





Induction of Genes Encoding Plant Cell Wall-Degrading Carbohydrate-Active Enzymes by Lignocellulose-Derived Monosaccharides and Cellobiose in the White-Rot Fungus *Dichomitus squalens*

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ABSTRACT Fungi can decompose plant biomass into small oligo- and monosaccharides to be used as carbon sources. Some of these small molecules may induce metabolic pathways and the production of extracellular enzymes targeted for degradation of plant cell wall polymers. Despite extensive studies in ascomycete fungi, little is known about the nature of inducers for the lignocellulolytic systems of basidiomycetes. In this study, we analyzed six sugars known to induce the expression of lignocellulolytic genes in ascomycetes for their role as inducers in the basidiomycete white-rot fungus *Dichomitus squalens* using a transcriptomic approach. This identified cellobiose and L-rhamnose as the main inducers of cellulolytic and pectinolytic genes, respectively, of *D. squalens*. Our results also identified differences in gene expression patterns between dikaryotic and monokaryotic strains of *D. squalens* cultivated on plant biomass-derived monosaccharides and the disaccharide cellobiose. This suggests that despite conservation of the induction between these two genetic forms of *D. squalens*, the fine-tuning in the gene regulation of lignocellulose conversion is differently organized in these strains.

IMPORTANCE Wood-decomposing basidiomycete fungi have a major role in the global carbon cycle and are promising candidates for lignocellulosic biorefinery applications. However, information on which components trigger enzyme production is currently lacking, which is crucial for the efficient use of these fungi in biotechnology. In this study, transcriptomes of the white-rot fungus *Dichomitus squalens* from plant biomass-derived monosaccharide and cellobiose cultures were studied to identify compounds that induce the expression of genes involved in plant biomass degradation.

KEYWORDS basidiomycetes, cellobiose, plant biomass degradation, regulation, rhamnose

Plant biomass is an emerging source of fuels and chemicals, since it is the most abundant renewable organic material on earth (1, 2). However, its depolymerization is usually required before it can be used in biotechnological applications. White-rot fungi are a group of basidiomycetes whose natural substrate is wood (3); therefore, they produce a set of enzymes that are ideally suited for the depolymerization of components present in wood.

Wood cell walls consist mainly of four different polymers, cellulose, hemicellulose, pectin, and lignin, with cellulose being the major structural component (4). All of these polymers form a complex and compact lignocellulose network. White-rot fungi have the unique ability to efficiently degrade all the polymeric components of the plant cell walls, including the recalcitrant aromatic lignin. This makes them attractive candidates for various applications in the biobased economy (5). The polysaccharide fraction of

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plant biomass, which comprises cellulose, hemicellulose, and pectin, is composed of several different monomeric sugars. While cellulose consists solely of D-glucose, hemicelluloses (xylan, xyloglucan, or mannan) and pectin consist of various sugar monomers, such as D-glucose, D-galactose, D-mannose, D-xylose, L-arabinose, L-rhamnose, D-galacturonic acid, D-glucuronic acid, and L-fucose (6).

Fungi produce a broad range of extracellular enzymes to facilitate the depolymerization of plant biomass (7), most of which are classified in the Carbohydrate-Active enZymes (CAZy) database (www.cazy.org) (8). The CAZy database divides the enzymes and associated modules into families and subfamilies and provides genomic, structural, and biochemical information on them. The action of carbohydrate-active enzymes (CAZymes) results in the formation of monomeric and small oligomeric components that are taken up into the fungal cells and catabolized for growth and reproduction.

The expression of the genes encoding plant biomass-degrading enzymes is tightly controlled to ensure efficient utilization of the carbon and energy sources available in fungal habitats (9). Production of the most suitable set of enzymes is achieved by inducing or repressing regulatory systems. Monosaccharides or oligosaccharides from plant biomass can trigger signaling pathways resulting in the activation of a transcriptional regulator. Several transcriptional activators and repressors related to lignocellulose degradation have been characterized from ascomycete fungi (10, 11); however, except for Ace3 and Cre1, none of these have orthologs in basidiomycete fungi (12). Interestingly, the sets of genes that are expressed in response to specific plant biomass substrates appear to be highly similar between ascomycetes and basidiomycetes, despite the absence of orthologous regulators (13, 14). This similarity in the expression profiles of the CAZyme-encoding genes together with the absence of orthologous regulators suggests parallel evolution of corresponding regulatory systems in these two fungal phyla (14).

Uncovering the regulatory systems driving wood degradation in the white-rot basidiomycetes is crucial to understanding the abilities of this group of fungi and to fully exploit them in biotechnological applications. A first step is the identification of the low-molecular-weight inducers of these regulators. In this study, the white-rot fungus *Dichomitus squalens* was cultivated on six monosaccharides derived from cellulose, hemicellulose, and pectin, as well as on the disaccharide cellobiose. Transcriptomics was used to identify which of these compounds induce the expression of genes encoding plant cell wall polymer-degrading enzymes. In nature, the predominant form of *D. squalens* is dikaryotic, but colonization of wood initiates from a spore that forms monokaryotic mycelium until it encounters a compatible mate. Previously, mono- and dikaryotic strains of *D. squalens* were suggested to have highly diverse abilities to degrade plant biomass (15). To evaluate if the diversity between these forms is due to regulatory-level differences, comparative transcriptomics of a monokaryotic progeny of the dikaryotic *D. squalens* strain was performed on the three carbon sources that were observed to induce plant biomass-degrading enzyme-encoding genes in the dikaryon.

