), 669-677. https://doi.org/10.1016/j.sbi.2013.05.005.

Gilbert, W., 1981. DNA sequencing and gene structure Nobel lecture, 8 December 1980. Biosci Rep 1(5), 353-375. https://doi.org/10.1007/bf01116186.

Gilkes, N.R., Warren, R.A.J., Miller, R.C., Kilburn, D.G., 1988. Precise excision of the cellulose binding domains from two *Cellulomonas fimi* cellulases by a homologous protease and the effect of catalysis. J. Biol. Chem. 263, 10401-10407.

Glass, S., Zelinka, S., 2010. Moisture Relations and Physical Properties of Wood.

Goodell, B., 2003. Brown-Rot Fungal Degradation of Wood: Our Evolving View. https://doi.org/10.1021/bk-2003-0845.ch006.

Goodell, B., 2020. Fungi Involved in the Biodeterioration and Bioconversion of Lignocellulose Substrates, in: Benz, J.P., Schipper, K. (Eds.), Genetics and Biotechnology. Springer International Publishing, Cham, pp. 369-397. https://doi.org/10.1007/978-3-030-49924-2_15. Goodell, B., Daniel, G., Jellison, J., Qian, Y., 2006. Iron-reducing capacity of low-molecular-weight compounds produced in wood by fungi. 60(6), 630-636. https://doi.org/doi:10.1515/HF.2006.106.

Goodell, B., Jellison, J., Liu, J., Daniel, G., Paszczynski, A., Fekete, F., Krishnamurthy, S., Jun, L., Xu, G., 1997. Low molecular weight chelators and phenolic compounds isolated from wood decay fungi and their role in the fungal biodegradation of wood. Journal of Biotechnology 53(2), 133-162. https://doi.org/https://doi.org/10.1016/S0168-1656(97)01681-7.

Goodell, B., Qian, Y., Jellison, J., Richard, M., Qi, W., 2002. Lignocellulose oxidation by low molecular weight metal-binding compounds isolated from wood degrading fungi: A comparison of brown rot and white rot systems and the potential application of chelator-mediated fenton reactions., in: Viikari, L., Lantto, R. (Eds.), Progress in Biotechnology. Elsevier, pp. 37-47. https://doi.org/https://doi.org/10.1016/S0921-0423(02)80006-5.

Goodell, B., Winandy, J.E., Morrell, J.J., 2020. Fungal Degradation of Wood: Emerging Data, New Insights and Changing Perceptions. Coatings 10(12), 1210.

Goodell, B., Zhu, Y., Kim, S., Kafle, K., Eastwood, D.C., Daniel, G., Jellison, J., Yoshida, M., Groom, L., Pingali, S.V., O´Neill, H., 2017. Modification of the nanostructure of lignocellulose cell walls via a non-enzymatic lignocellulose deconstruction system in brown rot wood-decay fungi. Biotechnology for Biofuels 10:179, 15. https://doi.org/10.1186/s13068-017-0865-2.

Gress, J., de Oliveira, L.M., da Silva, E.B., Lessl, J.M., Wilson, P.C., Townsend, T., Ma, L.Q., 2015. Cleaning-induced arsenic mobilization and chromium oxidation from CCA-wood deck: Potential risk to children. Environ Int 82, 35-40. https://doi.org/10.1016/j.envint.2015.04.012.

Hakkou, M., Pétrissans, M., Gérardin, P., Zoulalian, A., 2006. Investigations of the reasons for fungal durability of heat-treated beech wood. Polymer Degradation and Stability 91, 393-397. https://doi.org/10.1016/j.polymdegradstab.2005.04.042.

Hammel, K.E., Cullen, D., 2008. Role of fungal peroxidases in biological ligninolysis. Curr Opin Plant Biol 11(3), 349-355. https://doi.org/10.1016/j.pbi.2008.02.003.

Hammel, K.E., Kapich, A.N., Jensen, K.A., Ryan, Z.C., 2002. Reactive oxygen species as agents of wood decay by fungi. Enzyme and Microbial Technology 30(4), 445-453. https://doi.org/https://doi.org/10.1016/S0141-0229(02)00011-X.

Hervé, C., Rogowski, A., Blake, A.W., Marcus, S.E., Gilbert, H.J., Knox, J.P., 2010. Carbohydrate-binding modules promote the enzymatic deconstruction of intact plant cell walls by targeting and proximity effects. Proceedings of the National Academy of Sciences 107(34), 15293-15298. https://doi.org/10.1073/pnas.1005732107.

Hibbett, D., Binder, M., Bischoff, J., Blackwell, M., Cannon, P., Eriksson, O., Huhndorf, S.M., James, T., Kirk, P., Lücking, R., Lumbsch, T., Lutzoni, F., Matheny, P., McLaughlin, D., Powell, M., Redhead, S., Schoch, C., Spatafora, J., Stalpers, J., Zhang, N., 2007. A higher-level phylogenetic classification of the Fungi. Mycological Research 111, 509-547.

Hibbett, D., Donoghue, M., 2001. Analysis of Character Correlations Among Wood Decay Mechanisms, Mating Systems, and Substrate Ranges in Homobasidiomycetes. Systematic biology 50, 215-242. https://doi.org/10.1080/10635150151125879.

Hill, C., 2006. Wood Modification: Chemical, Thermal and other Processes. Stevens C.V., Hoboken, New Jersey, USA: John Wiley and Sons, Ltd.

Hill, C., 2009. Why does acetylation protect wood from microbiological attack? Wood Material Science & Engineering 4, 37-45. https://doi.org/10.1080/17480270903249409.

Hill, C., Norton, A., 2014. Environmental Standards and Embedded Carbon in the Built Environment, in: Schmitz-Hoffmann, C., Schmidt, M., Hansmann, B., Palekhov, D. (Eds.), Voluntary Standard Systems: A Contribution to Sustainable Development. Springer Berlin Heidelberg, Berlin, Heidelberg, pp. 145-163. https://doi.org/10.1007/978-3-642-35716-9_10.

Hill, C.A.S., Dibdiakova, J., 2016. The environmental impact of wood compared to other building materials. International Wood Products Journal 7(4), 215-219. https://doi.org/10.1080/20426445.2016.1190166.

Hill, C.A.S., Ormondroyd, G.A., 2004. Dimensional changes in Corsican pine (Pinus nigra Arnold) modified with acetic anhydride measured using a helium pycnometer. 58(5), 544-547. https://doi.org/doi:10.1515/HF.2004.082.

Hingston, J.A., Collins, C.D., Murphy, R.J., Lester, J.N., 2001. Leaching of chromated copper arsenate wood preservatives: a review. Environ Pollut 111(1), 53-66. https://doi.org/10.1016/s0269-7491(00)00030-0.

Hoffmeyer, P., Engelund, E.T., Thygesen, L.G., 2011. Equilibrium moisture content (EMC) in Norway spruce during the first and second desorptions. 65(6), 875-882. https://doi.org/doi:10.1515/HF.2011.112.

Hosseinpourpia, R., Mai, C., 2016b. Mode of action of brown rot decay resistance of acetylated wood: resistance to Fenton's reagent. Wood Science and Technology 50(2), 413-426. https://doi.org/10.1007/s00226-015-0790-0.

Hunt, C., Zelinka, S., Frihart, C., Lorenz, L., Yelle, D., Gleber, S.-C., Vogt, S., Jakes, J.E., 2018. Acetylation increases relative humidity threshold for ion transport in wood cell walls – A means to understanding decay resistance. International Biodeterioration & Biodegradation 133, 230-237. https://doi.org/10.1016/j.ibiod.2018.06.014.

Huß, W., Krötsch, S., 2013. Energieeffizientes Sanieren und Bauen mit Holz. LWF aktuell 97(6), 4.

Hyde, S., Wood, P., 1995. A model from the hyphae based on studies with the brown rot *Coniophora puteana*., Proceedings in: International Research Group Wood Preservation - IRG/WP: 95-10104.

Ibach, R.E., Rowell, R.M., 2000. Improvements in Decay Resistance Based on Moisture Exclusion. Molecular Crystals and Liquid Crystals Science and Technology. Section A. Molecular Crystals and Liquid Crystals 353(1), 23-33. https://doi.org/10.1080/10587250008025645.

Jakes, J., Arzola, X., Berman, R., Ciesielski, P., Hunt, C.G., Rahbar, N., Tshabalala, M., Wiedenhieft, A.C., Zelinka, S.L., 2016. Not Just Lumber - Using Wood in the Sustainable Future of Materials, Chemicals, and Fuels. The Minerals, Metals & Materials Society 68(9), 10. https://doi.org/DOI: 10.1007/s11837-016-2026-7.

Jakes, J.E., Arzola, X., Bergman, R., Ciesielski, P., Hunt, C.G., Rahbar, N., Tshabalala, M., Wiedenhoeft, A.C., Zelinka, S.L., 2016. Not Just Lumber—Using Wood in the Sustainable Future of Materials, Chemicals, and Fuels. JOM 68(9), 10. https://doi.org/10.1007/s11837-016-2026-7.

Jakes, J.E., Hunt, C.G., Zelinka, S.L., Ciesielski, P.N., Plaza, N.Z., 2019. Effects of Moisture on Diffusion in Unmodified Wood Cell Walls: A Phenomenological Polymer Science Approach. Forests 10(12), 1084.

Jensen, K., Ryan, Z., Marty, A., Cullen, D., E Hammel, K., 2002. An NADH:Quinone Oxidoreductase Active during Biodegradation by the Brown-Rot Basidiomycete Gloeophyllum trabeum. Applied and Environmental Microbiology 68, 2699-2703. https://doi.org/10.1128/AEM.68.6.2699-2703.2002.

Jensen, K.A., Houtman, C.J., Ryan, Z.C., Hammel, K.E., 2001. Pathways for Extracellular Fenton Chemistry in the Brown Rot Basidiomycete Gloeophyllum trabeum. Applied and Environmental Microbiology 67(6), 2705-2711. https://doi.org/10.1128/aem.67.6.2705-2711.2001.

Jones, D., Sandberg, D., 2020. A Review of Wood Modification Globally – Updated Findings from COST FP1407. Interdisciplinary Perspectives on the Built Environment 1. https://doi.org/10.37947/ipbe.2020.vol1.1.

Jones, D., Sandberg, D., Goli, G., Todaro, L., 2019. Wood Modification in Europe: a state-of-the-art about processes, products and applications.

Kerem, Z., hammel, Hammel, K.E., 1999. Biodegradative mechanism of the brown rot basidiomycete Gloeophyllum trabeum: evidence for an extracellular hydroquinone-driven fenton reaction. FEBS Lett 446(1), 49-54. https://doi.org/10.1016/s0014-5793(99)00180-5.

Kersten, P., Cullen, D., 2014. Copper radical oxidases and related extracellular oxidoreductases of wood-decay Agaricomycetes. Fungal Genetics and Biology 72, 124-130. https://doi.org/https://doi.org/10.1016/j.fgb.2014.05.011.

Kirk, P.M., Cannon, P.F., Minter, D.W., Stalpers, J.A., 2008. Dictionary of the Funghi, 10 ed. CAB International, Wallingford, UK.

Kirk, T.K., Farrell, R.L., 1987. Enzymatic "combustion": the microbial degradation of lignin. Annu Rev Microbiol 41, 465-505. https://doi.org/10.1146/annurev.mi.41.100187.002341.

Kirker, G., Zelinka, S., Gleber, S.-C., Vine, D., Finney, L., Chen, S., Hong, Y.P., Uyarte, O., Vogt, S., Jellison, J., Goodell, B., Jakes, J.E., 2017. Synchrotron-based X-ray fluorescence microscopy enables multiscale spatial visualization of ions involved in fungal lignocellulose deconstruction. Scientific Reports 7(1), 41798. https://doi.org/10.1038/srep41798.

Klutts, J., Yoneda, A., Reilly, M., Bose, I., Doering, T., 2006. Glycosyltransferases and their products: Cryptococcal variations on fungal themes. FEMS yeast research 6, 499-512. https://doi.org/10.1111/j.1567-1364.2006.00054.x.

Kojima, Y., Várnai, A., Ishida, T., Sunagawa, N., Petrovic, D.M., Igarashi, K., Jellison, J., Goodell, B., Alfredsen, G., Westereng, B., Eijsink, V.G.H., Yoshida, M., 2016. A Lytic Polysaccharide Monooxygenase with Broad Xyloglucan Specificity from the Brown-Rot Fungus Gloeophyllum trabeum and Its Action on Cellulose-Xyloglucan Complexes. Applied and Environmental Microbiology 82(22), 6557-6572. https://doi.org/10.1128/aem.01768-16.

Kühnen, R., Wagenführ, R., 2002. Werkstoffkunde für Restauratoren. E. A. Seemann, Leipzig. Lairson, L.L., Henrissat, B., Davies, G.J., Withers, S.G., 2008. Glycosyltransferases: Structures, Functions, and Mechanisms. Annual Review of Biochemistry 77(1), 521-555. https://doi.org/10.1146/annurev.biochem.76.061005.092322.

Lande, S., Eikenes, M., Westin, M., Schneider, M., 2008. Furfurylation of Wood: Chemistry, Properties, and Commercialization, pp. 337-355. https://doi.org/10.1021/bk-2008-0982.ch020.

Lande, S., Westin, M., Schneider, M., 2004. Properties of furfurylated wood. Scandinavian Journal of Forest Research 19(sup5), 22-30. https://doi.org/10.1080/0282758041001915.

Larnøy, E., Karaca, A., Gobakken, L., Hill, C., 2018. Polyesterification of wood using sorbitol and citric acid under aqueous conditions. International Wood Products Journal 9, 1-8. https://doi.org/10.1080/20426445.2018.1475918.

Larsson Brelid, P., Simonson, R., Bergman, Ö., Nilsson, T., 2000. Resistance of acetylated wood to biological degradation. Holz als Roh- und Werkstoff 58(5), 331-337. https://doi.org/10.1007/s001070050439.

Lebow, S., 2004. Alternatives to Chromated Copper Arsenate (CCA) for Residential Construction, Proceedings of the Environmental Impacts of Preservative-Treated Wood. February 8-10, 2004, Orlando, Florida, p. 12.

Levasseur, A., Drula, E., Lombard, V., Coutinho, P.M., Henrissat, B., 2013. Expansion of the enzymatic repertoire of the CAZy database to integrate auxiliary redox enzymes. Biotechnology for Biofuels 6(1), 41. https://doi.org/10.1186/1754-6834-6-41.

Li, F., Ma, F., Zhao, H., Zhang, S., Wang, L., Zhang, X., Yu, H., 2019. A Lytic Polysaccharide Monooxygenase from a White-Rot Fungus Drives the Degradation of Lignin by a Versatile Peroxidase. Appl Environ Microbiol 85(9). https://doi.org/10.1128/aem.02803-18.

Liese, W., 1970. Ultrastructural Aspects of Woody Tissue Disintegration. Annual Review of Phytopathology 8(1), 231-258. https://doi.org/10.1146/annurev.py.08.090170.001311.

Lombard, V., Bernard, T., Rancurel, C., Brumer, H., Coutinho, P.M., Henrissat, B., 2010. A hierarchical classification of polysaccharide lyases for glycogenomics. Biochem J 432(3), 437-444. https://doi.org/10.1042/bj20101185.

Lombard, V., Golaconda Ramulu, H., Drula, E., Coutinho, P.M., Henrissat, B., 2013. The carbohydrate-active enzymes database (CAZy) in 2013. Nucleic Acids Research 42(D1), D490-D495. https://doi.org/10.1093/nar/gkt1178.

Lombard, V., Golaconda Ramulu, H., Drula, E., Coutinho, P.M., Henrissat, B., 2014. The carbohydrate-active enzymes database (CAZy) in 2013. Nucleic Acids Res 42(Database issue), D490-495. https://doi.org/10.1093/nar/gkt1178.

MacPherson, S., Larochelle, M., Turcotte, B., 2006. A fungal family of transcriptional regulators: the zinc cluster proteins. Microbiol Mol Biol Rev 70(3), 583-604. https://doi.org/10.1128/MMBR.00015-06.

Mantau, U., Johnsson, R., Steierer, F., Verkerk, P.J., Oldenburger, J., Leek, N., Saal, U., Prins, K., 2010. EUwood - Real potential for changes in growth and use of EU forests. Methodology report. Hamburg/Germany, p. 165.

Martin, F., 2007. Fair Trade in the Underworld: the Ectomycorrhizal Symbiosis, in: Howard, R.J., Gow, N.A.R. (Eds.), Biology of the Fungal Cell. Springer Berlin Heidelberg, Berlin, Heidelberg, pp. 291-308. https://doi.org/10.1007/978-3-540-70618-2_12.

Martinez, A.T., 2002. Molecular biology and structure-function of lignin-degrading heme peroxidases. Enzyme Microb. Technol. 30, 425-444. Enzyme and Microbial Technology 30, 425-444. https://doi.org/10.1016/S0141-0229(01)00521-X.

Martínez, A.T., Rencoret, J., Nieto, L., Jiménez-Barbero, J., Gutiérrez, A., Del Río, J.C., 2011. Selective lignin and polysaccharide removal in natural fungal decay of wood as evidenced by in situ structural analyses. Environ Microbiol 13(1), 96-107. https://doi.org/10.1111/j.1462-2920.2010.02312.x.

Martinez, Á.T., Speranza, M., Ruiz-Dueñas, F.J., Ferreira, P., Camarero, S., Guillén, F., Martínez, M.J., Gutiérrez, A., Del Río, J.C., 2005. Biodegradation of lignocellulosics:

Microbial, chemical, and enzymatic aspects of the fungal attack of lignin. International Microbiology 8(3), 195-204.

Martinez, D., Challacombe, J., Morgenstern, I., Hibbett, D.S., Schmoll, M., Kubicek, C., P., Ferreira, P., Ruiz-Dueñas, F.J., Martinez, A.T., Kersten, P., Hammel, K.E., Vanden Wymelenberg, A., Gaskell, J., Lindquist, E., Sabat, G., BonDurant, S.S., Larrondo, L.F., Canessa, P., Vicuna, R., Yadav, J.S., Doddapaneni, H., Subramanian, V., Pisabarro, A., Lavin, J.L., Oquiza, J.A., Master, E., Henrissat, B., Coutinho, P.M., Harris, P., Magnuson, J., Karl, Baker, S.E., Bruno, K., Kenealy, W., Hoegger, P.J., Kües, U., Ramaiya, P., Lucas, S., Salamov, A., Shapiro, H., Tu, H., Chee, C.L., Misra, M., Xie, G., Teter, S., Yaver, D., James, T., Mokrejs, M., Pospisek, M., Grigoriev, I., Brettin, T., Rokshar, D., Berka, R., Cullen, D., 2009. Genome, transcriptome, and secretome analysis of wood decay fungus *Postia placenta* supports unique mechanisms of lignocellulose conversion. PNAS 106(6), 6.

Meyer-Veltrup, L., Brischke, C., 2015. Fungal decay at different moisture levels of selected European-grown wood species. International Biodeterioration & Biodegradation 103, 23-29. https://doi.org/10.1016/j.ibiod.2015.04.009.

Micales, J.A., 1997. Localization and induction of oxalate decarboxylase in the brown-rot wood decay fungus Postia placenta. International Biodeterioration & Biodegradation 39(2), 125-132. https://doi.org/https://doi.org/10.1016/S0964-8305(97)00009-7.

Militz, H., 2002. Thermal treatment of wood: European processes and their background., International Research Group on Wood Preservation. Cardiff, Wales, United Kingdom.

Mohareb, A., Sirmah, P., Pétrissans, M., Gérardin, P., 2012. Effect of heat treatment intensity on wood chemical composition and decay durability of Pinus patula. European Journal of Wood and Wood Products 70(4), 519-524. https://doi.org/10.1007/s00107-011-0582-7.

Morrell, J., 2005. Chapter 15 - Protection of wood-based materials, in: Kutz, M. (Ed.) Handbook of Environmental Degradation of Materials. William Andrew Publishing, Norwich, NY, pp. 299-317. https://doi.org/https://doi.org/10.1016/B978-081551500-5.50017-3.

Mueckler, M., Caruso, C., Baldwin, S.A., Panico, M., Blench, I., Morris, H.R., Allard, W.J., Lienhard, G.E., Lodish, H.F., 1985. Sequence and structure of a human glucose transporter. Science 229(4717), 941. https://doi.org/10.1126/science.3839598.

Munir, E., Yoon, J.-J., Tokimatsu, T., Hattori, T., Shimada, M., 2001a. New role for glyoxylate cycle enzymes in wood-rotting basidiomycetes in relation to biosynthesis of oxalic acid. Journal of Wood Science 47(5), 368-373. https://doi.org/10.1007/bf00766787.

Munir, E., Yoon, J., Tokimatsu, T., Hattori, T., Shimada, M., 2001b. A physiological role for oxalic acid biosynthesis in the wood-rotting basidiomycete Fomitopsis palutris. Proceedings of the National Academy of Sciences of the United States of America 98, 11126-11130. https://doi.org/10.1073/pnas.191389598.

Nagy, L.G., Riley, R., Tritt, A., Adam, C., Daum, C., Floudas, D., Sun, H., Yadav, J.S., Pangilinan, J., Larsson, K.-H., Matsuura, K., Barry, K., Labutti, K., Kuo, R., Ohm, R.A., Bhattacharya, S.S., Shirouzu, T., Yoshinaga, Y., Martin, F.M., Grigoriev, I.V., Hibbett, D.S., 2015. Comparative Genomics of Early-Diverging Mushroom-Forming Fungi Provides Insights

into the Origins of Lignocellulose Decay Capabilities. Molecular Biology and Evolution 33(4), 959-970. https://doi.org/10.1093/molbev/msv337.

Nakamura, A.M., Nascimento, A.S., Polikarpov, I., 2017. Structural diversity of carbohydrate esterases. Biotechnology Research and Innovation 1(1), 35-51. https://doi.org/https://doi.org/10.1016/j.biori.2017.02.001.

Ng, P.C., Kirkness, E.F., 2010. Whole genome sequencing. Methods Mol Biol 628, 215-226. https://doi.org/10.1007/978-1-60327-367-1_12.

Niemelä, T., Kinnunen, J., Larsson, K., Schigel, D., Larsson, E., 2005. Genus revisions and new combination of some North European polypores. Karstenia 45, 75-80. https://doi.org/10.29203/ka.2005.406.

Niemenmaa, O., Uusi-Rauva, A., Hatakka, A., 2007. Demethoxylation of [O14CH3]-labelled lignin model compounds by the brown-rot fungi Gloeophyllum trabeum and Poria (Postia) placenta. Biodegradation 19(4), 555. https://doi.org/10.1007/s10532-007-9161-3.

Ohm, R.A., Riley, R., Salamov, A., Min, B., Choi, I.-G., Grigoriev, I.V., 2014. Genomics of wood-degrading fungi. Fungal Genetics and Biology 72, 82-90. https://doi.org/https://doi.org/10.1016/j.fgb.2014.05.001.

Papadopoulos, A.N., Hill, C.A.S., 2003. The sorption of water vapour by anhydride modified softwood. Wood Science and Technology 37(3), 221-231. https://doi.org/10.1007/s00226-003-0192-6.

Park, S.T., Kim, J., 2016. Trends in Next-Generation Sequencing and a New Era for Whole Genome Sequencing. Int Neurourol J 20(Suppl 2), S76-83. https://doi.org/10.5213/inj.1632742.371.

Paszczynski, A., Crawford, R., Funk, D., Goodell, B., 1999. De novo synthesis of 4,5-dimethoxycatechol and 2, 5-dimethoxyhydroquinone by the brown rot fungus Gloeophyllum trabeum. Applied and environmental microbiology 65(2), 674-679.

Payne, C.M., Knott, B.C., Mayes, H.B., Hansson, H., Himmel, M.E., Sandgren, M., Ståhlberg, J., Beckham, G.T., 2015. Fungal Cellulases. Chemical Reviews 115(3), 1308-1448. https://doi.org/10.1021/cr500351c.

Pedrini, N., Ortiz-Urquiza, A., Huarte-Bonnet, C., Fan, Y., Juárez, M.P., Keyhani, N.O., 2015. Tenebrionid secretions and a fungal benzoquinone oxidoreductase form competing components of an arms race between a host and pathogen. Proc Natl Acad Sci U S A 112(28), E3651-3660. https://doi.org/10.1073/pnas.1504552112.

Pfriem, A., Zauer, M., Wagenführ, A., 2010. Alteration of the unsteady sorption behaviour of maple (Acer pseudoplatanus L.) and spruce (Picea abies (L.) Karst.) due to thermal modification. 64(2), 235-241. https://doi.org/doi:10.1515/hf.2010.029.

Pilgård, A., Alfredsen, G., Björdal, C., Fossdal, C.G., Børja, I., 2011. QPCR as a tool to study basidiomycete colonization in wooden field stakes. Holzforschung 65, 889-895. https://doi.org/10.1515/HF.2011.079.

Pilgård, A., Alfredsen, G., Fossdal, C.G., Long, I.C.J., 2012. The effects of acetylation on the growth of Postia placenta over 36 weeks, Proceedings IRG Annual Meeting, IRG/WP 12-40589.

Pilgård, A., Alfredsen, G., Hietala, A., 2010. Quantification of fungal colonization in modified wood: Quantitative real-time PCR as a tool for studies on Trametes versicolor. 64(5), 645-651. https://doi.org/doi:10.1515/hf.2010.074.

Pollegioni, L., Tonin, F., Rosini, E., 2015. Lignin-degrading enzymes. The FEBS Journal 282(7), 1190-1213. https://doi.org/https://doi.org/10.1111/febs.13224.

Popescu, C.-M., Hill, C.A.S., Curling, S., Ormondroyd, G., Xie, Y., 2014. The water vapour sorption behaviour of acetylated birch wood: how acetylation affects the sorption isotherm and accessible hydroxyl content. Journal of Materials Science 49(5), 2362-2371. https://doi.org/10.1007/s10853-013-7937-x.

Presley, G.N., Panisko, E., Purvine, S.O., Schilling, J.S., 2018. Coupling Secretomics with Enzyme Activities To Compare the Temporal Processes of Wood Metabolism among White and Brown Rot Fungi. Applied and environmental microbiology 84(16), e00159-00118. https://doi.org/10.1128/AEM.00159-18.

Presley, G.N., Schilling, J.S., 2017. Distinct Growth and Secretome Strategies for Two Taxonomically Divergent Brown Rot Fungi. Applied and environmental microbiology 83(7), e02987-02916. https://doi.org/10.1128/AEM.02987-16.

Presley, G.N., Zhang, J., Purvine, S.O., Schilling, J.S., 2020. Functional Genomics, Transcriptomics, and Proteomics Reveal Distinct Combat Strategies Between Lineages of Wood-Degrading Fungi With Redundant Wood Decay Mechanisms. Frontiers in microbiology 11, 1646-1646. https://doi.org/10.3389/fmicb.2020.01646.

Puls, J., 1997. Chemistry and biochemistry of hemicelluloses: Relationship between hemicellulose structure and enzymes required for hydrolysis. Macromolecular Symposia 120(1), 183-196. https://doi.org/10.1002/masy.19971200119.

Qi, W., Jellison, J., 2004. Characterization of a transplasma membrane redox system of the brown rot fungus Gloeophyllum trabeum. International Biodeterioration & Biodegradation 53(1), 37-42. https://doi.org/https://doi.org/10.1016/j.ibiod.2003.09.002.

Rättö, M., Ritschkoff, A.C., Viikari, L., 1997. The effect of oxidative pretreatment on cellulose degradation by Poria placenta and Trichoderma reesei cellulases. Applied Microbiology and Biotechnology 48(1), 53-57. https://doi.org/10.1007/s002530051014.

Ray, M.J., Leak, D.J., Spanu, P.D., Murphy, R.J., 2010. Brown rot fungal early stage decay mechanism as a biological pretreatment for softwood biomass in biofuel production. Biomass and Bioenergy 34(8), 1257-1262. https://doi.org/https://doi.org/10.1016/j.biombioe.2010.03.015.

Riley, R., Salamov, A.A., Brown, D.W., Nagy, L.G., Floudas, D., Held, B.W., Levasseur, A., Lombard, V., Morin, E., Otillar, R., Lindquist, E.A., Sun, H., LaButti, K.M., Schmutz, J., Jabbour, D., Luo, H., Baker, S.E., Pisabarro, A.G., Walton, J.D., Blanchette, R.A., Henrissat,

B., Martin, F., Cullen, D., Hibbett, D.S., Grigoriev, I.V., 2014. Extensive sampling of basidiomycete genomes demonstrates inadequacy of the white-rot/brown-rot paradigm for wood decay fungi. Proceedings of the National Academy of Sciences 111(27), 9923-9928. https://doi.org/10.1073/pnas.1400592111.

Ringman, R., Beck, G., Pilgård, A., 2019. The Importance of Moisture for Brown Rot Degradation of Modified Wood: A Critical Discussion. Forests 10(6), 522.

Ringman, R., Pilgård, A., Brischke, C., Richter, K., 2014a. Mode of action of brown rot decay resistance in modified wood: a review. Holzforschung 68(2), 239. https://doi.org/10.1515/hf-2013-0057.

Ringman, R., Pilgård, A., Brischke, C., Windeisen, E., Richter, K., 2017. Incipient brown rot decay in modified wood: patterns of mass loss, structural integrity, moisture and acetyl content in high resolution. International Wood Products Journal, 11. https://doi.org/10.1080/20426445.2017.1344382.

Ringman, R., Pilgård, A., Kölle, M., Brischke, C., Richter, K., 2016. Effects of thermal modification on Postia placenta wood degradation dynamics: measurements of mass loss, structural integrity and gene expression. Wood Science and Technology 50(2), 385-397. https://doi.org/10.1007/s00226-015-0791-z.

Ringman, R., Pilgård, A., Kölle, M., Richter, K., 2018. Expression patterns of Postia placenta genes involved in the chelator mediated Fenton degradation in modified wood, European Conference on Wood Modification. Arnhem, Netherlands, p. 3.

Ringman, R., Pilgård, A., Richter, K., 2014b. Effect of wood modification on gene expression during incipient Postia placenta decay. International Biodeterioration & Biodegradation 86, 86-91. https://doi.org/https://doi.org/10.1016/j.ibiod.2013.09.002.

Ringman, R., Pilgård, A., Richter, K., 2020. Brown rot gene expression and regulation in acetylated and furfurylated wood: a complex picture, Holzforschung.

Risse, M., Weber-Blaschke, G., Richter, K., 2017. Resource efficiency of multifunctional wood cascade chains using LCA and exergy analysis, exemplified by a case study for Germany. Resources, Conservation and Recycling 126, 141-152. https://doi.org/https://doi.org/10.1016/j.resconrec.2017.07.045.

Risse, M., Weber-Blaschke, G., Richter, K., 2019. Eco-efficiency analysis of recycling recovered solid wood from construction into laminated timber products. Science of The Total Environment 661, 107-119. https://doi.org/https://doi.org/10.1016/j.scitotenv.2019.01.117.

Rowell, R., Bongers, F., 2015. Coating Acetylated Wood. Coatings 5, 792-801. https://doi.org/10.3390/coatings5040792.

Rowell, R., Simonson, R., Hess, S., Plackett, D., Cronshaw, D., Dunningham, E., 1994. Acetyl distribution in acetylated whole wood and reactivity of isolated wood cell-wall components to acetic anhydride. Wood Fiber Sci. 26, 11-18.

Rowell, R.M., 2005. Wood Chemistry and Wood Composites. Taylor & Francis, Boca, Raton. https://doi.org/https://doi.org/10.1201/b12487.

Rowell, R.M., 2006. Chemical modification of wood: A short review. Wood Material Science & Engineering 1(1), 29-33. https://doi.org/10.1080/17480270600670923.

Ryu, J.S., Shary, S., Houtman, C.J., Panisko, E.A., Korripally, P., St John, F.J., Crooks, C., Siika-Aho, M., Magnuson, J.K., Hammel, K.E., 2011. Proteomic and functional analysis of the cellulase system expressed by Postia placenta during brown rot of solid wood. Appl Environ Microbiol 77(22), 7933-7941. https://doi.org/10.1128/AEM.05496-11.

Schilling, J.S., Duncan, S.M., Presley, G.N., Filley, T.R., Jurgens, J.A., Blanchette, R.A., 2013. Colocalizing incipient reactions in wood degraded by the brown rot fungus Postia placenta. International Biodeterioration & Biodegradation 83, 56-62. https://doi.org/https://doi.org/10.1016/j.ibiod.2013.04.006.

Schmidt, B., Hildebrandt, A., 2017. Next-generation sequencing: big data meets high performance computing. Drug Discovery Today 22(4), 712-717. https://doi.org/https://doi.org/10.1016/j.drudis.2017.01.014.

Schmidt, O., 2006. Wood and Tree Fungi: Biology, Damage, Protection, and Use. https://doi.org/10.1007/3-540-32139-X.

Schmidt, O., 2007. Indoor wood-decay basidiomycetes: damage, causal fungi, physiology, identification and characterization, prevention and control. Mycological Progress 6(4), 261. https://doi.org/10.1007/s11557-007-0534-0.

Schmöllerl, B., Alfredsen, G., Fossdal, C.G., Westin, M., Steitz, A., 2011. Molecular investigation of Postia placenta growing in modified wood, Proceedings IRG Annual Meeting, IRG/WP 11-10756. Queenstown, New Zealand, p. 10.

Schneider, M.H., 1995. New cell wall and cell lumen wood polymer composites. Wood Science and Technology 29.

Shelest, E., 2008. Transcription factors in fungi. FEMS Microbiology Letters 286(2), 145-151. https://doi.org/10.1111/j.1574-6968.2008.01293.x.

Shelest, E., 2017. Transcription Factors in Fungi: TFome Dynamics, Three Major Families, and Dual-Specificity TFs. Frontiers in Genetics 8(53). https://doi.org/10.3389/fgene.2017.00053.

Simpson, W., 1980. Sorption theories applied to wood. Wood and fiber 12.

Singh, A.P., Singh, T., 2014. Biotechnological applications of wood-rotting fungi: A review. Biomass and Bioenergy 62, 198-206. https://doi.org/https://doi.org/10.1016/j.biombioe.2013.12.013.

Sinnott, M.L., 1990. Catalytic mechanism of enzymic glycosyl transfer. Chemical Reviews 90(7), 1171-1202. https://doi.org/10.1021/cr00105a006.

Sista Kameshwar, A.K., Qin, W., 2018. Understanding the structural and functional properties of carbohydrate esterases with a special focus on hemicellulose deacetylating acetyl xylan esterases. Mycology 9(4), 273-295. https://doi.org/10.1080/21501203.2018.1492979.

Stamm, A.J., Baechler, R.H., 1960. Decay resistance and dimensional stability of five modified woods. Forest Products Journal 10(1), 22-26.

Suzuki, M.R., Hunt, C.G., Houtman, C.J., Dalebroux, Z.D., Hammel, K.E., 2006. Fungal hydroquinones contribute to brown rot of wood. Environmental Microbiology 8(12), 2214-2223. https://doi.org/10.1111/j.1462-2920.2006.01160.x.

Takao, S., 1965. Organic Acid Production by Basidiomycetes. Applied Microbiology 13(5), 732.

Temiz, A., Terziev, N., Eikenes, M., Hafren, J., 2007. Effect of accelerated weathering on surface chemistry of modified wood. Applied Surface Science 253, 5355-5362. https://doi.org/10.1016/j.apsusc.2006.12.005.

Thaler, N., Alfredsen, G., Fossdal, C.G., 2012. Variation in two Postia placenta strains, MAD-698-R and FPRL280 - mass loss, DNA content and gene expression, Proceedings IRG Annual Meeting, IRG/WP 12-10781. Kuala Lumpur, Malaysia.

Thybring, E.E., 2013. The decay resistance of modified wood influenced by moisture exclusion and swelling reduction. International Biodeterioration & Biodegradation 82, 87-95. https://doi.org/https://doi.org/10.1016/j.ibiod.2013.02.004.

Thybring, E.E., 2017. Water relations in untreated and modified wood under brown-rot and white-rot decay. International Biodeterioration & Biodegradation 118, 134-142. https://doi.org/https://doi.org/10.1016/j.ibiod.2017.01.034.

Thygesen, L.G., Barsberg, S., Venås, T.M., 2010. The fluorescence characteristics of furfurylated wood studied by fluorescence spectroscopy and confocal laser scanning microscopy. Wood Science and Technology 44(1), 51-65. https://doi.org/10.1007/s00226-009-0255-4.

Tiemann, H.D., United, S., Forest, S., United, S., Department of, A., 1906. Effect of moisture upon the strength and stiffness of wood. U.S. Dept. of Agriculture, Forest Service, Washington, D.C.

