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1 The molecular response of the white-rot fungus *Dichomitus squalens* to wood and non-woody
2 biomass as examined by transcriptome and exoproteome analyses
3

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21 Running title: Molecular response of *D. squalens* to biomass
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27 **Significance statement**

28 White-rot fungi are commonly found on wood in nature where they have a significant role in wood
29 decomposition. Considering earlier studies in which it was shown that the genomes of ascomycete
30 fungi with highly defined habitats (e.g. the dung fungus *Podospora anserina* and the plant pathogen
31 *Botrytis cinerea*) are well tailored to the carbon sources in these habitats, we aimed to evaluate if a
32 similar correlation could be observed for the wood decaying white-rot basidiomycete fungus
33 *Dichomitus squalens*. For this we evaluated the molecular response of *D. squalens* during growth
34 on two (natural) wood substrates and two non-woody substrates. To our knowledge this is the first
35 time when two natural and two non-natural substrates are compared in detail for a basidiomycete
36 fungus. Surprisingly, *D. squalens* was able to express a well-tailored set of genes in both the wood
37 and non-woody substrates, suggesting that the fungus apparently has preserved its ability to respond
38 adequately to a variety of plant biomass, even to those it would not be expected to encounter in
39 nature. It is therefore possible that the restriction of white-rot fungi to wood environments is due to
40 being outcompeted in other environments, rather than a choice to live in wood. These finding
41 therefore have a major impact on our understanding of the limitations of fungal habitats.

42

Summary

The ability to obtain carbon and energy is a major requirement to exist in any environment. For several ascomycete fungi (post-)genomic analyses have shown that species that occupy a large variety of habitats possess a diverse enzymatic machinery, while species with a specific habitat have a more focused enzyme repertoire that is well-adapted to the prevailing substrate. White-rot basidiomycete fungi also live in a specific habitat, as they are found exclusively in wood. In this study, we evaluated how well the enzymatic machinery of the white-rot fungus *Dichomitus squalens* is tailored to degrade its natural wood substrate. The transcriptome and exoproteome of *D. squalens* were analysed after cultivation on two natural substrates, aspen and spruce wood, and two non-woody substrates, wheat bran and cotton seed hulls. *D. squalens* produced ligninolytic enzymes mainly at the early time point of the wood cultures, indicating the need to degrade lignin to get access to wood polysaccharides. Surprisingly, the response of the fungus to the non-woody polysaccharides was nearly as good a match to the substrate composition as observed for the wood polysaccharides. This indicates that *D. squalens* has preserved its ability to efficiently degrade plant biomass types not present in its natural habitat.

Introduction

The natural habitats of fungi are highly diverse and strongly dependent on the biological traits of the individual species. One of these traits is the ability to obtain carbon from plant biomass, which is a complex substrate, consisting of cellulose, various hemicelluloses, pectin and the aromatic polymer lignin (Sjöström, 1993; Harris and Stone, 2008; Vogel, 2008). Depending on the plant species and tissue, the relative amounts and structure of these polymers can differ markedly. For example, wood contains higher amounts of lignin than non-woody plant biomass (Sjöström, 1993). In softwood, guaiacyl units of lignin dominate and low amounts of *p*-hydroxyphenyl units are present, whereas hardwood lignin consists mainly of guaiacyl and syringyl units and traces of *p*-

68 hydoxyphenyl units can also be detected. In non-woody plants, all the three phenylpropanoid lignin
69 units are present in comparable amounts (Billa and Monties, 1995; Vanholme *et al.*, 2010). Also,
70 the hemicellulose fraction differs significantly between plant biomasses (Ebringerová, 2006; Vogel,
71 2008). Softwood contains mainly acetylated galactoglucomannan with predominatly single
72 galactose side-chains and smaller amounts of acetylated 4-*O*-methylglucuronoxylan (Capek *et al.*,
73 2000; Capek *et al.*, 2002). In contrast, the latter is the dominant hemicellulose in hardwood, which
74 only contains small amounts of acetylated glucomannan without galactose side-chains (Teleman *et*
75 *al.*, 2000; Jacobs *et al.*, 2002; Teleman *et al.*, 2003). Furthermore, structurally different lignins are
76 connected to xylan and glucomannan via different linkages (Lawoko *et al.*, 2005; Du *et al.*, 2014;).
77 Cotton xylan (dicot) is similar to wood xylan, it contains both glucuronic acid and 4-*O*-
78 methylglucuronic acid as side chains (Matsuo *et al.,* 1991; Akpinar *et al.*, 2007). In contrast, cereals
79 (monocots) contain mainly arabinoxylans with a low amount of (4-*O*-methyl-)glucuronic acid
80 (Rumpagaporn *et al.*, 2015), but do not contain (galacto-)glucomannan. These variations indicate
81 that while all plant cell walls are complex structures consisting of multiple components, the
82 degradation of each type of plant biomass requires a different set of enzyme activities with different
83 substrate specificities. For instance, the level of side-chains on the xylan polymers will affect the
84 functionality of xylanases that require different numbers of unsubstituted residues (Kormelink *et*
85 *al.*, 1993).
86
87 In ascomycete fungi, clear correlations between the plant-biomass-degrading abilities of different
88 species and their natural habitats have been reported. For example, *Aspergillus niger* is commonly
89 found in many habitats around the world and is able to degrade all plant biomass polysaccharides,
90 which correlates well with genes encoding a broad range of plant-biomass-degrading enzymes in its
91 genome (Pel *et al.*, 2007; Benoit *et al.*, 2015). In contrast, *Podospora anserina*, exclusively a late
92 colonizer of herbivore dung, efficiently degrades cellulose and xylan, but is mostly unable to