RESULTS

Transcriptome analysis reveals cellobiose and L-rhamnose to be the main inducers of CAZyme-encoding genes in a *D. squalens* dikaryon. The dikaryotic and monokaryotic strains of *D. squalens* showed varied morphologies after 5 days of growth on the main plant polysaccharide-derived monomers and cellobiose (Fig. 1) (15). Both strains grew well on D-glucose, D-galactose, D-mannose, D-xylose, L-arabinose, and L-rhamnose. While the mycelium of the dikaryon FBCC312 fully covered those plates, the radial growth of the monokaryon CBS 464.89 was overall slower, except on L-rhamnose, and it formed a less dense mycelium. Compared to the dikaryon, the colony diameter of the monokaryon was smaller also on cellobiose, but the mycelium was denser, thus possibly indicating a better ability of CBS 464.89 to convert this carbon source. In contrast, compared to the monokaryon CBS 464.89, growth of the dikaryon FBCC312 was slow on D-galacturonic acid and especially poor on D-glucuronic acid. Most possibly due to the poor growth, extraction of high-quality RNA was not successful from these cultures, which were therefore omitted from the RNA sequencing (RNA-seq) analysis.

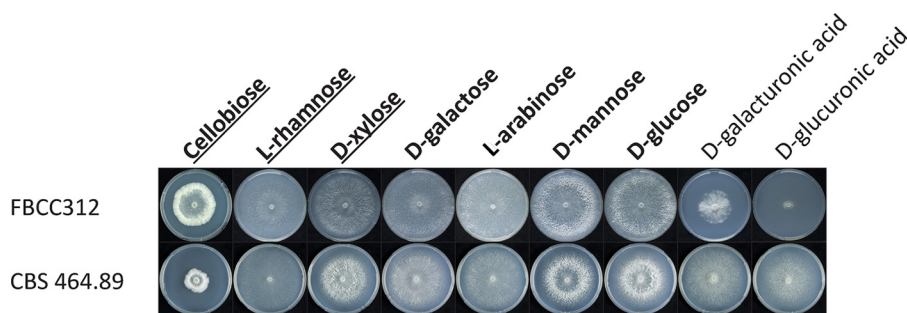


FIG 1 Growth profiles of *D. squalens* dikaryon FBCC312 and monokaryon CBS 464.89 on different sugars that were considered potential inducers of plant cell wall-degrading CAZymes, based on studies in ascomycetes (12). The plate cultures that were subsequently used for transcriptomics in FBCC312 are in bold, and those for CBS 464.89 are in bold and underlined.

When the putative plant biomass-degrading CAZyme-encoding genes induced by the different sugars in *D. squalens* FBCC312 were compared to D-glucose (fold change, ≥ 2.5 ; $P \leq 0.01$), 83 significantly upregulated genes were detected among all the sugars (Fig. 2; see also Table S1A to F in the supplemental material). Of these, 26 genes were unique to L-rhamnose and 27 genes were unique to cellobiose. In contrast, 28 genes were shared at least in two different sugars. On L-arabinose, D-xylose, L-rhamnose, D-galactose, and D-mannose, a smaller number of upregulated genes were present (Fig. 2). On one of the two pentoses studied, L-arabinose, 18 genes were upregulated, and all of them were also present in some of the other tested sugars. Similarly, none of the 13 genes upregulated in D-galactose were unique to this sugar. The growth of *D. squalens* on D-xylose, the other pentose studied, resulted in 16 upregulated CAZyme genes, with only one gene unique to this monosaccharide. On the hexose D-mannose, only three genes were upregulated, and two of them were also found on D-xylose or L-rhamnose. No upregulated CAZyme genes common to all the sugars were detected (Fig. 2). Downregulated genes were not analyzed.

The proportion of the induced genes encoding CAZymes putatively acting on different polymeric components of plant biomass was determined from the transcriptome data (Fig. 2 and Tables 1 to 3). Cellobiose induced 34 CAZyme genes, of which most (68%) encode enzymes that putatively act on cellulose (Tables 1 and S1A and Fig. S1A). CAZyme family glycoside hydrolase 6 (GH6) and GH7 cellobiohydrolase-encoding genes were highly expressed in the presence of cellulose, with *cel7c* (JGI MycoCosm protein identification [ID] 944872, https://genome.jgi.doe.gov/Dicsqu464_1/Dicsqu464_1.home.html) showing 27-fold higher expression than D-glucose (Tables 1 and S1A). In contrast, the only highly expressed cellulolytic genes in the presence of other sugars were cellobiohydrolase genes *cel6a* (protein ID 803735) on L-arabinose (Fig. 2 and Table S1A and C) and *cel7a* (protein ID 918783) on L-rhamnose, D-xylose (Tables 1 to 3), and L-arabinose (Fig. 2 and Table S1). On L-rhamnose, 38% of the 49 induced genes encoded enzymes putatively involved in pectin degradation (Tables 2 and S1B and Fig. S1B). The number of genes with increased expression on D-xylose was lower (16), and thus, its role as an inducer was less evident than with cellobiose and L-rhamnose. However, 25% of the CAZyme genes induced on D-xylose were related to xylan degradation (Tables 3 and S1D and Fig. S1C). Lignin degradation-related CAZyme genes were induced on L-rhamnose (27%) and D-xylose (25%) cultures of *D. squalens* FBCC312 but not on cellobiose (Fig. 2 and Tables 1 to 3).