Tjeerdsma, B.F., Boonstra, M., Pizzi, A., Tekely, P., Militz, H., 1998. Characterisation of thermally modified wood: molecular reasons for wood performance improvement. Holz als Roh- und Werkstoff 56(3), 149-153. https://doi.org/10.1007/s001070050287.

Tokimatsu, T., Nagai, Y., Hattori, T., Shimada, M., 1998. Purification and characteristics of a novel cytochrome c dependent glyoxylate dehydrogenase from a wood-destroying fungus Tyromyces palustris 1. FEBS Letters 437(1-2), 117-121. https://doi.org/10.1016/S0014-5793(98)01104-1.

Tomme, P., Boraston, A., McLean, B., Kormos, J., Creagh, A.L., Sturch, K., Gilkes, N.R., Haynes, C.A., Warren, R.A., Kilburn, D.G., 1998. Characterization and affinity applications of

cellulose-binding domains. J Chromatogr B Biomed Sci Appl 715(1), 283-296. https://doi.org/10.1016/s0378-4347(98)00053-x.

Tomme, P., Van Tilbeurgh, H., Pettersson, G., Van Damme, J., Vandekerckhove, J., Knowles, J., Teeri, T., Claeyssens, M., 1988. Studies of the cellulolytic system of Trichoderma reesei QM 9414. Analysis of domain function in two cellobiohydrolases by limited proteolysis. Eur J Biochem 170(3), 575-581. https://doi.org/10.1111/j.1432-1033.1988.tb13736.x.

Townsend, T., Dubey, B., Tolaymat, T., Solo-Gabriele, H., 2005. Preservative leaching from weathered CCA-treated wood. Journal of Environmental Management 75(2), 105-113. https://doi.org/https://doi.org/10.1016/j.jenvman.2004.11.009.

Umezawa, K., Niikura, M., Kojima, Y., Goodell, B., Yoshida, M., 2020. Transcriptome analysis of the brown rot fungus Gloeophyllum trabeum during lignocellulose degradation. PLoS One 15(12), e0243984. https://doi.org/10.1371/journal.pone.0243984.

van den Brink, J., de Vries, R.P., 2011. Fungal enzyme sets for plant polysaccharide degradation. Appl Microbiol Biotechnol 91(6), 1477-1492. https://doi.org/10.1007/s00253-011-3473-2.

Vanden Wymelenberg, A., Gaskell, J., Mozuch, M., BonDurant, S.S., Sabat, G., Ralph, J., Skyba, O., Mansfield, S.D., Blanchette, R.A., Grigoriev, I.V., Kersten, P.J., Cullen, D., 2011. Significant alteration of gene expression in wood decay fungi *Postia placenta* and *Phanerochaete chrysosporium* by plant species. Applied and environmental microbiology 77(13), 4499-4507. https://doi.org/10.1128/AEM.00508-11.

Vanden Wymelenberg, A., Gaskell, J., Mozuch, M., Sabat, G., Ralph, J., Skyba, O., Mansfield, S.D., Blanchette, R.A., Martinez, D., Grigoriev, I., Kersten, P.J., Cullen, D., 2010. Comparative Transcriptome and Secretome Analysis of Wood Decay Fungi *Postia placenta* and *Phanerochaete chrysosporium*. Applied and Environmental Microbiology 76(11), 3599-3610. https://doi.org/10.1128/aem.00058-10.

Wagenführ, A., Scholz, F., 2012. Taschenbuch der Holztechnik. Fachbuchverlag Leipzig im Carl Hanser Verlag, München.

Wang, Z., Gerstein, M., Snyder, M., 2009. RNA-Seq: a revolutionary tool for transcriptomics. Nat Rev Genet 10(1), 57-63. https://doi.org/10.1038/nrg2484.

Wardman, P., 1989. Reduction Potentials of One-Electron Couples Involving Free Radicals in Aqueous Solution. Journal of Physical and Chemical Reference Data 18(4), 1637-1755. https://doi.org/10.1063/1.555843.

Wei, D., Houtman, C.J., Kapich, A., Hunt, C., Cullen, D., Hammel, K., E., 2010. Laccase and Its Role in Production of Extracellular Reactive Oxygen Species during Wood Decay by the Brown Rot Basidiomycete Postia placenta. Applied and Environmental Microbiology 76, 2091-2097. https://doi.org/10.1128/AEM.02929-09.

Welzbacher, C.R., 2007. Verhalten von nach neuen thermischen Modifikationsverfahren behandelter Fichte und Kiefer unter besonderer Berücksichtigung der Dauerhaftigkeit gegenüber holzzerstörenden Mikroorganismen. University of Hamburg, Hamburg, Germany.

Wilcox, W., 1978. Review of literature on the effects of early stages of decy on wood strength. Wood Fiber 9(4), 6.

Windeisen, E., Bächle, H., Zimmer, B., Wegener, G., 2009. Relations between chemical changes and mechanical properties of thermally treated wood 10th EWLP, Stockholm, Sweden, August 25–28, 2008. 63(6), 773-778. https://doi.org/doi:10.1515/HF.2009.084.

Wu, B., Xu, Z., Knudson, A., Carlson, A., Chen, N., Kovaka, S., LaButti, K., Lipzen, A., Pennachio, C., Riley, R., Schakwitz, W., Umezawa, K., Ohm, R.A., Grigoriev, I.V., Nagy, L.G., Gibbons, J., Hibbett, D., 2018. Genomics and Development of Lentinus tigrinus: A White-Rot Wood-Decaying Mushroom with Dimorphic Fruiting Bodies. Genome Biol Evol 10(12), 3250-3261. https://doi.org/10.1093/gbe/evy246.

Xiao, C., Anderson, C., 2013. Roles of pectin in biomass yield and processing for biofuels. Frontiers in Plant Science 4(67). https://doi.org/10.3389/fpls.2013.00067.

Yan, N., 2015. Structural Biology of the Major Facilitator Superfamily Transporters. Annual Review of Biophysics 44(1), 257-283. https://doi.org/10.1146/annurev-biophys-060414-033901.

Yelle, D.J., Ralph, J., Lu, F., Hammel, K.E., 2008. Evidence for cleavage of lignin by a brown rot basidiomycete. Environ Microbiol 10(7), 1844-1849. https://doi.org/10.1111/j.1462-2920.2008.01605.x.

Yelle, D.J., Wei, D., Ralph, J., Hammel, K.E., 2011. Multidimensional NMR analysis reveals truncated lignin structures in wood decayed by the brown rot basidiomycete Postia placenta. Environmental Microbiology 13(4), 1091-1100. https://doi.org/10.1111/j.1462-2920.2010.02417.x.

Zabel, R., Morrell, J.J., 1992. Wood Microbiology: Decay and its Prevention. Academic Press, San Diego California.

Zabel, R., Morrell, J.J., 2020. Wood Microbiology, 2 ed. Academic Press.

Zelinka, S., Glass, S., Stone, D., 2008. A percolation model for electrical conduction in wood with implications for wood-water relations. Wood and fiber science: journal of the Society of Wood Science and Technology 40, 544-552.

Zelinka, S., Jakes, J., Kirker, G., Bishell, A., Boardman, C., Lai, B., Sterbinsky, G., Jellison, J., Goodell, B., 2021. Oxidation states of iron and manganese in lignocellulose altered by the brown rot fungus Gloeophyllum trabeum measured in-situ using X-ray absorption near edge spectroscopy (XANES). International Biodeterioration & Biodegradation 148, 105162. https://doi.org/10.1016/j.ibiod.2020.105162.

Zelinka, S.L., Gleber, S.C., Vogt, S., López, G.M.R., Jakes, J.E., 2015. Threshold for ion movements in wood cell walls below fiber saturation observed by X-ray fluorescence microscopy (XFM). Holzforschung 69(4), 441-448. https://doi.org/10.1515/hf-2014-0138.

Zelinka, S.L., Houtman, C.J., Hirth, K., Lacher, S., Lorenz, L., Engelund Thybring, E., Hunt, C.G., 2020. The Effect of Acetylation on Iron Uptake and Diffusion in Water Saturated Wood Cell Walls and Implications for Decay. Forests 11(10), 1121.

Zelinka, S.L., Ringman, R., Pilgård, A., Thybring, E.E., Jakes, J.E., Richter, K., 2016. The role of chemical transport in the brown-rot decay resistance of modified wood AU - Zelinka, S. L. International Wood Products Journal 7(2), 66-70. https://doi.org/10.1080/20426445.2016.1161867.

Zhang, J., Presley, G.N., Hammel, K.E., Ryu, J.-S., Menke, J.R., Figueroa, M., Hu, D., Orr, G., Schilling, J.S., 2016. Localizing gene regulation reveals a staggered wood decay mechanism for the brown rot fungus Postia placenta. Proceedings of the National Academy of Sciences 113(39), 10968-10973. https://doi.org/10.1073/pnas.1608454113.

Zhang, J., Silverstein, K.A.T., Castaño, J.D., Figueroa, M., Schilling, J.S., 2019. Gene Regulation Shifts Shed Light on Fungal Adaption in Plant Biomass Decomposers. mBio 10(6), e02176-02119. https://doi.org/10.1128/mBio.02176-19.

Zhou, X., Ren, L., Meng, Q., Li, Y., Yu, Y., Yu, J., 2010. The next-generation sequencing technology and application. Protein Cell 1(6), 520-536. https://doi.org/10.1007/s13238-010-0065-3.

Zhu, Y., Plaza, N., Kojima, Y., Yoshida, M., Zhang, J., Jellison, J., Pingali, S.V., O'Neill, H., Goodell, B., 2020. Nanostructural Analysis of Enzymatic and Non-enzymatic Brown Rot Fungal Deconstruction of the Lignocellulose Cell Wall. Frontiers in microbiology 11, 1389-1389. https://doi.org/10.3389/fmicb.2020.01389.

Zhu, Y., Zhuang, L., Goodell, B., Cao, J., Mahaney, J., 2016. Iron sequestration in brown-rot fungi by oxalate and the production of reactive oxygen species (ROS). International Biodeterioration & Biodegradation 109, 185-190. https://doi.org/https://doi.org/10.1016/j.ibiod.2016.01.023.

Zhuang, L., Guo, W., Yoshida, M., Feng, X., Goodell, B., 2015. Investigating oxalate biosynthesis in the wood-decaying fungus Gloeophyllum trabeum using 13C metabolic flux analysis. RSC Advances 5(126), 104043-104047. https://doi.org/10.1039/C5RA19203J.

8 Statement made in lieu of an oath

(Eidesstattliche Erklärung)

Ich erkläre an Eides statt, dass ich die bei der promotionsführenden Einrichtung, dem Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt der TUM zur Promotionsprüfung vorgelegte Arbeit mit dem Titel:

Genetic and regulatory differences between three brown rot strains and the impact of acetylation on decay mechanisms

am Lehrstuhl für Holzwissenschaft unter der Anleitung und Betreuung durch Prof. Dr. Johann Philipp Benz ohne sonstige Hilfe erstellt und bei der Abfassung nur die gemäß § 6 Abs. 6 und 7 Satz 2 angegebenen Hilfsmittel benutzt habe.

- Ich habe keine Organisation eingeschaltet, die gegen Entgelt Betreuerinnen und Betreuer für die Anfertigung von Dissertationen sucht, oder die mir obliegenden Pflichten hinsichtlich der Prüfungsleistungen für mich ganz oder teilweise erledigt.

- Ich habe die Dissertation in dieser oder ähnlicher Form in keinem anderen Prüfungsverfahren als Prüfungsleistung vorgelegt.

- Ich habe den angestrebten Doktorgrad noch nicht erworben und bin nicht in einem früheren Promotionsverfahren für den angestrebten Doktorgrad endgültig gescheitert.

Die öffentlich zugängliche Promotionsordnung der TUM ist mir bekannt, insbesondere habe ich die Bedeutung von § 28 (Nichtigkeit der Promotion) und § 29 (Entzug des Doktorgrades) zur Kenntnis genommen. Ich bin mir der Konsequenzen einer falschen Eidesstattlichen Erklärung bewusst.

München, den 21. 12. 2021   Martina Kölle

9 List of publications

9.1 Peer-reviewed publications

Kölle M., Ringman R., Pilgård A. (2019) Initial *Rhodonia placenta* gene expression in acetylated wood: group-wise upregulation of non-enzymatic oxidative wood degradation genes depending on the treatment level. *Forests*, 10, 1117. doi:10.3390/f10121117

Kölle M., Crivelente Horta MA., Nowrousian M., Ohm R., Benz JP., Pilgård A. (2020) Degradative capacity of two strains of *Rhodonia placenta*: from phenotype to genotype. *Frontiers in Microbiology,* 11, 1338. doi: 10.3389/fmicb.2020.01338

Kölle M., Crivelente Horta MA., Benz JP., Pilgård A. (2021) Comparative transcriptomics during brown rot decay in three fungi reveals strain-specific degradative strategies and responses to wood acetylation. *Frontiers in Fungal Biology,* 2, 701579. doi: 10.3389/ffunb.2021.701579

9.2 Other publications

Ringman R., Pilgård A., Kölle M., Richter K. (2018) Expression patterns of *Postia placenta* genes involved in the chelator mediated Fenton degradation in modified wood. *European Conference on Wood Modification 2018,* Arnhem, Netherlands.

Kölle M., Ringman, R., Pilgård, A. (2020) Different levels of acetylation lead to groupwise upregulation of non-enzymatic wood degradation genes of *Rhodonia placenta* during initial brown-rot decay. *Proceedings IRG Annual Meeting IRG/WP 20-10958*, Section Biology, Remote

10 Oral presentations

Kölle, M. (2019) Comparison of two methods for the investigation of initial brown rot wood decay. *Wood Science and Engineering Conference (WSE)* (2019), Lund, Sweden.

Kölle, M. (2020) Different levels of acetylation lead to groupwise upregulation of non-enzymatic wood degradation genes of *Rhodonia placenta* during initial brown-rot decay. *International Research Group on Wood Protection – Annual Meeting* (2020), Remote.

11 Annex

11.1 Article I

Degradative capacity of two strains of Rhodonia placenta: from phenotype to genotype. Martina Kölle, Maria Augusta Crivelente Horta, Minou Nowrousian, Robin A. Ohm, J. Philipp Benz, Annica Pilgård. 2020, Frontiers in Microbiology, Volume 11, Article 1338.

The original can be accessed at doi.org/10.3389/fmicb.2020.01338

11.2 Article II

Initial Rhodonia placenta gene expression in acetylated wood: group-wise upregulation of non-enzymatic oxidative wood degradation genes depending on the treatment level. Martina Kölle, Rebecka Ringman, Annica Pilgård. 2019, forests, Volume 10, Article 1117.

The original can be accessed at doi.org/10.3390/f10121117

11.3 Article III

Comparative transcriptomics during brown rot decay in three fungi reveals strain-specific degradative strategies and responses to wood acetylation. Martina Kölle, Maria Augusta Crivelente Horta, J. Philipp Benz, Annica Pilgård.

The original can be accessed at doi: 10.3389/ffunb.2021.701579

I

Degradative Capacity of Two Strains of *Rhodonia placenta*: From Phenotype to Genotype

Martina Kölle[1], Maria Augusta Crivelente Horta[2], Minou Nowrousian[3], Robin A. Ohm[4], J. Philipp Benz[2,5]* and Annica Pilgård[1,6]

[1] Chair of Wood Science, TUM School of Life Sciences Weihenstephan, Technical University of Munich, Munich, Germany, [2] Professorship for Wood Bioprocesses, TUM School of Life Sciences Weihenstephan, Technical University of Munich, Freising, Germany, [3] Department of Molecular and Cellular Botany, Ruhr University Bochum, Bochum, Germany, [4] Department of Biology, Microbiology, Utrecht University, Utrecht, Netherlands, [5] Institute of Advanced Study, Technical University of Munich, Garching, Germany, [6] Biobased Materials, Bioeconomy, RISE Research Institutes of Sweden, Borås, Sweden

Brown rot fungi, such as *Rhodonia placenta* (previously *Postia placenta*), occur naturally in northern coniferous forest ecosystems and are known to be the most destructive group of decay fungi, degrading wood faster and more effectively than other wood-degrading organisms. It has been shown that brown rot fungi not only rely on enzymatic degradation of lignocellulose, but also use low molecular weight oxidative agents in a non-enzymatic degradation step prior to the enzymatic degradation. *R. placenta* is used in standardized decay tests in both Europe and North America. However, two different strains are employed (FPRL280 and MAD-698, respectively) for which differences in colonization-rate, mass loss, as well as in gene expression have been observed, limiting the comparability of results. To elucidate the divergence between both strains, we investigated the phenotypes in more detail and compared their genomes. Significant phenotypic differences were found between the two strains, and no fusion was possible. MAD-698 degraded scots pine more aggressively, had a more constant growth rate and produced mycelia faster than FPRL280. After sequencing the genome of FPRL280 and comparing it with the published MAD-698 genome we found 660,566 SNPs, resulting in 98.4% genome identity. Specific analysis of the carbohydrate-active enzymes, encoded by the genome (CAZome) identified differences in many families related to plant biomass degradation, including SNPs, indels, gaps or insertions within structural domains. Four genes belonging to the AA3_2 family could not be found in or amplified from FPRL280 gDNA, suggesting the absence of these genes. Differences in other CAZy encoding genes that could potentially affect the lignocellulolytic activity of the strains were also predicted by comparison of genome assemblies (e.g., GH2, GH3, GH5, GH10, GH16, GH78, GT2, GT15, and CBM13). Overall, these mutations help to explain the phenotypic differences observed between both strains as they could interfere with the enzymatic activities, substrate binding ability or protein folding. The investigation of the molecular reasons that make these two strains distinct contributes to the understanding of the development of this important brown rot reference species and will help to put the data obtained from standardized decay tests across the globe into a better biological context.

Keywords: *Rhodonia placenta*, *Postia placenta*, brown rot, genome comparison, standardized decay tests, wood degradation, hydrolytic enzymes

INTRODUCTION

Brown rot fungi such as *Rhodonia placenta* (Fr.) *Niemelä, K.H. Larss. & Schigel* (previously *Postia placenta*) naturally occur in the northern forest ecosystems and are known to be the most destructive species, albeit representing only a small group within wood decay fungi (Zabel and Morrell, 1992; Goodell, 2003; Vanden Wymelenberg et al., 2010). It has been suggested that this is because brown rot fungi circumvent the lignin, leaving it behind in a highly modified state (Cowling, 1961; Kleman-Leyer et al., 1992; Suzuki et al., 2006; Schwarze, 2007; Arantes et al., 2011; Yelle et al., 2011; Schilling et al., 2015). Brown rot fungi do not only rely on enzymatic degradation of lignocellulose but use low molecular weight oxidative agents prior to the enzymatic degradation (Goodell et al., 1997; Arantes et al., 2012; Arantes and Goodell, 2014).

It is still not fully understood how the non-enzymatic oxidative degradation phase proceeds in detail. The currently most accepted theory is that brown rot fungi secrete oxalic acid, which diffuses into the lumen, where it sequesters Fe^{3+} as a chelator (Goodell et al., 1997; Eastwood et al., 2011; Arantes et al., 2012). Fe^{2+} is then formed through reduction by hydroquinones and other reducing agents, while additionally hydrogen peroxide (H_2O_2) is formed; likely through a reaction between hydroquinones and oxygen (Paszczynski et al., 1999; Jensen et al., 2001; Eastwood et al., 2011; Arantes et al., 2012; Melin et al., 2015). Hydroxyl radicals are then generated through the reaction of H_2O_2 and Fe^{2+} (Fenton reaction), causing cellulose and hemicellulose depolymerization and lignin modification (Fenton, 1894; Baldrian and Valášková, 2008; Arantes et al., 2012). Oligosaccharides, solubilized during this process, diffuse through the cell walls into the lumen, where they become accessible to cellulases and hemicellulases (Martinez et al., 2005; Goodell et al., 2017).

The number of genes encoding lignin-related enzymes is typically extremely reduced in the genomes of brown rot fungi. Most do not encode class II lignin-modifying peroxidases (AA2) (Riley et al., 2014) and laccase genes are either completely missing, as in *Gloeophyllum trabeum* (Floudas et al., 2012), or very limited in number in *R. placenta* (Martinez et al., 2009; Wei et al., 2010). Cellobiohydrolases, belonging to the GH families 6 and 7, attack cellulose and are often accompanied by a carbohydrate-binding module (mostly CBM1). In most brown rot fungi (including *Rhodonia placenta*) cellobiohydrolases are either absent or lacking a CBM1 domain (Lombard et al., 2013; Riley et al., 2014). Moreover cellobiose dehydrogenases (family AA3_1) are absent in the majority of brown rot fungi, and genes from cellulolytic families (GH5, GH12, GH44, GH45) are reduced. Still they are able to depolymerize and degrade polysaccharides from the wood cell wall (Martinez et al., 2009; Eastwood et al., 2011; Floudas et al., 2012; Riley et al., 2014). It is noteworthy that the number of enzymes active on hemicelluloses is not reduced to the same extent as cellulose-active enzymes (Martinez et al., 2009; Wei et al., 2010).

The dikaryotic strain MAD-698 of *Rhodonia placenta* was first sequenced by Martinez et al. (2009). Since then, there have been several studies on transcriptomics, proteomics and expression of single genes likely involved in wood decay (Vanden Wymelenberg et al., 2010; Ryu et al., 2011; Ringman et al., 2014; Alfredsen et al., 2016a; Zhang et al., 2016; Pilgård et al., 2017; Zhang and Schilling, 2017; Beck et al., 2018). The results of these studies imply that there is a time-wise and spatial separation between the non-enzymatic oxidative and the enzymatic degradation phase. Especially genes involved in the non-enzymatic oxidative degradation are significantly upregulated during early decay. Later decay stages are dominated by expression of GH family genes (Zhang et al., 2016). The transition from non-enzymatic oxidative to enzymatic degradation is triggered by the release of inducer molecules, particularly cellobiose (Zhang and Schilling, 2017). Investigations of the secretome seemed to confirm these trends (Presley et al., 2018). Findings by us and others (Ringman et al., 2014; Alfredsen et al., 2016a,b; Ringman et al., 2016; Pilgård et al., 2017; Kölle et al., 2019; Ringman et al., 2020), also imply that there might be additional mechanisms in the regulation of the non-enzymatic degradation beyond the cellobiose switch (Zhang and Schilling, 2017).

Since brown rot fungi can cause massive damage in a short period of time, wood products and wood protection systems need to be vigorously tested prior to permission for commercial use. This is performed according to national standardized tests (Cowling, 1961; Filley et al., 2002; Niemenmaa et al., 2007; Arantes et al., 2012; Arantes and Goodell, 2014). In standardized decay tests, *R. placenta* is used as a representative brown rot fungus. Two widely used strains of *R. placenta* are MAD-698 and FPRL280. MAD-698 is the recommended strain in the US American Wood-preservers' Association Standard (AWPA E10-16, 2016), while FPRL280 is the recommended strain in the European standard EN 113 (CEN EN 113, 1996).

Differences in mass loss, colonization-rate, as well as in gene expression between the *R. placenta* MAD-698 and the FPRL280 strains have been observed (Thaler et al., 2012), leading to the assumption that there might be significant differences in either the genome, gene regulation, or in post-transcriptional mechanisms involved in the degradation process. Importantly, these observations raise fundamental concerns about the comparability of results obtained with these two strains.

While the corresponding monokaryotic strain to MAD-698, MAD-SB12, was sequenced more recently (Gaskell et al., 2017), the genome of the monokaryotic *R. placenta* strain FPRL280 has not been analyzed so far. To investigate the degradative capacity of the "European" *R. placenta* strain FPRL280 in comparison with the "American" *R. placenta* strain MAD-698/MAD-SB12, we thus sequenced its genome and performed parallel standardized decay tests, aiming to identify differences between the two strains that might help to explain the observed variances in phenotype. Comparisons of the genomes of different *Rhodonia* strains has to our knowledge not been performed before. In this study, our goal was to deliver solid genomic data on differences between MAD-SB12 and FPRL280 that can be used for further functional analysis. While contributing to a better understanding of the species' lineage and hopefully providing a

reference for future studies using either one of the two strains, the knowledge gained may also be of great interest for new biomass conversion technologies (Mester et al., 2004; Goodell et al., 2008; Schilling et al., 2012).

MATERIALS AND METHODS

Strains

An isolate of *Rhodonia placenta* (Fr.) Niemelä, K.H. Larss. & Schigel (previously *Postia placenta*) FPRL280 was used in this study. MAD-698 is a dikaryotic strain (Martinez et al., 2009) and was used for phenotype tests and phenotype comparisons. MAD-SB12, a monokaryotic strain (Gaskell et al., 2017), was used for a fusion test, mapping of the FPRL280 sequence and for genotype comparisons. FPRL280 is a monokaryotic strain and was used for phenotype tests, sequencing as well as for genotype comparisons.

Phenotype Analysis

Growth Test and Appearance

To investigate growth speed and growth behavior, 4% malt agar plates were inoculated with either *R. placenta* FPRL280 or *R. placenta* MAD-698. Measurement of the growth progress started on day two and the hyphal front was measured daily until the mycelium reached the edges of the plate (8 days). The diameter was measured in two directions, and a mean was calculated. Growth appearance of *R. placenta* strain FPRL280 and MAD-698 was observed and evaluated subjectively, when growing either on 4% malt agar dishes or on wood samples.

Mass Loss Test

The mass loss test was done according to the method presented by Bravery (Bravery, 1979). Sterile nets were placed on 4% malt agar petri dishes and inoculated with either *R. placenta* FPRL280 or *R. placenta* MAD-698. When the plates were completely overgrown, miniblock wood samples (10 mm × 5 mm × 30 mm) of scots pine (*Pinus sylvestris* L.) were placed on top of the nets. Samples were harvested after 1, 2, 3, 4, 5, 6, and 7 weeks of incubation at 22°C and 70% relative humidity, dried and weighted.

Fusion Test

Possible fusion between *R. placenta* strain FPRL280 and strains MAD-698 and MAD-SB12 was tested on 4% malt agar petri dishes.

Genotype Analysis

Sequencing, Mapping, and *de novo* Assembly

Rhodonia placenta (Fr.) FPRL280 was cultivated on 4% malt agar plates for 14 days at room temperature (25°C). The DNA was extracted following the protocol by Traeger et al. (2013) (see Appendix). Library prep was done using the Illumina TruSeq PCR free kit (insert size 350 bp), according to the TruSeq DNA Sample Preparation Guide (Part #15036187, Rev.D, June 2015). The DNA libraries were paired-end (2 × 126) sequenced on an Illumina HiSeq2500 equipment. Paired-end reads were quality-trimmed to remove reads with undefined bases and trim reads

from the 5′ and 3′ends until a base with phred-quality ≥ 10 was reached, with minimum length 60 bp.

The reads were mapped against the dikaryotic *R. placenta* MAD-698 genome, but the described comparison was done with the monokaryotic *R. placenta* MAD-SB12 genome[1] (Gaskell et al., 2017) using Bowtie 2 (Langmead and Salzberg, 2012) version 2.2.6 for comparison, and SAMtools version 1.4 (Li et al., 2009) and BCFtools version 1.4 for variant calling. The reads were also *de novo* assembled using SPAdes (Bankevich et al., 2012) version 3.10. Gene prediction on SPAdes assembly was done with Maker (Cantarel et al., 2008). The following options were set in the CTL file of maker: protein2genome = 1, always_complete = 1. Maker was run with the [-RM_off] option, otherwise default parameters were used. The assembly was annotated based on the combined predicted proteins from the previously published assembly (Martinez et al., 2009; Gaskell et al., 2017). The predicted proteins from the *de novo* annotation were used for prediction of the 11,486 orthologous genes using reciprocal blast analysis and MAD-SB12 (Gaskell et al., 2017) as reference genome.

Annotation of CAZy-Encoding Genes

The public CAZy list determined by Gaskell et al. (2017) with 317 CAZy genes was used to find the corresponding CAZy genes in the *de novo* assembly of FPRL280. The CAZy gene sequences in coding sequences (CDSs) from MAD-SB12 were used to find the corresponding gene sequences within the entire set of CDSs from FPRL280, and only the best hits were selected, based on *e*-value, score and identity (**Supplementary Data Sheet 1**). A total of 298 genes were identified this way and another 16 sequences were when searching against the full *de novo* genome assembly. For four CAZy genes from MAD-SB12 no significant *e*-values could be retrieved by both methods, indicating that these genes do not exist in the FPRL280 *de novo* genome assembly (POSPLADRAFT_1141676, POSPLADRAFT_1141705, POSPLADRAFT_1042744, and POSPLADRAFT_1121407).

Validation of Genome Assembly

Polymerase chain reaction (PCR)-based Sanger sequencing of seven CAZy-encoding genes was performed to validate the differences found in the *de novo* assembled FPRL280 genome compared to MAD-SB12. Primers were designed based on the FPRL280 *de novo* assembly, and the sequences from the PCR product of the gDNA amplification were compared with the published sequence of MAD-SB12 (**Supplementary Table 1** and **Supplementary Figure 2**). Sequences were compared using CLC Genomics Workbench 20.0 (QIAGEN, Aarhus, Denmark). For those genes which were not found in the FPRL280 *de novo* assembly, primers were designed based on MAD-SB12 sequence and used to probe for the corresponding genes in the gDNA of both strains.

The whole genome project has been deposited in the Sequence Read Archive (SRA) of NCBI under accession numbers PRJNA606481 and SAMN14091738 to the sample reads and to the assembly.

---

[1]https://mycocosm.jgi.doe.gov/PosplRSB12_1/PosplRSB12_1.home.html

Phylogenetic Analysis

The species phylogeny was reconstructed using highly conserved gene products of the previously published genome annotations of the species indicated in **Figure 5** (Martinez et al., 2009; Eastwood et al., 2011; Fernandez-Fueyo et al., 2012; Floudas et al., 2012; Olson et al., 2012; Suzuki et al., 2012; Tang et al., 2012; Binder et al., 2013; Ohm et al., 2014; Nagy et al., 2015; Miettinen et al., 2016; Wu et al., 2018; Casado López et al., 2019). BUSCO v2 (dataset "fungi_odb9") was used to select 179 highly conserved proteins for the species phylogeny (Simão et al., 2015). These sequences were concatenated and aligned with MAFFT 7.307 (Katoh and Standley, 2013) and well-aligned regions were identified with Gblocks 0.91b (Talavera and Castresana, 2007). This resulted in 86,531 amino acid positions. RAxML version 8.1.16 was used for the phylogenetic tree reconstruction using the PROTGAMMAWAG similarity matrix and 100 bootstraps (Stamatakis, 2014). The phylogenetic tree was visualized and rooted on the outgroups *S. lacrymans* and *H. annosum* using Dendroscope (Huson and Scornavacca, 2012).

RESULTS

Phenotypic Comparison Between FPRL280 and MAD-698

When incubated on malt extract agar plates, MAD-698 was found to grow faster during the first 5 days, whereas FPRL280 started its growth delayed, but accelerated and caught up with MAD-698 between days 5 and 6 (**Figure 1**).

Differences were also observed in the general appearance, when growing on petri dishes with 4% malt agar. MAD-698 grew more multidirectional than FPRL280, which also grew closer to the surface of the medium, directed to the edges of the plates (**Figures 2**, **4**). The mycelium of MAD-698 produced more aerial hyphae growing upwards, giving the mycelium a more voluminous appearance (**Figures 2**, **4**). When growing on
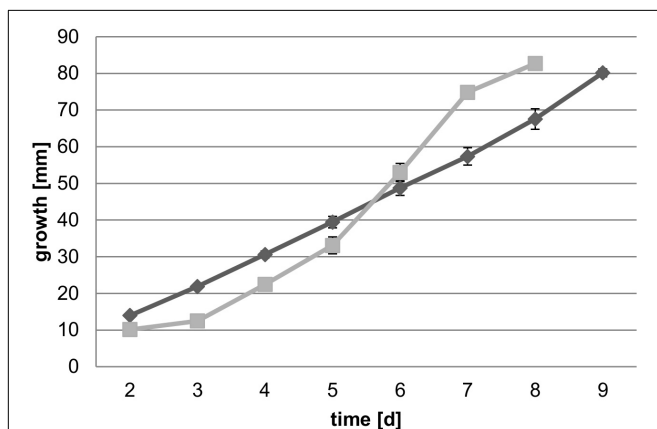
FIGURE 1 | Growth speed of both strains on 4% malt agar plates over 9 days (*n* = 5). Average daily growth rates of FPRL280 (gray squares) and MAD-698 (black diamonds) are shown with standard deviations in the graph and imply that MAD is starting growth earlier and grows more evenly than FPRL.

wood samples, there were also differences between the two fungi. MAD-698 clearly produced more mycelium during the 8-week degradation test (**Figure 2**).

To determine differences in wood degradation capacity a mass loss test on wooden miniblock samples was performed over 7 weeks (**Figure 3**). Despite showing a delayed growth phenotype on malt agar, FPRL280 started degrading the wood earlier than MAD-698 but then slowed down. After 6 and 7 weeks, mass loss by MAD-698 was significantly stronger, suggesting that MAD-698 is degrading the wood in a more aggressive manner than FPRL280 in the long term.

Fusion Test

To test whether FPRL280 and MAD-698 are able to fuse, the strains were confronted with each other on the same plate. A border was immediately built up between the two *R. placenta* strains when the mycelia met, which both fungi did not overgrow (**Figure 4A**). The same was observed for FPRL280 and the corresponding monokaryotic strain of MAD, MAD-SB12 (**Supplementary Figure 3**). Further days of investigation showed that even on the edges of the plates, the fungi grew upwards, but not over the demarcation line. After several weeks, there was a broad band of mycelium looking like a wall built up between the two strains (**Figure 4B**).

Sequencing of FPRL280 and Comparison With MAD-SB12

Next, we sequenced the genome of *R. placenta* strain FPRL280. Since the genome assembly of strain MAD-698 is much more fragmented and gap-rich than the more recent MAD-SB12 assembly, mapping rates of the genome reads of FPRL280 were much lower, reducing the quality of downstream variant analysis (**Table 1**). We therefore decided to focus our genomic comparison on the monokaryotic strain MAD-SB12 and a *de novo* assembly. *In* the *de novo* genome assembly, 12,997 genes were predicted for FPRL280, which is about the same number as reported for MAD-SB12 (12,541), 11,486 of these being putative orthologs (**Supplementary Data Sheet 1** and **Table 2**).

A comparison with the MAD-SB12 genome showed an overall identity of 98.4% with 660,566 SNPs and 25,837 indels, translating roughly into one difference per 60 basepairs on average (**Table 3** and **Supplementary Data Sheet 2**). In addition, precise classification is presented in **Supplementary Data Sheet 2**, showing the affected genes, changes in amino acid sequences, the position and the kind of variance. An overview on how the SNPs are distributed over introns, exons, UTRs, or if they occur in intergenic regions can be found in **Supplementary Figure 4**. A total of 686,402 variants were detected within the whole genome (introns included), 16,299 variants (2.37%) residing in CAZy genes. A phylogenetic tree based on 179 highly conserved gene products shows that both *R. placenta* strains are closely related. The evolutionary distance between them is similar to the distance between strains of *Lentinus tigrinus* and *Dichomitus squalens* (**Figure 5**).

Next, we compared all detected variants between FPRL280 and MAD-SB12 vs. variants that are present in predicted CAZyme genes (**Table 4** and **Supplementary Data Sheet 3**). **Table 4**

FIGURE 2 | Wood degradation test to observe appearance of both strains (FPRL280 and MAD-698) when growing on miniblock wood samples. **(A–D)** Show growth of FPRL280 after 2–8 weeks. **(E–H)** Show growth of MAD-698 after 2–8 weeks. Clear differences can be seen in these pictures, not only in appearance of the mycelium, but also in growth speed and mycelium production.

FIGURE 3 | Mass loss of pine wood samples over time when incubated with FPRL280 (gray) and MAD-698 (black). Data show means of biological ($n = 4$) and technical replicates ($n = 8$) with standard deviations, showing that MAD is causing a higher mass loss than FPRL after 6 weeks. *T*-test was used to determine the significance, with $p < 0.05$. "*" means significant differenced between the strains ($p < 0.05$).

presents results for the variant analysis between FPRL280 and MAD-SB12 as well as the number of coding sequences (CDS) and CAZy genes being affected. Within the CDS, we found a total of 10,027 genes with amino acid changes (AACs), of which 69% had between one and ten AACs and 1.2% having more than 50 AACs. Of all CAZy-encoding genes, almost all (92.43%) had at least one AAC, including 46 AAs, 136 GHs and 24 CBMs. Additionally, 17 genes with deletions, 30 with insertions and 20 frame shifts through indels were detected within the CAZy genes. Thirty-three CAZy genes were identified that had variants in consensus splice sites (VCSSs), the largest group here being the AA3_2 subfamily with eight genes and the GH5_5 subfamily with two genes. Overall, insertions appeared in 9.46% and deletions

in 5.36% of all CAZy genes. Summarized results of the variant analysis, considering the sizes of the CAZy subfamilies, can be seen in **Figures 6A,B**. For an overview of variants in all CAZy families see **Supplementary Figure 1**.