93 degrade pectin (Espagne *et al.*, 2008). This indicates that specialization of *P. anserina* to its habitat
94 resulted in an adaptation of its enzymatic machinery to the chemical composition of the prevalent
95 substrate. Similarly, the genome content of *Pyricularia oryzae*, a major pathogen of rice and other
96 grasses, corresponds well with the cell wall composition of monocots, and displays a reduced
97 ability to degrade polysaccharides not commonly present in these plants (Battaglia *et al.*, 2011). In
98 addition, the dicot pathogen *Botrytis cinerea* is particularly specialized to the degradation of the
99 main components of dicots, i.e. pectin and xyloglucan (Amselem *et al.*, 2011).
100
101 White-rot basidiomycete fungi also have a highly specific habitat, as they are usually exclusively
102 found on wood, a substrate rich in lignin. They are the only organisms that can degrade and
103 mineralize all components of wood and therefore play an important role in the carbon cycle in
104 woody environments. The ability of white-rot fungi to degrade wood is in part due to the production
105 of lignin-modifying peroxidases, which are unique to this group of fungi (Martinez *et al.*, 2004,
106 2009; Hatakka and Hammel, 2011; Floudas *et al.*, 2012). We hypothesize that the evolutionary
107 adaptation of white-rot fungi to wood implies that they have modified their complete enzymatic
108 machinery to match closely with the macromolecular composition of wood.
109
110 The white-rot fungus *Dichomitus squalens* is commonly found in the northern regions of Europe,
111 Asia and North America (Andrews and Gill, 1943). Its genome possesses genes predicted to encode
112 diverse plant cell wall-modifying enzymes, and it has been shown to produce an extensive set of
113 lignocellulose-degrading enzymes (Floudas *et al.*, 2012; Rytioja *et al.*, 2014a, 2015; Table 1). In
114 this study, we aimed to evaluate whether specialization of *D. squalens* to wood also resulted in a
115 better molecular response in terms of a set of genes that fits better to the composition of wood
116 substrates than to other plant biomass.
117

118 According to this hypothesis, the set of enzymes the fungus produces during growth on wood would
119 better correspond to the composition of wood than the enzymes produced on other types of plant
120 biomass. To test this hypothesis, we cultivated *D. squalens* on two wood and two non-woody plant
121 biomasses and evaluated the response of the fungus at the transcriptome and exoproteome level.
122 The analysis focused on genes and enzymes involved in plant biomass degradation as this is the
123 crucial step in obtaining carbon from plant cell wall. Most of these enzymes have been catalogued
124 in the Carbohydrate Enzyme Database (CAZy, http://www.cazy.org/, (Lombard *et al.*, 2014)),
125 allowing rapid identification and function assignment. We analysed this at two levels: the number
126 of genes expressed or proteins produced of a specific enzyme class, and the changes in gene
127 expression and protein production per individual gene/protein. These two datasets were separately
128 analysed to generate patterns across the samples.
129

130 **Results**

131 ***Selection of the substrates***

132 In this study, we aimed to analyse the molecular response of *D. squalens* to its natural carbon
133 source (wood) and compare it to other plant biomass types that not only have a much lower lignin
134 content and different lignin structure, but also differ in the structure of the hemicelluloses in their
135 cell walls. To select appropriate substrates and time points, an initial experiment was performed in
136 which the fungus was grown on seven plant-derived biomass substrates (microcrystalline cellulose,
137 spruce, wheat bran, cotton seed hulls, apple pectin, beech xylan and guar gum) and two control
138 carbon sources (glucose and cellobiose) for 21 days. The pure polysaccharides were included to
139 determine if any of them would be responsible for production of a significant number of the
140 dominant proteins in the profile of the complex substrates. The cultures were analysed by SDS-
141 PAGE at two to three day intervals. Extracellular proteins were observed in all culture liquids after
142 nine days of cultivation and therefore this time point was chosen as an early phase of substrate

143 degradation (Fig. S1). After 16 days of cultivation the protein profile was visibly different from day
144 nine profile in most substrates, and therefore this time point was selected as a later phase of
145 substrate degradation. *D. squalens* produced the most complex set of extracellular proteins in the
146 cultures supplemented with spruce sawdust, and powdered wheat bran and cotton seed hulls. These
147 lignocellulosic substrates from softwood, and monocot and dicot plants were therefore selected as
148 carbon sources for cultivation for RNA-seq and exoproteome analyses. As a representative of
149 hardwood, aspen sawdust was also included to complete the plant biomass substrate repertoire.
150 Substrate compositions are presented in Supplementary Table S2.
151

152 ***Transcriptomic and proteomic response of D. squalens to different plant biomasses***
153 In total, 297 different putative CAZyme-encoding genes showed expression levels above our cut-off
154 (>20 RPKM) in the *D. squalens* cultures (Fig. 1A, Table S3). Of these, 135 were related to plant
155 cell wall degradation. In addition, 12 genes distantly related to plant expansins and two genes
156 encoding feruloyl esterases were expressed and added to the analysis. *D. squalens* expressed ten
157 ligninolytic genes at the early phase and six at the later time point in spruce-containing medium
158 (Fig. 2A), while in aspen cultures the number of ligninolytic genes was similar (9-10) at both
159 phases. In contrast, in non-woody substrates only three to four ligninolytic genes were expressed.
160 Extracellular H_2O_2 is essential for ligninolytic peroxidase activity and therefore needed for efficient
161 lignin degradation. The number of H_2O_2-production related genes was also higher in wood (20-21)
162 than in non-woody substrates (14-15) during the cultivation. Overall, significant changes in
163 expression were observed for a large proportion of the genes across all the samples (Supplementary
164 Table S4).
165