L-Rhamnose resulted in significant differences in gene expression of mono- and dikaryotic strains of *D. squalens*. To compare possible regulatory differences in plant cell wall degradation between mono- and dikaryotic strains of *D. squalens*, the *D. squalens* monokaryon CBS 464.89 was cultivated on cellobiose, L-rhamnose, and D-xylose. When the transcriptomes from the three sugars were analyzed, a considerably smaller amount of highly expressed (fold change, ≥ 2.5 ; $P \leq 0.01$; fragments per

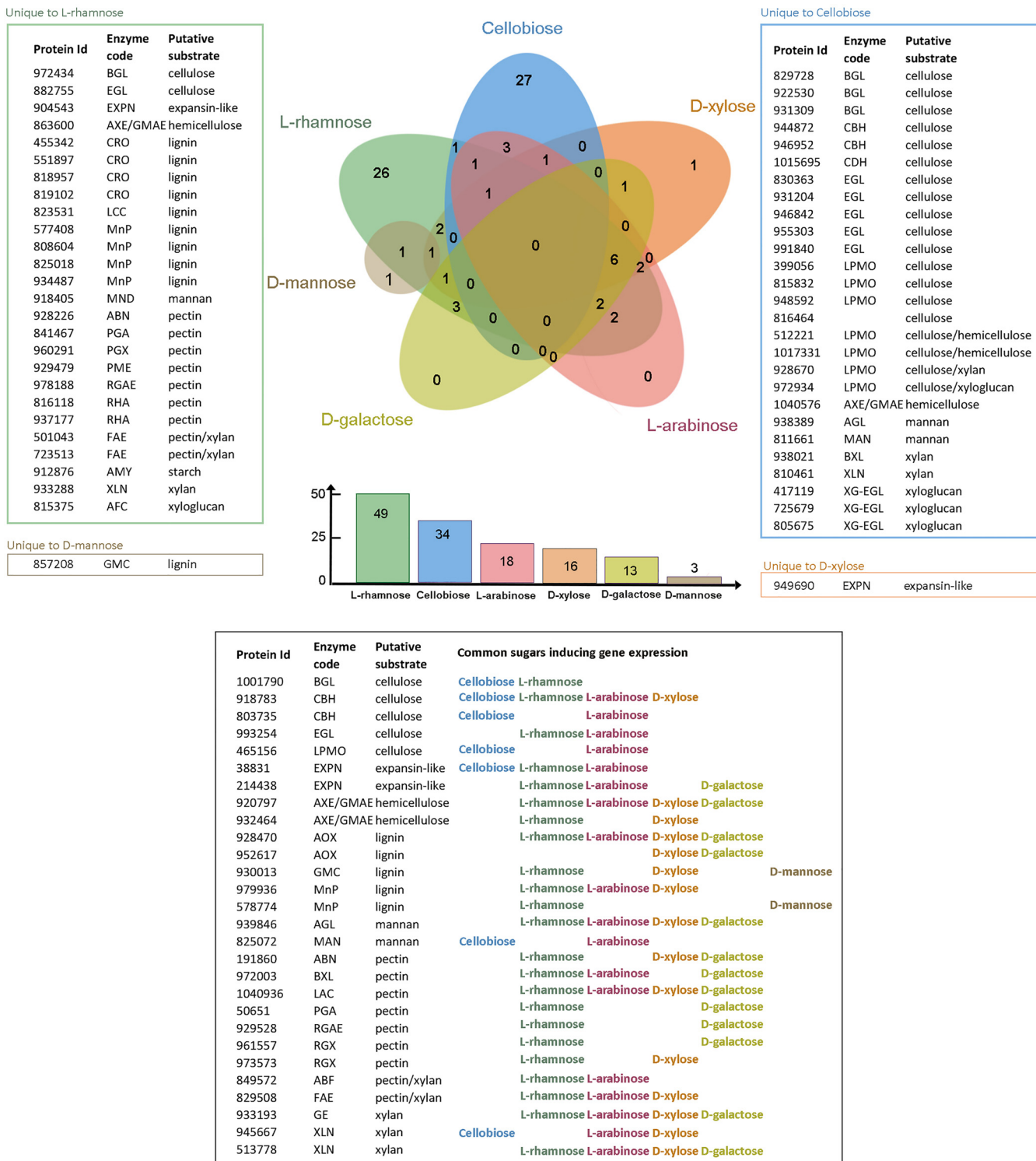


FIG 2 Comparison of the number of the plant cell wall-degrading CAZyme genes induced by the tested sugars in *D. squalens* FBCC312. The total number of induced CAZyme genes by each sugar is listed in the bar graph below the Venn diagram. CAZyme genes considered to be induced had a fold change value of ≥ 2.5 compared to D-glucose, a *P* value of ≤ 0.01 , and an FPKM value of > 10 . See Fig. S1 for an explanation of the enzyme codes.

kilobase per million [FPKM], ≥ 10) CAZyme genes was detected in D-xylose (7–11) (Table S2) than in cellobiose (21) and L-rhamnose (45) (Tables S3B and S4B); therefore, the D-xylose transcriptome was excluded from further analyses. When the putative plant biomass-degrading CAZyme-encoding genes induced by cellobiose (FPKM, > 10) were

TABLE 1 Expression of genes encoding putative plant biomass polysaccharide-degrading enzymes during the growth of *D. squalens* dikaryon FBCC312 in the presence of cellobiose compared to D-glucose^a