Of all genes having AACs, 162 genes (1.67%) had $\geq$ 50 instances and 17 (0.18%) even $\geq$ 100 (**Figure 7**). Genes with 50 and more AACs were looked at in more detail, which showed that 23 of these were affected over more than 10% of their length, 107 genes were affected between 5 and 10% and 29 genes $\leq$ 5%. The GO terms regarding the annotated molecular functions of the genes were investigated and grouped. Proteins with associated functions that might contribute the observed phenotypic differences were present in this group. For example, 15 genes had RNA-, DNA or nucleic acid binding functions, based on UniProt search of the respective *R. placenta* MAD-SB12 homologs, or WD-repeat regions involved in a wide range of protein-protein interactions in signal transduction processes, cell division, RNA processing and so on (Yu et al., 2000; Smith, 2008; Stirnimann et al., 2010). The list furthermore was enriched for F-box-domain containing proteins (further referred to as F-box proteins; eight of a total of 164 predicted in the genome; *p*-value $< 5 \times 10^{-3}$), and showed an overrepresentation of proteins with kinase function (15 out of a predicted total of 398; *p*-value $< 5 \times 10^{-4}$). Further alignment of the protein sequences showed that the domains of two F-box proteins, four protein kinases as well as two WD-repeats regions are heavily affected (**Table 5**). Screening all variants showed four genes which had additional variants besides AACs: FPRL280_424_1 (POSPLADRAFT_1046092) had three VCSSs as well as a one-base insertion leading to a frameshift; FPRL280_272 (POSPLADRAFT_1065282) had an additional VCSS, FPRL280_188_6 (POSPLADRAFT_1035374) showed one five-base, one one-base and one two-base deletion, leading to frameshifts, three one-base and two four-base insertions, also leading to frameshifts as well as one VCSS. Further
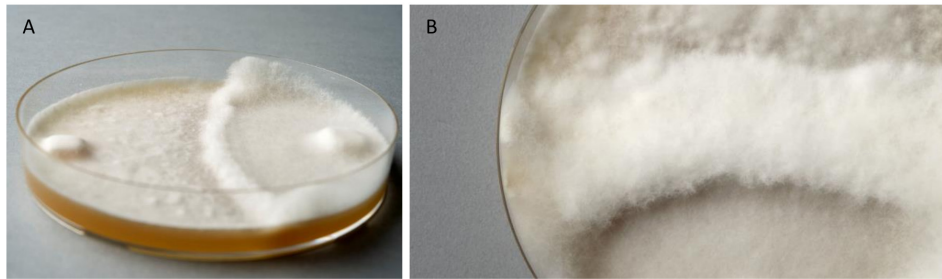
FIGURE 4 | Fusion test of the two *Rhodonia placenta* strains growing on one plate. **(A)** FPRL280 (left side) and MAD-698 (right side), 2 weeks after incubation, clearly showing the formation of a barrier, which is not overgrown. **(B)** The barrier got thicker and neither of the fungi overgrew it.

FPRL280_104_5 (POSPLADRAFT_1046501) had additionally two VCSSs and a three-base insertion, not leading to a frameshift.

Identification and Validation of Variants in CAZyme-Encoding Genes

Genomic regions of selected genes from GH31, AA3_2, GH16, CBM18, GH5, and CE15 family members were amplified and sequenced to verify the observed variances in the FPRL280 genome (**Supplementary Data Sheet 4**). All mentioned genes are consecutively numbered and shown in **Table 6** with

TABLE 1 | Comparison of genome assemblies for MAD-698 (Martinez et al., 2009), MAD-SB12 (Gaskell et al., 2017), and FPRL280.

	MAD-698 assembly	SB12 assembly	FPRL280
Assembly size (Mb)	90.9	42.5	36.3
No. of scaffolds	1243	549	1848
No. of gaps within scaffolds	10184	897	192
Total size of gaps (Mb)	21.9	2.6	0.007
% of scaffold length in gaps	24.1	6.1	0.02
Mapping rate of FPRL280 reads (%)	64	80	NA

TABLE 2 | Assembly and annotation features for *R. placenta* FPRL280.

Feature	Value
Genome assembly size (Mbp)	39
No. of contigs	3,158
No. of scaffolds	2,948
No. of scaffolds ≥ 1000 bp	1,848
Scaffold N50	74 kb
Gene models	12,997

TABLE 3 | Overview of the variant analysis between FPRL280 and MAD-SB12 genomes.

	All variants	Variants in CAZy genes
Total no. of variants	686, 403 (100%)	16, 299 (2.37%)
SNPs	660, 566 (96.24%)	15, 888 (2.40%)
Indels	25, 837 (3.76%)	411 (1.61%)

corresponding IDs for MAD-SB12 and FPRL280, as well as the predicted effects of the variants. The regions tested by PCR amplification are indicated in **Figure 8**.

Within the GH31 domain of gene FPRL280_88_4, as well as in the cellulose domain of gene FPRL280_14_15 several SNPs and AACs could be confirmed (**Figures 8A,B**). Thirteen SNPs were verified to exist in gene FPRL280_46_15, a predicted oxidoreductase (AA3_2; PFAM00732/PFAM05199) with putative cellobiose dehydrogenase or alcohol oxidase activity (EC 1.1.99.18 or EC 1.1.3.13), in a small region of the domain (**Figure 7C**) and the RicinB_lectin domain (PFAM14200) of gene FPRL280_3_68 (**Figure 7D**). High number of variants were furthermore detected in gene FPRL280_327_3, with predicted 4-O-methyl-glucuronoyl methylesterase activity (EC 3.1.1) in a region close to the functional domain (**Figure 7E**), predict to allow the frameshift.

In addition to the selected genes shown in **Figure 7**, it was possible to confirm SNPs (including two AACs) within CBM13 domain-containing gene, and further AACs in CBM18, CBM21, CBM48, and CBM50 genes (**Table 5** and **Supplementary Data Sheet 3**). Other CBM13-encoding genes (FPRL280_235_10 and FPRL280_174_14) presented variances in consensus splice sites and insertions/deletions leading to putative frameshifts. Mutations leading to frameshifts were also detected in genes belonging to the CAZy families CBM21 and CBM50, as well as members of GH95, GH31, GH30_3, GH71, GH47, GH79, GH18, and GH16 (**Table 5** and **Supplementary Data Sheet 3**). Fourteen GH families were affected by mutations within predicted consensus splice sites: GH10; GH12; GH128; GH16; GH2; GH28; GH3; GH30_3; GH47; GH5_5; GH5_9; GH55; GH92; GH95 (**Table 5**).

Four genes (POSPLADRAFT_1141676, POSPLADRAFT_11 41705, POSPLADRAFT_1042744, and POSPLADRAFT_112 1407) present in the MAD-SB12 genome could not be detected in or amplified from the FPRL280 genome. All four genes belong to the CAZy subfamily AA3_2 (**Supplementary Figure 2**).

DISCUSSION

In the present study, we performed a direct comparison of both phenotype and genotype of two commonly used strains of *R. placenta*, FPRL280 and MAD-698 (or MAD-SB12,

FIGURE 5 | Phylogenetic tree of members of the order Polyporales based on 179 highly conserved proteins. *S. lacrymans* (order Russulales) and *H. annosum* (order Boletales) were used as outgroup to root the tree. The strains of *R. placenta* cluster closely together, as expected for closely related strains (orange box).
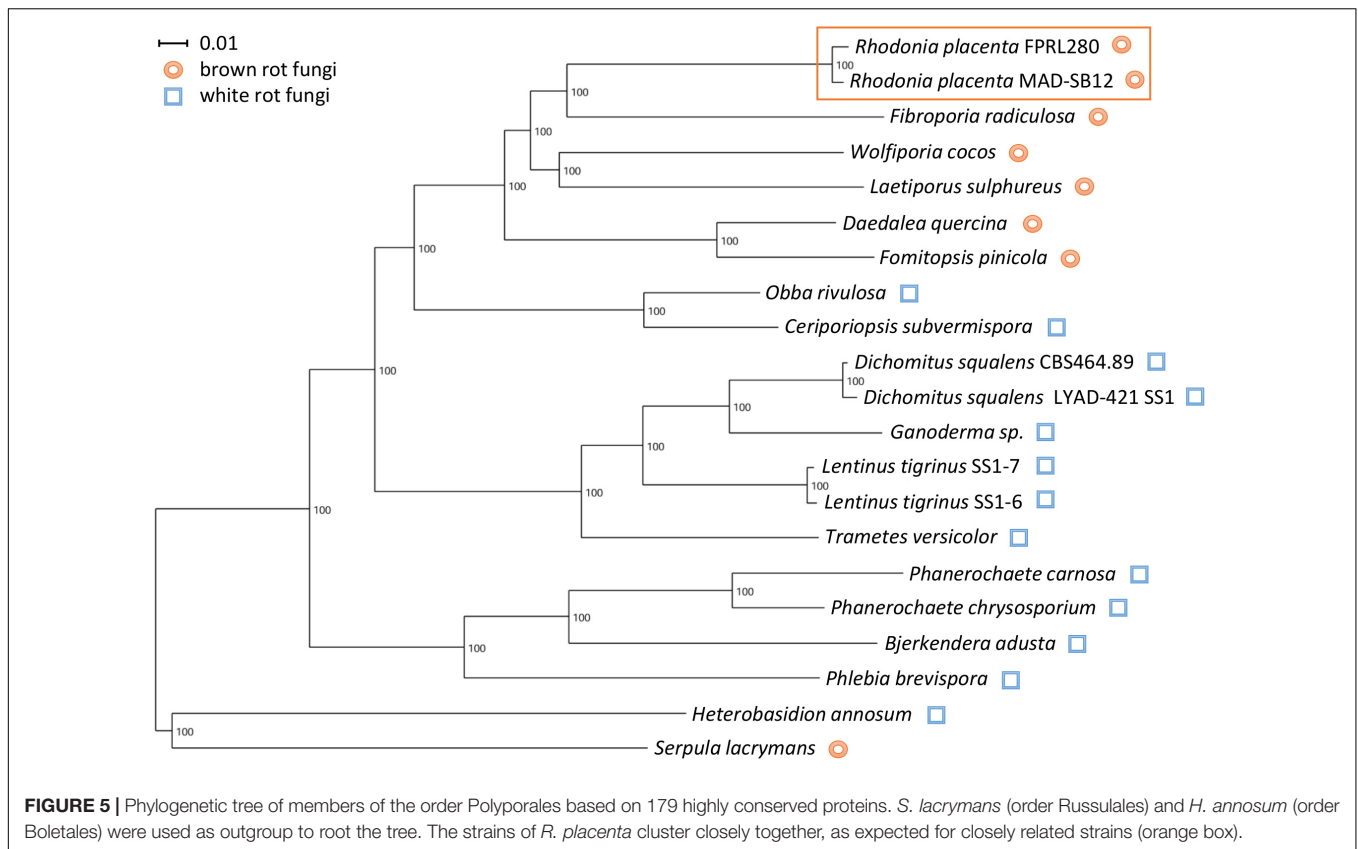
TABLE 4 | Detailed variant analysis and number of affected genes with corresponding percentage according to total number of CDS genes (all variants) or total number of CAZy genes (variants in CAZy genes).

	All variants		Variants in CAZy genes	
	Total No. of variants	No. of genes	Total No. of variants	No. of genes
AACs (in CDS)	115,302	10, 027 (79.95%)	3,301	293 (92.43%)
Deletions (in CDS)	1,177	929 (7.41%)	20	17 (5.36%)
Insertions (in CDS)	2,123	1, 233 (9.83%)	48	30 (9.48%)
Frameshifts through indels	1,499	954 (7.61%)	25	20 (7.89%)
VCSSs (in CDS)	1,194	984 (7.85%)	38	33 (10.41%)

respectively). We chose to directly compare the phenotypes of MAD-698 (a dikaryon) and FPRL280 (which, suggested by the genome assembly and by absence of microscopically visible clamps, is a monokaryon), since these are the two strains being used in laboratories in the US and Europe for standardized decay test of wood products (AWPA E10-16, 2016). However, for the genome comparison with FPRL280, we chose to use the monokaryotic MAD-SB12 genome. The genome size determined for FPRL280 was much closer to that of MAD-SB12 and mapping efficiencies were substantially higher (**Table 1**). Since the genome of MAD-SB12 was isolated from a basidiospore of the fruiting dikaryon MAD-698, the genome is as close to the formely reported genome of MAD-698 as possible (Gaskell et al., 2017).

Differences in growth behavior, wood decomposition effectiveness and mycelial appearance were observed and quantified as well as vegetative incompatibility between the

strains. Since it has been shown that monokaryons and dikaryons of *Trametes versicolor* have similar decay rate, combative ability and ligninolytic enzymes production (Hiscox et al., 2010), we assume that this does not account as a major influencing factor in *R. placenta* as well. While the observed vegetative incompatibility might have been caused by the different nuclear status nevertheless, this behavior was also seen for FPRL280 and MAD-SB12 (the respective monokaryon; **Supplementary Figure 3**), which indicates that the incompatibility depends on genetic differences rather than on differences in nuclear status.

One to two percent of the gene products in eukaryotic organisms belong to the GT family (Lairson et al., 2008), which are necessary, among others, for the biosynthesis of the fungal cell wall (Klutts et al., 2006). GTs are enzymes that utilize an activated sugar substrate, containing a phosphate leaving group (Lairson et al., 2008) and catalyze the transfer of sugar moieties from

FIGURE 6 | CAZy classification. **(A)** Variant analysis of CAZy genes of FPRL280 compared to MAD-SB12 (only CDS). The graphic only shows CAZy families mentioned in the text, a graphic with results for all CAZy families can be found as **Supplementary Figure 1**. The blue bar shows the number of CAZy genes with variants within the CAZy subfamilies. The green line shows the number of all variants occurring in the CAZy subfamilies. The red line shows the variances within the CAZy subfamilies normalized to their size. **(B)** Entire classification of CAZy-encoding genes in both genomes, highlighting class AA, with the subfamily AA3_2, in which many variants were found and four genes were missing in FPRL280 vs. MAD-SB12 (red line pattern). The piecharts contain CAZy families AA, CBM, CE, GH, GT, and PL, as well as expansins (EXP).

donor molecules to specific acceptors, leading to the formation of glycosidic bonds (Campbell et al., 1997; Coutinho et al., 2003). Five putative GT genes were found having AACs in FPRL280. Potentially therefore, these mutations could be part of the reason why the MAD-698 mycelia have a different appearance than FPRL280. However, since the GTs include 110 families and we don't know which effects the VCSS have on the enzymes (Lairson et al., 2008), this remains speculative until further analysis.

The differences in phenotype make the comparison interesting both in terms of genomic research and industrial applications. Mutations in important genes are one explanation for the differences seen in phenotype between the two species, but also regulatory mechanisms are likely having a powerful impact. The two genomes are closely related with a total identity of 98.4% and as confirmed by a phylogenetic analysis (**Figure 5**). The results of the variant analysis nevertheless showed a high number of

FIGURE 7 | All genes with amino acid changes (AACs) after comparing MAD-SB12 and FPRL280, according to amount of AACs (*y*-axis) and number of genes with corresponding percentage (*x*-axis).
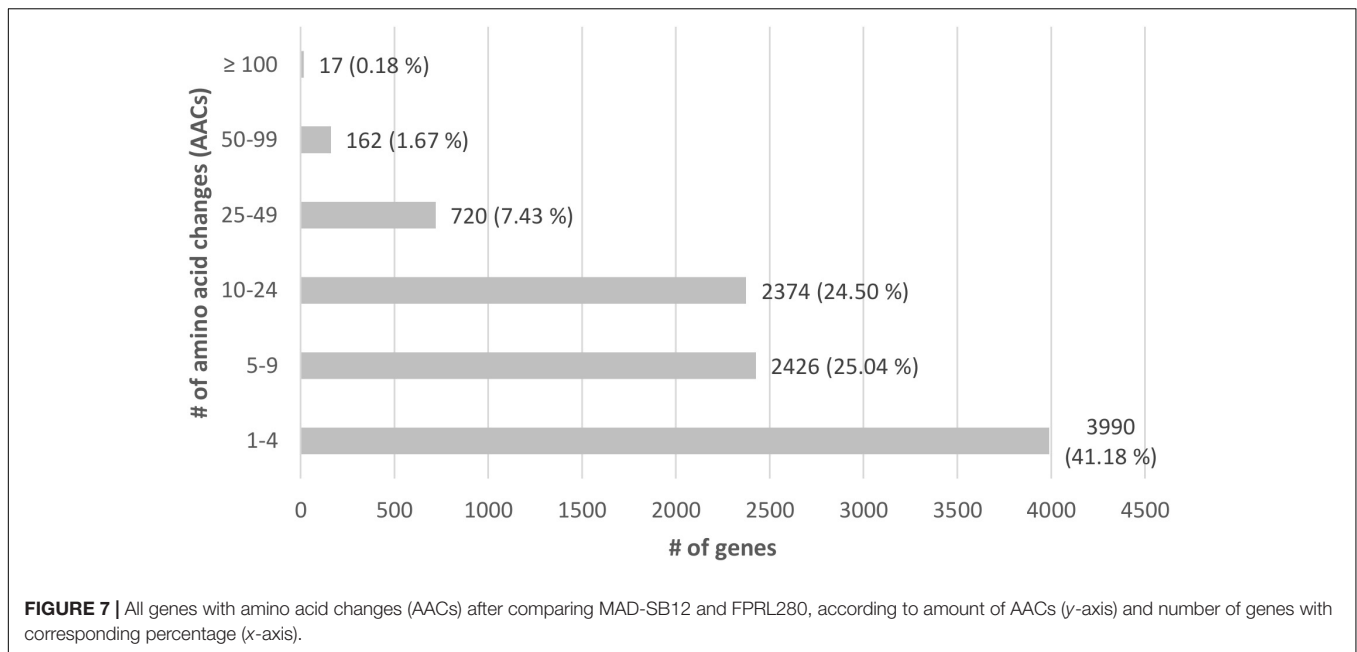
TABLE 5 | Genes with ≥ 50 AACs, which were looked at in more detail due to their predicted effect on the phenotype.

Protein	Affected genes	Avg. degree of affection (% of the gene length)	No. of AACs (Avg.)
F-Box protein	8	10.24	69.25
Kinases	15	6.13	62.4
WD repeats	3	7.75	107.7
DNA binding	7	4.87	56.43
Nucleic acid binding	4	5.92	71
RNA binding	4	5.01	66

The table includes the percentage of bases affected, relative to the total protein length and the number of AACs, as well as the protein.

potentially impactful differences between the two strains. For example, amino acid changes (AAC) were found in 92% of all predicted CAZy genes, as well as deletions in 5% and insertions in 9%. Moreover, variants in consensus splicing sites (VCSS) were found in 10% of all CAZYs. Overall, a higher percentage of AACs and VCSSs were found in CAZy genes compared to the entire complement of CDSs (**Table 2**), often affecting structural domains. We also found that four genes belonging to the CAZy family AA3_2 could not be detected in the FPRL280 genome, potentially explaining part of the observed differences regarding wood decay rates (see also below).

Brown rot fungi possess less GHs than white-rot fungi, but they seem to compensate this by secreting higher amounts of their remaining GHs (Presley et al., 2018). Brown rot fungi generally lack processive cellobiohydrolases and instead rely more on endoglucanases, which are thought to cleave cellulose randomly (Eriksson et al., 1990; Vanden Wymelenberg et al., 2010). Putative endo-acting cellulases belong to the CAZy families GH5 and GH12 (Ryu et al., 2011; Floudas et al., 2012;

Lombard et al., 2013). Zhang et al. (2016) found only three endoglucanases (GH5 and GH12) and one putative endoglucanase (GH12) in *R. placenta*. We found mutations in the well-characterized endo-1,4-β-D-glucanase PpCel5A (FPRL280_14_15; Ppl1| 115648; POSPLADRAFT_1164613; XP_024344095) belonging to the GH5 family and including the endo-1,4-β-glucanase FPRL280_142_19 (Ppl1| 52805/Ppl1| 112669; POSPLADRAFT_1050186; XP_024333913) belonging to the GH12 family in FPRL280. Mutations were also found in several putative hemicellulases, such as the β-mannosidase FPRL280_NODE_261 (Ppl1| 57564; POSPLADRAFT_1043339; XP_024342514), the α-1,2-mannosidase FPRL280_294_2 (Ppl1| 62385; POSPLADRAFT_1043572; XP_024342867) and the β-xylosidase FPRL280_9_74 (Ppl1| 127469; POSPLADRAFT_1069652; XP_024341044). In addition, we found that several putative β-glucosidase-encoding genes in FPRL280 have mutations (**Supplementary Data Sheet 3**).

Hemicellulases can be found in several GH families and are often co-operating to break down complex hemicelluloses (Ryu et al., 2011; Brigham et al., 2018). Moreover, β-glucosidases are relatively non-specific in brown rot fungi (Herr et al., 1978; Valášková and Baldrian, 2006). It could be hypothesized that the detected mutations in hemicellulases and β-glucosidases will not affect the decay capability of FPRL280 as much as mutations in the endoglucanases since several β-glucosidases have been found in *R. placenta* (Martinez et al., 2009) and only three to four endoglucanases (Zhang et al., 2016). The sequence variances seen in GH family enzymes, in particularly the endoglucanases, could thus be one part of a possible explanation to the overall lower decomposition rate by FPRL280 compared to MAD-698, since these enzymes are responsible for the depolymerization of the structural carbohydrates in the wood cell wall (Ryu et al., 2011; Floudas et al., 2012; Lombard et al., 2013).
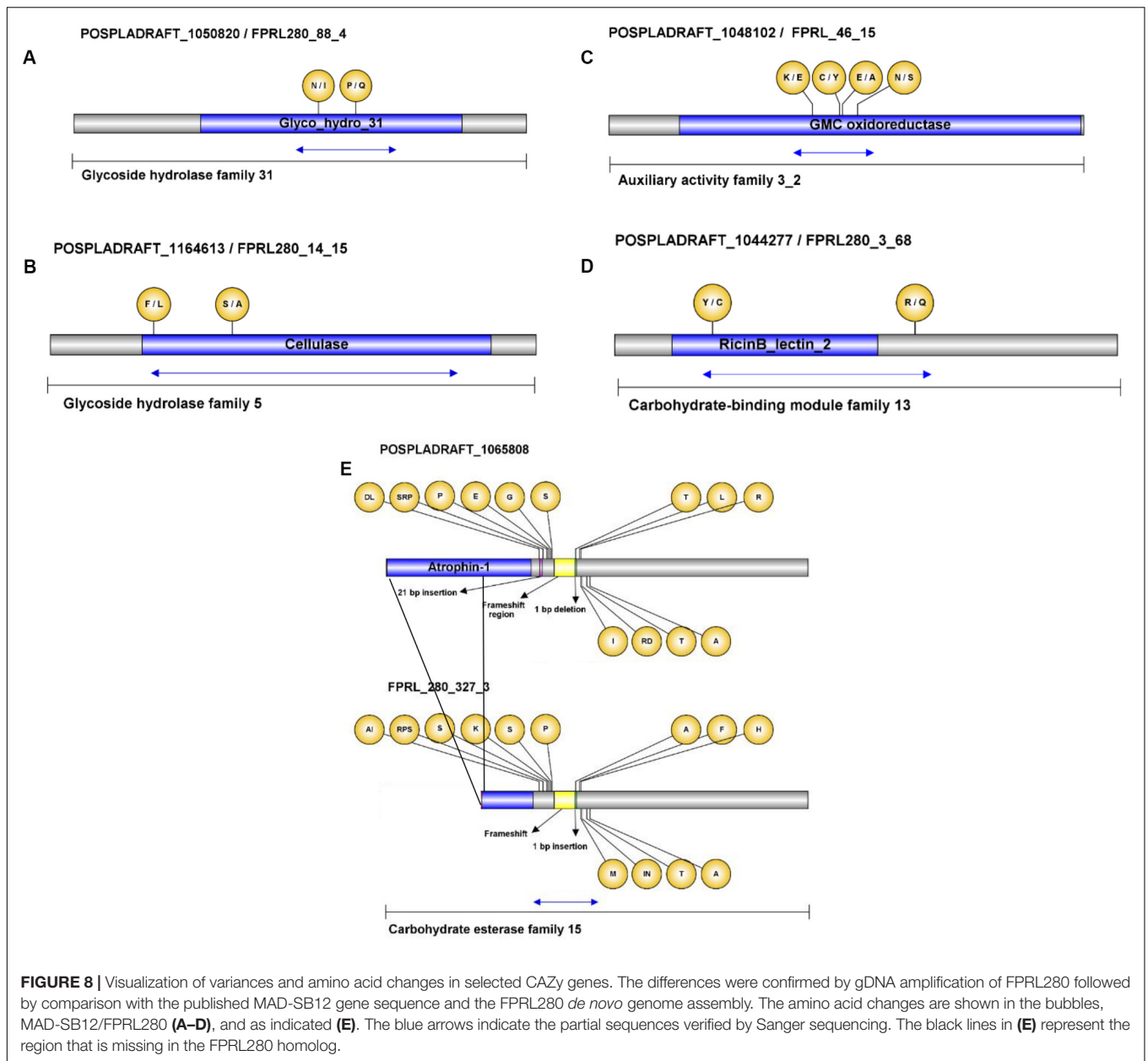
TABLE 6 | All genes mentioned in the results are numbered with both IDs (MAD-SB12 and FPRL280), the CAZy family these genes belong to and the predicted effects of variances, causing AACs, VCSSs or frameshifts.

ID MAD-SB12 "POSPLADRAFT_"	ID FPRL280 "FPRL280_"	CAZy family	Predicted effect
1050820	88_4	GH31	AAC (positions 480 and 553) in coding protein XP_024333100.1, mutations, leading to frameshifts
1164613	14_15	GH5_5	AAC (positions 72 and 126) in protein XP_024344095.1, VCSS
1048102	46_15	AA3_2	4 AACs (positions 272, 308, 312, and 332) in protein XM_024479440.1
1044277	3_68	CBM13	AAC (positions 30 and 90) in protein XP_024341629.1
1065808	327_3	CE15	Mutations, leading to frameshifts
1046599	156_2	GH95	Mutations, leading to frameshifts, VCSS
1057601	47_16	GH30_3	Mutations, leading to frameshifts, VCSS
1066962	238_7	GH71	Mutations, leading to frameshifts, deletion (4 bases) in EC 3.2.1.59
1138061	218_2	GH47	Mutations, leading to frameshifts, insertion (8 bases) and deletion (4 bases) in enzyme-encoding region (EC 3.2.1.113); VCSS
1155254	318_2	GH79	Mutations, leading to frameshifts
1168110	279_6	GH18	Mutations, leading to frameshifts
1181115	252_4	GH16	Mutations, leading to frameshifts
1064814	264_7	GH10	VCSS
1050186	142_19	GH12	VCSS
1183855	4_136	GH128	VCSS
1142572	640_1	GH16	VCSS
1043339	NODE_261	GH2	VCSS
1049546	348_3	GH28	VCSS
1174812	49_4	GH3	VCSS
1042537	14_27	GH5_5	VCSS
1181612	432_1	GH5_9	VCSS
1131418	269_3	GH55	VCSS
1043572	294_2	GH92	VCSS

During early stages of brown rot decay, CEs are required for hemicellulose removal, for example to remove acetyl groups from xylan in hardwoods (Cowling, 1961; Puls, 1997). Presley et al. (2018) found an increased spectrum of genes attributed to CEs in brown rot secretomes, particularly in early stages of decay. In one protein putatively belonging to the CE15 family (POSPLADRAFT_1065808/FPRL280_327_2; XP_024339306) we found a high number of AACs in the FPRL280 ortholog compared to MAD-SB12 and VCSSs. These mutations might affect the hemicellulose depolymerization in FPRL280 negatively.

As mentioned above, brown rot fungi rely on non-enzymatic break-down of lignocellulose using low molecular weight compounds, such as H_2O_2, Fe^{2+} and oxalate for Fenton chemistry (Goodell et al., 1997; Arantes et al., 2009; Arantes and Goodell, 2014), for an efficient lignocellulose degradation. The genomes of brown rot fungi suggest the presence of a number of AA enzymes that are known to generate H_2O_2. Among these are AA3 GMC oxidoreductases and AA5 copper radical oxidases (Floudas et al., 2012; Levasseur et al., 2013). AA3 are a family of flavoenzymes that oxidize aliphatic alcohols, aryl alcohols and mono- and disaccharides. This oxidation is coupled with the reduction of a variety of electron acceptors, including

O_2 (resulting in the formation of H_2O_2), quinones and other enzymes. Enzymes belonging to the AA3_2 subfamily include two closely related FAD-dependent enzymes, aryl-alcohol oxidase and glucose-1-oxidase (Levasseur et al., 2013; Sützl et al., 2018). Flavoproteins, such as GMC oxidoreductases, form the base of a wide array of biological processes, for example removal of radicals, which contribute to oxidative stress adaptation. The CAZy group with the highest number of variances in FPRL280 was the AA3_2 subfamily. We found eight putative genes with VCSS in the FPRL280 genome. In addition to this, four AA3_2-encoding genes were missing in the FPRL280 genome. Taking into account the suggested importance of hydroquinones for the H_2O_2 production, mutations in AA3_2 proteins, or the absence of entire proteins, could thus have severe effects on the degradation capacity of FPRL280. Enzymes belonging to the AA5 family are copper radical oxidases and are known to be a major constituent of the secretome of several brown rot fungi (Kersten and Cullen, 2014). AA5s oxidize a variety of substrates resulting in the production of H_2O_2 via the reduction of O_2 (Jensen et al., 2002). The AA5 family includes two subfamilies, AA5_1 containing characterized glyoxal oxidase and AA5_2 containing galactose oxidase, raffinose oxidase and alcohol oxidase enzymes (Ito et al., 1991). In a copper radical oxidase (Ppl1| 56703

FIGURE 8 | Visualization of variances and amino acid changes in selected CAZy genes. The differences were confirmed by gDNA amplification of FPRL280 followed by comparison with the published MAD-SB12 gene sequence and the FPRL280 *de novo* genome assembly. The amino acid changes are shown in the bubbles, MAD-SB12/FPRL280 **(A–D)**, and as indicated **(E)**. The blue arrows indicate the partial sequences verified by Sanger sequencing. The black lines in **(E)** represent the region that is missing in the FPRL280 homolog.

POSPLADRAFT_1046361/FPRL280_259_1;   XP_024339806) belonging to the subfamily AA5_2, we found VCSS. The fact that four AA3_2 genes are missing in FPRL280 and that parts of the domains are missing in enzymes in both AA3_2 and AA5_2, in addition to high numbers of gaps and point mutations, also in VCSS, might lead to changes with possible effects on the protein functions. Since members of the AA3 family do not directly act on polymeric constituents of lignocellulosic material, but support degradation by reducing low-molecular weight components, with one of the main products being H_2O_2 (Sützl et al., 2018), changes in AA3_2 genes could affect this support substantially. The effect of this might be a less effective Fenton reaction in FPRL280 compared to MAD-698, which could explain the phenotypical findings that MAD-698 is more potent

during prolonged decay, producing higher mass losses than FPRL280. Brown rot fungi have most likely a fine-tuned complex network of gene products working together to deliver radicals via the Fenton reaction. Perhaps even small changes in these gene products could lead to measurable effects. Whether this is indeed the case, however, needs to be verified by future experiments.

A proportionally large part of the variances found in FPRL280 are located in regulatory genes which could have a huge impact on the phenotype far beyond the mere differences of the two genomes.

Nucleic acid binding proteins, for example, are important factors involved in gene expression (Latchman, 1997). Zinc-finger proteins belonging to this group can act as transcription factors and form one of the largest families if transcriptional

regulators in eukaryotes, with an enormous functional diversity (Miller et al., 1985; MacPherson et al., 2006). One representative (POSPLADRAFT_1155119/FPRL280_66_2) was found to be affected by more than 50 AACs in this study. Mutations in genes coding for these proteins are therefore likely to contribute to the overall complexity of the phenotypes of both strains, since their functional differences will affect entire downstream regulons.

Kinases, mediating phosphorylation reactions of proteins and other cellular constituents, are another example of important regulatory proteins involved in many signaling cascades. Within the pool of proteins found to have > 50 AACs between FPRL280 and MAD-SB12, kinases were found to be overrepresented (p-value $< 5 \times 10^{-4}$) indicating that the function of several signaling pathways might be affected. However, the diversity of kinases is immense, and future efforts are necessary to identify exactly which pathways these are.

F-box motifs, also found to be overrepresented (p-value $< 5 \times 10^{-3}$), function as a site of protein-protein interaction and the respective proteins are key factors involved in protein ubiquination proteasomal degradation (Jonkers and Rep, 2009). As part of the Skp, Cullin, F-box containing complex (SCF complex; a multi-protein E3 ubiquitin ligase), F-box proteins have several target proteins, which suggests that mutations may also have a pleiotropic effect on the phenotype. In filamentous fungi, F-box proteins can be involved in several cellular processes, including control of the cell division cycle, sugar sensing, mitochondrial connectivity, and control of the circadian clock (Jonkers and Rep, 2009). The proteolytic function of the ubiquitin-proteasome system is furthermore important for virulence regulation in pathogenic fungi (Liu and Xue, 2011) and the variations in F-box domains in some proteins in FPRL280 might thus contribute to the lower virulence seen in FPRL280 compared to MAD-698.

Besides AACs, frame shift mutations and VCSSs can potentially have even more drastic effects on the amino acid sequences of proteins. However, for most CAZy genes we looked at in more detail, there variances did not affect the conserved functional domains. Moreover, splicing can be better observed by a transcriptional analysis. This is currently ongoing, including a gene expression regulation analysis, and will be part of another manuscript.

We limited the scope of this paper to a comparative genome study, since we wanted to highlight the phenotypic differences between the two *R. placenta* strains and the underlying genomes. A careful genomics analysis is very important, as it forms the basis for further research. However, genome analysis can clearly explain the differences between the strains only partially, and functional genomics studies, including transcription, translation, gene regulation and protein-protein-interactions, need to be included in the future for a more profound comparison.

CONCLUSION

The initial reason for comparing the two strains was to gain insight into the genetic differences between two economically relevant strains of *R. placenta* (FPRL280 and MAD-698) showing different phenotypes. The specific mutations discussed in this paper might contribute to these observed differences as they are found in relevant domains of many potentially important genes especially regulatory genes, and thus might affect the function of the respective proteins. However, with 98.4% overall identity, the genomic variances cannot explain all observations. Differences in regulatory mechanisms (signaling cascades etc.) are likely also present and impactful, and therefore need to be further investigated.

The results from this paper show the importance of a united strain selection of decay fungi in standardized decay tests. Two strains of one species can behave differently even though the genomes appear similar. The investigation of the reasons that make these two strains distinct is useful for the understanding of the degradation mechanisms employed by brown rot fungi and the development of this important brown rot reference species.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the NCBI databank under BioProject number PRJNA606481, and BioSample SAMN14091738.

AUTHOR CONTRIBUTIONS

AP, MK, JB, and MH initiated and designed the research. MK, MN, RO, and MH performed the analyses. AP, MK, JB, and MH co-wrote the manuscript with support of MN and RO. All authors were included in the interpretation of the data, read and approved the final manuscript.