166 The number of expressed cellulolytic genes increased from eight to 27 and seven to 17 in spruce
167 and aspen, respectively, in the course of the cultivation. In wheat bran the number of expressed

168 cellulolytic genes decreased slightly (from 18 to 13), while this stayed the same (17-18) in cotton
169 seed hulls. In both softwood and hardwood, the number of expressed xylanolytic genes increased
170 from four up to 24 during the cultivation, but slightly decreased in wheat bran and cotton seed hulls
171 (from 17 to 14 and from 21 to 19, respectively). The number of expressed mannanolytic genes (6-8)
172 was more similar among all the substrates and time points than that of the xylanolytic genes.
173 Pectinolytic genes increased in wood from the early to the later phase (from 4 to 18), whereas
174 roughly the same number of genes were expressed at both time points for non-woody substrates (11
175 in wheat bran and 17-18 in cotton seed hulls). Most of the expansin-like genes (8-12 out of 13) were
176 expressed in all the studied cultivation conditions.
177 When the CAZyme and expansin-like protein encoding genes expressed on all the substrates at the
178 two time points were analysed, the time point representing early degradation of spruce and aspen
179 clustered together, while the later time point of spruce and aspen clustered with the non-woody
180 substrates, with aspen being more similar to the non-woody substrates than spruce (Fig. 3). The
181 larger distance for the late spruce samples could be due to some clusters (G, K and R) of CAZyme-
182 encoding genes that had higher expression levels in this sample compared to all other samples.
183

184 In the *D. squalens* cultures, 175 different CAZymes were secreted (>0.02% of total exoproteome),
185 of which 120 were related to plant cell wall degradation (Fig. 1B, Table S5). The number of
186 extracellular enzymes was lower at the early degradation phase in spruce compared to other
187 substrates (Fig. 2B). The number of different ligninolytic enzymes increases from the early to later
188 degradation phase in spruce (from 5 to 11) and aspen (from 10 to 15). In contrast, their number
189 decreased in wheat bran (from 11 to 7) and remained similar in cotton seed hulls (5-6). Similarly,
190 the number of H_2O_2-production related enzymes increased in wood but stayed at the same level in
191 non-woody substrates.
192

193 The number of cellulolytic enzymes found in the extracellular media increased in spruce (from 10
194 to 23), but was similar (between 19 to 24) in the other substrates at both time points. Similar
195 patterns were detected for xylanolytic, mannanolytic and pectinolytic enzymes (Fig. 2B).
196
197 Transcriptome and exoproteome data correlated well with respect to the CAZyme-encoding
198 transcripts and enzymes (Fig. 4, Fig. S2, Table 1). However, despite the high expression level of
199 several expansin-like genes, only two expansin-like proteins were present in the *D. squalens*
200 exoproteome from aspen cultures and one in wheat bran. In contrast, some other enzymes were
201 detected that lacked expression above our cut-off (>20 RPKM) of their corresponding genes (e.g.
202 three GH27 α-galactosidases (AGLs) and four AA5_1 copper radical oxidases (CROs)).
203
204 ***Molecular response to wood***
205 In accordance with the high lignin content of spruce and aspen (25-33% and 20-25%, respectively;
206 (Sjöström, 1993)), AA2 lignin-modifying peroxidases and AA1_1 laccases were expressed on these
207 substrates. Overall, the majority (68%) of the ligninolytic genes showed the highest expression at
208 the early time point of the wood cultures (Fig. 4A, Figs. S3A, S4A). Genes encoding extra-long
209 manganese peroxidases (MnPs) and the corresponding enzymes were expressed and produced more
210 abundantly than short MnPs or versatile peroxidases (VPs) in both wood substrates. However,
211 several short MnP and VP isoenzymes were present in the exoproteomes for which the
212 corresponding genes were not detected in the transcriptomes, especially in aspen cultures. Three
213 laccase-encoding genes were highly expressed in aspen and two in spruce. Laccases were also the
214 most abundant proteins, representing up to 31% and 26% of all the detected proteins at day 16 in
215 spruce and aspen, respectively.
216

217 H_2O_2-supply related gene expression was similar in both wood substrates. The genes encoding
218 AA3_3 alcohol/methanol oxidases (AOX/MOX) oxidoreductases and AA5_1 CROs were among
219 the highest expressed genes in both softwood and hardwood (Fig. S2AB, Fig. S5). Several CROs
220 were produced in wood, while only one AOX/MOX was secreted in aspen. AA3_2 glucose-
221 methanol-choline (GMC) oxidoreductase-encoding genes showed low or moderate expression and
222 most of them (58%) displayed reduced expression at the later time point on both softwood and
223 hardwood. Ten out of 24 putative GMC oxidoreductases were secreted at low level.
224
225 In contrast with ligninolytic genes, transcript levels encoding most cellulases were significantly
226 higher at day 16 compared to day nine in spruce and aspen (Fig. S2B, Figs. S3B, S4B). However,
227 GH7 cellobiohydrolase I enzymes (CBHIs) were present at high level at both time points and were
228 the second most abundant proteins detected in the wood cultures (6-10% in spruce and 8-16% in
229 aspen). Endoglucanases and β-glucosidases represented a smaller proportion of the total proteins
230 than CBHs. Expression of AA9 lytic polysaccharide monooxygenase (LPMO)-encoding genes was
231 also higher at day 16 compared to day nine of growth. Eight and six out of 15 LPMO isoenzymes
232 were produced at low level in spruce and aspen, respectively.
233
234 Expression of the majority of hemicellulolytic CAZyme-encoding genes was higher at day 16 than
235 at day 9 in wood (Fig. 4B, C, Figs. S3C, D, S4C, D). The hemicelluloses in spruce consist of
236 galactomannan and glucuronoxylan, with similar amounts of mannose and xylose residues
237 (Supplementary Table S2). Aspen contains less mannan (5 mol%) but more xylan (29 mol%) than
238 spruce (17 and 12 mol%, respectively). Despite these structural differences, no clear correlation of
239 the *D. squalens* transcriptome or exoproteome was found to the composition of the softwood and
240 hardwood substrates.
241