Protein ID	Enzyme code	Gene name	Fold change over D-glucose	P value over D-glucose	FPKM cellobiose	Putative substrate	CAZyme family ^b
829728	BGL		18.7	1.32E-75	229.2	Cellulose	GH1
1001790	BGL		7.2	5.90E-30	38.5	Cellulose	CBM1-GH3
922530	BGL		3.5	1.79E-15	44.3	Cellulose	GH3
931309	BGL		3.0	4.16E-20	96.5	Cellulose	GH3
944872	CBH	<i>cel7c</i>	27.3	3.19E-88	969.9	Cellulose	GH7
918783	CBH	<i>cel7a</i>	14.6	1.83E-19	203.0	Cellulose	GH7
803735	CBH	<i>cel6a</i>	9.6	7.73E-46	451.4	Cellulose	CBM1-GH6
946952	CBH	<i>cel7b</i>	8.6	3.96E-11	252.4	Cellulose	GH7
1015695	CDH		12.4	6.64E-47	107.7	Cellulose	AA8-AA3_1
931204	EGL		19.7	7.98E-44	182.3	Cellulose	GH131-CBM1
830363	EGL		18.1	6.75E-69	199.8	Cellulose	CBM1-GH5_5
955303	EGL		9.6	1.09E-63	426.1	Cellulose	CBM1-GH5_5
946842	EGL		8.2	5.82E-23	52.8	Cellulose	CBM1-GH5_5
991840	EGL		5.0	3.33E-12	140.6	Cellulose	GH45
399056	LPMO	<i>lpmo2</i>	20.3	1.86E-56	417.2	Cellulose	AA9
815832	LPMO		14.3	5.92E-34	99.1	Cellulose	AA9-CBM1
948592	LPMO		14.2	1.73E-45	269.7	Cellulose	AA9-CBM1
465156	LPMO		9.1	1.09E-33	340.1	Cellulose	AA9
816464			23.8	3.88E-40	160.6	Cellulose	CBM1
1017331	LPMO		20.7	8.23E-52	280.7	Cellulose/hemicellulose	AA9
512221	LPMO		18.3	3.22E-54	417.8	Cellulose/hemicellulose	AA9
928670	LPMO		12.0	6.85E-26	195.7	Cellulose/xylan	AA9
972934	LPMO		2.8	0.003	17.6	Cellulose/xyloglucan	AA9
38831	EXPN		2.8	0.0001	24.5	Expansin-like	NA
1040576	AXE/GMAE	16.2	1.08E-33	79.1	Hemicellulose	CBM1-CE16	
938389	AGL		3.4	5.43E-13	109.6	Mannan	GH27
825072	MAN		3.5	8.65E-18	82.4	Mannan	CBM1-GH5_7
811661	MAN		2.8	1.99E-11	134.1	Mannan	GH5_7
938021	BXL		7.0	4.32E-22	101.1	Xylan	GH5_22
945667	XLN		9.7	9.97E-34	82.2	Xylan	CBM1-GH10
810461	XLN		6.4	7.48E-07	11.5	Xylan	CBM1-GH10
725679	XG-EGL		36.0	7.12E-53	200.8	Xyloglucan	GH12
805675	XG-EGL		13.8	5.31E-48	243.5	Xyloglucan	GH74-CBM1
417119	XG-EGL		3.8	8.38E-07	67.7	Xyloglucan	GH12

^aOnly genes for which expression was at least 2.5-fold higher than on D-glucose with a *P* value of ≤ 0.01 and an FPKM value of > 10 are included.

^bNA, not applicable.

compared to those induced by L-rhamnose (fold change, ≥ 2.5 ; $P \leq 0.01$) in the two *D. squalens* strains, 20 genes were highly expressed in the dikaryon, 11 (55%) of which are related to cellulose degradation (Table S3A). In the monokaryon, the number of genes (17) induced by cellobiose and the proportion of the genes putatively targeted for cellulose degradation were similar (12 [57%]), but 1/3 of these were different genes in the two strains (Fig. 3 and Table S3B). Overall, when the CAZyme gene expression of the mono- and dikaryotic strains in the presence of cellobiose was compared to that in the presence of L-rhamnose, no large differences were detected in the number of highly expressed genes targeting different putative plant cell wall compounds (Fig. 3).

When the putative plant biomass-degrading CAZyme-encoding genes of *D. squalens* induced by L-rhamnose (FPKM, > 10) were compared to those induced by cellobiose (fold change, ≤ 2.5 ; $P \leq 0.01$), the differences between the mono- and dikaryotic strains were evident. The number of expressed genes was higher in the dikaryon (55) than in the monokaryon (45) (Fig. 4 and Table S4). Also, the proportion of the induced pectinolytic genes was higher in the monokaryon (44%) than in the dikaryon (25%) (Fig. 4 and Table S4). Nearly all pectinolytic genes upregulated in the dikaryon were also upregulated in the monokaryon, indicating that in the monokaryon, an additional set of genes responds to L-rhamnose. Interestingly, the proportion of the lignin degradation-related genes varied notably between the strains, as 31% and 18% of the induced genes in the dikaryon and the monokaryon, respectively, were putatively

TABLE 2 Expression of genes encoding putative plant biomass polysaccharide-degrading enzymes during the growth of *D. squalens* dikaryon FBCC312 on L-rhamnose compared to D-glucose^a