FUNDING

ACKNOWLEDGMENTS

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb. 2020.01338/full#supplementary-material

REFERENCES

Alfredsen, G., Fossdal, C. G., Nahy, N. E., Jellison, J., and Goodell, B. (2016a). Furfurylated wood: impact on *Postia placenta* gene expression and oxalate crystal formation. *Holzforschung* 70:947. doi: 10.1515/hf-2015-0203

Alfredsen, G., Pilgård, A., and Fossdal, C. G. (2016b). Characterisation of *Postia placenta* colonisation during 36 weeks in acetylated southern yellow pine sapwood at three acetylation levels including genomic DNA and gene expression quantification of the fungus. *Holzforschung* 70, 1055–1065. doi: 10.1515/hf-2016-0009

Arantes, V., and Goodell, B. (2014). "Current understanding of brown-rot fungal biodegradation mechanisms: a review," in *Deterioration and Protection of Sustainable Biomaterials*, eds T. P. Schultz, B. Goodell, and D. D. Nicholas (Washington, DC: American Chemical Society), 3–21. doi: 10.1021/bk-2014-1158.ch001

Arantes, V., Jellison, J., and Goodell, B. (2012). Peculiarities of brown-rot fungi and biochemical Fenton reaction with regard to their potential as a model for bioprocessing biomass. *Appl. Microbiol. Biotechnol.* 94, 323–338. doi: 10.1007/s00253-012-3954-y

Arantes, V., Milagres, A. M. F., Filley, T. R., and Goodell, B. (2011). Lignocellulosic polysaccharides and lignin degradation by wood decay fungi: the relevance of nonenzymatic Fenton-based reactions. *J. Ind. Microbiol. Biotechnol.* 38, 541–555. doi: 10.1007/s10295-010-0798-2

Arantes, V., Qian, Y., Milagres, A. M. F., Jellison, J., and Goodell, B. (2009). Effect of pH and oxalic acid on the reduction of Fe3+ by a biomimetic chelator and on Fe3+ desorption/adsorption onto wood: implications for brown-rot decay. *Int. Biodeterior. Biodegradation* 63, 478–483. doi: 10.1016/j.ibiod.2009.01.004

AWPA E10-16 (2016). *Laboratory Method for Evaluating the Decay Resistance of Wood-Based Materials Against Pure Basidiomycete Cultures: Soil/Block Test*. Birmingham, AL: AWPA.

Baldrian, P., and Valášková, V. (2008). Degradation of cellulose by basidiomycetous fungi. *FEMS Microbiol. Rev.* 32, 501–521. doi: 10.1111/j.1574-6976.2008.00106.x

Bankevich, A., Nurk, S., Antipov, D., Gurevich, A. A., Dvorkin, M., Kulikov, A. S., et al. (2012). SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. *J. Comput. Biol.* 19, 455–477. doi: 10.1089/cmb.2012.0021

Beck, G., Hegnar, O. A., Fossdal, C. G., and Alfredsen, G. (2018). Acetylation of Pinus radiata delays hydrolytic depolymerisation by the brown-rot fungus *Rhondonia placenta*. *Int. Biodeterior. Biodegradation* 135, 39–52. doi: 10.1016/j.ibiod.2018.09.003

Binder, M., Justo, A., Riley, R., Salamov, A., Lopez-Giraldez, F., Sjökvist, E., et al. (2013). Phylogenetic and phylogenomic overview of the Polyporales. *Mycologia* 105, 1350–1373. doi: 10.3852/13-003

Bravery, A. F. (1979). "A miniaturised wood-block test for the rapid evaluation of preservative fungicides. In: Screening Techniques for Potential Wood Preservative Chemicals," in *Proceedings of a Special Seminar Held in Association with the 10th Annual Meeting of IRG, Peebles 1978*, South Hill, VA: Peebles.

Brigham, J. S., Adney, W. S., and Himmel, M. E. (2018). "Hemicellulases: diversity and applications," in *Handbook on Bioethanol - Production and Utilization*, ed. C. Wyman (Washington, DC: Taylor & Francis).

Campbell, J. A., Davies, G. J., Bulone, V., and Henrissat, B. (1997). A classification of nucleotide-diphospho-sugar glycosyltransferases based on amino acid sequence similarities. *Biochem. J.* 326(Pt 3), 929–939. doi: 10.1042/bj3260929u

Cantarel, B. L., Korf, I., Robb, S. M. C., Parra, G., Ross, E., Moore, B., et al. (2008). MAKER: an easy-to-use annotation pipeline designed for emerging model organism genomes. *Genome Res.* 18, 188–196. doi: 10.1101/gr.6743907

Casado López, S., Peng, M., Daly, P., Andreopoulos, B., Pangilinan, J., Lipzen, A., et al. (2019). Draft genome sequences of three Monokaryotic isolates of the white-rot basidiomycete fungus *Dichomitus squalens*. *Microbiol. Resour. Announc.* 8:e00264-19.

CEN EN 113 (1996). *Wood Preservatives. Test method for determining the protective effectiveness against wood destroying Basidiomycetes. Determination of the Toxic Values*. Brussels: European Committee for Standardisation.

Coutinho, P. M., Deleury, E., Davies, G. J., and Henrissat, B. (2003). An evolving hierarchical family classification for glycosyltransferases. *J. Mol. Biol.* 328, 307–317. doi: 10.1016/s0022-2836(03)00307-3

Cowling, E. B. (1961). *Comparative Biochemistry of the Decay of Sweetgum Sapwood by White-Rot and Brown-Rot Fungi*. Washington, DC: United States Department of Agriculture.

Eastwood, D. C., Floudas, D., Binder, M., Majcherczyk, A., Schneider, P., Aerts, A., et al. (2011). The plant cell wall–decomposing machinery underlies the functional diversity of forest fungi. *Science* 333, 762–765.

Eriksson, K., Blanchette, R. A., and Ander, P. (1990). *Microbial and Enzymatic Degradation of Wood and Wood Components*. Berlin: Springer.

Fenton, H. (1894). Oxidation of tartaric acid in the presence of iron. *J. Chem. Soc. Trans.* 65, 899–910. doi: 10.1039/ct8946500899

Fernandez-Fueyo, E., Ruiz-Dueñas, F. J., Ferreira, P., Floudas, D., Hibbett, D. S., Canessa, P., et al. (2012). Comparative genomics of *Ceriporiopsis subvermispora* and *Phanerochaete chrysosporium* provide insight into selective ligninolysis. *Proc. Natl. Acad. Sci. U.S.A.* 109, 5458–5463.

Filley, T. R., Cody, G. D., Goodell, B., Jellison, J., Noser, C., and Ostrofsky, A. (2002). Lignin demethylation and polysaccharide decomposition in spruce sapwood degraded by brown rot fungi. *Organ. Geochem.* 33, 111–124. doi: 10.1016/s0146-6380(01)00144-9

Floudas, D., Binder, M., Riley, R., Barry, K., Blanchette, R. A., Henrissat, B., et al. (2012). The paleozoic origin of enzymatic lignin decomposition reconstructed from 31 fungal genomes. *Science* 336, 1715–1719.

Gaskell, J., Kersten, P., Larrondo, L. F., Canessa, P., Martinez, D., Hibbett, D. S., et al. (2017). Draft genome sequence of a monokaryotic model brown-rot fungus *Postia (Rhodonia) placenta* SB12. *Genomics Data* 14, 21–23. doi: 10.1016/j.gdata.2017.08.003

Goodell, B. (2003). "Brown-rot fungal degradation of wood: our evolving view," in *Wood Deterioration and Preservation: Advances in Our Changing World*, eds B. Goodell, D. D. Nicholas, and T. P. Schultz (Washington, DC: ACS Publications).

Goodell, B., Jellison, J., Liu, J., Daniel, G., Paszczynski, A., Fekete, F., et al. (1997). Low molecular weight chelators and phenolic compounds isolated from wood decay fungi and their role in the fungal biodegradation of wood. *J. Biotechnol.* 53, 133–162. doi: 10.1016/s0168-1656(97)01681-7

Goodell, B., Qian, Y., and Jellison, J. (2008). "Fungal decay of wood: soft rot - brown rot - white rot. Development of commercial wood preservatives," in *ACS Symposium Series*, ed. T. E. A. Schultz (Washington, DC: American Chemical Society).

Goodell, B., Zhu, Y., Kim, S., Kafle, K., Eastwood, D. C., Daniel, G., et al. (2017). Modification of the nanostructure of lignocellulose cell walls via a non-enzymatic lignocellulose deconstruction system in brown rot wood-decay fungi. *Biotechnol. Biofuels* 10:179.

Herr, D., Baumer, F., and Dellweg, H. (1978). Purification and properties of an extracellular veta-glucosidase from *Lenzites trabea*. *Eur. J. Appl. Microbiol. Biotechnol.* 5, 29–36. doi: 10.1007/bf00515684

Hiscox, J., Hibbert, C., Rogers, H., and Boddy, L. (2010). Monokaryons and dikaryons of *Trametes versicolor* have similar combative, enzyme and decay ability. *Fungal Ecol.* 3, 347–356. doi: 10.1016/j.funeco.2010.02.003

Huson, D. H., and Scornavacca, C. (2012). Dendroscope 3: an interactive tool for rooted phylogenetic trees and networks. *Syst. Biol.* 61, 1061–1067. doi: 10.1093/sysbio/sys062

Ito, N., Phillips, S. E. V., Stevens, C., Ogel, Z. B., Mcpherson, M. J., Keen, J. N., et al. (1991). Novel thioether bond revealed by a 1.7 Å crystal structure of galactose oxidase. *Nature* 350, 87–90. doi: 10.1038/350087a0

Jensen, K., Ryan, Z., Marty, A., Cullen, D., and Hammel, K. E. (2002). An NADH:quinone oxidoreductase active during biodegradation by the brown-rot basidiomycete *Gloeophyllum trabeum*. *Appl. Environ. Microbiol.* 68, 2699–2703. doi: 10.1128/aem.68.6.2699-2703.2002

Jensen, K. A., Houtman, C. J., Ryan, Z. C., and Hammel, K. E. (2001). Pathways for extracellular Fenton chemistry in the brown rot basidiomycete *Gloeophyllum trabeum*. *Appl. Environ. Microbiol.* 67, 2705–2711. doi: 10.1128/aem.67.6.2705-2711.2001

Jonkers, W., and Rep, M. (2009). Lessons from fungal F-box proteins. *Eukaryot. Cell* 8, 677–695. doi: 10.1128/ec.00386-08

Katoh, K., and Standley, D. M. (2013). MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Mol. Biol. Evol.* 30, 772–780. doi: 10.1093/molbev/mst010

Kersten, P., and Cullen, D. (2014). Copper radical oxidases and related extracellular oxidoreductases of wood-decay Agaricomycetes. *Fungal Genet. Biol.* 72, 124–130. doi: 10.1016/j.fgb.2014.05.011

Kleman-Leyer, K., Agosin, E., Conner, A. H., and Kirk, T. K. (1992). Changes in molecular size distribution of cellulose during attack by white rot and brown rot fungi. *Appl. Environ. Microbiol.* 58, 1266–1270. doi: 10.1128/aem.58.4.1266-1270.1992

Klutts, J., Yoneda, A., Reilly, M., Bose, I., and Doering, T. (2006). Glycosyltransferases and their products: Cryptococcal variations on fungal themes. *FEMS Yeast Res.* 6, 499–512. doi: 10.1111/j.1567-1364.2006.00054.x

Kölle, M., Ringman, R., and Pilgård, A. (2019). Initial *Rhodonia placenta* gene expression in acetylated wood: group-wise upregulation of non-enzymatic oxidative wood degradation genes depending on the treatment level. *Forests* 10:1117. doi: 10.3390/f10121117

Lairson, L. L., Henrissat, B., Davies, G. J., and Withers, S. G. (2008). Glycosyltransferases: structures, functions, and mechanisms. *Annu. Rev. Biochem.* 77, 521–555. doi: 10.1146/annurev.biochem.76.061005.092322

Langmead, B., and Salzberg, S. L. (2012). Fast gapped-read alignment with Bowtie 2. *Nat. Methods* 9, 357–359. doi: 10.1038/nmeth.1923

Latchman, D. S. (1997). Transcription factors: an overview. *Int. J. Biochem. Cell Biol.* 29, 1305–1312.

Levasseur, A., Drula, E., Lombard, V., Coutinho, P. M., and Henrissat, B. (2013). Expansion of the enzymatic repertoire of the CAZy database to integrate auxiliary redox enzymes. *Biotechnol. Biofuels* 6:41. doi: 10.1186/1754-6834-6-41

Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., et al. (2009). The Sequence Alignment/Map format and SAMtools. *Bioinformatics* 25, 2078–2079. doi: 10.1093/bioinformatics/btp352

Liu, T.-B., and Xue, C. (2011). The ubiquitin-proteasome system and f-box proteins in pathogenic fungi. *Mycobiology* 39, 243–248. doi: 10.5941/myco.2011.39.4.243

Lombard, V., Golaconda Ramulu, H., Drula, E., Coutinho, P. M., and Henrissat, B. (2013). The carbohydrate-active enzymes database (CAZy) in 2013. *Nucleic Acids Res.* 42, D490–D495.

MacPherson, S., Larochelle, M., and Turcotte, B. (2006). A fungal family of transcriptional regulators: the zinc cluster proteins. *Microbiol. Mol. Biol. Rev.* 70, 583–604. doi: 10.1128/MMBR.00015-06

Martinez, Á. T., Speranza, M., Ruiz-Dueñas, F. J., Ferreira, P., Camarero, S., Guillén, F., et al. (2005). Biodegradation of lignocellulosics: microbial, chemical, and enzymatic aspects of the fungal attack of lignin. *Int. Microbiol.* 8, 195–204.

Martinez, D., Challacombe, J., Morgenstern, I., Hibbett, D. S., Schmoll, M., Kubicek, C., et al. (2009). Genome, transcriptome, and secretome analysis of wood decay fungus *Postia placenta* supports unique mechanisms of lignocellulose conversion. *Proc. Natl. Acad. Sci. U.S.A.* 106, 1954–1959.

Melin, V., Henríquez, A., Freer, J., and Contreras, D. (2015). Reactivity of catecholamine-driven Fenton reaction and its relationships with iron(III) speciation. *Redox Rep.* 20, 89–96. doi: 10.1179/1351000214y.0000000119

Mester, T., Varela, E., and Tien, M. (2004). "Wood degradation by brown-rot and white-rot fungi," in *Genetics and Biotechnology*, ed. U. Kück (Berlin: Springer), 355–368. doi: 10.1007/978-3-662-07426-8_17

Miettinen, O., Riley, R., Barry, K., Cullen, D., De Vries, R. P., Hainaut, M., et al. (2016). Draft genome sequence of the white-rot fungus *Obba rivulosa* 3A-2. *Genome Announc.* 4:e00976-16.

Miller, J., Mclachlan, A. D., and Klug, A. (1985). Repetitive zinc-binding domains in the protein transcription factor IIIA from Xenopus oocytes. *EMBO J.* 4, 1609–1614. doi: 10.1002/j.1460-2075.1985.tb03825.x

Nagy, L. G., Riley, R., Tritt, A., Adam, C., Daum, C., Floudas, D., et al. (2015). Comparative genomics of early-diverging mushroom-forming fungi provides insights into the origins of lignocellulose decay capabilities. *Mol. Biol. Evol.* 33, 959–970. doi: 10.1093/molbev/msv337

Niemenmaa, O., Uusi-Rauva, A., and Hatakka, A. (2007). Demethoxylation of [O14CH3]-labelled lignin model compounds by the brown-rot fungi *Gloeophyllum trabeum* and *Poria (Postia) placenta*. *Biodegradation* 19, 555–565. doi: 10.1007/s10532-007-9161-3

Ohm, R. A., Riley, R., Salamov, A., Min, B., Choi, I.-G., and Grigoriev, I. V. (2014). Genomics of wood-degrading fungi. *Fungal Genet. Biol.* 72, 82–90. doi: 10.1016/j.fgb.2014.05.001

Olson, Å., Aerts, A., Asiegbu, F., Belbahri, L., Bouzid, O., Broberg, A., et al. (2012). Insight into trade-off between wood decay and parasitism from the genome of a fungal forest pathogen. *New Phytol.* 194, 1001–1013. doi: 10.1111/j.1469-8137.2012.04128.x

Paszczynski, A., Crawford, R., Funk, D., and Goodell, B. (1999). De novo synthesis of 4,5-dimethoxycatechol and 2, 5-dimethoxyhydroquinone by the brown rot fungus *Gloeophyllum trabeum*. *Appl. Environ. Microbiol.* 65, 674–679. doi: 10.1128/aem.65.2.674-679.1999

Pilgård, A., Schmöllerl, B., Risse, M., Fossdal, C. G., and Alfredsen, G. (2017). "Profiling *Postia placenta* colonisation in modified wood - microscopy, DNA quantification and gene expression," in *Proceedings of the Wood Science and Engineering*, Copenhagen, 17–18.

Presley, G. N., Panisko, E., Purvine, S. O., and Schilling, J. S. (2018). Coupling Secretomics with enzyme activities to compare the temporal processes of wood metabolism among white and brown rot fungi. *Appl. Environ. Microbiol.* 84:e00159-18.

Puls, J. (1997). Chemistry and biochemistry of hemicelluloses: relationship between hemicellulose structure and enzymes required for hydrolysis. *Macromol. Symp.* 120, 183–196. doi: 10.1002/masy.19971200119

Riley, R., Salamov, A. A., Brown, D. W., Nagy, L. G., Floudas, D., Held, B. W., et al. (2014). Extensive sampling of basidiomycete genomes demonstrates inadequacy of the white-rot/brown-rot paradigm for wood decay fungi. *Proc. Natl. Acad. Sci. U.S.A.* 111, 9923–9928. doi: 10.1073/pnas.1400592111

Ringman, R., Pilgård, A., Kölle, M., Brischke, C., and Richter, K. (2016). Effects of thermal modification on *Postia placenta* wood degradation dynamics: measurements of mass loss, structural integrity and gene expression. *Wood Sci. Technol.* 50, 385–397. doi: 10.1007/s00226-015-0791-z

Ringman, R., Pilgård, A., and Richter, K. (2014). Effect of wood modification on gene expression during incipient *Postia placenta* decay. *Int. Biodeterior. Biodegradation* 86, 86–91. doi: 10.1016/j.ibiod.2013.09.002

Ringman, R., Pilgård, A., and Richter, K. (2020). Brown rot gene expression and regulation in acetylated and furfurylated wood: a complex picture. *Holzforschung* 74, 391–399. doi: 10.1515/hf-2019-0031

Ryu, J. S., Shary, S., Houtman, C. J., Panisko, E. A., Korripally, P., St John, F. J., et al. (2011). Proteomic and functional analysis of the cellulase system expressed by *Postia placenta* during brown rot of solid wood. *Appl. Environ. Microbiol.* 77, 7933–7941. doi: 10.1128/aem.05496-11

Schilling, J. S., Ai, J., Blanchette, R. A., Duncan, S. M., Filley, T. R., and Tschirner, U. W. (2012). Lignocellulose modifications by brown rot fungi and their effects, as pretreatments, on cellulolysis. *Bioresour. Technol.* 116, 147–154. doi: 10.1016/j.biortech.2012.04.018

Schilling, J. S., Kaffenberger, J. T., Liew, F. J., and Song, Z. (2015). Signature wood modifications reveal decomposer community history. *PLoS One* 10:e0120679. doi: 10.1371/journal.pone.0120679

Schwarze, F. W. M. R. (2007). Wood decay under the microscope. *Fungal Biol. Rev.* 21, 133–170. doi: 10.1016/j.fbr.2007.09.001

Simão, F. A., Waterhouse, R. M., Ioannidis, P., Kriventseva, E. V., and Zdobnov, E. M. (2015). BUSCO: assessing genome assembly and annotation completeness with single-copy orthologs. *Bioinformatics* 31, 3210–3212. doi: 10.1093/bioinformatics/btv351

Smith, T. F. (2008). "Diversity of WD-repeat proteins," in *The Coronin Family of Proteins: Subcellular Biochemistry*, eds C. S. Clemen, L. Eichinger, and V. Rybakin (New York, NY: Springer), 20–30.

Stamatakis, A. (2014). RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics* 30, 1312–1313. doi: 10.1093/bioinformatics/btu033

Stirnimann, C. U., Petsalaki, E., Russell, R. B., and Müller, C. W. (2010). WD40 proteins propel cellular networks. *Trends Biochem. Sci.* 35, 565–574. doi: 10.1016/j.tibs.2010.04.003

Sützl, L., Laurent, C. V. F. P., Abrera, A. T., Schütz, G., Ludwig, R., and Haltrich, D. (2018). Multiplicity of enzymatic functions in the CAZy AA3 family. *Appl. Microbiol. Biotechnol.* 102, 2477–2492. doi: 10.1007/s00253-018-8784-0

Suzuki, H., Macdonald, J., Syed, K., Salamov, A., Hori, C., Aerts, A., et al. (2012). Comparative genomics of the white-rot fungi, *Phanerochaete carnosa* and *P. chrysosporium*, to elucidate the genetic basis of the distinct wood types they colonize. *BMC Genomics* 13:444. doi: 10.1186/1471-2164-13-444

Suzuki, M. R., Hunt, C. G., Houtman, C. J., Dalebroux, Z. D., and Hammel, K. E. (2006). Fungal hydroquinones contribute to brown rot of wood. *Environ. Microbiol.* 8, 2214–2223. doi: 10.1111/j.1462-2920.2006.01160.x

Talavera, G., and Castresana, J. (2007). Improvement of phylogenies after removing divergent and ambiguously aligned blocks from protein sequence alignments. *Syst. Biol.* 56, 564–577. doi: 10.1080/10635150701472164

Tang, J. D., Perkins, A. D., Sonstegard, T. S., Schroeder, S. G., Burgess, S. C., and Diehl, S. V. (2012). Short-read sequencing for genomic analysis of the brown rot fungus Fibroporia radiculosa. *Appl. Environ. Microbiol.* 78, 2272–2281. doi: 10.1128/aem.06745-11

Thaler, N., Alfredsen, G., and Fossdal, C. G. (2012). "Variation in two *Postia placenta* strains, MAD-698-R and FPRL280 - mass loss, DNA content and gene expression," in *Proceedings of the IRG Annual Meeting, IRG/WP 12-10781*, Kuala Lumpur.

Traeger, S., Altegoer, F., Freitag, M., Gabaldon, T., Kempken, F., Kumar, A., et al. (2013). The genome and development-dependent transcriptomes of *Pyronema confluens*: a window into fungal evolution. *PLoS Genet.* 9:e1003820. doi: 10.1371/journal.pgen.1003820

Valášková, V., and Baldrian, P. (2006). Degradation of cellulose and hemicelluloses by the brown rot fungus Piptoporus betulinus – production of extracellular enzymes and characterization of the major cellulases. *Microbiology* 152, 3613–3622. doi: 10.1099/mic.0.29149-0

Vanden Wymelenberg, A., Gaskell, J., Mozuch, M., Sabat, G., Ralph, J., Skyba, O., et al. (2010). Comparative transcriptome and secretome analysis of wood decay fungi *Postia placenta* and *Phanerochaete chrysosporium*. *Appl. Environ. Microbiol.* 76, 3599–3610. doi: 10.1128/aem.00058-10

Wei, D., Houtman, C. J., Kapich, A., Hunt, C., Cullen, D., and Hammel, K. E. (2010). Laccase and its role in production of extracellular reactive oxygen species during wood decay by the brown rot basidiomycete *Postia placenta*. *Appl. Environ. Microbiol.* 76, 2091–2097. doi: 10.1128/aem.02929-09

Wu, B., Xu, Z., Knudson, A., Carlson, A., Chen, N., Kovaka, S., et al. (2018). Genomics and development of *Lentinus tigrinus*: a white-rot wood-decaying mushroom with dimorphic fruiting bodies. *Genome Biol. Evol.* 10, 3250–3261. doi: 10.1093/gbe/evy246

Yelle, D. J., Wei, D., Ralph, J., and Hammel, K. E. (2011). Multidimensional NMR analysis reveals truncated lignin structures in wood decayed by the brown rot basidiomycete *Postia placenta*. *Environ. Microbiol.* 13, 1091–1100. doi: 10.1111/j.1462-2920.2010.02417.x

Yu, L., Gaitatzes, C., Neer, E., and Smith, T. F. (2000). Thirty-plus functional families from a single motif. *Protein Sci.* 9, 2470–2476. doi: 10.1110/ps.9.12.2470

Zabel, R., and Morrell, J. J. (1992). *Wood Microbiology: Decay and its Prevention*. San Diego, CA: Academic Press.

Zhang, J., Presley, G. N., Hammel, K. E., Ryu, J.-S., Menke, J. R., Figueroa, M., et al. (2016). Localizing gene regulation reveals a staggered wood decay mechanism for the brown rot fungus *Postia placenta*. *Proc. Natl. Acad. Sci. U.S.A.* 113, 10968–10973. doi: 10.1073/pnas.1608454113

Zhang, J., and Schilling, J. (2017). Role of carbon source in the shift from oxidative to hydrolytic wood decomposition by *Postia placenta*. *Fungal Genet. Biol.* 106, 1–8. doi: 10.1016/j.fgb.2017.06.003

II

forests **MDPI**

Article

Initial *Rhodonia Placenta* Gene Expression in Acetylated Wood: Group-Wise Upregulation of Non-Enzymatic Oxidative Wood Degradation Genes Depending on the Treatment Level

Martina Kölle [1,*], Rebecka Ringman [2] and Annica Pilgård [1,2]

[1] TUM School of Life Sciences Weihenstephan, Technical University of Munich, 85354 Freising, Germany; annica.pilgard@ri.se

[2] RISE, Research Institutes of Sweden, Bioeceonmy, Box 857, 501 15 Borås, Sweden; rebecka.ringman@ri.se

* Correspondence: koelle@hfm.tum.de

Abstract: Acetylation has been shown to delay fungal decay, but the underlying mechanisms are poorly understood. Brown-rot fungi, such as *Rhodonia placenta* (Fr.) *Niemelä*, *K.H. Larss.* & *Schigel*, degrade wood in two steps, i.e., oxidative depolymerization followed by secretion of hydrolytic enzymes. Since separating the two degradation steps has been proven challenging, a new sample design was applied to the task. The aim of this study was to compare the expression of 10 genes during the initial decay phase in wood and wood acetylated to three different weight percentage gains (WPG). The results showed that not all genes thought to play a role in initiating brown-rot decay are upregulated. Furthermore, the results indicate that *R. placenta* upregulates an increasing number of genes involved in the oxidative degradation phase with increasing WPG.

1. Introduction

Wood is an attractive building material that is biodegradable, renewable, stores carbon [1,2], and can serve as an alternative to concrete and steel due to its mechanical properties [3,4]. Biodegradability is a disadvantage when wood is used in outdoor construction [5,6], if no adequate preservative method is applied. Hence, wood protection is necessary to prolong the service life of wooden products made of nondurable wood. Traditionally, wood is treated with copper-based preservatives, however, ecological and health issues surrounding copper-based preservatives have led to restrictions and regulations [7–9]. An alternative approach to traditional preservatives is wood modification, which alters the properties of the wood to enhance its resistance to degradation [8]. Wood can be either physically (heat-treated wood) or chemically (for example wood acetylation and furfurylation) modified. Acetylation is a widely studied wood modification method [8,10–13]. During acetylation, hydroxyl groups in the wood are replaced by acetyl groups [1,8], leading to reduced hygroscopicity and volumetric swelling which bulks the cell walls and reduces water absorption [8,14–16]. Several studies have found, that a weight percent gain (WPG) of 10% leads to limited decay inhibition, whereas a WPG of 20% significantly increases decay resistance [8,17–20].

The exact mechanisms behind the decay resistance in acetylated wood is not known. However, recent results indicate that diffusion of fungal depolymerizing agents through the wood cell wall may be inhibited due to decreased equilibrium moisture content [8,21–25]. Hunt et al. [26] reported a decreased diffusion of K^+ ions with an increasing level of acetylation, which supports the theory

that acetylation inhibits diffusion of hydrolytic reductants due to moisture exclusion [25]. Zelinka et al. [24,27,28] hypothesized in their studies that an interrupted moisture network inhibits diffusion. This conclusion was supported by Beck et al. [16], who characterized moisture in acetylated wood with low-field nuclear magnetic resonance relaxometry (LF-NMR).

Thirty-two percent of the described fungi in the world belong to the Basidiomycota [29]. Wood decaying fungi, belonging to the Basidiomycota, are filamentous fungi and can be divided into two groups, white-rot and brown-rot fungi [30–32]. However, because decay mechanisms are much more diverse than assumed, such a distinction may be too simplistic [33]. Although more species of white-rot fungi are known, approximately 80% of the wood decay fungi found in wooden constructions belong to the brown-rot subgroup [34–37]. Because of their preference for softwoods, brown-rot fungi are the main recyclers of lignocellulose in Northern Hemisphere coniferous forests, and decay associated with brown-rot is reportedly the most destructive type [5,38,39]. Brown-rot decay rapidly leads to significant strength loss through depolymerization of the cellulose and hemicellulose fractions through non-enzymatic oxidative degradation processes [40–44], followed by the secretion of hydrolyzing enzymes [44–46]. Zhang et al. found, in 2016, that the two decay phases are in fact spatially separated and that the presence of sugars solubilized during oxidative decay triggers the transition from oxidative to enzymatic degradation [47].

Brown-rot wood degradation has been studied mainly in the organism *Gloeophyllum trabeum* (Pers.) *Murrill* and verified to some degree in *Rhodonia placenta* (Fr.) *Niemelä, K.H. Larss. & Schigel* (also known as *Postia placenta*), *Coniophora puteana* (Schumach.) *P. Karst.*, and *Serpula lacrymans* (Wulfen) *J. Schröt*. The details regarding the non-enzymatic oxidative degradation phase still remains unknown. The current theory is that during decay brown-rot fungi secrete oxalic acid, which diffuses into the lumen, where it functions as a chelator to sequester Fe^{3+} [46,48,49]. Fe^{2+} is formed through reduction by hydroquinones, and H_2O_2 is believed to be formed through a reaction between hydroquinones and oxygen [50,51]. Hydrogen peroxide and Fe^{2+} react to hydroxyl radicals, which polymerize cellulose and hemicellulose, and modifies lignin [45,46,52]. This process solubilizes sugars, which can diffuse through the cell walls into the lumen, to become accessible to cellulases and hemicellulases [42,53].

Zhang et al. [47] found that thirty-three genes likely associated with redox processes were upregulated during early stages of decay, and 21 of them may be involved in generating H_2O_2 and Fe^{2+}. Martinez et al. [54] identified a putative quinone reductase in *R. placenta* that is believed to recover iron reductants (hydroquinones) [50,54–56] and is also putatively mediating the reduction of iron chelators (oxalic acid) [55]. *R. placenta* has only one quinone reductase (QRD Ppl124517), compared with the two in *Gloeophyllum trabeum*, which have been shown to have different functions; one is involved in wood degradation and the other in stress defense [57]. *R. placenta* quinone reductase belongs to the Carbohydrate-Active enZYmes (CAZy) family AA6 and is likely involved in oxidative processes and the formation of intracellular enzymes for fungal protection. Mueckler et al. [58] suggested that Ppl44553 is a quinate transporter (PQT), belonging to the major facilitator superfamily domain. This domain includes sugar transporters, which bind and transport a variety of carbohydrates, organic alcohols, and acids. A major part of this family catalyzes sugar transport [59]. The exact function of the *R. placenta* PQT remains unclear. Laccases appears to play an important role by oxidizing methoxyhydroquinones into semiquinones that reduce Fe^{2+} [60] and are believed to be effective producers of H_2O_2 [61]. Four putative laccases have been found in *R. placenta* [54] and one of them is included in this present study (Lac1 Ppl111314). Extracellular H_2O_2 has also been suggested to be produced by copper radical oxidases (Cro), gluco-oligosaccharide oxidases, and glucose-methanol-choline (GMC) oxidoreductases, including alcohol oxidases (AlOx) and glucose oxidases (GOx) [54,62,63]. Alcohol oxidase is upregulated in the presence of cellulose and wood [38,54]. *R. placenta* has three genes encoding alcohol oxidases, belonging to the CAZy family AA3, AlOx1 Ppl44331, AlOx2 Ppl129158, and AlOx3 Ppl118723. Copper radical oxidase (Cro1 Ppl56703) belongs to the CAZy family AA5. Cro1, together with glucose oxidase 2 (GOx2 Ppl108489), are oxidoreductases involved in the H_2O_2 production [47]. Brown-rot fungi accumulate significant amounts of oxalic acid, which has been shown

to be involved in their wood decaying system [64]. Oxaloacetate dehydrogenase (CyOx Ppl112832) is presumed to be involved in oxalic acid synthesis, which is important for sequestering and reducing Fe^{3+} to Fe^{2+} [13]. Glyoxylate dehydroxygenase (GlyD Ppl121561) is likely involved in oxalic acid synthesis, by catalyzing the production of oxalate through oxidation of glyoxalate [65]. It is also involved in the glyoxylate cycle [66]. Munir et al. [66,67] purified a GlyD that catalyzes dehydrogenation of glyoxylate to oxalate in the presence of cytochrome c. Munir et al. [66] also found that GlyD exhibited a strong correlation with the biosynthesis of oxalic acid and fungal growth.

Previous studies (mostly on *R. placenta*) have shown that brown-rot fungi are not killed when growing on acetylated wood and can express genes needed for both the non-enzymatic oxidative degradation process and the enzymatic wood degradation process [11,13,68–70]. Even though the fungi express the genes needed for degradation, the wood remains intact for a prolonged period of time as compared with untreated wood materials. Since the fungi express genes involved in wood degradation in modified wood, before mass loss occurs, at similar levels as in untreated wood where degradation has begun, it is clearly shown that the fungi are attempting to degrade the wood but fail [11,13,69–71]. Despite previous research, it is still not known which regulatory mechanisms in the brown-rot degradation machinery are prevalent during degradation of acetylated wood. In order to improve the decay resistance of acetylated wood, it is of crucial importance to understand the decay mechanisms of incipient decay.

To investigate and improve wood modification methods, degradation tests are often used, for example, the miniblock test [72]. *Rhodonia placenta* (Fr.) M.J. *Larsen & Lombard* is used as a model fungus for standardized degradation tests in Europe (FPRL280) and the USA (MAD-698R) (AWPA E10-16, 1991; EN 113, 1996). Previous comparisons of gene expression when growing on untreated and modified wood supply hints on fungal behavior [11,24,25,73]. Most studies used only a small number of biological replicates and often encountered difficulties differentiating the non-enzymatic oxidative degradation phase from the enzymatic degradation phase. Zhang et al. presented in 2016 a method making it possible to separate the two phases. They used wood wafers (with the largest area being the cross-section) placed in an upward position on previously inoculated feeder strips. Fungi were allowed to grow up along the wafers forming a clear hyphal front. This method has previously never been used on modified wood.

The aim of this study was to investigate the differences in gene expression of 10 different genes in *R. placenta* during the non-enzymatic oxidative degradation phase, when grown on wafers of untreated wood and wood acetylated to three different levels.

2. Material and Methods

2.1. Wood Samples

Wood boards from Scots pine sapwood (*Pinus sylvestris* L.) were cut into wafers ($80 \times 18 \times 2.5$ mm^3), with the largest area being the cross section [74]. The samples were dried at 103 °C for 24 h and their dry weights were determined. Dried wafers were acetylated for 15 min, 30 min, and 60 min to achieve three different acetylation levels; 10 (AC10), 15 (AC15), and 20 (AC20) weight percent gain (WPG) (Table 1). Then, 50 samples (only 25 for AC10) were put in a glass flask (1 L) and a vacuum was attached for 30 min. Then, 50 mL of acetic anhydride was injected, followed by 50 mL of pyridine. After vacuuming for another minute, the samples were incubated at room temperature for 3 h, followed by lowering the flask into an 80 °C water bath for the corresponding reaction time. The reaction was stopped by washing the samples twice with ice-cold acetone and twice with an acetone-water mixture with a ratio of 1:1. Samples were rinsed in distilled water several times for 3 days, vacuum-impregnated with water, to remove all accessible chemicals, and dried again before the weight gain was measured. All samples were packed and autoclaved before the decay test.

Table 1. Mean weight percent gain (WPG) and standard deviation of the wood samples.

Mean WPG	Standard Deviation
10	0.18
15	0.13
20	0.14

2.2. Decay Test

R. placenta FPRL 280 (Fr.) was used for the decay test previously described by Zhang et al. [47]. As growth medium, 50 g of soil, 25 g of sand, 20 g of vermiculite, and 45 mL of water were mixed for each specimen container. The jars were autoclaved. Feeder strips of *P. sylvestris* sapwood were autoclaved, and three per glass were placed on the soil medium and inoculated with agar plugs from pregrown 4% malt agar plates. For each treatment, untreated (UT), 10% acetylated (AC10), 15% acetylated (AC15), and 20% acetylated (AC20), 16 glasses (*n* = 16) with three samples each were prepared. After the feeder strips were completely overgrown, one wood wafer was placed on each feeder strip. The jars were stored in a climate chamber at 22 °C and 70% relative humidity. Samples were harvested according to the height of the hyphal front (3/4 of the wood wafer overgrown). Only samples with even hyphal growth, around the whole sample, were included in the test. Since the cross section was the largest area, the mycelia growing up the sample could easily grow into the sample through the wood cell lumen. With this sample design, it was possible to assure that the fungi had reached the same level inside the sample as outside the sample [47]. Sections with a size of five millimeters, including the hyphal front, were cut out of the wafers, immediately frozen in liquid nitrogen and stored at −80 °C awaiting further analysis. The section size was chosen due to an expected growth rates of about 2.5 mm/day, with the first 5 mm representing approximately a 48 h window, during which the non-enzymatic degradation phase has been shown to take place [47]. Additional tests were done to obtain data on mass loss and growth rates for all treatments. For these tests a different set of samples was used.