242 Pectin is a minor component of wood, and expression of most of pectinolytic genes was higher at
243 day 16 than at day 9 in *D. squalens* wood cultures (Fig. 2C, Figs. S3F, S4F). Enzymes acting on the
244 side-chains of pectin, such as GH43 endoarabinanase (ABN) and GH35 β-galactosidase (LAC),
245 were expressed at high level at day 16. LACs were also the most abundantly secreted pectinases.
246
247 Transcripts corresponding to 12 out of 13 expansin-like genes were detected in *D. squalens* wood
248 cultures and of these six were highly expressed (Fig. S2E). In aspen, most of the expansin-like
249 transcripts had higher values at the later time point, while in spruce their pattern was more variable
250 including one gene with significantly lower expression at day 16. In contrast, only two expansin-
251 like proteins were detected in the aspen exoproteome.
252
253 ***Molecular response to non-woody substrates***
254 The expression profiles of the genes related to plant cell wall decomposition during early and later
255 degradation of wheat bran and cotton seed hulls were highly similar (Fig. 4). In accordance with the
256 low lignin content of wheat bran and cotton seed hulls, the amount of expressed AA2 and AA1_1
257 ligninolytic genes in these cultures was lower than in the wood cultures (Fig. 4A, Figs. S6A, S7A).
258 Short MnP-encoding genes were the most highly expressed ligninolytic genes, and low protein
259 levels of extra-long MnPs, short MnPs and VPs were detected in the non-woody cultures. No
260 expression above our cut-off (>20 RPKM) of laccase-encoding genes was detected on wheat bran,
261 and only low or moderate amounts of up to four laccase isoenzymes were produced in both non-
262 woody substrates. *D. squalens* expressed and produced most of the AA3_2 GMC oxidoreductase
263 genes and corresponding enzymes at low level in the non-woody substrates (Fig. S2A, Fig. S5).
264 Interestingly, transcript and exoproteome patterns of other putative H_2O_2-producing enzymes were
265 similar in wood and non-woody substrates.
266

267 Similar to the wood cultures, a complete set of cellulase-encoding genes was expressed and the
268 corresponding enzymes produced during the growth of *D. squalens* in non-woody substrates (Fig.
269 S2B, Figs. S6B, S7B). However, in contrast to wood substrates, expression of most of the GH
270 cellulase-encoding genes (64%) was higher at day 9 than at day 16 in wheat bran and half of them
271 in cotton seed hulls. GH7 CBHIs were the most highly secreted proteins by *D. squalens* in non-
272 woody substrates (up to 15% and 16% in wheat bran and cotton seed hulls, respectively). LPMO-
273 encoding genes were either expressed at the same level in both time points or at lower levels at day
274 16 in non-woody cultures. They were also the second most abundant secreted proteins (6%) at the
275 early phase of degradation in wheat bran cultures. In cotton seed hulls, LPMOs were present in
276 lower amounts.
277
278 Xylan is present in both wheat bran and cotton seed hulls and resulted in the expression of a
279 complete set of xylanolytic genes (Fig. 4B, Figs. S6D, S7D). During the early degradation, the
280 highest transcript level was detected for a GH10 endoxylanase (XLN), and it represented 2.8% and
281 2.4% of total proteins in wheat bran and cotton seed hulls, respectively. Despite the low mannan
282 content of wheat bran (1 mol%) and cotton seed hulls (3 mol%) (Table S2), several mannanolytic
283 genes were expressed in non-woody substrates and one of the six AGL genes was highly expressed
284 at both time points in cotton seed hulls (Fig. 4C, Figs. S6C, S7C). In addition, the amount of
285 mannanolytic enzymes was relatively high (3-7%) in non-woody substrates.
286
287 Pectinolytic genes showed variable expression patterns in the non-woody cultures of *D. squalens*
288 (Fig. S2C, Figs. S6F, S7F). In both substrates, the highest amount of transcripts was detected for a
289 GH43 ABN at day 16. Furthermore, genes encoding GH28 endopolygalacturonase and CE8 pectin
290 methyl esterase were highly expressed in cotton seed hull cultures. Despite the higher pectin content

291 of cotton seed hulls, the overall set of secreted pectinases was similar for both non-woody
292 substrates.
293
294 High-level constant expression was observed for up to five out of the ten detected expansin-like
295 genes in the non-woody cultures, but only one expansin-like protein was detected in the
296 exoproteome in wheat bran (Fig. S2E).
297

298 **Discussion**

299 In this study, we aimed to evaluate whether *D. squalens*, as a reference basidiomycete white-rot
300 species, has an enzymatic machinery that is ideally suited for the degradation of the substrate it is
301 commonly found on in nature, i.e. wood. This phenomenon has been demonstrated in ascomycetes
302 in that species with a broad habitat range (e.g. *Aspergillus*) harbour a large variety of polymer-
303 degrading enzymes encoded in their genome (Benoit *et al.*, 2015). In contrast, fungi with narrower
304 habitats possess a less extensive set of different enzymes, although they often have a higher number
305 of isoenzymes that target the prevalent substrates in their natural environment (Espagne *et al.*, 2008;
306 Battaglia *et al.*, 2011). Even though basidiomycete white-rot fungi also have a highly defined
307 habitat, the topic of molecular response to their natural substrate and correlation of their enzyme
308 sets with wood composition has not been addressed in detail before. Considerable focus has been
309 placed on their unique lignin-modifying enzymatic machinery including peroxidases, but less
310 attention has been given to their arsenal of (hemi-)cellulose-acting enzymes (Rytioja *et al.*, 2014b).
311 Genomic studies revealed that the white-rot genomes contain fewer genes for enzymes targeting
312 polymers less commonly present in wood biomass (Floudas *et al.*, 2012; Rytioja *et al.*, 2014b), e.g.
313 the enzyme set related to xylan degradation. Wood xylan contains significantly less arabinose than
314 monocot xylan and white-rot fungi typically lack many of the arabinose-releasing enzymes found in
315 ascomycetes, such as family GH54 and GH62 enzymes (Ebringerová, 2006; Floudas *et al.*, 2012).