Protein ID	Enzyme code	Gene name	Fold change over D-glucose	P value over D-glucose	FPKM L-rhamnose	Putative substrate	CAZyme family
972434	BGL		2.7	5.64E-09	36.4	Cellulose	GH3
1001790	BGL		3.7	5.41E-13	18.1	Cellulose	CBM1-GH3
918783	CBH	<i>cel7a</i>	3.3	1.66E-04	33.5	Cellulose	GH7
882755	EGL		7.5	3.31E-68	714.4	Cellulose	GH45
993254	EGL		3.4	7.38E-05	21.6	Cellulose	GH45
214438	EXPN		2.7	5.02E-13	543.1	Expansin-like	
904543	EXPN		4.0	1.56E-06	130.5	Expansin-like	
38831	EXPN		2.8	1.44E-04	22.4	Expansin-like	
920797	AXE/GMAE		3.5	1.67E-11	93.9	Hemicellulose	CE16
932464	AXE/GMAE		2.6	1.64E-07	69.8	Hemicellulose	CE16
863600	AXE/GMAE		3.6	1.05E-04	15.5	Hemicellulose	CE16
818957	CRO		22.6	2.94E-65	567.6	Lignin	AA5_1
455342	CRO		11.0	1.85E-49	432.9	Lignin	AA5_1
819102	CRO		5.9	1.18E-19	186.2	Lignin	AA5_1
551897	CRO		40.8	5.43E-63	153.0	Lignin	AA5_1
930013	GMC		3.3	3.20E-06	20.1	Lignin	AA3_2
823531	LCC	<i>lcc8</i>	2.6	2.79E-05	19.9	Lignin	AA1_1
979936	MnP	<i>mnp7_short</i>	76.4	2.19E-153	3,594.2	Lignin	AA2
808604	MnP	<i>mnp4_extralong</i>	7.2	4.16E-14	2,539.5	Lignin	AA2
825018	MnP	<i>mnp5_short</i>	6.6	1.04E-10	2,001.9	Lignin	AA2
578774	MnP	<i>mnp2_extralong</i>	2.8	6.53E-03	560.0	Lignin	AA2
577408	MnP	<i>mnp3_extralong</i>	7.2	2.31E-26	153.4	Lignin	AA2
934487	MnP	<i>mnp1_extralong</i>	2.7	1.19E-03	13.9	Lignin	AA2
928470	AOX		6.2	1.09E-65	4,376.5	Lignin	AA3_3
939846	AGL		7.2	7.47E-40	448.6	Mannan	GH27
918405	MND		3.3	4.75E-08	14.2	Mannan	GH2
191860	ABN		6.0	6.28E-34	744.9	Pectin	GH43
972003	BXL		7.6	2.06E-41	142.4	Pectin	GH43-CBM35
928226	ABN		2.5	1.53E-03	16.5	Pectin	GH43
1040936	LAC		11.1	4.39E-104	1,101.3	Pectin	GH35
841467	PGA		4.0	2.30E-07	49.1	Pectin	GH28
960291	PGX		3.7	1.68E-09	59.3	Pectin	GH28
929479	PME		2.8	7.92E-08	50.6	Pectin	CE8
978188	RGAE		2.7	7.88E-04	26.2	Pectin	CE12
929528	RGAE		3.5	1.66E-04	21.7	Pectin	CE12
961557	RGX		3.7	5.27E-13	89.4	Pectin	GH28
973573	RGX		4.0	6.67E-10	27.6	Pectin	GH28
937177	RHA		2.9	5.64E-08	23.4	Pectin	GH78
816118	RHA		3.1	2.31E-08	21.5	Pectin	GH78
50651	PGA		2.9	2.11E-06	39.6	Pectin	GH28
849572	ABF		2.6	1.78E-10	75.9	Pectin/xylan	GH51
501043	FAE		3.1	1.59E-16	211.5	Pectin/xylan	
723513	FAE		3.2	1.82E-18	208.0	Pectin/xylan	
829508	FAE		4.5	2.20E-34	152.1	Pectin/xylan	
912876	AMY		3.0	1.16E-23	296.9	Starch	GH13_1
933193	GE		20.4	8.94E-50	94.5	Xylan	CBM1-CE15
513778	XLN		31.9	2.07E-66	161.1	Xylan	CBM1-GH10
933288	XLN		14.4	2.35E-17	33.3	Xylan	GH10
815375	AFC		2.5	2.04E-17	275.5	Xyloglucan	GH95

^aOnly genes for which expression was at least 2.5-fold higher than on D-glucose with a *P* value of ≤ 0.01 and an FPKM value of > 10 are included.

involved in lignin modification (Fig. 4). Here, the opposite pattern was observed from that for the pectinolytic genes, as all induced genes in the monokaryon were also induced in the dikaryon, suggesting that in the dikaryon, an additional set of ligninolytic genes responds to L-rhamnose.

The largest difference in gene expression between the mono- and dikaryon was observed on L-rhamnose, since despite the similar number of genes induced by L-rhamnose and by cellobiose, only 36 of those genes were common in the two strains. From the nine genes unique to the monokaryon, seven (78%) genes were related to pectin degradation, whereas almost half (47%) of the 19 unique dikaryotic genes were related to lignin degradation (Fig. 4 and Table S4).

TABLE 3 Expression of genes encoding putative plant biomass polysaccharide-degrading enzymes during growth of *D. squalens* dikaryon FBCC312 on D-xylose compared to D-glucose^a

Protein ID	Enzyme code	Gene name	Fold change over D-glucose	P value over D-glucose	FPKM L-rhamnose	Putative substrate	CAZyme family
918783	CBH	<i>cel7a</i>	4.0	3.1E-05	44.555	Cellulose	GH7
949690	EXPN		3.5	9.3E-04	21.47	Expansin-like	EXPN
920797	AXE/GMAE		2.6	2.2E-06	72.855	Hemicellulose	CE16
932464	AXE/GMAE		2.5	2.5E-06	70.8	Hemicellulose	CE16
930013	GMC		4.2	5.6E-08	27.8	Lignin	AA3_2
979936	MnP	<i>mp7_short</i>	4.0	5.5E-15	177.31	Lignin	AA2
952617	AOX		2.8	2.1E-08	346.085	Lignin	AA3_3
928470	AOX		2.8	2.5E-20	2,079.17	Lignin	AA3_3
939846	AGL		3.4	7.3E-15	221.745	Mannan	GH27
1040936	LAC		3.2	5.7E-24	335.375	Pectin	GH35
973573	RGX		2.5	2.6E-04	17.97	Pectin	GH28
191860	ABN		3.1	1.8E-13	409.23	Pectin	GH43
829508	FAE		3.0	2.0E-17	107.605	Pectin/xylan	
933193	GE		4.2	2.4E-10	19.25	Xylan	CBM1-CE15
513778	XLN		5.5	7.2E-15	27.48	Xylan	CBM1-GH10
945667	XLN		2.7	4.1E-06	22.06	Xylan	CBM1-GH10

^aOnly genes for which expression was at least 2.5-fold higher than on D-glucose with a P value of ≤0.01 and an FPKM value of >10 are included.

DISCUSSION

Gene regulation has been extensively studied in fungi, especially in the representatives from the phylum Ascomycota (10, 11). In contrast, the understanding of gene regulation in basidiomycetes is less extensive, largely due to the lack of characterized regulators and complications with gene transformation systems in these fungi. With the recent development of a transformation system (18), the white-rot fungus *D. squalens* has become an interesting species to study the regulation of plant biomass degradation in basidiomycetes. This species can degrade both hardwood and softwood (3), and several dikaryotic and related monokaryotic strains are available (15), as well as genome sequences for four monokaryotic strains (19).