2.3. RNA Purification and cDNA Synthesis

All 5 mm sections from one jar (three samples) were pooled into one biological replicate. A Mixer Mill MM 400 (Retsch GmbH, Haan, Germany) using one 1.5 cm steel ball and 30 Hz for 2 min was used to produce wood powder. Containers, beads, and samples were frozen with liquid nitrogen. Sixty milligrams of each sample were taken for RNA purification. Total RNA was extracted using a MasterPureTM RNA Purification Kit (Lucigen, Middleton, USA) [24]. RNA was converted to cDNA using TaqMan Reverse Transcription Reagents using Oligo d(T)16 (Applied Biosystems, Foster City, USA) with 10 times the standard dNTP concentration.

2.4. Quantitative Real-Time Polymerase Chain Reaction

A rotor-gene SYBRGreen polymerase chain reaction (PCR) kit (Qiagen, Hilden, Germany) was used according to the manufacturer's protocol for quantitative real-time PCR. Each sample was run with three technical replicates. β-tubulin was used as an endogenous control [13]. A list with all used primers including the number of the Joint Genome Institute (JGI) can be found in Table 2. The ten genes used in this study were chosen because of their assumed importance during the non-enzymatic degradation phase. This selection was based on previous findings [11,13,47,68–70,75–79]. Most importantly, the ten genes included in this paper have never been studied with this sample design before. Rotor-gene Q series software (Version 2.3.1, Qiagen, Hilden, Germany) was used to evaluate the runs. Expression levels for each technical replicate of the target genes (Tg) were calculated and normalized to the endogenous control according to the formula [80]:

$$\text{Expression level} = 10^4 \times 2^{C_t\beta t - C_t Tg} \tag{1}$$

Table 2. Primer sequences and JGI number of the target genes.

Gene	JGI no.	Primer Sequence
β-tubulin (bT)	113871	CAGGATCTTGTCGCCGAGTAC/ CCTCATACTCGCCCTCCTCTT
Quinone oxidoreductase (QRD)	124517	CGACGACAAGCCCAACAAG/ GATGACGATGATGGCGATTTTAGG
Alcohol oxidase 1 (AlOx1)	44331	GGAGGTACAGACGGACGAAC/ AGAGTCGACGACACCGTTCT
Alcohol oxidase 2 (AlOx2)	129158	TACTCGACGGCCCTCACTAT/ CCGCTTGAGACTGAACACTG
Alcohol oxidase 3 (AlOx3)	118723	ACACCAAGGAGGACGACGAG/ GACGAGCAAGGCAGACGAGTA
Putative quinate transporter (PQT)	44553	ACTGACCTTTTGCGCAGACT/ CAATGTTGATTGTGGCGAAC
Laccase (Lac)	111314	CGGTGCTCTTGGCCACTTAG/ CCATTGGTTATGGGCAGCTC
Copper radical oxidase (Cro1)	56703	CCTACCAGCTGCTTCCTGAC/ AACGTTCGGCTGTATGAACC
Glucose oxidase (GOx2)	108489	GTCCGCTCTAACGTTGCTTC/ CCGGCGTTATTGGAGAGATA
Glyoxylate dehydrogenase (GlyD)	121561	CGGAGCTGGACCTTTGTTAC/ GCGCGAAGGCAAATCTAATA
Oxaloacetate dehydrogenase (CyOx)	112832	AAGGCGTTCTTCGAGGTCAT/ AAAGCAGCAACCCGAGAAG

A mean concentration was calculated for each sample (n = 11–16) and normalized. Significance ($p < 0.05$) was calculated using the Student's *t*-test. Expression levels of the four different treatment levels (UT, AC10, AC15 and AC20) were compared.

2.5. Statistical Evaluation

A simple linear regression in Excel (Version 2016) was constructed for each of the four treatments for growth rate calculations.

3. Results

3.1. Mass Loss and Growth Rates

Average mass loss data and linear regression values of the growth test for all treatments are supplied in Table 3. Significantly lower mass loss was found in treated samples as compared with that in untreated samples. In AC20, a negative mass loss was detected.

Table 3. Mass loss and growth rates of all treatments during the period of colonizing the wood wafers.

Treatment	Mass Loss (%)	Growth Rate [mm/day]	Growth Rate R^2 Values
untreated	9.36	0.23	0.9955
10% acetylated	1.63	0.24	0.9955
15% acetylated	0.25	0.22	0.91
20% acetylated	−0.16	0.25	0.9897

The growth rates of *R. placenta* growing on untreated samples, as well as on acetylated samples, can be seen in Table 3. These correlate well with the findings of Zhang et al. (2016) [47]. However,

R. placenta growing on AC15 behaved differently with a slower and more uneven growth rate as compared with the other acetylation levels.

3.2. Expression of Target Genes

3.2.1. Upregulated Genes

The results of all upregulated genes can be found in detail in Figure 1. Note that the figure shows logarithmic values and that the *y*-axis scale varies between the different genes.
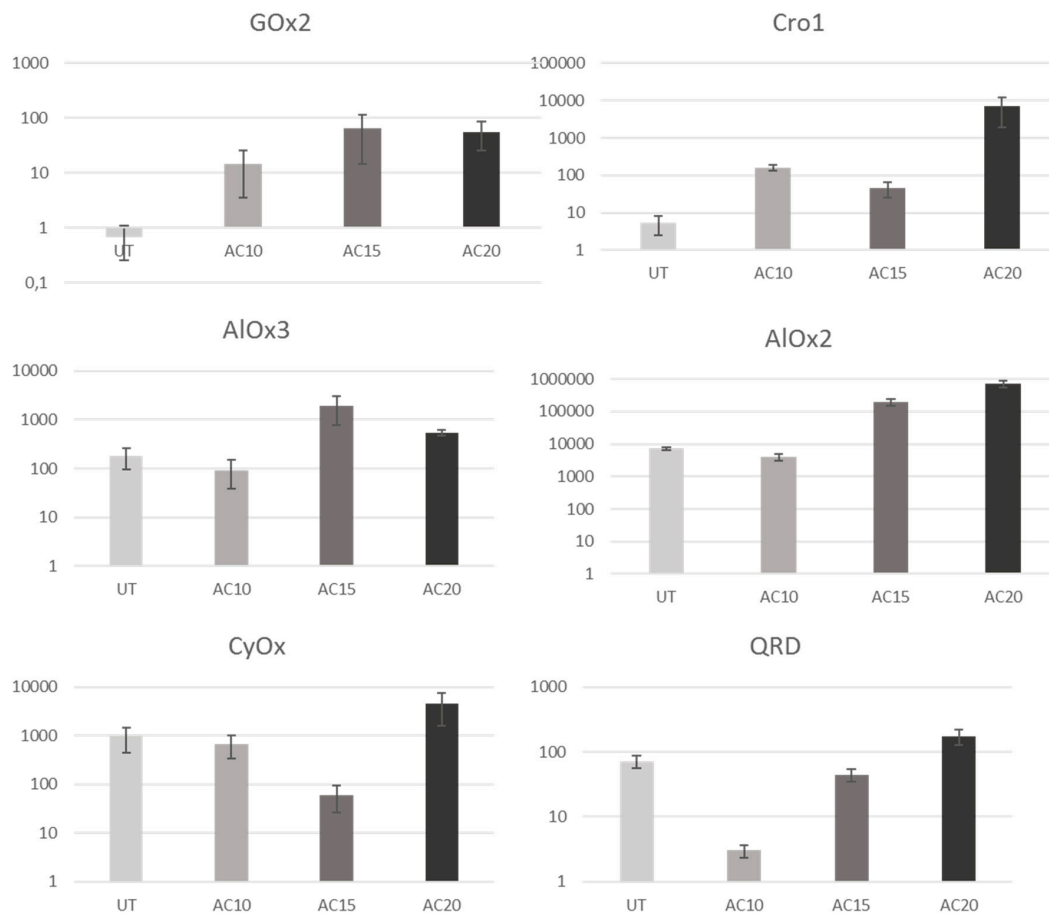
Figure 1. Logarithmic values for gene expression for upregulated genes (UT, untreated; AC10, 10 WPG acetylated; AC15, 15 WPG acetylated; and AC20, 20 WPG acetylated samples; GOx2, glucose oxidase 2; Cro1, copper radical oxidase 1; AlOx3 & AlOx2, alcohol oxidases 2 & 3; CyOx, oxaloacetate dehydrogenase; QRD, quinone oxidoreductase).

Expression levels for AlOx2 on acetylated samples were significantly upregulated as were those for AlOx3 (except for AC10) as compared with those on untreated samples. For Cro1, all gene expression levels were upregulated in modified samples as compared with those in untreated samples, with an extreme upregulation seen in the AC20 samples. All treatments showed highly significant differences in gene expression for CyOx as compared with each other, except for the untreated samples as compared with the AC10 samples. The AC15 samples showed the lowest levels of CyOx expression, whereas expression was clearly upregulated in AC20 samples. Upregulation of GOx2 was unambiguous when comparing treated samples with untreated samples. AC15 and AC20 did not differ significantly for this gene, but all other treatments showed highly significant differences. Differences in gene expression of QRD were highly significant between all treatments. A clear upregulation of QRD was not found

when comparing treated and untreated samples, although a strong upregulation in samples with high acetylation was observed.

3.2.2. Downregulated Genes

Figure 2 illustrates the results for all genes that were downregulated in treated samples as compared with those in untreated samples. As well, Figure 1 shows the logarithmic values and the *y*-axis scale differs between the genes. Lac1 showed the highest expression levels among untreated samples as compared with all treated samples. The lowest values were observed in AC10, increasing with increased intensity of acetylation. PQT was clearly downregulated in all three acetylation levels as compared with the untreated samples. Results for AlOx1 showed no significant differences between untreated and AC15 samples. AC10 and AC20 showed highly significant lower levels of gene expression as compared the untreated samples. Highly significant differences were also revealed for expression levels of GlyD in all sample treatments. All treated samples had lower expression rates for GlyD as compared with the untreated samples.
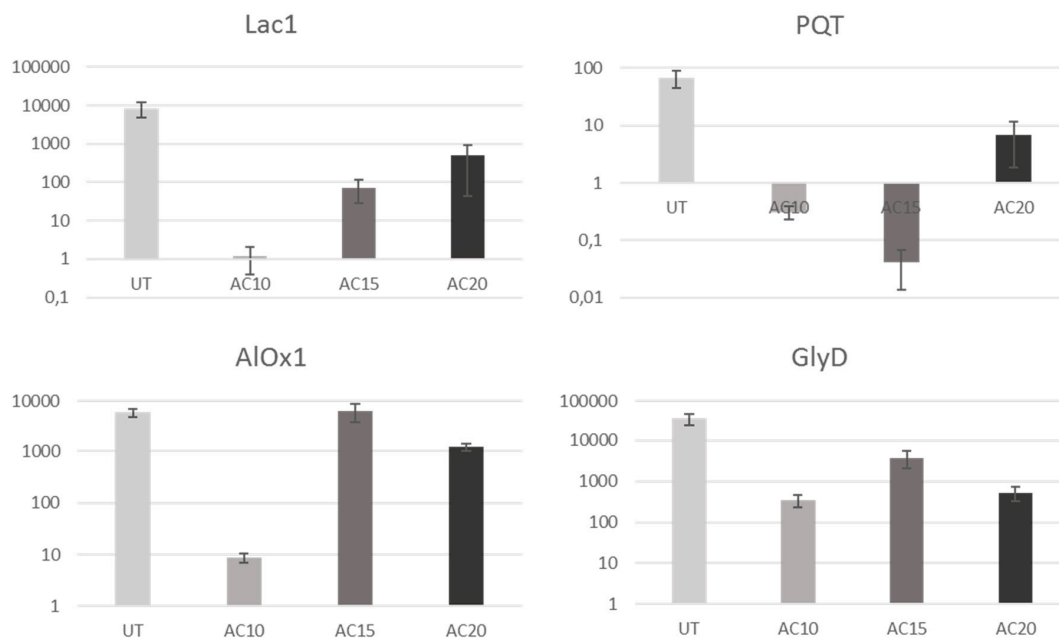

Figure 2. Logarithmic values for gene expression for downregulated genes (UT, untreated; AC10, 10 WPG acetylated; AC15, 15 WPG acetylated; and AC20, 20 WPG acetylated samples; Lac1, laccase 1; PQT, putative quinate transporter; AlOx1, alcohol oxidase 1; GlyD, glyoxylate dehydrogenase).

3.2.3. Effect of Acetylation on Overall Gene Expression

For all genes, significant differences were found between treatments, except in three cases (AlOx1: UT-AC15; CyOx: UT-AC10; and GOx2: AC15-AC20) (Table 4). When UT and AC10 samples were compared, upregulation was observed for only two genes, Cro1 and GOx2, which are likely involved in H_2O_2-production. The comparison of untreated samples with AC15 showned an upregulation of Cro1, GOx2 and, additionally, AlOx2 and AlOx3 were seen. AlOx2 and AlOx3 are also thought to be involved in the production of H_2O_2. The same four genes were also upregulated in the AC20 samples. In addition to those, an upregulation was also seen for CyOx and QRD, which are involved in Fe^{2+} supply for the Fenton reaction.

Table 4. *P*-values for all genes and treatments calculated with t-test. *P*-values are "not significant" for $p > 0.05$; "significant" for $0.05 \geq p < 0.01$, and highly significant for $p \leq 0.01$. Genes in bold are upregulated, others are downregulated.

	AC10	AC15	AC20	Gene
UT	2.08×10^{-9}	7.33×10^{-9}	2.27×10^{-9}	
AC10		7.3×10^{-6}	9.81×10^{-9}	GlyD
AC15			1.14×10^{-5}	
UT	3.60×10^{-5}	5.85×10^{-4}	4.07×10^{-5}	
AC10		3.48×10^{-4}	5.19×10^{-4}	**GOx2**
AC15			1.37×10^{-1}	
UT	1.29×10^{-1}	3.14×10^{-6}	4.03×10^{-4}	
AC10		3.16×10^{-6}	2.21×10^{-4}	**CyOx**
AC15			4.53×10^{-5}	
UT	8.95×10^{-9}	3.26×10^{-6}	1.50×10^{-4}	
AC10		9.69×10^{-9}	1.88×10^{-4}	**Cro1**
AC15			1.60×10^{-4}	
UT	5.64×10^{-7}	6.44×10^{-7}	1.21×10^{-6}	
AC10		7.38×10^{-6}	3.18×10^{-4}	Lac1
AC15			1.41×10^{-3}	
UT	1.32×10^{-6}	1.26×10^{-6}	3.13×10^{-6}	
AC10		2.25×10^{-7}	6.08×10^{-4}	PQT
AC15			4.23×10^{-4}	
UT	7.86×10^{-3}	7.71×10^{-5}	1.19×10^{-12}	
AC10		5.48×10^{-5}	3.75×10^{-14}	**AlOx3**
AC15			5.32×10^{-4}	
UT	4.70×10^{-11}	1.59×10^{-10}	3.72×10^{-10}	
AC10		1.24×10^{-10}	3.51×10^{-10}	**AlOx2**
AC15			7.04×10^{-9}	
UT	1.83×10^{-12}	3.5×10^{-1}	1.71×10^{-11}	
AC10		1.39×10^{-8}	6.56×10^{-12}	AlOx1
AC15			1.63×10^{-7}	
UT	1.26×10^{-11}	4.99×10^{-6}	3.48×10^{-8}	
AC10		1.79×10^{-8}	1.52×10^{-10}	**QRD**
AC15			2.27×10^{-9}	
Highly significant		Significant		Not significant

4. Discussion

4.1. Mass Loss and Growth Rates

The negative value for the mass loss in AC20 samples could be due to the weight gain through fungal hyphae. This phenomenon has been seen in previous studies [71,77,81].

4.2. Expression of Target Genes

4.2.1. Upregulated Genes

The results of this study show that *R. placenta* upregulates parts of the genes involved in non-enzymatic oxidative degradation when growing on acetylated samples as compared with untreated wood, especially on higher levels of acetylation. An upregulation of GOx2 on acetylated samples was clearly shown as compared with untreated wood (Figure 1). GOx2 belongs to the GMC oxidoreductases, just as AlOx3. Ringman et al. [76] did not find any significant differences between untreated and modified samples for GOx2 expression which differs from our findings. This might be explained by the different sample design and that not only the non-enzymatic degradation phase was captured in the samples from Ringman et al. [76]. An upregulation in treated samples may be of importance for production of higher amounts of H_2O_2 for the Fenton reaction.

The highest levels of Cro1 were found in AC20 samples, but no clear upward trend was observed with increasing acetylation level. An upregulation of Cro1 and GOx2 appeared already in the AC10 samples. This differs from the upregulation of AlOx2 and AlOx3 which were lower in AC10 samples as compared with untreated samples. The reason for the upregulation of Cro1 could be that the fungus increases the H_2O_2 production. Maybe the fungus can upregulate Cro1 and GOx2 faster and with lower effort than the two AlOx genes. Beck et al. [13] found a significant upregulation in samples with a WPG of 21 during initial decay as compared with other treatments, which supports our findings, but the values were higher in the present study. In the study by Ringman et al. [76], Cro1 levels were significantly lower in acetylated samples which is contrary to our findings for all acetylation levels. It should, however, be noted that the sample and experiment design differ between these studies. In Beck et al. [13] and Ringman et al. [76] the samples may have contained mycelia that were in both the non-enzymatic and in the enzymatic phase.

AlOx2 and AlOx3 are both significantly upregulated in AC15 and AC20 samples as compared with untreated samples. Alfredsen et al. [11] compared three different acetylation levels at the same mass loss level with untreated samples and found a significant upregulation in AlOx3 between high-level acetylated and untreated samples. This supports our findings, even though it cannot be decided with certainty that only the oxidative degradation phase was detected due to the sample design in Alfredsen et al. [11]. Beck et al. [13] also reported upregulation of AlOx2 and AlOx3 in acetylated samples as compared with untreated samples, at initial decay. They also found a significantly higher upregulation in the highest level of acetylated samples when looking at samples in early stages of decay, which is similar to our results [13], assuming that Beck et al. [13] only looked at mycelia in the non-enzymatic degradation stage. Ringman et al. [70] reported a small, but not significant, upregulation of AlOx3 in acetylated samples, which differs from our findings. In another study from Ringman et al. [76], an upregulation of AlOx3 in modified samples as compared with untreated samples was seen, as well as in Schmöllerl et al. [69]. This indicates a higher investment in the production of H_2O_2 on modified samples as compared with untreated samples.

AC15 showed the lowest values of CyOx gene expression while the highest values were seen in AC20 [13]. Beck et al. [13] did not find an upregulation in CyOx in acetylated samples, except for the AC17 samples. The reason for these differences might be the different sample design. They also found mass loss in the AC21 samples, showing that the fungus had started the enzymatic degradation phase, which may be another reason for the differences in gene expression. CyOx functions as a catalysator to form acetate and oxalate in filamentous fungi. Enhanced oxalate production forms soluble Fe^{2+}-oxalate complexes needed for the Fenton reaction [82]. This could explain the high expression, especially in samples with high levels of acetylation [83].

R. placenta appears to downregulate the formation of QRD in samples with lower acetylation levels as compared with untreated samples and upregulates it to a level high than that of the untreated samples when growing on higher-level acetylated samples. These results indicate that only higher levels of acetylation induce upregulation of QRD as compared with untreated samples. This suggests that the *R. placenta* QRD could be involved in stress defense rather than in the reduction of chelators. The lower levels of gene expression in AC10 and AC15 could be explained by less stress, since the fungus is still able to degrade the wood. Ringman et al. [70] showed a significant upregulation of QRD in acetylated samples after two and 14 days as compared with untreated samples. The results for QRD gene expression during the initial decay phase differ from the results found in the present study [70], clearly demonstrating the importance of studying the exact function of the QRD found in *R. placenta* in comparison to the two QRD genes found in *G. trabeum*. Beck et al. [13] found no significant upregulation of QRD (BqR) between different harvesting points. Alfredsen et al. [11] observed an increase of QRD expression on treated samples with increasing incubation time which most likely is a result of the enzymatic degradation.

4.2.2. Downregulated Genes

The downregulation of the genes Lac1, PQT, GlyD, and AlOx1 clearly shows that not all genes presumably involved in the non-enzymatic oxidative degradation are upregulated when *R. placenta* is growing on acetylated wood as compared with untreated wood. This leads to the assumption that the downregulated genes may not be as important for the non-enzymatic degradation as assumed or that the acetylation affects the regulation of the genes.

Significantly lower levels of Lac1 expression were shown, in this study, for acetylated samples as compared with untreated, which differs from previous studies. Ringman et al. [77] studied Lac1 on thermally modified wood as compared with untreated wood and no significant differences were shown between treatments and different mass loss levels. In addition, previously, no significant differences have been shown by Zhang et al. [47] for gene expression of Lac1 between oxidative and enzymatic degradation which could indicate that Lac1 does not play an important role during the non-enzymatic oxidative degradation phase or the expression is inhibited through acetylation. However, they found the highest activity for laccase in the hyphal front.

Clearly higher amounts of PQT were found in untreated samples; downregulation in AC10 and further downregulation in AC15 seem to show a tendency. However, levels of PQT expression were higher in AC20, which does not follow any trendline. Since the differences in expression levels between the treatments in this study do not show any clear trends, they are difficult to interpret. The downregulation of PQT in acetylated samples seen in our study, may be due to the fact that this gene may not be involved in oxidative wood degradation after all. On the basis of its sequence, it was proposed to be a quinate transporter [58], however, it is also possible that it is a sugar transporter [59]. Ringman et al. [76] did not find significant differences in expression of PQT between treatments or time points during the non-enzymatic degradation phase but an induction of PQT was seen at 3% mass loss. The results from Ringman et al. [76] indicate that PQT is not induced before the enzymatic degradation phase.

Expression levels of GlyD were significantly lower in all treated samples as compared with untreated. AC10 showed the lowest expression levels and gene expression in AC15 samples was upregulated as compared with the other two treatment levels. Since GlyD levels were lower in AC20 samples as compared with AC15 samples, a clear trend cannot be observed. No significant differences were shown for GlyD in Beck et al. [13], except at the first harvesting point, where untreated samples showed higher expression of GlyD as compared with all levels of acetylation. However, Munir et al. [84] suggested that the major enzyme involved in oxalate production in wood rotting basidiomycetes could be oxaloacetase, which makes the interpretation of our GlyD results difficult. Determination of the total oxalic acid production for comparison of the amounts of different treatments and treatment levels would be a useful goal for further research [85].

In this study, a downregulation of AlOx1 was seen for AC20, and for AC10 markedly lower rates as compared with untreated samples were seen. However, AC15 behaved differently, since there was no significant difference between expression levels of AC15 as compared with untreated samples in this study. Beck et al. [13] reported a downregulation of AlOx1 in AC17 and AC21 samples as compared with AC10 and untreated samples. The results imply that AlOx1 does not serve the same purpose as AlOx2 and AlOx3.

4.2.3. Effect of Acetylation on Overall Gene Expression

In this study, not all genes previously proposed to be involved in oxidative processes were upregulated in acetylated samples as compared with untreated samples. Instead, some were associated with much lower expression levels in acetylated samples as compared with untreated samples. Because of a high number of replicates ($n \geq 11$) and the high significance of the results, these findings can be considered reliable.

Zhang et al. [86] suggested that the shift between non-enzymatic and enzymatic wood degradation is mediated by cellobiose, which is formed through degradation of cellulose. In wood not experiencing

mass loss, the levels of cellobiose are assumed to be low, keeping the expression of the genes in the enzymatic degradation phase at a low level. However, the absence of glucose, upregulates the expression of non-enzymatic genes [86]. Therefore, it is assumed, in modified wood, that there is a general upregulation of the genes involved in the non-enzymatic degradation phase, since the fungus is struggling to degrade it. Previous studies have shown that *R. placenta* both up- and downregulates the expression of some genes assumed to be involved in oxidative degradation processes when growing on modified wood, which is in accordance with our results [68–70]. These results, together with previously published gene expression studies on modified wood [11,69,70,75,76,78,79], imply that there might be mechanisms in the regulation of the non-enzymatic degradation in addition to the cellobiose switch proposed by Zhang et al. [86] that are important also in untreated wood. However, it should be noted that in modified wood, degradation products, such as cellobiose, presumably will be modified as well during the wood modification process, which may affect their function as gene expression regulators. Hence, it should be noted that in acetylated wood, the degradation products might look different from the degradation products in untreated wood since they come from wood constituents that were acetylated.

The expression of genes involved in the non-enzymatic degradation phase appeared to be enhanced group-wise according to an increasing level of acetylation. An upregulation of GOx2 and Cro1 (involved in the H_2O_2- production) was seen in AC10 as compared with untreated wood. In AC15, the same two genes were upregulated including two more, also involved in the production of H_2O_2, AlOx2, and AlOx3. In AC20, in addition to GOx2, Cro1, AlOx2, and AlOx3, also CyOx and QRD (both involved in the oxalic acid production) were upregulated. Perhaps the fungus is trying to increase the output of the Fenton reaction by increasing the input of H_2O_2. Perhaps this works for AC10 and AC15, and more sugars are diffusing out into the lumen, but it does not seem to be the case for AC20. Here the fungus must do something more, so it additionally upregulates the production of oxalic acid, trying to set more iron free, and thereby increasing the output of the Fenton reaction. It is possible that upregulating the genes involved in H_2O_2 production presents the lowest "cost" for the fungus and that it is a more direct way to control the Fenton reaction. However, when that does not work, other measures have to be taken.

A clear downregulation of at least some genes, presumably involved in the non-enzymatic oxidative degradation mechanism, implies that the downregulated genes either do not have the function they were assumed to have or that they do not play an important role during the first degradation step. Comparisons with previous studies proved difficult, due to the differences in sample designs. A complete picture of all transcribed genes (transcriptome sequencing) during the early stages of decay and a comparison between untreated samples and treated samples would be of great interest. Useful data on gene expression levels during enzymatic degradation from the same wood wafers for comparison would also be valuable.

5. Conclusions

In this study the gene expression of 10 *R. placenta* genes, presumed to be important in the non-enzymatic degradation phase, were studied in untreated and wood acetylated to three different levels. The expression of the genes involved in the non-enzymatic degradation phase appeared to be enhanced group-wise according to an increasing level of acetylation. *R. placenta* seemed to first increase the output of the Fenton reaction by increasing the input of H_2O_2, trying to establish a satisfying level of degradation, and when this was not possible, the production of oxalic acid was enhanced. Highly interesting is the fact that even though growth and degradation were not inhibited in AC10 samples, results on gene expression showed a relatively strong reaction of the fungus. Furthermore, the results of this study show that *R. placenta* upregulates parts of the genes involved in non-enzymatic oxidative degradation when growing on acetylated samples as compared with untreated wood, especially on higher levels of acetylation. However, only six of the 10 genes are as important for the non-enzymatic degradation phase as previously assumed. The results imply, for example, that AlOx1 does not

serve the same purpose as AlOx2 and AlOx3. This confirms previous findings by Beck et al. [13]. Furthermore, the results indicate that only higher levels of acetylation induce upregulation of QRD as compared with untreated samples. This suggests that the *R. placenta* QRD may be involved in stress defense rather than in the reduction of chelators. Highlighting the importance of finding the true function of the QRD gene in *R. placenta*. The results from this study, together with previously published gene expression studies on modified wood [11,69,70,75,76,78,79], imply that there might be mechanisms in the regulation of the non-enzymatic degradation in addition to the cellobiose switch proposed by Zhang et al. [86] in both untreated and modified wood. It would be interesting to apply this method to other modification methods, such as furfurylated and thermally-modified wood. Further research on the whole transcriptome including both the non-enzymatic and the enzymatic degradation phase would be of great interest for an increased understanding of the wood degrading capacities of *R. placenta*.

References

1. Rowell, R.M. *Wood Chemistry and Wood Composites*; Rowell, R.M., Ed.; Taylor & Francis: Boca Raton, FL, USA, 2005; p. 703. [CrossRef]
2. Huß, W.; Krötsch, S. Energieeffizientes Sanieren und Bauen mit Holz. *LWF Aktuell* **2013**, *97*, 4.
3. Jakes, J.E.; Arzola, X.; Bergman, R.; Ciesielski, P.; Hunt, C.G.; Rahbar, N.; Tshabalala, M.; Wiedenhoeft, A.C.; Zelinka, S.L. Not Just Lumber—Using Wood in the Sustainable Future of Materials, Chemicals, and Fuels. *JOM* **2016**, *68*, 10. [CrossRef]
4. Bergman, R.; Puettmann, M.; Taylor, A.; Skog, K. The Carbon Impacts of Wood Products. *For. Prod. J.* **2014**, *64*, 13. [CrossRef]
5. Zabel, R.A.; Morrell, J.J. *Wood Microbiology: Decay and Its Prevention*; Academic Press: San Diego, CA, USA, 1992.
6. Eaton, R.; Hale, M. *Decay, Pests and Protection*; Chapman & Hall: Cambridge, UK, 1993.
7. Lebow, S. Alternatives to Chromated Copper Arsenate (CCA) for Residential Construction. In Proceedings of the Environmental Impacts of Preservative-Treated Wood, Orlando, FL, USA, 8–10 February 2004; p. 12.
8. Hill, C. *Wood Modification: Chemical, Thermal and other Processes*; Stevens, C.V., Ed.; John Wiley and Sons, Ltd.: Hoboken, NJ, USA, 2006.
9. Townsend, T.; Dubey, B.; Tolaymat, T.; Solo-Gabriele, H. Preservative leaching from weathered CCA-treated wood. *J. Environ. Manag.* **2005**, *75*, 105–113. [CrossRef]
10. Mantanis, G.I. Chemical modification of wood by acetylation or furfurylation: A review of the present scaled-up technologies. *Bioresources* **2017**, *12*, 12. [CrossRef]
11. Alfredsen, G.; Pilgård, A.; Fossdal, C.G. Characterisation of Postia placenta colonisation during 36 weeks in acetylated southern yellow pine sapwood at three acetylation levels including genomic DNA and gene expression quantification of the fungus. *Holzforschung* **2016**, *70*, 11. [CrossRef]
12. Hosseinpourpia, R.; Mai, C. Mode of action of brown rot decay resistance of acetylated wood: Resistance to Fenton's reagent. *Wood Sci. Technol.* **2016**, *50*, 413–426. [CrossRef]
13. Beck, G.; Hegnar, O.A.; Fossdal, C.G.; Alfredsen, G. Acetylation of Pinus radiata delays hydrolytic depolymerisation by the brown-rot fungus Rhondonia placenta. *Int. Biodeterior. Biodegrad.* **2018**, *135*, 39–52. [CrossRef]

14. Engelund, E.; Thygesen, L.G.; Svensson, S.; Hill, C. A critical discussion of the physics of wood-water interactions. *Wood Sci. Technol.* **2013**, *47*, 21. [CrossRef]

15. Popescu, C.-M.; Hill, C.A.S.; Curling, S.; Ormondroyd, G.; Xie, Y. The water vapour sorption behaviour of acetylated birch wood: How acetylation affects the sorption isotherm and accessible hydroxyl content. *J. Mater. Sci.* **2014**, *49*, 2362–2371. [CrossRef]

16. Beck, G.; Thybring, E.; Thygesen, L.; Hill, C. Characterization of moisture in acetylated and propionylated radiata pine using low-field nuclear magnetic resonance (LFNMR) relaxometry. *Holzforschung* **2017**. [CrossRef]

17. Stamm, A.J.; Baechler, R.H. Decay resistance and dimensional stability of five modified woods. *For. Prod. J.* **1960**, *10*, 22–26.

18. Ibach, R.E.; Rowell, R.M. Improvements in Decay Resistance Based on Moisture Exclusion. *Mol. Cryst. Liq. Cryst. Sci. Technol. Sect. A Mol. Cryst. Liq. Cryst.* **2000**, *353*, 23–33. [CrossRef]

19. Larsson Brelid, P.; Simonson, R.; Bergman, Ö.; Nilsson, T. Resistance of acetylated wood to biological degradation. *Holz Als Roh- Und Werkst.* **2000**, *58*, 331–337. [CrossRef]

20. Hill, C. Why does acetylation protect wood from microbiological attack? *Wood Mater. Sci. Eng.* **2009**, *4*, 37–45. [CrossRef]

21. Papadopoulos, A.N.; Hill, C.A.S. The biological effectiveness of wood modified with linear chain carboxylic acid anhydrides against Coniophora puteana. *Holz Als Roh- Und Werkst.* **2002**, *60*, 329–332. [CrossRef]

22. Jakes, J.; Plaza, N.; Stone, D.S.; Hunt, C.; Glass, S.; Zelinka, S. Mechanism of Transport Through Wood Cell Wall Polymers. *J. For. Prod. Ind.* **2013**, *2*, 10–13.

23. Xie, Y.; Xiao, Z.; Mai, C. Degradation of chemically modified Scots pine (Pinus sylvestris L.) with Fenton reagent. *Holzforschung* **2015**, *69*, 153. [CrossRef]

24. Zelinka, S.L.; Ringman, R.; Pilgård, A.; Thybring, E.E.; Jakes, J.E.; Richter, K. The role of chemical transport in the brown-rot decay resistance of modified wood AU - Zelinka, S.L. *Int. Wood Prod. J.* **2016**, *7*, 66–70. [CrossRef]

25. Ringman, R.; Pilgård, A.; Brischke, C.; Richter, K. Mode of action of brown rot decay resistance in modified wood: A review. *Holzforschung* **2014**, *68*, 239. [CrossRef]

26. Hunt, C.; Zelinka, S.; Frihart, C.; Lorenz, L.; Yelle, D.; Gleber, S.-C.; Vogt, S.; Jakes, J.E. Acetylation increases relative humidity threshold for ion transport in wood cell walls—A means to understanding decay resistance. *Int. Biodeterior. Biodegrad.* **2018**, *133*, 230–237. [CrossRef]

27. Zelinka, S.; Glass, S.; Stone, D. A percolation model for electrical conduction in wood with implications for wood-water relations. *Wood Fiber Sci. J. Soc. Wood Sci. Technol.* **2008**, *40*, 544–552.

28. Zelinka, S.L.; Gleber, S.C.; Vogt, S.; López, G.M.R.; Jakes, J.E. Threshold for ion movements in wood cell walls below fiber saturation observed by X-ray fluorescence microscopy (XFM). *Holzforschung* **2015**, *69*, 441–448. [CrossRef]

29. Kirk, P.M.; Cannon, P.F.; Minter, D.W.; Stalpers, J.A. *Dictionary of the Funghi*, 10th ed.; CAB International: Wallingford, UK, 2008.

30. Eriksson, K.; Blanchette, R.A.; Ander, P. *Microbial and Enzymatic Degradation of Wood and Wood Components*; Springer: Berlin, Germany, 1990.

31. Blanchette, R. Delignification by Wood-Decay Fungi. *Annu. Rev. Phytopathol.* **1991**, *29*, 18. [CrossRef]

32. Daniel, G. Use of electron microscopy for aiding our understanding of wood biodegradation. *FEMS Microbiol. Rev.* **1994**, *13*, 199–233. [CrossRef]

33. Riley, R.; Salamov, A.A.; Brown, D.W.; Nagy, L.G.; Floudas, D.; Held, B.W.; Levasseur, A.; Lombard, V.; Morin, E.; Otillar, R.; et al. Extensive sampling of basidiomycete genomes demonstrates inadequacy of the white-rot/brown-rot paradigm for wood decay fungi. *Proc. Natl. Acad. Sci. USA* **2014**, *111*, 9923–9928. [CrossRef]

34. Liese, W. Ultrastructural Aspects of Woody Tissue Disintegration. *Annu. Rev. Phytopathol.* **1970**, *8*, 231–258. [CrossRef]

35. Martin, F. Fair Trade in the Underworld: The Ectomycorrhizal Symbiosis. In *Biology of the Fungal Cell*; Howard, R.J., Gow, N.A.R., Eds.; Springer: Berlin/Heidelberg, Germany, 2007; pp. 291–308. [CrossRef]

36. Schmidt, O. Indoor wood-decay basidiomycetes: Damage, causal fungi, physiology, identification and characterization, prevention and control. *Mycol. Prog.* **2007**, *6*, 261. [CrossRef]

37. Alfredsen, G.; Solheim, H.; Mohn Jenssen, K. Evaluation of decay fungi in Norwegian buildings. In Proceedings of the IRG Annual Meeting, IRG/WP 09-10701, Bangalore, India, 24–28 April 2015; p. 11.