316 Interestingly, the genome of the coprophilic basidiomycete *Coprinopsis cinerea* contains three
317 GH62 enzyme-encoding genes, which correlates well with its habitat that also includes meadows
318 (Stajich *et al.*, 2010). Wood xylans can be abundantly acetylated and contain significant amounts of
319 (4-*O*-methyl-)glucuronic acid (Ebringerová, 2006). This likely explains why white-rot genomes
320 typically contain more GH10 than GH11 XLNs, as the latter group is more inhibited by such
321 substitutions (Paës *et al.*, 2012). This is again different for *C. cinerea* as its genome contains six
322 GH11 XLNs (Stajich *et al.*, 2010), which are more suitable for monocot xylans. In addition, white-
323 rot fungi also contain less pectin- and starch-degrading enzymes, targeting polysaccharides that are
324 minor components of wood (Timell, 1967).
325
326 *D. squalens* is a typical white-rot basidiomycete and its genome contains all the features mentioned
327 above (Floudas *et al.*, 2012). To evaluate in more detail how this fungus is able to respond to its
328 natural and non-natural substrates, we analysed its transcriptome and exoproteome during growth
329 on two wood and two non-woody substrates, focusing on the genes and enzymes required to
330 degrade the polymeric carbon sources present in these substrates. Overall, the transcriptome and
331 exoproteome results were consistent, although differences were detected for expansins, AGLs and
332 CROs. We also did not detect several starch-related enzymes in some of the proteomics samples,
333 even though their corresponding genes were expressed. Interestingly, the same phenomeon has been
334 observed in a previous study for the *A. niger* glucoamylase-encoding gene (*glaA*), where expression
335 was high on several substrates, but protein and enzyme activity was only observed on a subset of
336 them (R.P. de Vries *et al.*, unpublished). This can be due to various reasons, such as translation and
337 secretion, the rate of the transcription and translation, stability of mRNA and protein, and
338 biochemical characters of the protein (Vogel and Marcotte, 2012; Patyshakuliyeva *et al.*, 2015). It is
339 also possible that the expansin-like proteins are bound to either the fungal cell wall or to the
340 biomass (Quiroz-Castañeda *et al.*, 2011; Veneault-Fourrey *et al.*, 2014; Tovar-Herrera *et al.*, 2015).

341

342 Interestingly, only a very small set of genes was highly expressed in the early time point (Fig. 3,
343 clusters A, B, C, E and F), putatively encoding one AOX/MOX, seven expansin-like proteins, one
344 LPMO, three laccases, two CROs, one MnP and one β-1,4-galactosidase (LAC). Several of these
345 enzymes are involved in the degradation of lignin (laccases, MnP, AOX/MOX, CROs), which
346 would fit well with the rapid degradation of lignin reported for *D. squalens* grown on spruce wood
347 lignin (Fackler *et al.*, 2006). Several *D. squalens* strains have been reported to selectively remove
348 the lignin-rich middle lamella from wood cell walls (Blanchette, 1984), but in long term cultivation
349 other white-rot species have been shown to be more selective in terms of lignin degradation (Hakala
350 *et al.*, 2004). While the role of the expansin-like proteins has not been functionally described,
351 expansins in plants mediate cell wall loosening during growth (Marowa *et al.*, 2016), so it could be
352 speculated that these proteins from fungi have a similar role during plant wall degradation. The role
353 of LPMO could be in initial cleavage of either cellulose or hemicellulose, to open these structures
354 for the hydrolytic enzymes. The role of LAC is more difficult to speculate upon, as this enzyme
355 mainly removes D-galactose side-chains from xylan, pectin, galactomannan and xyloglucan and it
356 is surprising to see this gene highly expressed already at the early time point, whereas no other
357 genes involved in degradation of these polymers were detectable.

358

359 In general, the repertoire of the expressed CAZyme encoding genes and secreted CAZymes by *D.*
360 *squalens* was comparable in softwood and hardwood corresponding to typical white-rot degradation
361 of lignocellulose reported e.g. from the white-rot fungus *Pycnoporus coccineus* (Riley *et al.*, 2014;
362 Couturier *et al.*, 2015) and *Phlebia radiata* (Kuuskeri *et al.*, 2016). Abundant expression and
363 production of ligninolytic genes and enzymes on both wood substrates, especially in the early phase
364 of degradation, fits well with the white-rot decay mechanism in which lignin depolymerisation
365 precedes cellulose degradation. The same has been observed for instance with production of