In response to different conditions, fungi have the ability to up- or downregulate the expression of specific genes in order to adapt to their environment. Since the two major fungal phyla, Ascomycota and Basidiomycota, separated 500 million years ago (20), we may expect different responses in their gene expression. In addition, that dichotomy is possibly a reason for the scarcity of orthologous transcriptional regulators between

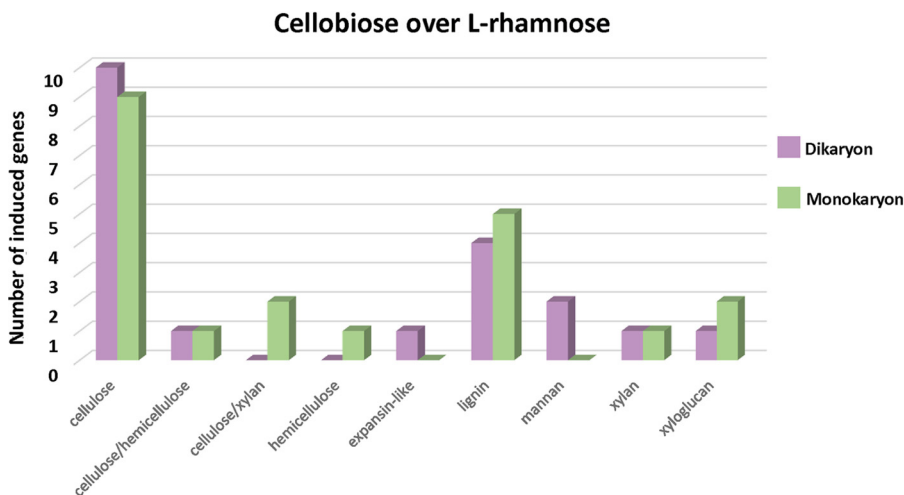
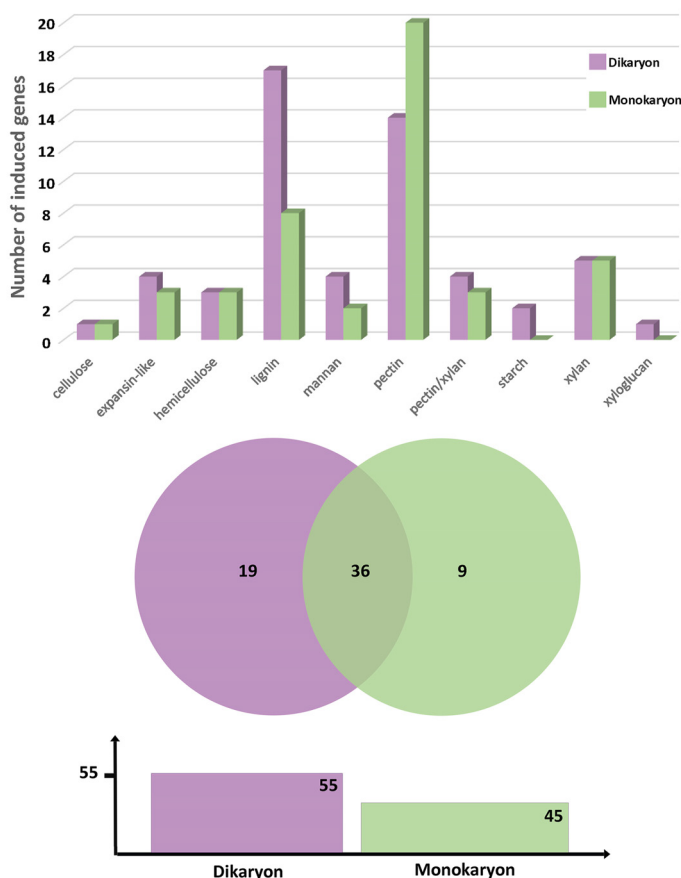


FIG 3 Plant biomass degradation-related CAZyme genes induced by cellobiose in comparison to L-rhamnose in the *D. squalens* dikaryon FBCC312 and monokaryon CBS 464.89. CAZyme genes considered to be induced by cellobiose were expressed at a 2.5-fold higher level ($P \leq 0.01$) than on L-rhamnose with an FPKM value of >10.

Common genes

Protein Id	Enzyme code	Putative substrate
882755	EGL	cellulose
803581	EXPN	expansin-like
822418	EXPN	expansin-like
904543	EXPN	expansin-like
863600	AXE/GMAE	hemicellulose
920797	AXE/GMAE	hemicellulose
928470	AOX	lignin
952617	AOX	lignin
455342	CRO	lignin
551897	CRO	lignin
819102	CRO	lignin
808604	MnP	lignin
825018	MnP	lignin
979936	MnP	lignin
939846	AGL	mannan
981883	MND	mannan
191860	ABN	pectin
313068	ABN	pectin
17723	ABX	pectin
972003	BXL	pectin
1040936	LAC	pectin
50651	PGA	pectin
841467	PGA	pectin
960291	PGX	pectin
929479	PME	pectin
978188	RGAE	pectin
961557	RGX	pectin
973573	RGX	pectin
937177	RHA	pectin
849572	ABF	pectin/xylan
723513	FAE	pectin/xylan
829508	FAE	pectin/xylan
1043122	BGL	xylan
933193	GE	xylan
513778	XLN	xylan
933288	XLN	xylan

L-rhamnose over cellobiose



Unique to monokaryon

Protein Id	Enzyme code	Putative substrate
920795	AXE/GMAE	hemicellulose
928226	ABN	pectin
856119	LAC	pectin
944781	PME	pectin
915707	PME	pectin
929528	RGAE	pectin
914697	RHG	pectin
962448	URGH	pectin
930449	AXE/FAE	xylan