38. Vanden Wymelenberg, A.; Gaskell, J.; Mozuch, M.; Sabat, G.; Ralph, J.; Skyba, O.; Mansfield, S.D.; Blanchette, R.A.; Martinez, D.; Grigoriev, I.; et al. Comparative Transcriptome and Secretome Analysis of Wood Decay Fungi *Postia placenta* and *Phanerochaete chrysosporium*. *Appl. Environ. Microbiol.* **2010**, *76*, 3599–3610. [CrossRef]

39. Goodell, B. Brown-Rot Fungal Degradation of Wood: Our Evolving View. In *Wood Deterioration and Preservation*; American Chemical Society: Washington, DC, USA, 2003; Volume 845, pp. 97–118.

40. Filley, T.R.; Cody, G.D.; Goodell, B.; Jellison, J.; Noser, C.; Ostrofsky, A. Lignin demethylation and polysaccharide decomposition in spruce sapwood degraded by brown rot fungi. *Org. Geochem.* **2002**, *33*, 111–124. [CrossRef]

41. Curling, S.F.; Clausen, C.A.; Winandy, J.E. Relationships between mechanical properties, weight loss, and chemical composition of wood during incipient brown-rot decay. *For. Prod. J.* **2002**, *52*, 34–39.

42. Martínez, Á.T.; Speranza, M.; Ruiz-Dueñas, F.J.; Ferreira, P.; Camarero, S.; Guillén, F.; Martínez, M.J.; Gutiérrez, A.; Del Río, J.C. Biodegradation of lignocellulosics: Microbial, chemical, and enzymatic aspects of the fungal attack of lignin. *Int. Microbiol.* **2005**, *8*, 195–204. [PubMed]

43. Niemenmaa, O.; Uusi-Rauva, A.; Hatakka, A. Demethoxylation of [O14CH3]-labelled lignin model compounds by the brown-rot fungi Gloeophyllum trabeum and Poria (Postia) placenta. *Biodegradation* **2007**, *19*, 555. [CrossRef] [PubMed]

44. Arantes, V.; Goodell, B. Current Understanding of Brown-Rot Fungal Biodegradation Mechanisms: A Review. In *Deterioration and Protection of Sustainable Biomaterials*; American Chemical Society: Washington, DC, USA, 2014; Volume 1158, pp. 3–21.

45. Baldrian, P.; Valášková, V. Degradation of cellulose by basidiomycetous fungi. *FEMS Microbiol. Rev.* **2008**, *32*, 501–521. [CrossRef]

46. Arantes, V.; Jellison, J.; Goodell, B. Peculiarities of brown-rot fungi and biochemical Fenton reaction with regard to their potential as a model for bioprocessing biomass. *Appl. Microbiol. Biotechnol.* **2012**, *94*, 323–338. [CrossRef]

47. Zhang, J.; Presley, G.N.; Hammel, K.E.; Ryu, J.-S.; Menke, J.R.; Figueroa, M.; Hu, D.; Orr, G.; Schilling, J.S. Localizing gene regulation reveals a staggered wood decay mechanism for the brown rot fungus Postia placenta. *Proc. Natl. Acad. Sci. USA* **2016**, *113*, 10968–10973. [CrossRef]

48. Eastwood, D.C.; Floudas, D.; Binder, M.; Majcherczyk, A.; Schneider, P.; Aerts, A.; Asiegbu, F.O.; Baker, S.E.; Barry, K.; Bendiksby, M.; et al. The Plant Cell Wall–Decomposing Machinery Underlies the Functional Diversity of Forest Fungi. *Science* **2011**, *333*, 762–765. [CrossRef]

49. Goodell, B.; Jellison, J.; Liu, J.; Daniel, G.; Paszczynski, A.; Fekete, F.; Krishnamurthy, S.; Jun, L.; Xu, G. Low molecular weight chelators and phenolic compounds isolated from wood decay fungi and their role in the fungal biodegradation of wood1This is paper 2084 of the Maine Agricultural and Forest Experiment Station.1. *J. Biotechnol.* **1997**, *53*, 133–162. [CrossRef]

50. Paszczynski, A.; Crawford, R.; Funk, D.; Goodell, B. De novo synthesis of 4,5-dimethoxycatechol and 2, 5-dimethoxyhydroquinone by the brown rot fungus Gloeophyllum trabeum. *Appl. Environ. Microbiol.* **1999**, *65*, 674–679.

51. Jensen, K.A.; Houtman, C.J.; Ryan, Z.C.; Hammel, K.E. Pathways for Extracellular Fenton Chemistry in the Brown Rot Basidiomycete Gloeophyllum trabeum. *Appl. Environ. Microbiol.* **2001**, *67*, 2705–2711. [CrossRef]

52. Fenton, H. Oxidation of tartaric acid in the presence of iron. *J. Chem. Soc. Trans.* **1894**, *65*, 12. [CrossRef]

53. Goodell, B.; Zhu, Y.; Kim, S.; Kafle, K.; Eastwood, D.C.; Daniel, G.; Jellison, J.; Yoshida, M.; Groom, L.; Pingali, S.V.; et al. Modification of the nanostructure of lignocellulose cell walls via a non-enzymatic lignocellulose deconstruction system in brown rot wood-decay fungi. *Biotechnol. Biofuels* **2017**, *10*, 15. [CrossRef] [PubMed]

54. Martinez, D.; Challacombe, J.; Morgenstern, I.; Hibbett, D.S.; Schmoll, M.; Kubicek, C.P.; Ferreira, P.; Ruiz-Dueñas, F.J.; Martinez, A.T.; Kersten, P.; et al. Genome, transcriptome, and secretome analysis of wood decay fungus *Postia placenta* supports unique mechanisms of lignocellulose conversion. *Proc. Natl. Acad. Sci. USA* **2009**, *106*, 6. [CrossRef] [PubMed]

55. Jensen, K.; Ryan, Z.; Marty, A.; Cullen, D.; Hammel, K.E. An NADH: Quinone Oxidoreductase Active during Biodegradation by the Brown-Rot Basidiomycete Gloeophyllum trabeum. *Appl. Environ. Microbiol.* **2002**, *68*, 2699–2703. [CrossRef] [PubMed]

56. Qi, W.; Jellison, J. Characterization of a transplasma membrane redox system of the brown rot fungus Gloeophyllum trabeum. *Int. Biodeterior. Biodegrad.* **2004**, *53*, 37–42. [CrossRef]

57. Cohen, R.; Suzuki, M.R.; Hammel, K.E. Differential stress-induced regulation of two quinone reductases in the brown rot basidiomycete Gloeophyllum trabeum. *Appl. Environ. Microbiol.* **2004**, *70*, 324–331. [CrossRef] [PubMed]

58. Mueckler, M.; Caruso, C.; Baldwin, S.A.; Panico, M.; Blench, I.; Morris, H.R.; Allard, W.J.; Lienhard, G.E.; Lodish, H.F. Sequence and structure of a human glucose transporter. *Science* **1985**, *229*, 941. [CrossRef]

59. Yan, N. Structural Biology of the Major Facilitator Superfamily Transporters. *Annu. Rev. Biophys.* **2015**, *44*, 257–283. [CrossRef]

60. Wardman, P. Reduction Potentials of One-Electron Couples Involving Free Radicals in Aqueous Solution. *J. Phys. Chem. Ref. Data* **1989**, *18*, 1637–1755. [CrossRef]

61. Wei, D.; Houtman, C.J.; Kapich, A.; Hunt, C.; Cullen, D.; Hammel, K.E. Laccase and Its Role in Production of Extracellular Reactive Oxygen Species during Wood Decay by the Brown Rot Basidiomycete Postia placenta. *Appl. Environ. Microbiol.* **2010**, *76*, 2091–2097. [CrossRef]

62. Floudas, D.; Binder, M.; Riley, R.; Barry, K.; Blanchette, R.A.; Henrissat, B.; Martínez, A.T.; Otillar, R.; Spatafora, J.W.; Yadav, J.S.; et al. The Paleozoic Origin of Enzymatic Lignin Decomposition Reconstructed from 31 Fungal Genomes. *Science* **2012**, *336*, 1715–1719. [CrossRef] [PubMed]

63. Levasseur, A.; Drula, E.; Lombard, V.; Coutinho, P.M.; Henrissat, B. Expansion of the enzymatic repertoire of the CAZy database to integrate auxiliary redox enzymes. *Biotechnol. Biofuels* **2013**, *6*, 41. [CrossRef] [PubMed]

64. Takao, S. Organic Acid Production by Basidiomycetes. *Appl. Microbiol.* **1965**, *13*, 732. [PubMed]

65. Akamatsu, Y.; Shimada, M. Partial purification and characterization of glyoxylate oxidase from the brown-rot basidiomycete Tyromyces palustris. *Phytochemistry* **1994**, *37*, 649–653. [CrossRef]

66. Munir, E.; Yoon, J.-J.; Tokimatsu, T.; Hattori, T.; Shimada, M. New role for glyoxylate cycle enzymes in wood-rotting basidiomycetes in relation to biosynthesis of oxalic acid. *J. Wood Sci.* **2001**, *47*, 368–373. [CrossRef]

67. Tokimatsu, T.; Nagai, Y.; Hattori, T.; Shimada, M. Purification and characteristics of a novel cytochrome c dependent glyoxylate dehydrogenase from a wood-destroying fungus Tyromyces palustris 1. *FEBS Lett.* **1998**, *437*, 117–121. [CrossRef]

68. Pilgård, A.; Alfredsen, G.; Fossdal, C.G.; Long, I.C.J. The effects of acetylation on the growth of Postia placenta over 36 weeks. In Proceedings of the IRG Annual Meeting, Kuala Lumpur, Malaysia, 6–10 May 2012. IRG/WP 12-40589.

69. Schmöllerl, B.; Alfredsen, G.; Fossdal, C.G.; Westin, M.; Steitz, A. Molecular investigation of Postia placenta growing in modified wood. In Proceedings of the IRG Annual Meeting, IRG/WP 11-10756, Queenstown, New Zealand, 8–12 May 2018; p. 10.

70. Ringman, R.; Pilgård, A.; Richter, K. Effect of wood modification on gene expression during incipient Postia placenta decay. *Int. Biodeterior. Biodegrad.* **2014**, *86*, 86–91. [CrossRef]

71. Ringman, R.; Pilgård, A.; Brischke, C.; Windeisen, E.; Richter, K. Incipient brown rot decay in modified wood: Patterns of mass loss, structural integrity, moisture and acetyl content in high resolution. *Int. Wood Prod. J.* **2017**. [CrossRef]

72. Bravery, A.F. A miniaturised wood-block test for the rapid evaluation of preservative fungicides. In *Screening Techniques for Potential Wood Preservative Chemicals. Proceedings of the a Special Seminar Held in Association with the 10th Annual Meeting of IRG, Peebles, UK, 18−22 September 1978*; IRG: Washington, DC, USA, 1978.

73. Alfredsen, G.; Pilgård, A. Postia placenta decay of acetic anhydride modified wood—Effect of leaching. *Wood Mater. Sci. Eng.* **2014**. [CrossRef]

74. Schilling, J.S.; Duncan, S.M.; Presley, G.N.; Filley, T.R.; Jurgens, J.A.; Blanchette, R.A. Colocalizing incipient reactions in wood degraded by the brown rot fungus Postia placenta. *Int. Biodeterior. Biodegrad.* **2013**, *83*, 56–62. [CrossRef]

75. Alfredsen, G.; Ringman, R.; Pilgård, A.; Fossdal, C.G. New insight regarding mode of action of brown rot decay of modified wood based on DNA and gene expression studies: A review. *Int. Wood Prod. J.* **2015**, *6*, 5–7. [CrossRef]

76. Ringman, R.; Pilgård, A.; Kölle, M.; Richter, K. Expression patterns of Postia placenta genes involved in the chelator mediated Fenton degradation in modified wood. In Proceedings of the European Conference on Wood Modification, Arnhem, The Netherlands, 17–18 September 2019; p. 3.

77. Ringman, R.; Pilgård, A.; Kölle, M.; Brischke, C.; Richter, K. Effects of thermal modification on Postia placenta wood degradation dynamics: Measurements of mass loss, structural integrity and gene expression. *Wood Sci. Technol.* **2016**, *50*, 385–397. [CrossRef]

78. Alfredsen, G.; Fossdal, C.G.; Nahy, N.E.; Jellison, J.; Goodell, B. Furfurylated wood: Impact on Postia placenta gene expression and oxalate crystal formation. *Holzforschung* **2016**, *70*, 16. [CrossRef]

79. Pilgård, A.; Schmöllerl, B.; Risse, M.; Fossdal, C.G.; Alfredsen, G. Profiling Postia placenta colonisation in modified wood—microscopy, DNA quantification and gene expression. In Proceedings of the Wood Science and Engineering Conference, Copenhagen, Denmark, 17–18 September 2019.

80. Salame, T.M.; Knop, D.; Levinson, D.; Yarden, O.; Hadar, Y. Redundancy among manganese peroxidases in *Pleurotus ostreatus*. *Appl. Environ. Microbiol.* **2013**, *79*, 2405. [PubMed]

81. Ringman, R.; Beck, G.; Pilgård, A. The Importance of Moisture for Brown Rot Degradation of Modified Wood: A Critical Discussion. *Forests* **2019**, *10*, 522. [CrossRef]

82. Arantes, V.; Qian, Y.; Milagres, A.M.F.; Jellison, J.; Goodell, B. Effect of pH and oxalic acid on the reduction of Fe3+ by a biomimetic chelator and on Fe3+ desorption/adsorption onto wood: Implications for brown-rot decay. *Int. Biodeterior. Biodegrad.* **2009**, *63*, 478–483. [CrossRef]

83. Narayanan, B.C.; Niu, W.; Han, Y.; Zou, J.; Mariano, P.S.; Dunaway-Mariano, D.; Herzberg, O. Structure and function of PA4872 from Pseudomonas aeruginosa, a novel class of oxaloacetate decarboxylase from the PEP mutase/isocitrate lyase superfamily. *Biochemistry* **2008**, *47*, 167–182. [CrossRef]

84. Munir, E.; Yoon, J.; Tokimatsu, T.; Hattori, T.; Shimada, M. A physiological role for oxalic acid biosynthesis in the wood-rotting basidiomycete Fomitopsis palutris. *Proc. Natl. Acad. Sci. USA* **2001**, *98*, 11126–11130. [CrossRef]

85. Schilling Jonathan, S.; Jellison, J. High-performance liquid chromatographic analysis of soluble and total oxalate in Ca- and Mg-amended liquid cultures of three wood decay fungi. *Holzforschung* **2004**, *58*, 682. [CrossRef]

86. Zhang, J.; Schilling, J. Role of carbon source in the shift from oxidative to hydrolytic wood decomposition by Postia placenta. *Fungal Genet. Biol.* **2017**, *106*. [CrossRef]

III

Comparative Transcriptomics During Brown Rot Decay in Three Fungi Reveals Strain-Specific Degradative Strategies and Responses to Wood Acetylation

Martina Kölle[1], Maria Augusta Crivelente Horta[2], J. Philipp Benz[2,3] and Annica Pilgård[1,4]*

[1] Chair of Wood Science, TUM School of Life Sciences Weihenstephan, Technical University of Munich, Munich, Germany,
[2] Professorship of Fungal Biotechnology in Wood Science, TUM School of Life Sciences Weihenstephan, Technical University of Munich, Munich, Germany, [3] Institute of Advanced Study, Technical University of Munich, Munich, Germany, [4] Biobased Materials, Bioeconomy, RISE Research Institutes of Sweden, Borås, Sweden

Brown rot fungi degrade wood in a two-step process in which enzymatic hydrolysis is preceded by an oxidative degradation phase. While a detailed understanding of the molecular processes during brown rot decay is mandatory for being able to better protect wooden products from this type of degradation, the underlying mechanisms are still not fully understood. This is particularly true for wood that has been treated to increase its resistance against rot. In the present study, the two degradation phases were separated to study the impact of wood acetylation on the behavior of three brown rot fungi commonly used in wood durability testing. Transcriptomic data from two strains of *Rhodonia placenta* (FPRL280 and MAD-698) and *Gloeophyllum trabeum* were recorded to elucidate differences between the respective decay strategies. Clear differences were found between the two decay stages in all fungi. Moreover, strategies varied not only between species but also between the two strains of the same species. The responses to wood acetylation showed that decay is generally delayed and that parts of the process are attenuated. By hierarchical clustering, we could localize several transcription factors within gene clusters that were heavily affected by acetylation, especially in *G. trabeum*. The results suggest that regulatory circuits evolve rapidly and are probably the major cause behind the different decay strategies as observed even between the two strains of *R. placenta*. Identifying key genes in these processes can help in decay detection and identification of the fungi by biomarker selection, and also be informative for other fields, such as fiber modification by biocatalysts and the generation of biochemical platform chemicals for biorefinery applications.

Keywords: *Rhodonia placenta*, *Postia placenta*, *Gloeophyllum trabeum*, brown rot decay, transcriptome comparison, wood degradation and deterioration, acetylated wood

INTRODUCTION

Brown rot fungi occur naturally within the northern coniferous forest ecosystems, where they also represent the most dominant form of wood decay (Eriksson et al., 1990; Blanchette, 1991; Daniel, 1994). Due to their preference of growing mainly on softwood, they are one of the most important microorganisms when it comes to degradation of wooden products (Zabel and Morrell, 1992; Rowell, 2005). Brown rot degradation leads to massive strength loss through depolymerization of the cellulose fraction before mass loss is even detectable (Eaton and Hale, 1993; Goodell, 2003). Previous research suggests that brown rot degrades wood in a two-step mechanism (Goodell, 2003; Baldrian and Valášková, 2008; Arantes et al., 2012; Zhang et al., 2016). Oxidative, non-enzymatic processes solubilize sugars leading to the secretion of hydrolytic enzymes (Curling et al., 2002; Filley et al., 2002; Martinez et al., 2005; Niemenmaa et al., 2008; Arantes et al., 2012; Zhang et al., 2016; Zhang and Schilling, 2017). The currently most accepted course of action is as follows: Oxalic acid, secreted by the brown-rot fungi, diffuses through the cell wall into the lumen, where it chelates Fe^{3+} (Goodell et al., 1997; Eastwood et al., 2011; Arantes et al., 2012). Through reduction by hydroquinones and other reducing agents, Fe^{2+} is formed, which reacts with H_2O_2, likely generated by a reaction between oxygen and hydroquinone's (Paszczynski et al., 1999; Jensen et al., 2001). This step is key to the "chelator-mediated Fenton" reaction (CMF) producing hydroxyl radicals, which depolymerize cellulose and hemicellulose and are able to modify lignin (Fenton, 1894; Baldrian and Valášková, 2008; Arantes et al., 2012). Solubilized sugars diffuse into the plant cell lumen, where they become accessible for cellulases and hemicellulases (Martinez et al., 2005; Goodell et al., 2017). Since brown rot fungi evolved several times independently from white rot fungi, versatile mechanisms for polysaccharide degradation exist, meaning that different brown rot species use variable enzymatic cocktails during the degradation process. Compared to the molecular processes in white rot fungi, brown rot degradation seems to be more complex, as for example more recent studies found that the oxidative phase, driven by reactive oxygen species is also supported by GHs seemingly tolerating oxidative stress (Castaño et al., 2018). However, the details are still not fully understood, showing that more research is needed.

To protect wooden products from degradation and prolong service life, wood modification is an approach to substitute traditionally used copper-based preservatives (Hill, 2006). Acetylation is one type of modification (Hill, 2006) that has been widely studied (Hill, 2006; Alfredsen et al., 2016; Hosseinpourpia and Mai, 2016; Mantanis, 2017; Beck et al., 2018; Zelinka et al., 2020). It has been shown that acetylation enhances the resistance of wooden products against fungal decay, but the exact mode of action remains vague beyond that. The replacement of hydroxyl groups in the cell wall polysaccharides by acetyl groups is thought to create a bulking effect of the cell walls that reduces water absorption through reduced hygroscopicity and volumetric swelling (Rowell, 2005; Hill, 2006; Thybring, 2013; Popescu et al., 2014; Beck et al., 2017). One likely theory considers that the equilibrium moisture content of the wood is reduced by the acetylation, resulting in the inhibition of diffusion of fungal degradation agents through the wood cell wall (Papadopoulos and Hill, 2002; Hill, 2006; Jakes et al., 2013; Ringman et al., 2014; Xie et al., 2015; Zelinka et al., 2016). The inhibition of diffusion has been further observed by several studies on either ion mobility (Hunt et al., 2018), hydrolytic reductants (Ringman et al., 2014) or an interrupted moisture network (Zelinka et al., 2008, 2015, 2016; Beck et al., 2017).

It has been shown that degradation is delayed in modified wood, but once it has started, it appears to progress in a similar manner (Alfredsen et al., 2016; Beck et al., 2018). Since the accumulated data point to the initial decay phase as the crucial step where degradation is inhibited, a better understanding of how and where the fungus is inhibited is mandatory to further improve modification methods. Similarly important for an increased use of wood in construction is the development of biomarkers for detection and monitoring of fungal decay in untreated and modified wood (Gelhaye and Morel, 2009).

Here, we investigated differential gene expression between three brown rot fungi: two strains of *R. placenta* (Fr.) *Niemelä, K.H. Larss. &* Schigel (also known as *Postia placenta*) FPRL280 and MAD-698, as well as *G. trabeum* (Pers.) *Murrill*, all of them presenting different phenotypes (Thaler et al., 2012; Presley and Schilling, 2017; Kölle et al., 2020). The brown rot fungi *R. placenta* and *G. trabeum* are common inhabitants of forest ecosystems and are also largely responsible for the destructive decay of wooden structures (Niemenmaa et al., 2008). Indeed, they are considered the two major experimental organisms for studies of brown rot decay, representing distantly related species origins of brown rot (Hibbett and Donoghue, 2001; Martinez et al., 2009). According to observations *G. trabeum* and *R. placenta* have differences in the decay mechanisms, as during the demethoxylation reactions (Niemenmaa et al., 2008). In addition, they are largely used in standard wood durability testing (EN113) (CEN, 1996). Our aim was to reveal differences in brown rot degradation strategies to further elucidate the complexity of the ongoing processes during lignocellulose decay. This would be beneficial for fungal biotechnological applications as for example biomass pretreatment in bio refineries as well as the development of novel wood preservation methods. For this, the wafer degradation method (Schilling et al., 2013) was adopted to be able to separate non-enzymatic oxidative degradation from the enzymatic (hydrolytic) degradation phase. A transcriptomics approach was subsequently used to provide insight into differences in gene expression when growing on untreated vs. acetylated wood. Understanding how fungal behavior and growth is affected by wood modifications is important for the optimization and development of new wood protection methods. Furthermore, these findings will also be valuable for the development of new commercial applications for the degradation of lignocellulosic materials. Based on the previous genome comparison of the two *R. placenta* strains (Kölle et al., 2020), differences in overall gene expression should further elaborate the adapted degradation mechanisms between them.

MATERIALS AND METHODS

Wood Material

Wood wafers (80 × 18 × 2.3 mm^3) of Scots pine sapwood (*Pinus sylvestris*) were cut, dried and weighed (Zhang et al., 2016). Half of the samples were acetylated (15%) according to (Kölle et al., 2019). All samples were autoclaved prior to the decay test.

Decay Test

Weck jars were filled with a growth medium composed of 50 g soil, 25 g sand, 20 g vermiculite, and 45 mL of water for each sample container. Three feeder strips of pine wood were placed on the medium per glass and inoculated with either *R. placenta* (strain FPRL280), *R. placenta* (strain MAD-698) or *G. trabeum* (strain BAM 115), originally cultivated on potato dextrose agar (*R. placenta*) or 4% malt agar (*G. trabeum*) plates. After the feeder strips were completely overgrown with mycelium, one wafer was placed on each feeder strip. Three glasses per treatment and fungus were prepared. Jars were stored in a climate chamber (25°C, 65% rh) until wafers were ¾ overgrown. Since the growth speed was more or less the same for all strains and treatments, we assumed that these parameters (5 mm section for oxidative decay and ¾ line) are suitable for all three strains. A 5 mm section containing the hyphal front was taken representing the oxidative degradation zone. The rest of the mycelia was used for enzymatic degradation analysis. The section size of 5 mm was chosen due to a growth rate of about 2.5 mm/day, with the first 5 mm thus approximately representing a 48 h window, during which the non-enzymatic degradation phase has been shown to take place (Zhang et al., 2016). All three samples in one glass were pooled into one biological replicate.

RNA Purification

Samples were ground with an MM400 Mixer Mill (Retsch GmbH, Haan, Germany) using one 1.5 cm steel ball and 30 Hz for 2 min. All equipment was pre-cooled in liquid nitrogen. Sixty mg of wood powder was taken for RNA purification. Total RNA was extracted and purified using the MasterPure^{TM} RNA Purification Kit (Lucigen) (Ringman et al., 2016).

Sequencing

The quality of RNA extraction was measured with the Agilent 2,100 Bioanalyzer. The TruSeq stranded mRNA Library Prep Kit (Illumina) with TruSeq RNA Single Indexes Set A and Set B was used to generate the RNA-Seq library preparation 500 ng of input-RNA was quantified using a Qubit Fluorometer 2.0 and a Qubit RNA BR Assay Kit (both Invitrogen). Libraries were quantified with a Qubit Fluorometer 2.0 and a Qubit DNA HS Assay Kit. The fragment length of the libraries was reviewed and finally quantified with qPCR [QuantStudio 5 (Applied Biosystems)] according to the Illumina qPCR Guide using KAPA SYBR Fast Mastermix Low Rox (KAPA Biosystems). The libraries were normalized to 2nM with EB (Qiagen) and equally pooled (according to Illumina User Guide). An overview of the fragment lengths for all strains, treatments and decay stages is given in **Supplementary Figure 1**. The libraries were multiplexed using HiSeq Rapid single-read Cluster Kit v2 and sequenced on the Illumina HiSeq2500 sequencer using a HiSeq Rapid SBS Kit

v2 (single-indexed read 1:100 cycles index read for 7 cycles). HiSeq Control Software 2.2.70 was used, image analysis and base calling were performed with Real Time Analysis 1.18.66.4. Fastq-files were generated with the CASAVA BCL2FASTQ Conversion software v2.20.

Transcriptomic data are available on NCBI with the BioProject ID: PRJNA681134 (https://www.ncbi.nlm.nih.gov/sra/PRJNA681134).

Data Analysis

For data analysis, data were uploaded to the Galaxy Server (Afgan et al., 2018). The reads from all samples were mapped against the total genome using Hisat2 (Kim et al., 2015). For transcript prediction, the mapped reads of each sample were assembled by StringTie (v1.3.3b) (Pertea et al., 2015, 2016) using a reference-based approach. BCFtools was used to perform the variant calling against genome sequences. The variant annotation and the prediction of functional effects were performed with SnpEff v5.0. Gene expression was analyzed using CLC Genomic Workbench v. 20.0.4 (Qiagen). Reads were mapped against the corresponding genomes (Floudas et al., 2012; Kölle et al., 2020), while the MAD-698 transcriptome data were mapped to the monokaryotic genome of MAD-SB12 (Gaskell et al., 2017). Mapping statistics can be found in **Supplementary Material 3**. MAD-SB12 genome was used as reference following previous results by Kölle et al. (2020). the genome size determined for FPRL280 is similar to the monokaryotic condition of MAD-SB12, isolated from a basidiospore of the fruiting dikaryon MAD-698, (Gaskell et al., 2017). Expression analysis was normalized and measured in TPM Ortholog search obtained the corresponding orthologous genes of all three strains. In total, 9,581 orthologs were found between the two strains of *R. placenta*, 6,145 orthologs between FPRL280 and *G. trabeum* and 6,381 between MAD-SB12 and *G. trabeum* (**Supplementary Material 1**). The gene set of the two strains with the lowest number of orthologs was chosen to create a list with orthologs to compare all three strains (**Supplementary Material 2**). Differential expression analysis was performed between the four groups within each strain [untreated/oxidative (UT/Ox); untreated/enzymatic (UT/Enz); acetylated/oxidative (AC/Ox); acetylated/enzymatic (AC/Enz)], using a log2 fold change cutoff of ±2 and a *p*-value of ≤0.05 (**Supplementary Materials 2, 3**). The differential expression analysis compares the oxidative and the enzymatic state of untreated samples of the two *R. placenta* strains FPRL280 and MAD-698 (**Supplementary Material 4**). A functional enrichment analysis was done using the Gene Ontology (GO) terms of both *R. placenta* strains and *G. trabeum*. Functional groups were described according to the model of (Zhang et al., 2016). A separate analysis classified brown rot-specific BRS genes into two groups, genes that are active during brown rot decay or have oxidative functions (BRS "Ox") and those that are related to enzymatic brown rot decay or have functions related to sugar metabolism (as for example GHs) (BRS "Enz") (**Supplementary Material 5**). The BRS gene list was assembled based on previous reports from Martinez et al. (2009), Ryu et al. (2011), and Zhang et al. (2016) and was manually accurate with

genes with specific functional annotation that showed phase-specific expression behavior. Genes encoding carbohydrate active enzymes (CAZy) were also grouped separately as the following groups: transporters, cytochrome P450 genes (partly putative), proteases and aldo-keto reductases (**Supplementary Material 3**). Genes with other function were grouped ("others"), including genes encoding uncharacterized or unknown proteins. A Pearson-correlation was performed to correlate MAD-698 data with the published data from (Zhang et al., 2016). Expression levels were hierarchically clustered with the "Hierarchical Clustering Explorer" software v3.5 (Seo and Shneiderman, 2002). Genes with low expression (<10 FPKMs under all conditions) were excluded from the analysis. Data were initially normalized (i.e., the deviation from the mean was divided by the standard deviation). The average linkage method was used for cluster generation, with centered Pearson's correlation as distance/similarity measure. Nodes were arranged with the smaller subtrees kept to the right.

RESULTS

Reads Mapping to the Reference Genome

We checked the accuracy of the previously published *R. placenta* FPRL280 genome (Kölle et al., 2020) for the analysis of transcriptomes (**Supplementary Material 1**). A variance rate of only one variant every 6,926 bases, with a total of 4,576 variant events, was detected for the genome assembly when

all transcripts were mapped against the reference *R. placenta* FPRL280, confirming the reliability of the reference genome.

Overall Expression Analysis

To compare the fungal responses during both degradation stages to the treatments, a differential expression analysis was performed. The total number of genes expressed by each strain for each condition is shown in **Supplementary Figure 2**. Since the wafer method was adopted from a related study testing the two-step response of *R. placenta* MAD-698 to untreated spruce wood samples (Zhang et al., 2016), we compared the gene expression data and found an acceptable degree of correlation [$r = 0.56$; $p = 0.00$; log2 FC between 0 and 5 mm (Ox) and 15–20 mm (Enz)], considering the differences in the used substrates (Spruce vs. Scots Pine).

The results for the differential expression analysis are shown in **Figure 1**. The differential expression analysis (DE) between UT/Ox and UT/Enz revealed the majority of genes to be upregulated during enzymatic decay (**Figure 1A**). There were also more genes upregulated in AC/Enz compared to AC/Ox. When comparing untreated and acetylated samples, it can be seen that there are more genes upregulated in acetylated samples, both during oxidative [UT/Ox vs. AC/Ox: FPRL280: 104 vs. 144; MAD-698: 67 vs. 185; GloTrab: 87 vs. 248) and enzymatic decay (UT/Enz vs. AC/Enz: FPRL280: 62 vs. 238; MAD-698: 51 vs. 112; GloTrab: 214 vs. 253]. In total, a higher number of genes
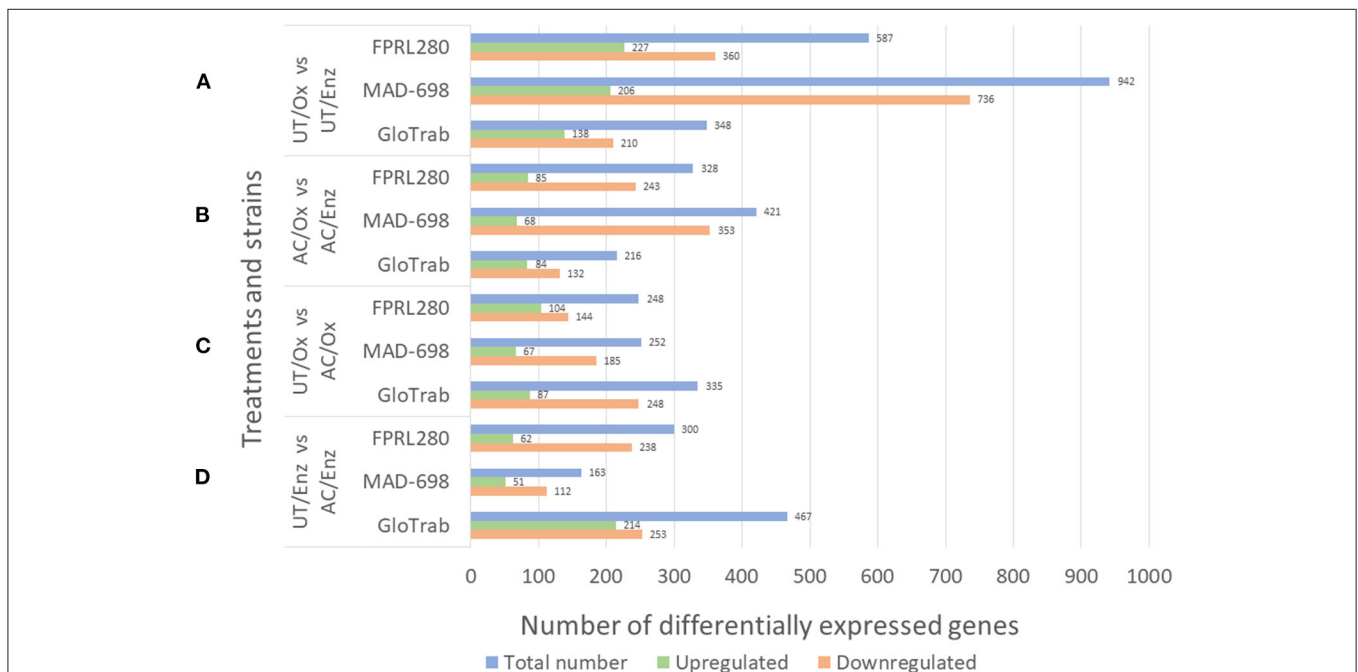
FIGURE 1 | Results for the differential expression analysis of the treatment and decay stage comparison of the three brown rot strains *R. placenta* FPRL280 and MAD-698 and *G. trabeum* (GloTrab). The treatments that were compared are untreated (UT) and 15 % acetylated (AC) samples, at different decay stages: early oxidative decay (Ox) and later enzymatic decay (Enz). **(A)** Shows the comparison of the oxidative (Ox) and the enzymatic (Enz) phase in untreated (UT) samples. **(B)** Compares the oxidative and the enzymatic phase, but in acetylated samples. **(C)** Compares the oxidative phases of untreated and acetylated samples. **(D)** Shows the comparison of untreated and acetylated samples during the enzymatic decay phase.

were differentially expressed in untreated samples compared to acetylated samples.

Comparison of the Non-enzymatic Oxidative and the Enzymatic Decay Phases

When comparing gene expression profiles between oxidative (early) and enzymatic (late) phase on untreated wood samples, different numbers of expressed genes were revealed between the strains, but with a similar grouping of the associated GO terms (**Figure 2**).

In all three strains, the number of CAZy-encoding genes and genes encoding proteins involved in sugar metabolism were substantially higher in the enzymatic phase. The total number of differentially expressed brown rot-specific (BRS) genes (**Supplementary Material 5**) varied between the strains: FPRL280 and *G. trabeum* expressed a higher number of BRS "Ox" genes during the oxidative state, and more BRS "Enz" genes during enzymatic decay. During the oxidative phase, no BRS "Enz" genes were expressed in all three strains in untreated samples. This clearly shows the separation and the delay of the enzymatic phase, as previously suggested by Zhang et al. (2016).

However, in MAD-698 the number of upregulated BRS "Ox" genes was increased during enzymatic decay compared to the oxidative phase. While the number of upregulated BRS "Enz" genes were almost the same in FPRL280 compared to MAD-698

during enzymatic decay, *G. trabeum* expressed the lowest number of genes in both stages. However, this observation can at least partly be attributed to the fact that a lower number of orthologs of *G. trabeum* could be used to find the corresponding BRS genes in this species.

These results show that both strains of *R. placenta* rely on a higher number of genes during enzymatic decay when compared to oxidative decay, as well as when compared to *G. trabeum*. MAD-698 seems to upregulate a more diverse cocktail of genes than FPRL280 during enzymatic decay as well as a higher number of BRS "Ox" genes. All three strains express more BRS "Enz" genes during enzymatic decay.