366 laccases during the initial response to wood by the white-rot fungus *P. coccineus* (Couturier *et al.*,
367 2015) and production of ligninolytic enzymes in compost by the litter-decomposing fungus
368 *Agaricus bisporus* (Patyshakuliyeva *et al.*, 2015). Ligninolytic enzymes have also been
369 overproduced in poplar and wheat bran cultures of *P. ostreatus* (Fernández-Fueyo *et al.*, 2016). The
370 barrier that lignin forms to impede the utilization of cellulose at the onset of wood degradation was
371 also evident from the results of our study as expression of polysaccharide-hydrolysing CAZyme-
372 and LPMO-encoding genes increased in time in wood. As reported previously (Rytioja *et al.*,
373 2014a), *D. squalens* expressed all three genes encoding CBHI isoenzymes and the gene encoding
374 CBHII in all the studied conditions. LPMO expression clustered with different sets of CAZymes
375 and expansin-like proteins, which is in line with their multifunctional nature in the degradation of
376 various lignocellulose polymers (Westereng *et al.*, 2015).
377 The change in expression profile of some genes between the two time points in all substrates likely
378 reflects the ability of the fungus to respond to the changes in substrate composition as the
379 degradation proceeds and provides insight into which components are being degraded. This would
380 imply that the later time point is mainly a reflection on the changes to the substrate due to partial
381 conversion by *D. squalens*. However, this does not mean that there is no effect of the natural
382 substrate composition on the gene expression at 16 days. These substrates not only contain different
383 polymers, but also the exact structure and composition of shared polymers (e.g. xylan) is different
384 between them (Matsuo *et al.*, 1991; Teleman *et al.*, 2000; Jacobs *et al.*, 2002; Ebringerová *et al.*,
385 2006). Therefore, even after partial degradation, the resulting carbohydrates will still reflect, at least
386 in part, the original difference in the fine-structure of the plant cell walls.
387
388 A low number of produced extracellular proteins was observed during the early phase of
389 degradation in the *D. squalens* spruce cultures. While it cannot be excluded that the differences

390 between the substrates are in part due to differences in growth, we did not see significant
391 differences in growth between the substrates on plates (data not shown).
392 The softwood lignin consisting of guaiacyl subunits has also been suggested to be more resistant to
393 enzymatic degradation than the guaiacyl-syringyl lignin in hardwoods (Ramos *et al.*, 1992;
394 Sjöström, 1993; Mooney *et al.*, 1998). However, this did not change the overall pattern of the
395 response of *D. squalens* to wood. Highest production level of extra-long MnPs of *D. squalens* was
396 detected in spruce. Extra-long MnPs have been reported to be extraordinary stable proteins due to
397 the polar tail structure (Li *et al.*, 1999, 2001) which may be beneficial in the degradation of
398 softwood lignin.
399
400 A lower number of ligninolytic genes and enzymes was detected during growth of *D. squalens* on
401 the non-woody than on the wood substrates. This indicates that the expression of ligninolytic genes
402 is specifically induced by the presence of lignin and not part of a general response to the presence
403 of plant biomass. However, genes encoding H_2O_2-producing enzymes from AA3_3 and AA5_1
404 were expressed at similar level in all substrates, thus suggesting that in addition to supporting
405 peroxidase catalysis these enzymes may also have other functions in plant biomass decay such as to
406 act in the electron transfer system together with LPMOs attacking lignocellulose (Kracher *et al.*
407 2016). Interestingly, the absence of lignin also resulted in an earlier expression of (hemi-
408)cellulolytic genes on the non-woody substrates, suggesting that the presence of lignin prevents
409 their induction. This would imply a mechanism in which *D. squalens* initially devotes most of its
410 energy to (partially) degrading lignin to ensure good access to the polysaccharides, before
411 producing high amounts of the polysaccharide-degrading enzymes. The energy required for this
412 initial stage of wood colonization is probably generated by the release of sugars from hemicellulose
413 by the small number of enzymes that is produced and target these polymers (Hori *et al.*, 2014). The
414 decreased expression of ligninolytic genes at the later time point shows that the need for lignin

415 degradation reduces once sufficient access is obtained, which is in line with earlier observations
416 (MacDonald and Master, 2012).
417
418 The hemicellulose and pectin fractions of the four substrates differ remarkably in their composition
419 as indicated in Supplementary Table S2. In summary, spruce contains mainly galactoglucomannan
420 and to a lesser extent glucuronoxylans, while the opposite is the case for aspen. Cotton seed hulls
421 contain significant amounts of pectin and xyloglucan, but also glucuronoxylan and
422 galactoglucomannan, while wheat bran contains mainly arabinoxylan. The number of
423 hemicellulolytic and pectinolytic genes expressed by *D. squalens* was in general uniform in both
424 spruce and aspen. However, in line with the higher mannan content of softwood, three
425 mannanolytic genes were highly expressed only in spruce. Similarly, endomannanases and AGL
426 were highly expressed in pine cultures of the white-rot fungus *Phlebiopsis gigantea* (Hori *et al.*,
427 2014). *D. squalens* displayed increased expression of xylanolytic genes in time in wood substrates.
428 Although in the cluster analysis the transcriptomes obtained from the wood cultures were all clearly
429 different from the transcriptomes obtained from the non-woody cultures, the later time point of
430 aspen was more similar to the transcriptomes from the non-woody cultures than to the other
431 transcriptomes from wood cultures. This is likely caused by the higher xylan content of aspen
432 compared to spruce. Surprisingly, the transcriptome and proteome profiles of wheat bran and cotton
433 seed hulls also corresponded well with the composition of these substrates, suggesting that *D.*
434 *squalens* has preserved its ability to respond adequately to these plant biomasses, even though they
435 are not part of its natural habitat. This indicates a much less specific match of the plant cell wall
436 degrading enzyme repertoire of this white-rot basidiomycete to the prevalent polymers in its natural
437 habitat than was previously observed for several ascomycete fungi, such as *P. anserina* and *M.*
438 *oryzae* (Espagne *et al.*, 2008; Battaglia *et al.*, 2011).
439