Unique to dikaryon

Protein Id	Enzyme code	Putative substrate
214438	EXPN	expansin-like
932464	AXE/GMAE	hemicellulose
818957	CRO	lignin
919297	GMC	lignin
932354	GMC	lignin
163347	GMC	lignin
958517	GMC	lignin
930013	GMC	lignin
857208	GMC	lignin
578774	MnP	lignin
577408	MnP	lignin
804815	AGL	mannan
918405	MND	mannan
816118	RHA	pectin
197845	FAE	pectin/xylan
843398		starch
912876	AMY	starch
937833	GE	xylan
815375	AFC	xyloglucan

FIG 4 Common and strain-specific plant cell wall degradation-related CAZyme genes of *D. squalens* dikaryon FBCC312 (purple) and monokaryon CBS 464.89 (green) induced by L-rhamnose in comparison to cellobiose. The genes considered to be induced by L-rhamnose were expressed at a 2.5-fold higher level ($P \leq 0.01$) than on cellobiose and an FPKM value of >10 . See Table S2 for an explanation of the enzyme codes.

ascomycetes and basidiomycetes (12, 14). The present study reveals detailed insights into the induction system of plant cell wall polymer degradation in *D. squalens*.

Similar to what has been observed in ascomycetes (21), genes encoding enzymes acting on a variety of plant polymers were upregulated in *D. squalens* when cultivated on cellobiose, L-rhamnose, and D-xylose. However, the distribution of these genes revealed a clear pattern for two of these compounds. On cellobiose, more than half of the upregulated genes encode enzymes acting on cellulose, while on L-rhamnose, more than 1/3 of the upregulated genes encoded enzymes acting on pectin. In contrast, on D-xylose, a more randomly distributed set of genes was upregulated, including a very small number of cellulolytic genes. At this point, it is not clear whether a specific inducer for (subsets of) hemicellulolytic genes exists in *D. squalens*.

Therefore, our results demonstrate that the dimer cellobiose is the primary inducer for cellulose degradation in *D. squalens*, while L-rhamnose is an inducer for pectin degradation. This role for cellobiose was previously suggested for the basidiomycete brown-rot fungus *Postia placenta*, where cellulases were induced by soluble sugars, especially cellobiose, after the repression of oxidoreductases (22).

In the well-studied white-rot fungus *Phanerochaete chrysosporium*, cellotriose and cellotetraose, but not cellobiose, were suggested to result in the strongest induction of cellulases (23). In light of the results of our study, we had a closer look at the data from Suzuki et al. (23) and do not think those results exclude a role for cellobiose as inducer. In that study, cellobiose is accumulating after 1 h cultivation of *P. chrysosporium* on cellotriose and cellotetraose. This is also the time point at which high induction of

the cellobiohydrolase-encoding genes *cel7C*, *cel7D*, *cel7F*, *celG*, and *cel6A* is observed on cellobiose and cellotetraose cultures (23), suggesting that, in fact, the accumulated cellobiose may have caused induction of these genes. Microcrystalline cellulose (Avicel) has been found to upregulate the expression of cellobiohydrolase-encoding genes in *D. squalens* FBCC312, with the strongest effect on the gene *cel7c* (JGI MycoCosm protein ID 944872) (24, 25). This observation is in agreement with our results determined for this particular gene in the cellobiose cultures of *D. squalens* FBCC312, indicating that the results from the Avicel cultures may in fact also reflect the induction by cellobiose released from this substrate. Cellobiose has also been proposed as an inducer for several, but not all, ascomycete species (12), suggesting that there possibly are some similarities in the signaling mechanism of ascomycete and basidiomycete fungi, even if no orthologs for ascomycete cellobiose-responsive regulators were identified in basidiomycete genomes.

Considering the broad range of pectinolytic genes that were upregulated in the presence of L-rhamnose, this sugar appears to be a main inducer for pectinolytic gene expression in *D. squalens*. An L-rhamnose-responsive pectinolytic regulator (RhaR) has been identified in the ascomycete fungus *Aspergillus niger* (26), but this regulator seems to have a narrower role in activating pectinolytic genes than our data suggest for *D. squalens* (27). Furthermore, *D. squalens* does not have an ortholog of RhaR, nor of the other two *A. niger* pectinolytic regulators GaaR and AraR (12), indicating significant differences in pectinolytic regulation in *D. squalens*. In *A. niger*, genes encoding pectinolytic enzymes have recently been shown to be induced mainly by D-galacturonic acid (27), while L-rhamnose and L-arabinose affect smaller sets of pectinolytic genes (28). Although we were not able to assess the role of D-galacturonic acid as a pectinolytic inducer in *D. squalens* due to the low quality of RNA extracted from those cultures, our results demonstrated L-rhamnose induction for a wide set of pectinolytic genes, especially from the glycoside hydrolase (GH) families. Considering the low percentage of pectin in wood (29), the development of an efficient regulatory system for pectin degradation in *D. squalens* may seem less important than that for cellulose degradation. However, it has been previously reported that pectin in wood is particularly present at high levels in bordered pits, which connect adjacent xylem parenchyma cells, and are often the point of entry for basidiomycete hyphae to the wood cell walls (30). Therefore, this polysaccharide may still be a significant target for white-rot fungi during the onset of wood colonization.

Differences in gene expression between the *D. squalens* mono- and dikaryon were observed especially on L-rhamnose. This effect was not limited to pectinolytic genes but was observed across all CAZyme genes. When both strains were exposed to L-rhamnose, the dikaryon showed higher upregulation for genes encoding lignin-modifying enzymes than did the monokaryon. The monokaryon instead showed higher upregulation for pectinolytic genes than the dikaryon. The proportion of induced ligninolytic genes during growth on L-rhamnose correlated with a previously reported comparison of enzymatic activities in mono- and dikaryotic strains of *D. squalens* (15). In addition, the dikaryon had a broader set of upregulated genes encoding CAZymes, like those active on starch and xyloglucan, which were not expressed in the monokaryon, as well as a larger number of genes involved in mannan degradation.