CAZy Genes and the Importance of GH5_5 (Cel5A, Cel5B) and GH12 (Cel12A)

Since genes encoding CAZymes displayed the most distinct regulatory shift from "Ox" to "Enz" stages (**Figure 2**), we analyzed their absolute expression levels in more detail (**Figure 3**, **Supplementary Material 3**, **Supplementary Figures 3A–F**).

During oxidative degradation, the expression profiles of CAZy genes of the three strains appeared similar. *G. trabeum* displayed slightly higher accumulated expression levels, which however can be partly attributed to the higher overall number of CAZy genes (352) compared to the *Rhodonia* strains (317). CAZy gene induction during enzymatic decay is then much

FIGURE 2 | Functional groups of genes significantly upregulated in untreated samples during oxidative and enzymatic brown rot decay. The figure shows the two *R. placenta* strains used for this study (FPRL280 and MAD-698) and *G. trabeum*. **(A)** For the grouping the categories "Transporters", "Cytochrome P450", "Aldo-keto" and "Proteases", genes encoding for enzymes involved in sugar metabolism as well as "CAZy", "BRS Ox" and "BRS Enz" (brown rot specific) were used. Genes with other functions, as well as uncharacterized genes are also shown. **(B)** For enhanced readability the categories of "Others" and "Uncharacterized" were exluded.

FIGURE 3 | Accumulated expression values [transcripts per million (TPM)] for all CAZy families in strains of *R. placenta* and *G. trabeum* showing the differences between the strains and the decay stages. The largest bar of FPRL280/UT/Enz and MAD-698/UT/Enz is the GH12 family. The second larger group represents the CAZy family GH5. More detailed graphics on the single CAZy families are shown in **Supplementary Figures 3A–F**.

more pronounced in the *R. placenta* strains. The most highly induced CAZy families were GH12 (predicted endoglucanase and xyloglucan hydrolase functions), GH5 (predicted endo-β-1,4-glucanase/cellulase, endo-β-1,4-xylanase, and β-glucosidases, among other functions), carbohydrate esterase (CE)16 (acetylesterase function on various carbohydrate acetyl esters), GH10 (endo-1,4-β-xylanase and endo-1,3-β-xylanase function) and GH3 (β-glucosidase, xylan 1,4-β-xylosidase, and β-glucosylceramidase predicted functions). In *R. placenta*, particularly one GH12 family member, the endoglucanase Cel12A (FPRL280_170_10; POSPLADRAFT_1071715), contributes largely to this effect with a log2 FC of 7.98 (FPRL280) and 5.92 (MAD-698) between enzymatic and oxidative degradation. Within the GH5 family, several genes are most strongly induced from oxidative to enzymatic decay: GH5_22 (FPRL280_75_15; POSPLADRAFT_1169431), GH5_5 [FPRL280_14_15; POSPLADRAFT_1164613 (Cel5A) and FPRL280_14_16; POSPLADRAFT_1068430 (Cel5B)] and additionally in MAD-698 GH5_7 (POSPLADRAFT_1030481 and POSPLADRAFT_1177854). In *G. trabeum*, one

CE16-encoding gene (GLOTRDRAFT_48624) as well as one GH12- (GLOTRDRAFT_138821) and two GH5-encoding genes (GH5_5: GLOTRDRAFT_57704; GH5_7: GLOTRDRAFT_110405) are responsible for the differences, but not to the same level. These results show that all three strains enhance CAZy gene expression during later enzymatic decay. This shift is strongly pronounced in MAD-698 and FPRL280.

Differences in Decay Strategies Between FPRL280 and MAD-698

Rather minor genomic differences have been observed between the two strains of *R. placenta* (FPRL280 and MAD-698) (Kölle et al., 2020), raising the question whether the phenotypic differences between the strains (Thaler et al., 2012; Kölle et al., 2020) are dominated by transcriptional differences. Therefore, a differential expression analysis of the orthologous genes shared by the *R. placenta* strains was performed (**Figure 4**). This analysis resulted in 620 genes being upregulated (by over two-fold) by FPRL280 during the oxidative degradation phase compared to MAD-698, while MAD-698 had 315 genes upregulated

during oxidative phase. The enzymatic phase showed a reverse trend, since MAD-698 had 270 genes upregulated while only 149 genes were upregulated in FPRL280 during the enzymatic phase.

To determine the genes that differ most in terms of expression levels between the two strains, a log2 fold change cutoff of ±6 was applied (genes that were differentially expressed between the two *R. placenta* strains during oxidative decay can be found in **Supplementary Material 5**). The cutoff resulted in 26 genes being more highly expressed in FPRL280 vs. MAD-698 during growth on UT/Ox, with two of these genes exceeding thousand twenty four-fold expression. While most of these highly differentially expressed genes are uncharacterized, two proteases and one transporter were among this group. MAD-698 showed 41 genes extensively upregulated vs. FPRL280, one gene encoding a protease, two putative cytochrome P450s and one gene encoding a member of the CAZy family GH13, involved in alpha-amylase activity, calcium ion binding and carbohydrate catabolic processes. In the enzymatic phase of FPRL280, we found 10 genes with a log2 fold change ≥ 6 over MAD-698, four of them fc>10, including one protease-encoding gene and two encoding nucleic acid binding proteins.

In MAD-698, 24 genes were expressed with a fc ≥ 6 in the enzymatic phase, including a cytochrome P450-encoding gene and a GH13 family member. Overall, FPRL280 upregulated a higher number of BRS "Ox" genes during the oxidative phase compared to MAD-698, whereas in MAD-698 these genes are more highly expressed in number during enzymatic decay. FPRL280 upregulated more BRS "Enz" genes during the oxidative and the enzymatic phase compared to MAD-698 (**Supplementary Material 5**). It is noteworthy that in contrast to FPRL280, MAD-698 upregulated the same number of BRS "Ox" genes during oxidative and enzymatic decay.

In conclusion, FPRL280 and MAD-698 upregulated different genes during enzymatic decay. These differences are observed in CAZy-genes and genes with unknown functions. Furthermore, it could be observed that MAD-698 upregulated a relatively high number of BRS "Ox" genes during enzymatic decay compared to FPRL280.

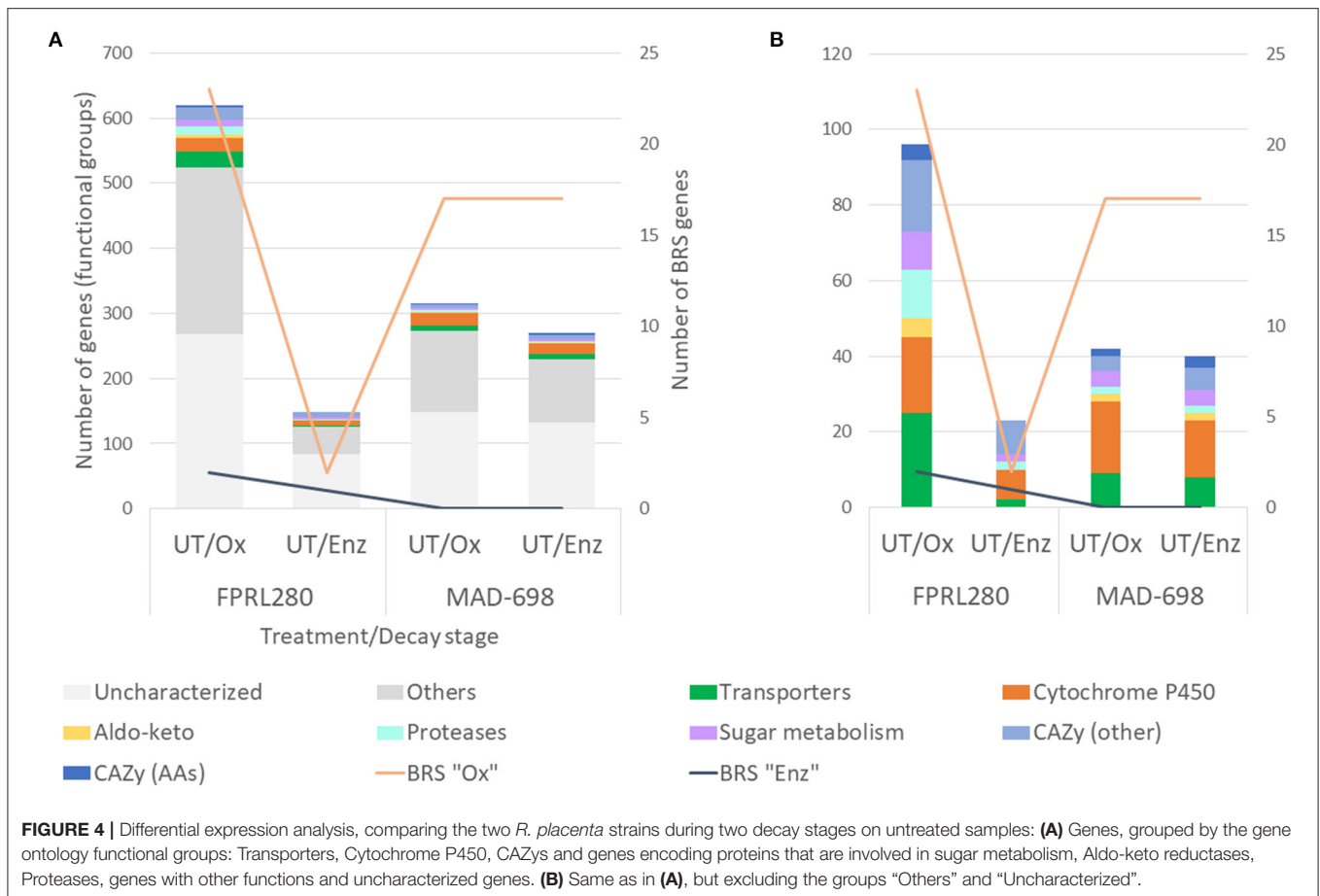## The Effect of Wood Acetylation on the Fungal Response

To elucidate how brown rot fungi react to wood acetylation on a genome-wide scale, we next analyzed the differences in gene expression of the same decay phases between treated and untreated wood samples (**Supplementary Material 4**). FPRL280 downregulated a larger number of genes encoding cytochrome P450s (11), as well as proteases (7) and aldo-keto reductases (3) in acetylated wood samples compared to untreated samples during oxidative decay. No aldo-keto reductases or cytochrome P450 and only one protease was upregulated in acetylated samples compared to untreated samples. In MAD-698, acetylation induced cytochrome P450-encoding genes (6) and, other than in FPRL280, a higher number of transporters (11). *G. trabeum* showed the largest differences between the treatments in the group of genes with "other" functions. Furthermore, *R.*

placenta upregulated a higher number of genes encoding for proteins involved in sugar metabolism in acetylated samples during oxidative degradation compared to untreated samples (FPRL280: 12; MAD-698: 5). This was different in *G. trabeum*, where the numbers were equal between the two treatments.

The TPMs of all genes of the differential expression analysis were accumulated to see differences in expression levels between the treatments in each functional group (**Figure 5**; for *G. trabeum* and a group-wise separation see **Supplementary Figures 4A–M**). Overall, FPRL280 expression levels were higher in acetylated samples during oxidative decay, but similar in the enzymatic phase, compared to untreated samples. While members of the CAZy families GH and AA9, as well as carbohydrate binding modules (CBMs) were not strongly affected during oxidative decay, one expansin-encoding gene (FPRL280_145_15) was clearly upregulated on acetylated wood. In the later enzymatic decay stage, FPRL280 exhibited a lower expression of genes encoding transporters, proteases, aldo-keto reductases, cytochrome P450s and proteins involved in sugar metabolism on acetylated samples compared to untreated samples. The expression levels of CAZy-encoding genes (GH + CBM + AA9) were reduced to about half in acetylated samples compared to untreated samples during enzymatic decay of FPRL280, similar to the levels of BRS "Enz" genes.

In MAD-698, the accumulated TPMs of differentially expressed genes were generally lower than in FPRL280 during both phases, mainly caused by genes of unknown function. As in FPRL280, expression levels of cytochrome P450-encoding genes were lower in acetylated samples during oxidative decay compared to untreated samples, but proteases and transporters were expressed more strongly. POSPLADRAFT_1071715 (Cel12A/GH5_5) is the protein that mainly causes this difference. It is noteworthy that the overall expression level of BRS "Enz" genes during enzymatic degradation was substantially lower in FPRL280 and MAD-698, when comparing acetylated to untreated samples.

Gene expression in *G. trabeum* was overall significantly lower than both strains of *R. placenta* during enzymatic decay, but during oxidative decay, expression levels were intermediate. Lower levels of gene expression were observed for cytochrome P450-, protease- and aldo-keto reductase-encoding genes as well as genes encoding for proteins involved in sugar metabolism during oxidative decay on acetylated samples, compared to untreated samples. The group of "CAZy (Other)"-encoding genes behaved differently when compared to the other two strains, showing lower levels of gene expression in acetylated samples during oxidative decay when compared to untreated samples. Also, genes encoding for AAs did not behave equally in all three strains. *G. trabeum* and FPRL280 showed higher expression levels in AC/Ox samples compared to UT/Ox, while MAD-698 showed the opposite. All three strains have in common that uncharacterized genes represent a large part of the accumulated TPMs, especially in acetylated samples. The genes that most differed between untreated and acetylated samples during oxidative decay in all three strains are listed in **Supplementary Material 4**.

FIGURE 4 | Differential expression analysis, comparing the two *R. placenta* strains during two decay stages on untreated samples: **(A)** Genes, grouped by the gene ontology functional groups: Transporters, Cytochrome P450, CAZys and genes encoding proteins that are involved in sugar metabolism, Aldo-keto reductases, Proteases, genes with other functions and uncharacterized genes. **(B)** Same as in **(A)**, but excluding the groups "Others" and "Uncharacterized".

In total, all three strains expressed higher levels of gene transcripts during initial brown rot degradation in acetylated samples, but similar levels during enzymatic degradation in both treatments. In FPRL280 and *G. trabeum* the differences between UT/Ox and AC/Ox were mainly caused by uncharacterized genes and were larger than in MAD-698. In contrast, the differences between the treatments during enzymatic decay were driven to a larger extent by the functional groups of GHs, CBMs and AA9, especially in *R. placenta*. In all three strains, the expression of BRS "Ox" genes was lower in acetylated samples, reaching out highest levels in UT/Enz samples. The expression of BRS "Enz" genes differed during oxidative decay, but was significantly higher in untreated samples during enzymatic decay in all three strains compared to acetylated samples.

Hierarchical Clustering and Transcription Factors

To achieve an additional overview of gene expression profiles between conditions, hierarchical clustering of the whole transcriptomes of *R. placenta* (FPRL280 and MAD-698) and *G. trabeum* was performed, resulting in 24 clusters for FPRL280, 23 clusters for MAD-698 and 16 clusters for *G. trabeum* (**Figure 6**, **Supplementary Material 7** and **Supplementary Figures 5A,B**).

In FPRL280 clustering, four interesting clusters were identified clustering together genes involved in oxidative decay partly or heavily affected by acetylation (cluster 1), oxidative decay-specific genes that were only slightly affected by acetylation (cluster 3), genes specific for oxidative decay that were enhanced by acetylation (cluster 12) and genes of the enzymatic phase that were affected by the acetylation (cluster 20). These clusters also contained the majority of BRS- and CAZy-encoding genes as well as transcription factors. Cluster 1 contained a high number of genes encoding for GH and GT proteins, as well as the AA3_2 family. Cluster 3 contained several transcription factors (TFs) and BRS "Ox" genes, some of them likely involved in oxalate production. Many GT-encoding genes were located in this cluster. Cluster 20 contained 20% of BRS "Ox"- and the majority of BRS "Enz"-genes, including two genes encoding the endoglucanases Cel12A (GH12) and Cel5A (GH5_5). The clustering results for MAD-698 and *G. trabeum* can be found in **Supplementary Material 7**. Clustering showed four large gene clusters of FRPL280 that were affected by acetylation, resulting in decreased levels of gene expression in acetylated samples (clusters 1, 3, 20) or in enhanced levels (cluster 12). The clusters 1 and 3 contained genes encoding for proteins of the CAZy families GH, GT and AA3_2 as well as BRS genes. Cluster 3 contained genes that are likely involved in oxalate production, indicating that the acetylation
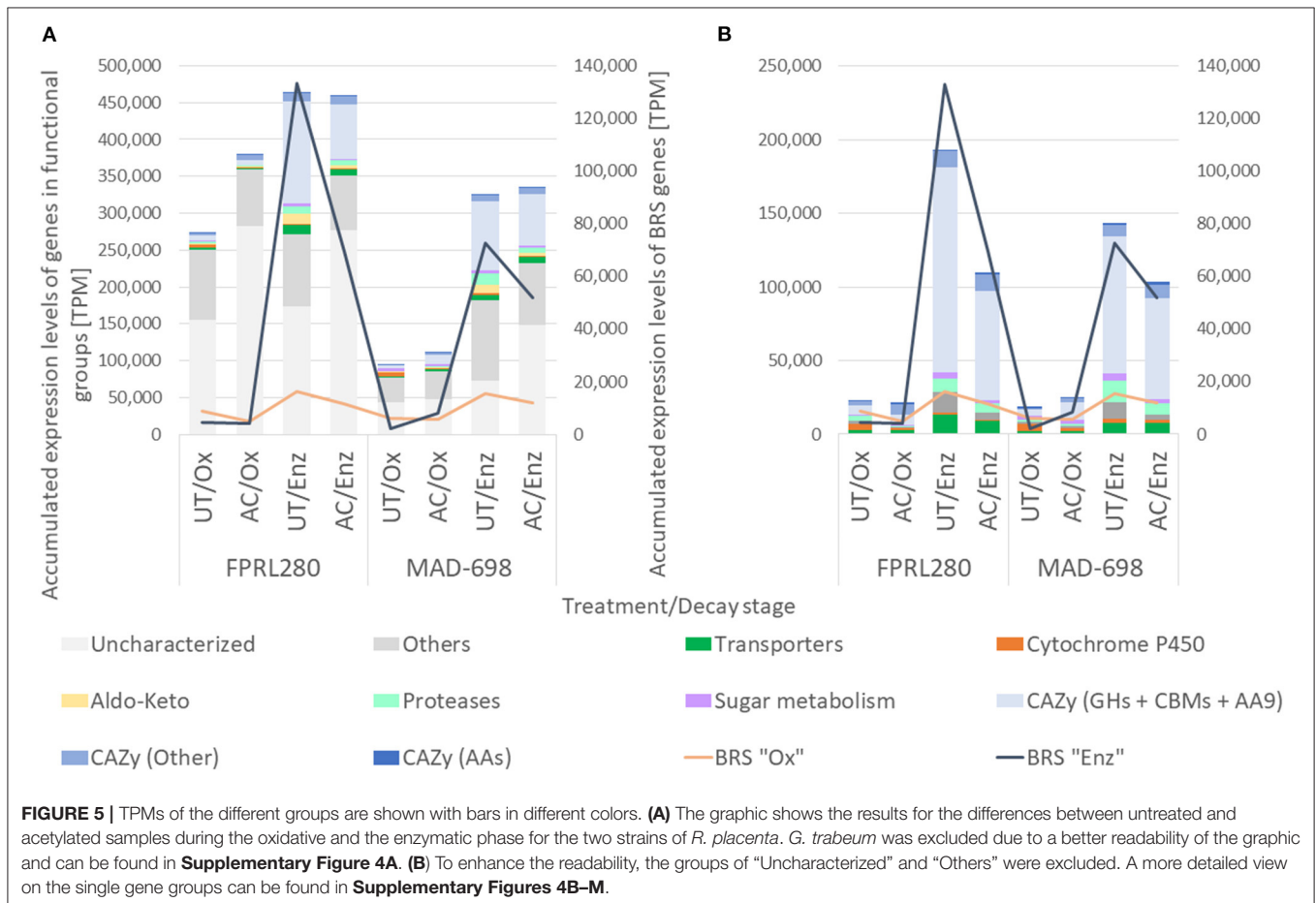
affects oxalate synthesis and thereby possibly indirectly the Fenton reaction. The cluster with highest numbers of BRS genes was cluster 20. Genes located in this cluster encode members of the GH family, including the two endoglucanases Cel5A and Cel12A. In MAD-698 the two endoglucanases-encoding genes (POSPLADRAFT_1164613; POSPLADRAFT_1071715) clustered together with one important gene for oxalate production, an oxalate decarboxylase (OxaD, POSPLADRAFT_ 1058890), strongly affected by the acetylation.

Three specific TFs (FPRL280: 384_5, 28_39 and 10_10), previously shown to be associated with the early oxidative degradation phase in MAD-698 (Zhang et al., 2019), behaved differently in all three fungi on both untreated and acetylated wood. Another Zn(II)2Cys6-type TF associated with early oxidative degradation (Zhang et al., 2019) (FPRL280_11_82, POSPLADRAFT_1043794,   GLOTRDRAFT_135433),   was detected specifically in the oxidative stage in untreated samples and all strains, but was expressed in acetylated samples only for MAD-698. It is noteworthy that these TFs clustered closely to other proteins potentially related to the degradative process in all three strains, as for example closely to genes encoding an expansin (EXPN), GH128 family members (FPRL280_11_82; POSPLADRAFT_1043794), as well as genes

encoding for members of the GH71, GH18, PL14_4, and GH5_9 (GLOTRDRAFT_135433).   Another   TF   (FPRL280_28_39, POSPLADRAFT_1174375) clustered closely to a BRS "Ox" gene encoding an oxaloacetate acetylhydrolase (OahA), a GT69 and a CBM48 and in MAD-698 to a GT48, CBM50 and an EXPN. In *G. trabeum* (GLOTRDRAFT_122681) it clustered together with the BRS "Ox" gene. The TF FPRL280_10_10 clustered closely together with a histidine kinase-encoding gene, a BRS "Ox" gene, a GH16 gene and a second TF, while in MAD-698 this TF (POSPLADRAFT_ 1043897) clustered with a CBM48 and an AA3_2, and in *G. trabeum* (GLOTRDRAFT_ 137651) it clustered with gene coding for GH5_12, GT2, BRS "Ox" and a CBM50. Details on the gene IDs can be found in **Supplementary Material 7**.

Important Genes During Brown Rot Decay

To identify the principal molecular differences during brown rot decay between the two *R. placenta* strains, we next analyzed the accumulated TPMs of brown rot-specific (BRS) genes between the tested conditions (**Figure 7**). In previous studies on brown rot, several genes had been proposed to be involved in the degradation mechanisms by either oxidative (BRS "Ox") or

FIGURE 5 | TPMs of the different groups are shown with bars in different colors. **(A)** The graphic shows the results for the differences between untreated and acetylated samples during the oxidative and the enzymatic phase for the two strains of *R. placenta*. *G. trabeum* was excluded due to a better readability of the graphic and can be found in **Supplementary Figure 4A**. **(B)** To enhance the readability, the groups of "Uncharacterized" and "Others" were excluded. A more detailed view on the single gene groups can be found in **Supplementary Figures 4B–M**.
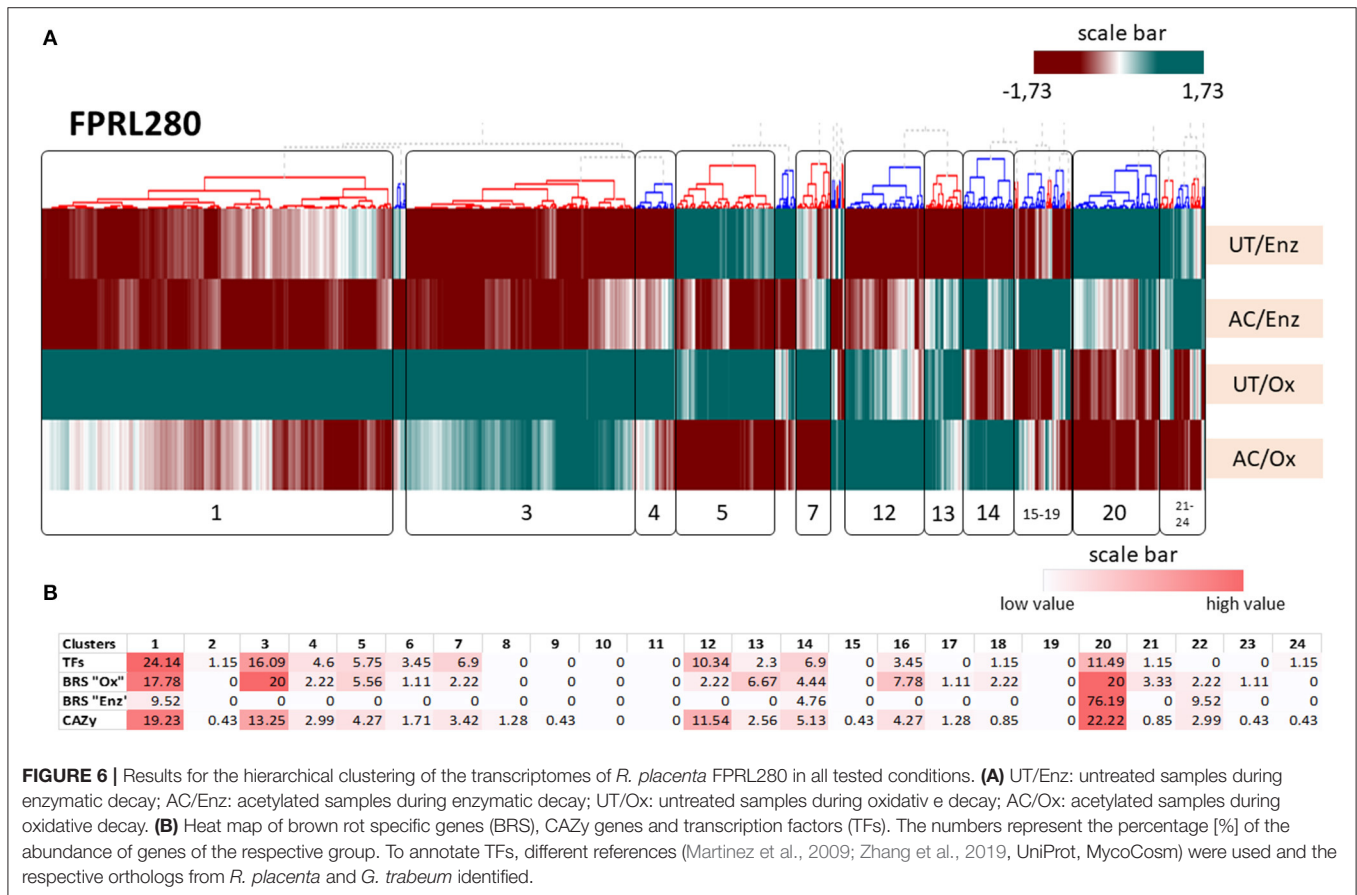
enzymatic (BRS "Enz") action. We collected these genes and created a new list including genes with interesting expression patterns in our analyses (**Supplementary Material 5**). As was previously observed (**Figure 2**), significantly higher expression of BRS genes was detected during the enzymatic phase in both strains as well as in both treatments, mainly driven by the high transcription levels of enzymatic BRS genes. From this data, it could also be seen that even though more differentially expressed BRS "Ox" genes are present in MAD-698 (**Figure 2**: FPRL280/UT/Enz vs. MAD-698/UT/Enz), the accumulated expression levels were actually higher in FPRL280 (**Figure 7**).

In the majority of cases, BRS genes were expressed at a lower level in acetylated samples compared to untreated samples by all strains, except for BRS "Enz" genes in MAD-698 during oxidative decay, which displayed a slightly elevated expression.

To identify genes that are consistently upregulated during either the oxidative or enzymatic decay stages, the combined results of the differential expression analysis for all three fungi were used. This resulted in a list of six genes being significantly upregulated in oxidative decay samples and 19 being upregulated during enzymatic decay in all three strains (**Table 1**). To further enlarge the list of genes, the orthologs were used to identify protein names of genes that were already characterized in one of the three strains (UniProt).

Next, we analyzed the expression of genes that are putatively involved in oxalic acid biosynthesis (for FPRL280 see **Figure 8**, for MAD-698 and *G. trabeum* see **Supplementary Figures 6A,B** respectively). Since oxalate plays an important role during early brown rot decay, it is important for the fungi to have working production cycles. We only considered the genes to be upregulated with a log2 fold change ≥ 2 to ensure a significant difference between the comparisons. FPRL280 showed an upregulation of five genes in untreated samples (**Figure 8**) comparing the two degradation phases. These genes encode isocitrate lyase (ISL; FPRL280_213_3), acetyl CoA-synthase, citrate synthase (CS; FPRL280_520_1), glyoxylate dehydrogenase (GlyD; FPRL280_17_38), as well as an OahA (FPRL280_17_43). MAD-698 upregulated five genes, four of these being orthologs to FPRL280, while *G. trabeum* only upregulated two genes during the oxidative phase, one encoding for an acetyl CoA-synthase and an OahA, also upregulated by FPRL280. Corresponding orthologs were not found for *G. trabeum*, however other genes with these functions can exist in *G. trabeum*.

During enzymatic decay, FPRL280 upregulated a malate synthase-encoding gene (malate synthase; FPRL280_197_5), also expressed by *G. trabeum*, and a malic enzyme, indeed upregulated in MAD-698. MAD-698 additionally upregulated a

Clusters	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
TFs	24.14	1.15	16.09	4.6	5.75	3.45	6.9	0	0	0	0	10.34	2.3	6.9	0	3.45	0	1.15	0	11.49	1.15	0	0	1.15
BRS "Ox"	17.78	0	20	2.22	5.56	1.11	2.22	0	0	0	0	2.22	6.67	4.44	0	7.78	1.11	2.22	0	20	3.33	2.22	1.11	0
BRS "Enz"	9.52	0	0	0	0	0	0	0	0	0	0	0	0	4.76	0	0	0	0	0	76.19	0	9.52	0	0
CAZy	19.23	0.43	13.25	2.99	4.27	1.71	3.42	1.28	0.43	0	0	11.54	2.56	5.13	0.43	4.27	1.28	0.85	0	22.22	0.85	2.99	0.43	0.43

FIGURE 6 | Results for the hierarchical clustering of the transcriptomes of *R. placenta* FPRL280 in all tested conditions. **(A)** UT/Enz: untreated samples during enzymatic decay; AC/Enz: acetylated samples during enzymatic decay; UT/Ox: untreated samples during oxidativ e decay; AC/Ox: acetylated samples during oxidative decay. **(B)** Heat map of brown rot specific genes (BRS), CAZy genes and transcription factors (TFs). The numbers represent the percentage [%] of the abundance of genes of the respective group. To annotate TFs, different references (Martinez et al., 2009; Zhang et al., 2019, UniProt, MycoCosm) were used and the respective orthologs from *R. placenta* and *G. trabeum* identified.

CS (POSPLADRAFT_ 1058962) and *G. trabeum* an OxaD during enzymatic decay.

Comparing both degradation phases ("Ox" and "Enz") in acetylated samples revealed an upregulation of a cytochrome c oxidase (FPRL280_30_30) in oxidative samples of FPRL280 and a downregulation of a gene encoding a malic enzyme during enzymatic decay (FPRL280_51_6), also in FPRL280. The other strains did not show significant differences between the samples. When comparing the enzymatic stages of untreated and acetylated samples, an upregulation of genes coding for an OahA (FPRL280_17_43), an ISL (FPRL280_213_3) as well as an acetyl CoA-synthase (FPRL280_74_8) was observed in acetylated samples of FPRL280. MAD-698 did not show significant differences between the treatments for the applied fold change. *G. trabeum* upregulated a malate synthase-encoding gene (GLOTRDRAFT_137388) in untreated samples, as well as an acetyl CoA-synthase like gene (GLOTRDRAFT_ 115850) and an OahA-encoding gene (GLOTRDRAFT_ 115850) in acetylated samples.

All the genes discussed above, the genes which were differentially expressed in all three strains and the genes used in the publication of (Zhang et al., 2016), helped to create a list of 139 genes with putatively important functions during brown rot decay (**Supplementary Material 6**).

DISCUSSION

In the present study, we compared the transcriptomes of two strains of *R. placenta* (FPRL280 and MAD-698) as well as another brown rot fungus, *G. trabeum,* during initial and proceeded wood decay, when growing on untreated and acetylated Scots pine sapwood. Differential expression analyses were performed to reach a better understanding of both ongoing processes during brown rot decay, as well as of the inhibitory processes induced by the wood modification.

Decay Strategies Differ Among All Tested Strains

The presented data strongly suggest that the phenotypic differences that have been reported between the two *R. placenta* strains (MAD-698 and FPRL280) (Thaler et al., 2012; Kölle et al., 2020) can be explained to a large extent by regulatory differences. In total, MAD-698 expressed around 1,000 more genes than FPRL280 across all treatments (**Supplementary Figure 2**) during wood degradation. Regarding CAZyme-encoding genes, the overall expressed gene set appeared quite similar during oxidative and enzymatic decay. However, a small number of genes showed dramatically differing expression levels, such as the endoglucanase Cel12A-encoding gene (FPRL280_170_10; POSPLADRAFT_1071715; **Figure 3**), which is an important protein during enzymatic brown rot decay (Ryu et al., 2011; Zhang et al., 2016; Umezawa et al., 2020) and was also highly differentially regulated between oxidative and enzymatic phases in all three tested fungi.

All three strains displayed a switch from the oxidative to the enzymatic decay stage, which was characterized by a clear shift in the gene expression profiles (**Figure 2**) supporting the theory of a two-stepped brown rot decay (Eaton and Hale, 1993; Goodell, 2003; Fackler et al., 2010; Arantes et al., 2012; Alfredsen et al., 2016; Ringman et al., 2016; Zhang
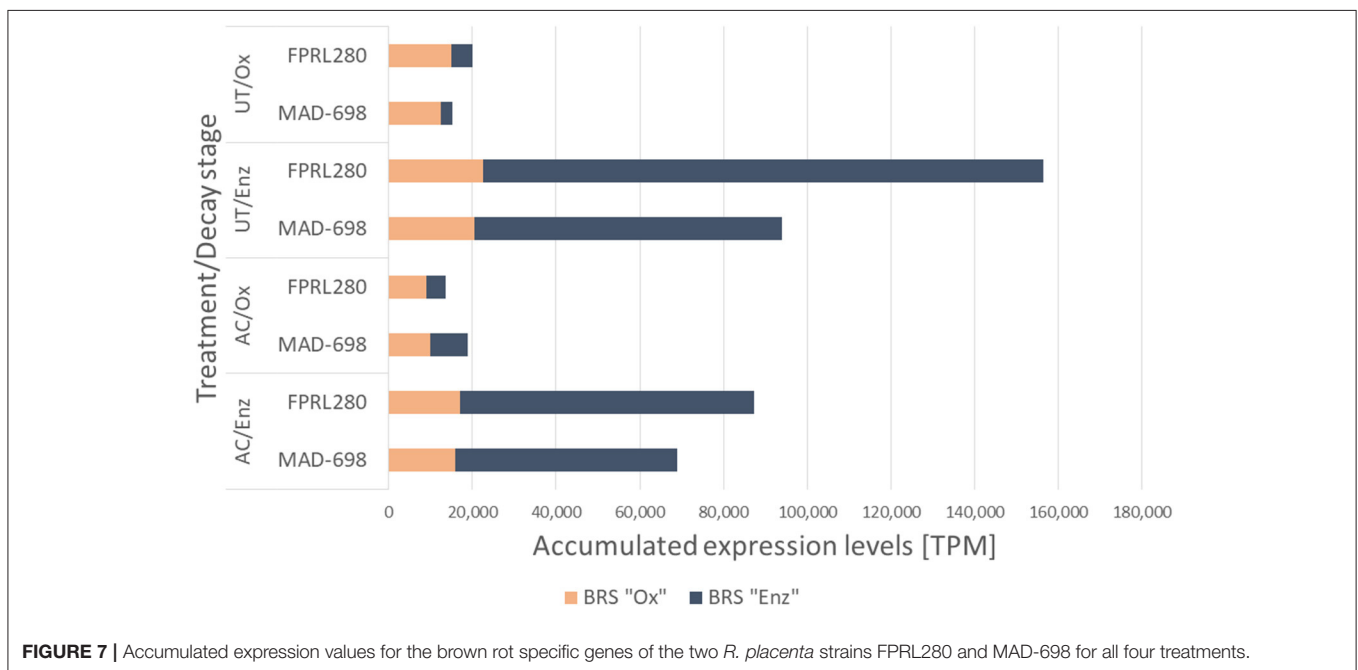

FIGURE 7 | Accumulated expression values for the brown rot specific genes of the two *R. placenta* strains FPRL280 and MAD-698 for all four treatments.