440 Another interesting feature is the presence of genes that are only expressed on specific substrates,
441 suggesting a highly fine-tuned regulatory system in *D. squalens*. A particularly high number of
442 genes were specifically expressed at the later time point on spruce, including a GH7 CBHI, an AA9
443 LPMO and a GH51 arabinofuranosidase. The genes specifically expressed on (one of the) non-
444 woody substrates include an AA2 MnP, a CE12 rhamnogalacturonan acetyl esterase and a GH10
445 XLN. While many transcriptional regulators of genes encoding plant-biomass-degrading enzymes
446 have been described in ascomycetes (Kowalczyk *et al.*, 2014), most of these lack orthologs in
447 basidiomycetes (Todd *et al.*, 2014). This suggests that basidiomycetes possess a separately evolved
448 regulatory systems for plant biomass degradation, which based on the results presented in this study
449 appears to be equally complex as the ascomycete system.
450
451 Our study indicates that *D. squalens* expressed and secreted a variable and efficient enzymatic
452 repertoire for conversion of softwood, hardwood and non-woody plant-derived biomasses. These
453 enzyme sets corresponded well with the composition of wood substrates, especially regarding
454 lignin, but also with the non-woody substrates. *D. squalens* therefore has a much less obvious
455 preference for the polymers available in its natural habitat than has been observed in ascomycetes
456 and should in fact be able to also live in non-woody substrates, based on its ability to use different
457 carbohydrates. Restriction of *D. squalens* to wood is therefore likely the result of other factors, such
458 as competition with other fungi. Ability to degrade lignin provides *D. squalens* with a competitive
459 edge in wood over other (not wood-rotting) fungi, which does not benefit its survival in non-woody
460 habitats. The dominant role of lignin on the expression of genes encoding plant biomass active
461 enzymes is very well consistent with the white-rot lifestyle of *D. squalens* and would suggest that
462 this is its main mechanisms to tackle the variation in composition of different wood types.
463
464 **Experimental Procedures**

465 ***Fungal strains and cultures***

466 *Dichomitus squalens* FBCC312 was obtained from the Fungal Biotechnology Culture Collection
467 (FBCC), Department of Food and Environmental Sciences, University of Helsinki, and maintained
468 on 2% (w/v) malt extract agar (MEA) plates (Biokar, France). The fungus was cultivated for 4 days
469 at 28ºC in 75 ml liquid low-nitrogen-asparagine-succinate medium (LN-AS) (pH 4.5) (Hatakka and
470 Uusi-Rauva, 1983) supplemented with 6.8 mM glycerol in 250 ml Erlenmeyer flasks inoculated with
471 five mycelium-covered plugs (diameter 7 mm) from MEA plates. These cultures were homogenized
472 with a Waring Blender (USA) and four millilitres of homogenized mycelial suspension (Mäkelä *et*
473 *al.*, 2002) was used to inoculate 100 ml LN-AS medium containing 0.25% (w/v) Tween20 (Sigma
474 Aldrich, Germany) and supplemented with 1 g of spruce wood (*Picea abies*) sawdust (1-2 mm in
475 diameter), aspen wood (*Populus tremula*) sawdust (1-2 mm in diameter), powdered wheat bran
476 (Windkorenmolen De Vlijt, Wageningen, The Netherlands) or powdered cotton seed hulls (a gift
477 from Garold Gresham, Interfacial Chemistry Idaho National Laboratory, Idaho Falls, ID). The
478 agitated (120 rpm) cultivations were performed as two biological replicate cultures in 250 ml baffled
479 Erlenmeyer flasks and incubated for 9 and 16 days at 28°C. Materials and methods for pre-test
480 cultures and substrate composition analysis are provided in Supplementary material.

481

482 ***Transcriptome analysis***

483 Total RNA was extracted from mycelia originated from two biological replicate cultures that were
484 ground in liquid N_2 with the N-cetyl-N,N,N-trimethylammonium bromide (CTAB, Sigma,
485 Germany) method (Chang *et al.*, 1993) and purified with RNeasy Plant Mini kit (Qiagen,
486 Germany). RNA quantity and integrity were determined spectrophotometrically (NanoDrop ND-
487 1000, NanoDrop Technologies Inc., USA) and with RNA6000 Nano Assay (Agilent 2100
488 Bioanalyzer, Agilent Technologies, USA). Purification of mRNA, synthesis of cDNA library and
489 sequencing reactions were conducted in the BGI Tech Solutions Co., Ltd. (Hong Kong, China) as

490 described previously (Patyshakuliyeva *et al.*, 2015). On average, 51 bp sequenced reads were
491 constituted, producing approximately 500 MB raw yields for each sample.
492
493 RNA-seq data was analysed and statistically treated as described previously (Patyshakuliyeva *et al.*,
494 2015). Raw reads were produced by base calling from the original image data. After that, data
495 filtering was performed. Adaptor sequences, reads with unknown bases (N) >10% and low quality
496 reads (more than 50% of the bases with quality values < 5%) were removed. Clean reads were
497 mapped to the genome sequence of *D. squalens* LYAD-421 SS1 (v1.0 annotation, the Joint Genome
498 Institute (JGI)) using SOAPALIGNER/SOAP2 (Li *et al.*, 2009) with two mismatches allowed in
499 the alignment. In average, 80% of the clean reads mapped to the genome. RNA-seq results were
500 quantified using RPKM method (Mortazavi, *et al.*, 2008). Genes with RPKM value from 20-100
501 were considered as lowly, 100-300 as moderately and over 300 as highly expressed (approximately
502 top 4% of the genes). Genes with RPKM value lower than 20 under all conditions were considered
503 as not expressed and filtered out of the cluster analysis. Differential expression was identified by
504 CyberT BayesianANOVA algorithm (Kayala and Baldi, 2012). A cut-off of fold change of >1.5
505 and *P*-value (corrected by multiple test) of <0.05 were used to identify differentially expressed
506 genes between the time points. The RT-qPCR results verified the RNA-seq data by showing similar
507 trends of expression for the selected genes (Supplementary information, Table S1, Figs. S9, S10).
508 The RNA-seq data were deposited to the National Center for Biotechnology Information (NCBI)
509 Gene Expression Omnibus (GEO) database (Edgar *et al.*, 2002) with accession number: GSE79674.
510
511 The clustering of the CAZyme-encoding genes based on the RNA-seq data was performed using
512 Genesis (Sturn *et al.*, 2002) with the Euclidean distance and complete linkage, using the expression
513 levels as the entry file.
514