On cellobiose, the strains showed a similar pattern of CAZyme gene expression, both having a large proportion of cellulolytic genes upregulated but showing differences between individual genes. However, genes encoding mannanases and expansin-like proteins were induced only in the dikaryon. The broader induction of CAZyme-encoding genes in the dikaryon indicates that this form of the fungus has better ability to grow or utilize plant biomass in nature than the monokaryon. This was not only observed during growth on plant biomass substrates for *D. squalens* (15) but also for another white-rot species (*Pleurotus ostreatus*), a litter-degrading species (*Agaricus bisporus*), and a brown-rot species (*Serpula lacrymans*) (www.fung-growth.org).

The results of this study are an important step toward dissecting the regulatory network driving the expression of genes encoding plant biomass-degrading enzymes

in the white-rot fungus *D. squalens* and, possibly, other basidiomycetes. Together with the recent development of a transformation system for *D. squalens* (18), this species is a good candidate as a model system for the molecular biological analysis of wood decay by white-rot fungi.

MATERIALS AND METHODS

Fungal strains and cultivation. The dikaryotic *D. squalens* strain FBCC312 (the Fungal Biotechnology Culture Collection [FBCC], Department of Microbiology, University of Helsinki) and monokaryotic *D. squalens* strain CBS 464.89 (the CBS collection, Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands), which is a direct offspring of the dikaryon FBCC312, were maintained on 2% (wt/vol) malt extract–1.5% (wt/vol) agar (MEA) plates. The strains were grown on low-nitrogen asparagine-succinate (LN-AS) medium (16) with 1.5% (wt/vol) agar plates supplemented with main monosaccharides derived from plant biomass polysaccharides, i.e., 25 mM D-glucose, D-glucuronic acid, D-galacturonic acid, L-rhamnose, D-galactose, D-xylose, D-mannose, or L-arabinose or 25 mM disaccharide cellobiose. For growth profiling, an agar plug (diameter, 0.5 cm) from an MEA plate that was covered with fresh fungal mycelium was placed at the center of the LN-AS plate and incubated at 28°C. As the mycelium of the dikaryon FBCC312 reached the edge of the D-glucose-supplemented plate after 5 days, this was chosen as the incubation time for all the plates.

RNA isolation. Based on the growth profiling, six plant cell wall polysaccharide-derived monosaccharides and cellobiose were selected to be used as carbon sources to test the fungal response at the gene expression level. The dikaryon FBCC312 was cultivated as mentioned before on 25 mM D-glucose, L-rhamnose, D-galactose, D-xylose, D-mannose, L-arabinose, or cellobiose, but this time using a polycarbonate membrane (Maine Manufacturing, LLC) on the top of the agar plate to facilitate harvesting of the mycelium. The monokaryon CBS 464.89 was cultivated similarly, with L-rhamnose, D-xylose, and cellobiose as carbon sources. After 5 days of growth at 28°C, the most external mycelium from the edge of the colony (1.5 cm) of each plate was harvested and transferred to 2-ml Eppendorf tubes containing two carbon steel balls (diameter, 3/16") and frozen in liquid nitrogen. The tubes were placed in precooled adapters (Qiagen) and ground for 1 min at a frequency of 25 s⁻¹ using a TissueLyser II (Qiagen). TRIzol (Ambion) and the Nucleospin RNA extraction kit (Macherey-Nagel) were used for RNA isolation according to the instructions of the manufacturers. RNA was eluted using RNase-free H₂O and stored at -45°C.

RNA sequencing analysis. The quantity and quality of RNA were checked with a RNA6000 Nano assay using the Agilent 2100 Bioanalyzer (Agilent Technologies). RNA samples were single-end sequenced using the Illumina HiSeq 2000 platform (Illumina). Purification of mRNA, synthesis of the cDNA library, and sequencing were conducted at BGI Tech Solutions Co., Ltd. (Hong Kong, China).

Raw reads were produced from the original image data by base calling. After data filtering, the adaptor sequences, reads with high "N" content (>10% of unknown bases), and low-quality reads (more than 50% bases with a quality value of <5%) were removed. After data filtering, on average, 99.9% clean reads remained in each sample. The clean reads were then mapped to the genome of *D. squalens* CBS 464.89 (http://genome.jgi.doe.gov/Dicsqu464_1) using Bowtie2 (31) and the BWA software (32). In average, 64.9% total mapped reads to the genome were achieved. The gene expression level was measured in FPKM (33) using the RSEM tool (34). Differential expression was identified by the DESeq2 (17), with a cutoff value of ≥ 2.5 -fold change, FPKM value of ≥ 10 , and adjusted *P* value of ≤ 0.01 . For the comparison of the plant cell wall CAZyme genes considered highly expressed with previous parameters, a Venn diagram was constructed using the online tool (35). For CAZyme annotations, the JGI MycoCosm website was used (https://genome.jgi.doe.gov/mycocosm/proteins-browser/browse;qLeIA4?p=Dicsqu464_1).

Accession number(s). The RNA-seq data have been submitted to the Gene Expression Omnibus (GEO) (36) with accession number [GSE105076](https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE105076).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AEM.00403-18>.

SUPPLEMENTAL FILE 1, PDF file, 3.2 MB.

SUPPLEMENTAL FILE 2, XLSX file, 0.1 MB.

SUPPLEMENTAL FILE 3, XLSX file, 0.1 MB.

SUPPLEMENTAL FILE 4, XLSX file, 0.1 MB.

SUPPLEMENTAL FILE 5, XLSX file, 0.1 MB.

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