TABLE 1 | Genes that are significantly upregulated during either oxidative or enzymatic decay in all three strains *R. placenta* FPRL280, MAD-698 and *G. trabeum* with their protein names.

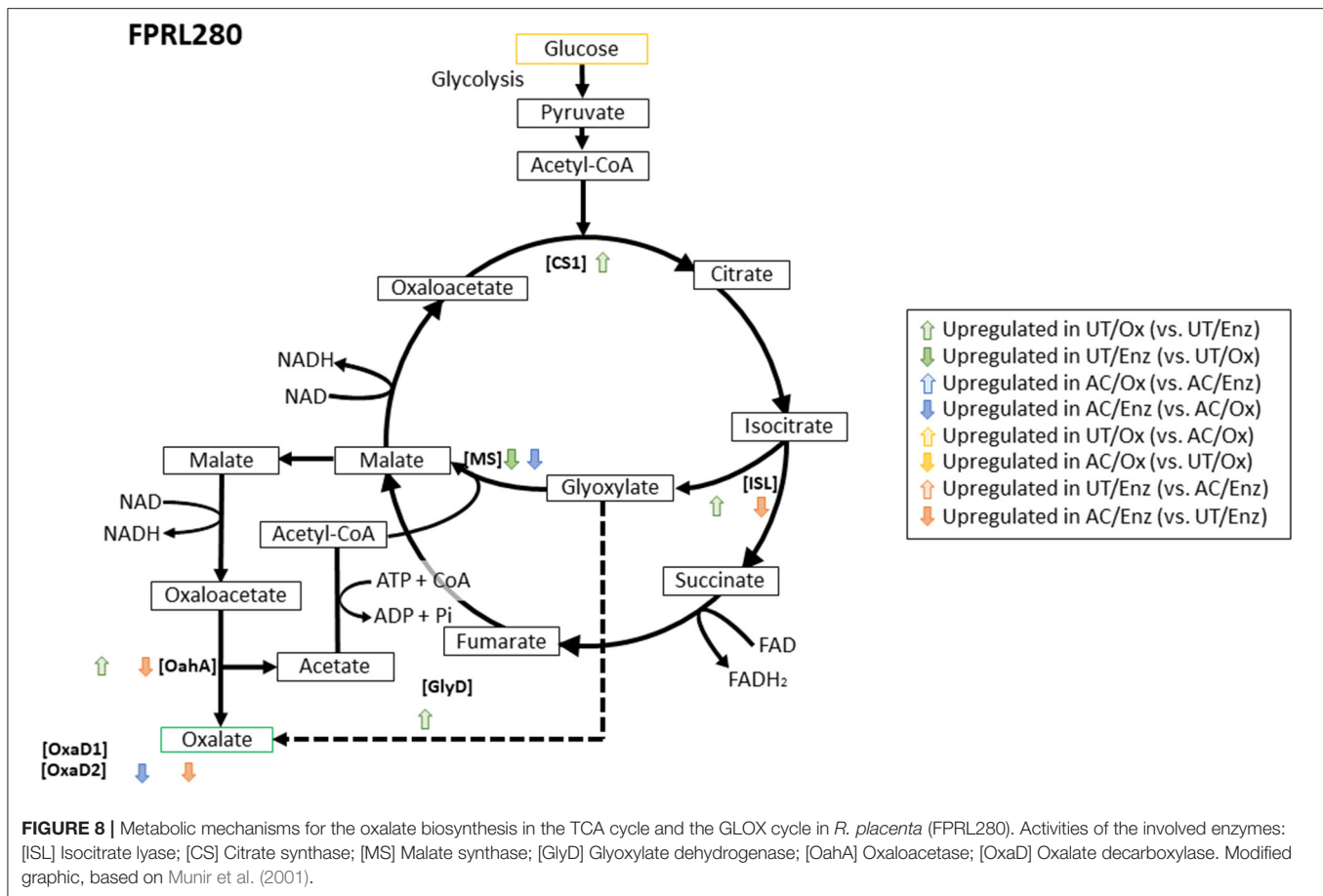| FPRL280-Id FPRL280_	MAD-698-Id (SB12) POSPLADRAFT_	GloTrab-Id GLOTRDRAFT_	Protein name
UPREGULATED IN ALL THREE STRAINS DURING OXIDATIVE DECAY			
120_7	1034711	107870	Uncharacterized protein
1_189	1165052	53242	Aspartate aminotransferase
39_6	1146207	80687	Serine/threonine-protein kinase
67_24	1151609	62779	Cyclin N-terminal domain-containing protein
70_40	1058125	111949	Uncharacterized protein
8_99	1037719	58342	Uncharacterized protein
UPREGULATED IN ALL THREE STRAINS DURING ENZYMATIC DECAY			
122_7	1067481	116093	MFS domain-containing protein
129_4	1064649	110464	Uncharacterized protein
14_15	1164613	57704	Glycoside hydrolase family 5, Cel5A
170_10	1071715	138821	Glycoside hydrolase family 12, Cel12A
21_14	1072224	76822	Aldo_ket_red domain-containing protein
220_7	1043996	46499	Glycoside hydrolase family 10, Beta-xylanase
221_13	1127293	108929	Uncharacterized protein
28_46	1156080	116882	Uncharacterized protein
327_2	1065808	46545	Carbohydrate esterase family 15
47_21	1047078	105888	Uncharacterized protein
49_4	1174812	69843	Glycoside hydrolase family 3
4_37	1044461	96567	COesterase domain-containing
5_124	1034806	90516	Uncharacterized protein
60_17	1048486	141198	Uncharacterized protein
65_19	1049710	21790	Uncharacterized protein
67_31	1152036	27801	Uncharacterized protein (Fragment)
75_17	1169431	79212	Glycoside hydrolase family 5
9_48	1065541	75159	GFO_IDH_MocA domain-containing protein
9_74	1069652	122002	Glycoside hydrolase family 3

et al., 2016; Beck et al., 2018). The functional annotation of the differentially expressed genes also differed between the two degradation stages. While more cytochrome P450s were upregulated during oxidative decay, aldo-keto reductases, transporters, proteases and genes encoding for proteins involved in sugar metabolism were upregulated during the enzymatic stages (**Figure 2**; **Supplementary Material 4**). In particular five CAZyme-encoding genes displayed a very strong induction in the enzymatic phase: the genes coding for the endoglucanases Cel5A, Cel5B, and Cel12A (for all three strains) as well as for the two CE16 carbohydrate esterases (for *R. placenta*)

(FPRL280_428_3, FPRL280_27_40; POSPLADRAFT_1067618, POSPLADRAFT_1179218) (**Figure 3**), supporting previous findings (Martinez et al., 2009; Vanden Wymelenberg et al., 2010; Ryu et al., 2011; Beck et al., 2018). Six other genes were significantly upregulated during oxidative decay in all three strains, including three uncharacterized genes, one encoding for an aspartate aminotransferase, one serine/threonine-protein kinase and one cyclin N-terminal domain-containing protein. These genes are clear candidates as common biomarkers for detection and monitoring of brown rot decay in untreated wood.

FPRL280 was shown to have a slower initial growth speed than MAD-698, while producing a higher mass loss during the 1st days of degradation (Thaler et al., 2012; Kölle et al., 2020). This might indicate that FPRL280 is able to recognize the substrate and start the degradation earlier than MAD-698 (Kölle et al., 2020). In line with these observations, FPRL280 was found to upregulate more CAZy and BRS genes during the initial, oxidative decay phase than MAD-698 (**Figure 5**). MAD-698, on the other hand, was found to express an overall higher number of CAZy encoding and BRS genes, albeit at lower levels, compared to FPRL280 (**Figures 2**, **3**), supporting the enzymatic degradation with more BRS "Ox" genes (**Figure 4**). These results, including the higher degradation rates found by Kölle et al. (2020), lead to the conclusion that the degradation approach in MAD-698 is the more effective one.

Looking at highly differentially expressed genes (log2 FC ≥ 6) between both strains of *R. placenta* during the initial, oxidative decay, FPRL280 upregulated more proteins with protease function, while the largest functional group in MAD-698 was cytochrome P450s (**Supplementary Material 5**). Cytochrome P450s are likely involved in reactions required for the biosynthesis of methoxyhydroquinones, as well as for hydroxylation of lignin fragments, which is important for lignocellulolytic processes (Ide et al., 2012) and could support the more effective degradation observed in MAD-698. An additional interesting gene found to be highly upregulated in MAD-698 encodes a citrate synthase (FPRL280_520_1/ POSPLADRAFT_1156738), which is assumed to play an important role in the oxalate cycle (Munir et al., 2001) and could suggest that oxalate production is more active in MAD-698 than in FPRL280. Together, these findings further strengthen the assumption that the two strains follow different regulatory routes.

G. trabeum seems to proceed with a radically different decay strategy than *R. placenta*. Overall, *G. trabeum* appears to be a more aggressive degrader growing on Spruce [*Picea abies* (L.) Karst.], since it was demonstrated that it needs only 28 days to gain a 16% mass loss, while *R. placenta* reached the same mass loss only after 56 days (Fackler et al., 2010). Surprisingly, however, although *G. trabeum* has a wider range of CAZyme-encoding genes in its genome (Floudas et al., 2012; Gaskell et al., 2017), these were not induced to the same extent from early to late decay stage as in *R. placenta* (**Figure 3**). Previous research showed that *G. trabeum* might have different carbohydrate preferences than *R. placenta* (Presley et al., 2018). In addition to this, it was also shown that *G. trabeum* expressed more oxidoreductases during late decay compared to early decay stages (Presley et al., 2018). This observation could not be confirmed

FIGURE 8 | Metabolic mechanisms for the oxalate biosynthesis in the TCA cycle and the GLOX cycle in *R. placenta* (FPRL280). Activities of the involved enzymes: [ISL] Isocitrate lyase; [CS] Citrate synthase; [MS] Malate synthase; [GlyD] Glyoxylate dehydrogenase; [OahA] Oxaloacetase; [OxaD] Oxalate decarboxylase. Modified graphic, based on Munir et al. (2001).

in our data, where *G. trabeum* expressed a similar number of genes with oxidoreductase activity during both phases, while *R. placenta* expressed more oxidoreductases during enzymatic decay (especially MAD-698). It has to be noted, however, that the test setup used in our study was developed for *R. placenta* and might not optimally capture the same degradation stages for *G. trabeum*, and thus it is possible that the enzymatic phase was not yet completely induced in our late time point samples.

Wood Acetylation Leads to Genome-Wide Expression Changes

Wood acetylation led to differential expression of several hundred genes in each degradation stage and in all three fungi (**Figures 1B,C**) indicating that the fungi are clearly reacting to the modification on a genome-wide scale. Previously, a group-wise upregulation of four oxidative genes [glucose oxidase (GOx2, CAZy family AA5), copper radical oxidase (Cro1, CAZy family AA3), alcohol oxidase 2 and 3 (AlOx2, AlOx3; CAZy family AA3)] was shown (Kölle et al., 2019). All corresponding proteins are putatively involved in the extracellular H_2O_2 production. This group-wise upregulation was observed on wood samples with increasing levels of acetylation (to a maximum level of 20%), compared to untreated samples during oxidative decay (Kölle et al., 2019). This was confirmed in the present study for GOx2 by

showing a clear upregulation of the corresponding ortholog in the acetylated samples in all three tested fungi (FPRL280_46_12; POSPLADRAFT_1040696; GLOTRDRAFT_139980). Upregulation of AlOx3 in the acetylated samples was also observed in transcriptomic data for FPRL280 and MAD-698 (FPRL280_40_14; POSPLADRAFT_1183136). Since these genes behave similarly for all three brown rot fungi and their importance during early brown rot decay have been proven, they could also be suitable as potential biomarkers for brown rot degradation in acetylated wood. Notably, proteins putatively involved in the oxalic acid synthesis were not as differentially expressed in acetylated samples between the two phases as in untreated samples, suggesting an impaired oxalate cycle due to the acetylation. This information could be an important part of the puzzle understanding the mode of action of acetylated wood. Furthermore noteworthy is the upregulation of an iron permease-encoding gene (FPRL280_195_6) in acetylated samples, since it seems to be upregulated during the enzymatic phase in untreated samples and is also strongly upregulated in acetylated samples in the oxidative decay phase.

Surprisingly, more genes related to sugar metabolism were upregulated in acetylated samples during oxidative decay in all three strains (**Supplementary Material 4**). An upregulation of these genes is typically part of the subsequent enzymatic decay phase and induced by polysaccharides solubilized during initial

decay (Zhang and Schilling, 2017). Nevertheless, the accumulated expression levels of these genes are rather low in acetylated samples—even in comparison to untreated samples during oxidative decay, and therefore resemble more of a scouting reaction. Previous studies demonstrated an extremely slow mass loss on acetylated samples during early decay (Alfredsen et al., 2016; Ringman et al., 2017; Beck et al., 2018), which was attributed to the protective function of the acetylation. However, this starving situation apparently induces a scouting reaction leading to the production of a broader range of enzymes involved in sugar metabolism (Van Munster et al., 2014). Nevertheless, the affected gene complements of the different strains varied in composition, showing that different groups of genes were either enhanced or inhibited by the acetylation. This further underlines the fact that the strains not only follow different decay strategies, but also seem to react differently to the modification. Expression profiles of all three strains in acetylated samples were furthermore dominated by many uncharacterized genes. Finding the functions of these genes might be crucial for the understanding on how acetylation inhibits fungal growth.

All three tested fungi displayed a high number of genes that were heavily affected by the acetylation treatment. Hierarchical clustering revealed that in total, more than 50% of the genes in all three strains are located in clusters that are more or less affected by the acetylation, showing the broad genome-wide effects of the modification. A very interesting observation was a cluster with genes in *G. trabeum* that were enhanced in AC/Enz samples, containing many BRS "Ox" genes as well as closely located TFs. This strongly indicates that *G. trabeum* did not reach a proper enzymatic degradation phase in acetylated samples. This phenomenon was not seen in *R. placenta*, demonstrating the differences in decay strategies and/or timing of the responses and the use of different approaches to overcome the inhibition associated with the wood modification.

In addition to this, *G. trabeum* upregulated eight hydrophobin-encoding genes in the oxidative degradation phase on acetylated wood. This might represent a stress-induced response, since hydrophobins are for example involved in the formation of fruiting bodies and aerial hyphae (Wessels et al., 1991). Filamentous fungi might also use hydrophobins for better adherence to hydrophobic surfaces (Wessels, 1996). In a previous study on the wettability and swelling of treated wood (Moghaddam et al., 2016), it was shown that acetylation renders the wood surface (of fresh cut veneers) more hydrophobic, which might explain the observed response.

Considering CAZyme-encoding genes, the majority of these were found in gene clusters affected by the acetylation in all three strains. Notably, a gene cluster including many BRS genes could be identified that was strongly affected in FPRL280 (Cluster 20) and *G. trabeum* (Cluster 7), albeit less so in MAD-698 (Cluster 14). Besides these, particularly AA3_2-and GT-encoding genes were identified in clusters that demonstrated a strong inhibition by acetylation (e.g., Cluster 1 in FPRL280). As already discussed, proteins from the AA3_2 family are important during brown rot decay and known to support cellulases and hemicellulases. GTs are, among others, necessary for the biosynthesis of the fungal

cell wall (Klutts et al., 2006), indicating that the formation of new mycelium is either inhibited by the acetylation or the lack of energy leads to decreased GT production.

The clusters containing genes that were affected by acetylation also contained higher percentages of TFs. Many TFs clustered closely to important CAZy-encoding genes or genes related to oxidative brown rot decay, indicating that these might be interesting targets for further studies aimed at gaining a better molecular understanding of brown rot gene regulation.

Genes Relevant for Brown Rot Decay

Previous studies have presented a wide range of genes that appear to be involved in brown rot decay (Martinez et al., 2009; Vanden Wymelenberg et al., 2010; Ryu et al., 2011; Zhang et al., 2016; Beck et al., 2018). We created a list of relevant BRS genes including previously identified genes and genes newly found in this study (**Supplementary Material 6**). Especially genes that were similarly expressed in all three strains during either the oxidative or the enzymatic degradation phase (**Table 1**) could be of interest for future studies or to be used as BRS biomarkers. Similar to the comparison of expression levels, this gene list contains a high number of uncharacterized genes (oxidative phase 3/6, enzymatic phase 8/19), highlighting the importance of further annotations.

The classification into BRS "Ox" and BRS "Enz" genes was conducted based on their expression data and their respective functions. The hierarchical clustering revealed many clusters related to enzymatic degradation, in which BRS "Ox" genes were located together with BRS "Enz" genes, indicating co-regulation despite differing functions. For future research, a phase-specific classification into "early" and "late response" might be more suitable than a function-based classification.

CONCLUSIONS

Gaining more insight into the two-stepped decay mechanism of brown rot fungi was one of the reasons to compare the transcriptomes of the two different strains of *R. placenta* (FPRL280 and MAD-698) and *G. trabeum*. The results confirm the presence of a two-stepped degradation mechanism in all three fungi. However, the underlying regulatory switch seems dynamic rather than a rigid bipartite system and different strains use varying compositions of genes active in parallel during oxidative and enzymatic decay. These findings indicate that the duration of the early phase, dominated by oxidative processes, and the start of the later phase, dominated by enzymatic processes, are highly strain-specific, as well as the transition time between both phases. Nevertheless, genes expressed during initial and later decay by all three strains could be used as biomarkers for the respective degradation phase.

Wood modification by acetylation had a profound effect on gene expression patterns on a genome-wide level. Overall, more genes were found to be upregulated on acetylated wood, such as CAZymes, but appeared to be attenuated in accordance with a starvation stress-induced scouting-like response. Glucose oxidase (GOx2) was upregulated in

acetylated samples in all three strains, making it a promising biomarker for the investigation of acetylation effects. The vast number of differentially expressed uncharacterized genes identified in this study highlights the importance of future efforts to enrich the annotation of brown rot fungal genomes.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, PRJNA681134 .

AUTHOR CONTRIBUTIONS

The research was initiated and designed by AP, MK, JB, and MC. MK and MC performed the analyses. MK and AP co-wrote the paper with support of MC and JB. All authors were included in the interpretation of the data, read and approved the final manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/ffunb.2021.701579/full#supplementary-material

REFERENCES

Afgan, E., Baker, D., Batut, B., Beek, M., Bouvier, D., Cech, M., et al. (2018). The Galaxy platform for accessible, reproducible and collaborative biomedical analyses: 2018 update. *Nucleic Acids Res.* 46:379. doi: 10.1093/nar/gky379

Alfredsen, G., Pilgård, A., and Fossdal, C. G. (2016). Characterisation of Postia placenta colonisation during 36 weeks in acetylated southern yellow pine sapwood at three acetylation levels including genomic DNA and gene expression quantification of the fungus. *Holzforschung* 70:11. doi: 10.1515/hf-2016-0009

Arantes, V., Jellison, J., and Goodell, B. (2012). Peculiarities of brown-rot fungi and biochemical Fenton reaction with regard to their potential as a model for bioprocessing biomass. *Appl. Microbiol. Biotechnol.* 94, 323–338. doi: 10.1007/s00253-012-3954-y

Baldrian, P., and Valášková, V. (2008). Degradation of cellulose by basidiomycetous fungi. *FEMS Microbiol. Rev.* 32, 501–521. doi: 10.1111/j.1574-6976.2008.00106.x

Beck, G., Hegnar, O. A., Fossdal, C. G., and Alfredsen, G. (2018). Acetylation of Pinus radiata delays hydrolytic depolymerisation by the brown-rot fungus Rhondonia placenta. *Int. Biodeteriorat. Biodegrad.* 135, 39–52. doi: 10.1016/j.ibiod.2018.09.003

Beck, G., Thybring, E., Thygesen, L., and Hill, C. (2017). *Characterization of Moisture in Acetylated and Propionylated Radiata pine Using Low-Field Nuclear Magnetic Resonance (LFNMR) Relaxometry*. Boston: Holzforschung.

Blanchette, R. (1991). Delignification by wood-decay fungi. *Annu. Rev. Phytopathol.* 29:18. doi: 10.1146/annurev.py.29.090191.002121

Castaño, J. D., Zhang, J., Anderson, C. E., and Schilling, J. S. (2018). Oxidative damage control during decay of wood by brown rot fungus using oxygen radicals. *Appl. Environ. Microbiol.* 84:18. doi: 10.1128/AEM.01937-18

CEN, E. C. F. S. (1996). "EN 113. Wood preservatives. Test method for determining the protective effectiveness against wood destroying Basidiomycetes," in *Determination of the Toxic Values*. Belgium: Brussels.

Curling, S. F., Clausen, C. A., and Winandy, J. E. (2002). Relationships between mechanical properties, weight loss, and chemical composition of wood during incipient brown-rot decay. *For. Produc. J.* 52, 34–39. doi: 10.1016/S0964-8305(01)00101-9

Daniel, G. (1994). Use of electron microscopy for aiding our understanding of wood biodegradation. *FEMS Microbiol. Rev.* 13, 199–233. doi: 10.1111/j.1574-6976.1994.tb00043.x

Eastwood, D. C., Floudas, D., Binder, M., Majcherczyk, A., Schneider, P., Aerts, A., et al. (2011). the plant cell wall–decomposing machinery underlies the functional diversity of forest fungi. *Science* 333, 762–765. doi: 10.1126/science.1205411

Eaton, R., and Hale, M. (1993). *Decay, Pests and Protection*. Cambridge: Chapman and Hall.

Eriksson, K., Blanchette, R. A., and Ander, P. (1990). *Microbial and Enzymatic Degradation of Wood and Wood Components*. Berlin: Springer.

Fackler, K., Stevanic, J. S., Ters, T., Hinterstoisser, B., Schwanninger, M., and Salmén, L. (2010). Localisation and characterisation of incipient brown-rot decay within spruce wood cell walls using FT-IR imaging microscopy. *Enzyme Microb. Technol.* 47, 257–267. doi: 10.1016/j.enzmictec.2010.07.009

Fenton, H. (1894). Oxidation of tartaric acid in the presence of iron. *J. Chem. Soc. Trans.* 65:12. doi: 10.1039/CT8946500899

Filley, T. R., Cody, G. D., Goodell, B., Jellison, J., Noser, C., and Ostrofsky, A. (2002). Lignin demethylation and polysaccharide decomposition in spruce sapwood degraded by brown rot fungi. *Org. Geochem.* 33, 111–124. doi: 10.1016/S0146-6380(01)00144-9

Floudas, D., Binder, M., Riley, R., Barry, K., Blanchette, R. A., Henrissat, B., et al. (2012). The paleozoic origin of enzymatic lignin decomposition reconstructed from 31 fungal genomes. *Science* 336, 1715–1719. doi: 10.1126/science.1221748

Gaskell, J., Kersten, P., Larrondo, L. F., Canessa, P., Martinez, D., Hibbett, D. S., et al. (2017). Draft genome sequence of a monokaryotic model brown-rot fungus *Postia (Rhodonia) placenta* SB12. *Genom. Data* 14:3. doi: 10.1016/j.gdata.2017.08.003

Gelhaye, E., and Morel, M. (2009). "Molecular Microbiology and Wood Protection," in *Proceedings IRG Annual Meeting, IRG/WP 09-20416*, Beijing.

Goodell, B. (2003). *Brown-Rot Fungal Degradation of Wood: Our Evolving View*.

Goodell, B., Jellison, J., Liu, J., Daniel, G., Paszczynski, A., Fekete, F., et al. (1997). Low molecular weight chelators and phenolic compounds isolated from wood decay fungi and their role in the fungal biodegradation of wood. *J. Biotechnol.* 53, 133–162. doi: 10.1016/S0168-1656(97)01681-7

Goodell, B., Zhu, Y., Kim, S., Kafle, K., Eastwood, D. C., Daniel, G., et al. (2017). Modification of the nanostructure of lignocellulose cell walls *via* a non-enzymatic lignocellulose deconstruction system in brown rot wood-decay fungi. *Biotechnol. Biofuels* 10:179. doi: 10.1186/s13068-017-0865-2

Hibbett, D. S., and Donoghue, M. J. (2001). Analysis of character correlations among wood decay mechanisms, mating systems, and substrate ranges in homobasidiomycetes. *Syst. Biol.* 50, 215–242. doi: 10.1080/10635150151125879

Hill, C. (2006). *Wood Modification: Chemical, Thermal and other Processes*, ed Stevens C.V. Hoboken, New Jersey: John Wiley and Sons, Ltd.

Hosseinpourpia, R., and Mai, C. (2016). Mode of action of brown rot decay resistance of acetylated wood: resistance to Fenton's reagent. *Wood Sci. Technol.* 50, 413–426. doi: 10.1007/s00226-015-0790-0

Hunt, C., Zelinka, S., Frihart, C., Lorenz, L., Yelle, D., Gleber, S.-C., et al. (2018). Acetylation increases relative humidity threshold for ion transport in wood cell walls—a means to understanding decay resistance. *Int. Biodeter. Biodegrad.* 133, 230–237. doi: 10.1016/j.ibiod.2018.06.014

Ide, M., Ichinose, H., and Wariishi, H. (2012). Molecular identification and functional characterization of cytochrome P450 monooxygenases from the brown-rot basidiomycete Postia placenta. *Arch. Microbiol.* 194, 243–253. doi: 10.1007/s00203-011-0753-2

Jakes, J., Plaza, N. S., Stone, D., Hunt, C., Glass, S., and Zelinka, S. (2013). Mechanism of transport through wood cell wall polymers. *J. For. Produc. Industr.* 2, 10–13.

Jensen, K. A., Houtman, C. J., Ryan, Z. C., and Hammel, K. E. (2001). Pathways for extracellular fenton chemistry in the brown rot basidiomycete *Gloeophyllum trabeum*. *Appl. Environ. Microbiol.* 67, 2705–2711. doi: 10.1128/AEM.67.6.2705-2711.2001

Kim, D., Langmead, B., and Salzberg, S. L. (2015). HISAT: a fast spliced aligner with low memory requirements. *Nat. Methods* 12, 357–360. doi: 10.1038/nmeth.3317

Klutts, J., Yoneda, A., Reilly, M., Bose, I., and Doering, T. (2006). Glycosyltransferases and their products: cryptococcal variations on fungal themes. *FEMS Yeast Res.* 6, 499–512. doi: 10.1111/j.1567-1364.2006.00054.x

Kölle, M., Horta, M., a,.C., Nowrousian, M., Ohm, R. A., Benz, J. P., et al. (2020). Degradative capacity of two strains of *Rhodonia* placenta: from phenotype to genotype. *Front. Microbiol.* 11:1338. doi: 10.3389/fmicb.2020.01338

Kölle, M., Ringman, R., and Pilgård, A. (2019). Initial *Rhodonia* placenta gene expression in acetylated wood: group-wise upregulation of non-enzymatic oxidative wood degradation genes depending on the treatment level. *Forests* 10:11117. doi: 10.3390/f10121117

Mantanis, G. I. (2017). Chemical modification of wood by acetylation or furfurylation: a review of the present scaled-up technologies. *Bioresources* 12:12. doi: 10.15376/biores.12.2.Mantanis

Martinez, Á. T., Speranza, M., Ruiz-Dueñas, F. J., Ferreira, P., Camarero, S., Guillén, F., et al. (2005). Biodegradation of lignocellulosics: microbial, chemical, and enzymatic aspects of the fungal attack of lignin. *Int. Microbiol.* 8, 195–204.

Martinez, D., Challacombe, J., Morgenstern, I., Hibbett, D. S., Schmoll, M., Kubicek, C., et al. (2009). Genome, transcriptome, and secretome analysis of wood decay fungus *Postia placenta* supports unique mechanisms of lignocellulose conversion. *PNAS* 106:6. doi: 10.1073/pnas.0809575106

Moghaddam, M. S., Wålinder, M. E. P., Claesson, P. M., and Swerin, A. (2016). Wettability and swelling of acetylated and furfurylated wood analyzed by multicycle Wilhelmy plate method. *Holzforschung* 70, 69–77. doi: 10.1515/hf-2014-0196

Munir, E., Yoon, J., Tokimatsu, T., Hattori, T., and Shimada, M. (2001). A physiological role for oxalic acid biosynthesis in the wood-rotting basidiomycete Fomitopsis palutris. *Proc. Natl. Acad. Sci. USA.* 98, 11126–11130. doi: 10.1073/pnas.191389598

Niemenmaa, O., Uusi-Rauva, A., and Hatakka, A. (2008). Demethoxylation of [O14CH3]-labelled lignin model compounds by the brown-rot fungi Gloeophyllum trabeum and Poria (Postia) placenta. *Biodegradation* 19:555. doi: 10.1007/s10532-007-9161-3

Papadopoulos, A. N., and Hill, C. A. S. (2002). The biological effectiveness of wood modified with linear chain carboxylic acid anhydrides against Coniophora puteana. *Holz als Roh- und Werkstoff* 60, 329–332. doi: 10.1007/s00107-002-0327-8

Paszczynski, A., Crawford, R., Funk, D., and Goodell, B. (1999). De novo synthesis of 4,5-dimethoxycatechol and 2, 5-dimethoxyhydroquinone by the brown

rot fungus Gloeophyllum trabeum. *Appl. Environ. Microbiol.* 65, 674–679. doi: 10.1128/AEM.65.2.674-679.1999

Pertea, M., Kim, D., Pertea, G. M., Leek, J. T., and Salzberg, S. L. (2016). Transcript-level expression analysis of RNA-seq experiments with HISAT, StringTie and Ballgown. *Nat. Protoc.* 11, 1650–1667. doi: 10.1038/nprot.2016.095

Pertea, M., Pertea, G. M., Antonescu, C. M., Chang, T.-C., Mendell, J. T., and Salzberg, S. L. (2015). StringTie enables improved reconstruction of a transcriptome from RNA-seq reads. *Nat. Biotechnol.* 33, 290–295. doi: 10.1038/nbt.3122

Popescu, C.-M., Hill, C. A. S., Curling, S., Ormondroyd, G., and Xie, Y. (2014). The water vapour sorption behaviour of acetylated birch wood: how acetylation affects the sorption isotherm and accessible hydroxyl content. *J. Mater. Sci.* 49, 2362–2371. doi: 10.1007/s10853-013-7937-x

Presley, G. N., Panisko, E., Purvine, S. O., and Schilling, J. S. (2018). Coupling secretomics with enzyme activities to compare the temporal processes of wood metabolism among white and brown rot fungi. *Appl. Environ. Microbiol.* 84, e00159–e00118. doi: 10.1128/AEM.00159-18

Presley, G. N., and Schilling, J. S. (2017). Distinct growth and secretome strategies for two taxonomically divergent brown rot fungi. *Appl. Environ. Microbiol.* 83, e02987–e02916. doi: 10.1128/AEM.02987-16

Ringman, R., Pilgård, A., Brischke, C., and Richter, K. (2014). Mode of action of brown rot decay resistance in modified wood: a review. *Holzforschung* 68:239. doi: 10.1515/hf-2013-0057

Ringman, R., Pilgård, A., Brischke, C., Windeisen, E., and Richter, K. (2017). Incipient brown rot decay in modified wood: patterns of mass loss, structural integrity, moisture and acetyl content in high resolution. *Int. Wood Produc. J.* 11:344382. doi: 10.1080/20426445.2017.1344382

Ringman, R., Pilgård, A., Kölle, M., Brischke, C., and Richter, K. (2016). Effects of thermal modification on Postia placenta wood degradation dynamics: measurements of mass loss, structural integrity and gene expression. *Wood Sci. Technol.* 50, 385–397. doi: 10.1007/s00226-015-0791-z

Rowell, R. M. (2005). *Wood Chemistry and Wood Composites*. Boca: Taylor and Francis.

Ryu, J. S., Shary, S., Houtman, C. J., Panisko, E. A., Korripally, P., St John, F. J., et al. (2011). Proteomic and functional analysis of the cellulase system expressed by Postia placenta during brown rot of solid wood. *Appl. Environ. Microbiol.* 77, 7933–7941. doi: 10.1128/AEM.05496-11

Schilling, J. S., Duncan, S. M., Presley, G. N., Filley, T. R., Jurgens, J. A., and Blanchette, R. A. (2013). Colocalizing incipient reactions in wood degraded by the brown rot fungus Postia placenta. *Int. Biodeter. Biodegrad.* 83, 56–62. doi: 10.1016/j.ibiod.2013.04.006

Seo, J., and Shneiderman, B. (2002). Interactively exploring hierarchical clustering results. *Computer* 35, 80–86. doi: 10.1109/MC.2002.1016905

Thaler, N., Alfredsen, G., and Fossdal, C. G. (2012). "Variation in two Postia placenta strains, MAD-698-R and FPRL280 - mass loss, DNA content and gene expression," in *Proceedings IRG Annual Meeting, IRG/WP 12-10781*. (Malaysia: Kuala Lumpur).

Thybring, E. E. (2013). The decay resistance of modified wood influenced by moisture exclusion and swelling reduction. *Int. Biodeter. Biodegrad.* 82, 87–95. doi: 10.1016/j.ibiod.2013.02.004

Umezawa, K., Niikura, M., Kojima, Y., Goodell, B., and Yoshida, M. (2020). Transcriptome analysis of the brown rot fungus Gloeophyllum trabeum during lignocellulose degradation. *PLoS ONE* 15:e0243984. doi: 10.1371/journal.pone.0243984

Van Munster, J. M., Daly, P., Delmas, S., Pullan, S. T., Blythe, M. J., Malla, S., et al. (2014). The role of carbon starvation in the induction of enzymes that degrade plant-derived carbohydrates in Aspergillus niger. *Fung. Genet. Biol.* 72, 34–47. doi: 10.1016/j.fgb.2014.04.006

Vanden Wymelenberg, A., Gaskell, J., Mozuch, M., Sabat, G., Ralph, J., Skyba, O., et al. (2010). Comparative transcriptome and secretome analysis of wood decay fungi *Postia placenta* and *Phanerochaete chrysosporium*. *Appl. Environ. Microbiol.* 76, 3599–3610. doi: 10.1128/AEM.00058-10

Wessels, J., De Vries, O., Asgeirsdottir, S. A., and Schuren, F. (1991). Hydrophobin genes involved in formation of aerial hyphae and fruit bodies in schizophyllum. *Plant Cell* 3, 793–799. doi: 10.2307/3869273

Wessels, J. G. H. (1996). Fungal hydrophobins: proteins that function at an interface. *Trends Plant Sci.* 1, 9–15. doi: 10.1016/S1360-1385(96)80017-3

Xie, Y., Xiao, Z., and Mai, C. (2015). *Degradation of Chemically Modified Scots Pine (Pinus Sylvestris l.) with Fenton reagent.* Holzforschung.

Zabel, R., and Morrell, J. J. (1992). *Wood Microbiology: Decay and its Prevention.* San Diego: Academic Press.

Zelinka, S., Glass, S., and Stone, D. (2008). A percolation model for electrical conduction in wood with implications for wood-water relations. *Wood Fiber Sci. J. Soc. Wood Sci. Technol.* 40, 544–552.

Zelinka, S. L., Gleber, S. C., Vogt, S., López, G. M. R., and Jakes, J. E. (2015). Threshold for ion movements in wood cell walls below fiber saturation observed by X-ray fluorescence microscopy (XFM). *Holzforschung* 69, 441–448. doi: 10.1515/hf-2014-0138

Zelinka, S. L., Houtman, C. J., Hirth, K., Lacher, S., Lorenz, L., Engelund Thybring, E., et al. (2020). The effect of acetylation on iron uptake and diffusion in water saturated wood cell walls and implications for decay. *Forests* 11:1121. doi: 10.3390/f11101121

Zelinka, S. L., Ringman, R., Pilgård, A., Thybring, E. E., Jakes, J. E., and Richter, K. (2016). The role of chemical transport in the brown-rot decay resistance of modified wood AU - Zelinka, S. L. *Int. Wood Product. J.* 7, 66–70. doi: 10.1080/20426445.2016.1161867

Zhang, J., Presley, G. N., Hammel, K. E., Ryu, J. -S., Menke, J. R., Figueroa, M., et al. (2016). Localizing gene regulation reveals a staggered wood decay mechanism for the brown rot fungus Postia placenta. *Proc. Nat. Acad. Sci.* 113, 10968–10973. doi: 10.1073/pnas.1608454113

Zhang, J., and Schilling, J. (2017). Role of carbon source in the shift from oxidative to hydrolytic wood decomposition by Postia placenta. *Fung. Genet. Biol.* 106:003. doi: 10.1016/j.fgb.2017.06.003

Zhang, J., Silverstein, K. T., Castaño, J. D., Figueroa, M., and Schilling, J. S. (2019). Gene regulation shifts shed light on fungal adaption in plant biomass decomposers. *MBio* 10, e02176–e02119. doi: 10.1128/mBio.02176-19