515 ***Protein extraction and LC-MS/MS-based protein identification***

516 For exoproteome analysis, fungal culture liquid was filtered through Miracloth (Millipore, Billerica,
517 MA) and centrifuged at 4ºC for 15 min at 2500×g after which the supernatant was re-centrifuged for
518 1 h at 10000×g (SL16R-centrifuge, Thermo-Scientific, Waltham, MA). Proteins in the supernatant
519 were precipitated with 20% trichloroacetic acid, 20 mM dithiothreitol and 80% acetone solution for
520 1 h on ice. Precipitated proteins were resuspended and reprecipitated with 20 mM DTT and 80%
521 acetone solution overnight at -20ºC. Ten micrograms of protein resuspended in 0.25% Anionic Acid
522 Labile Surfactant I (AALS I, ProgentaTM, Protea Biosciences, Inc., USA) detergent prepared in 200
523 mM ammonium bicarbonate buffer (pH 7.8) was loaded and separated on 12% SDS-PAGE gels for
524 subsequent in-gel trypsin digestion as previously described (Mahajan *et al.*, 2016). Protein separation
525 was stopped as soon as the 250 kDa marker could be seen entering the separating gel. The complete
526 lane, delimited by the bromophenol blue front at the bottom and the start of the separating gel at the
527 top (about 1.5 cm), was cut and processed for in-gel digestion as a whole.

528

529 In-gel digested peptide extracts from two biological replicate cultures were analysed on LTQ-
530 Velos-Orbitrap mass spectrometer (Thermo-Scientific, San Jose, CA) and the acquired MS-MS data
531 were searched against the *D. squalens* database from JGI containing 12290 protein sequences for
532 peptide/protein identification as previously reported (Ozturkoglu Budak *et al.*, 2014). Total
533 normalized ion profiles from the top three identified peptides for each protein as determined in
534 Scaffold (Proteome Software, Portland, OR) was used to quantify relative levels of secreted
535 proteins. The amount of protein was defined as percentages of total identified secreted proteins
536 within individual samples.

537

538 For analysis, secreted proteins representing higher than 0.02% of the total exoproteome were
539 considered as significantly produced and lower than that were removed. Proteins accumulated to

540 0.02-1% level of total exoproteome were considered low and 1-5% moderate while those
541 accumulated >5% were deemed high.
542
543 **Conflict of Interest**
544 Authors confirm that there are no competing interests.
545
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738 **Table and Figure Legends**

739 **Table 1.** List of abbreviations of selected *D. squalens* plant-biomass-degrading enzymes and
740 corresponding CAZy families.

741 **Fig. 1.** Total number of CAZy (A) transcripts and (B) extracellular proteins detected from *D.*
742 *squalens* cultivations on spruce wood, aspen wood, wheat bran and cotton seed hulls. CAZy
743 transcripts and proteins related to plant cell wall degradation are divided by their corresponding
744 substrates. PCWDE, plant cell wall-degrading enzymes; Xylan, heteroxylan; Man, heteromannan;
745 Xg, xyloglucan S, starch; AA, auxiliary activities; GH, glycoside hydrolases; CE, carbohydrate
746 esterases.

747 **Fig. 2.** Total amount of plant cell wall degradation-related (A) transcripts (RPKM>20) and (B)
748 extracellular proteins (total>0.02%) from the plant biomass cultures of *D. squalens*. Spr, spruce
749 wood; Asp, aspen wood, WB, wheat bran; CSH, cotton seed hulls.

750 **Fig. 3.** Cluster analysis of expression profiles of CAZyme- and expansin-like protein encoding
751 genes in spruce wood (Spr), aspen wood (Asp), wheat bran (WB) and cotton seed hull (CSH)
752 cultures. RPKM value ≥300 was considered as highly expressed and RPKM value <20 was not
753 included in to the cluster analysis (black). Gene abbreviations are presented in Table 1. NA; CAZy
754 classification not available.

755 **Fig. 4.** Transcripts and extracellular proteins divided by their predicted function towards (A) lignin,
756 and (B) heteroxylan and xyloglucan, and (C) heteromannan from spruce wood (Spr, green circles),
757 aspen wood (Asp, blue circles), wheat bran (WB, orange circles) and cotton seed hull (CSH, grey
758 circles) cultures of *D. squalens* after nine and 16 days of cultivation. Transcripts and extracellular
759 proteins, which putatively function in H_2O_2-supply and degradation of cellulose, pectin and starch,
760 as well as expansin-like proteins are provided in Supplementary material. Difference in gene
761 expression is significant (fold change>1.5, p<0.05) if the size of the circles corresponding to one

762 gene varies; otherwise significant upregulation is marked with + or no change with =. ID numbers

763 for the gene and predicted protein model are according to the Protein ID numbers of JGI. LCC,

764 laccase. Other enzyme abbreviations are presented in Table 1. Circles were drawn with iTol

765 (Letunic and Bork, 2011).