Pectin as Carbon Source for *Monilinia laxa* Exoproteome and Expression Profiles of Related Genes

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Pectin, as part of the fruit cell wall, can be degraded by brown rot fungi by coordinating the production, secretion, and action of extracellular enzymes. In this study, pectin utilization by the necrotroph *Monilinia laxa* 8L was studied by in vitro and in silico approaches. A total of 403 genes encoding carbohydrate-active enzymes (CAZymes) were identified, including 38 coding a predicted pectin-degrading activity. Analyzing the differences between *M. laxa* 8L exoproteomes in media containing glucose and pectin as sole carbon sources, we identified 107 pectin-specific proteins, among them, 64.48% harbor a classical secretory activity, including 42 CAZymes and six pectin-degrading proteins. Analyzing the gene-expression patterns of some pectinase families revealed their possible sequential action in pectin disassembly. We found, in vitro, an early pectin-dependent induction of *MIRGAEL1*, *MIPG1*, and three members of the rhamnosidase family (*MlaRHA2*, *MlaRHA3*, and *MlaRHA6*) and late response of *MIPG2* and *MIPNL3*. *M. laxa* 8L has the ability to use both pectin and byproducts as carbon sources, based on a functional pectinolytic machinery encoded in its genome, subjected to pectin-dependent regulation and appropriate secretion mechanisms of these pectinolytic enzymes. Differences in the secretion and transcription profile of *M. laxa* 8L provided insights into the different mechanisms that contribute to brown rot development.

Keywords: CAZymes, cell wall-degrading enzymes, necrotroph, pectin degradation, Sclerotiniaceae

Nucleotide sequence data reported are available in the Third Party Annotation Section of the DDBJ/ENA/GenBank database under accession numbers TPA: BK012065 to BK012083.

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Monilinia laxa (Aderhold and Ruhland) Honey, *M. fructicola* (Winter) Honey, and *M. fructigena* (Aderhold and Ruhland) are important necrotrophic plant pathogens that cause brown rot on stone fruit (Byrde and Willetts 1977). Necrotrophic phytopathogens establish disease and obtain nourishment from dead cells by means of releasing lytic enzymes, such as cell wall-degrading enzymes (CWDEs) and phytotoxins, into host tissues, both prior to and during colonization (Walton 1996). But it has been observed that plant–necrotrophic fungi interactions are more subtle than merely killing cells and feeding on them. Throughout infection, these fungi also actively hijack host cellular machinery and selectively silence host immunity genes, consequently promoting disease progression (Weiberg et al. 2013). Through different strategies, some necrotrophs release effectors to trigger host-programmed cell death (Mukhtar et al. 2016) as well as to influence phytohormone levels by disrupting defense signaling leading to necrotrophic colonization (Prins et al. 2000; Sharon et al. 2007).

In the Sclerotiniaceae fungal family, including genera *Monilinia*, *Botrytis*, and *Sclerotinia*, the production of a wide range of degrading enzymes and toxins is key, helping to overcome plant defenses to guarantee the success of colonization of diverse hosts (Laluk and Mengiste 2010; van Kan 2006). Others have adapted mechanisms to detoxify host metabolites that interfere with fungal virulence (Morrissey and Osbourn 1999). Production and release of virulence factors into the host is a common strategy in Sclerotiniaceae necrotrophic fungi regardless of the mechanism of action. Some of these factors include pectinolytic enzymes, phytotoxins, and necrosis-inducing-proteins that lead to rot diseases (Collado et al. 2005; Gijzen and Nürnberger 2006; Pemberton and Salmond 2004; Reignault et al. 2008).

Pectin is one of the main components of the middle lamella, acting as a dynamic matrix embedding the cellulose-hemicellulose primary cell wall and providing strength and support to fruit (Caffall and Mohnen 2009; Mohnen 2008; ten Have et al. 2002). Pectin also influences cell-wall properties and texture in fruit (Brummell et al. 2004; Hegde and Maness 1996; Muramatsu et al. 2004). Pectinases are those enzymes able to degrade pectic components. They are carbohydrate-active enzymes (CAZymes) classified into four groups: glycosyl hydrolase 28 (GH28), glycosyl hydrolase 78 (GH78), carbohydrate esterase 8 and 12 (CE8, CE12), and polysaccharide lyases 1 and 3 (PL1, PL3) (Blanco-Ulate et al. 2014). These groups include polygalacturonases (PGs), rhamnogalacturonan hydrolases, pectin and pectate lyases, pectin methylesterase, α -L-rhamnosidases, and rhamnogalacturonan acetyltransferase (RGAE) protein families.

CWDEs of *Botrytis cinerea* including laccases, proteases, pectinases, and, specifically, endoPGs that break the epidermal host cells (Blanco-Ulate et al. 2014; Kars et al. 2005), while oxidases, cutinases, and lipases dismantled the plant cutin and wax layers during the penetration step (van Kan et al. 1997). *Sclerotinia sclerotiorum* also produces multiple CWDEs, such as cellulases, cutinases, glucanases, glycosidases, pectinases, proteases, and xylanases, that facilitate the colonization and degradation of host tissues (Bolton et al. 2006). CWDE activities by *M. laxa*, *M. fructicola*, and *M. fructigena* have been detected in culture media (Garcia-Benitez et al. 2018), and recent transcriptomic *Monilinia* data also support an important role of these extracellular activities in pathogenesis (De Miccolis Angelini et al. 2018). Fruit cuticle is the primary fruit external barrier being the target of cutinase enzymes. Those strains of *M. fructicola* overexpressing the cutinase gene *MfCUT1* cause more severe symptoms (Lee et al. 2010). Additionally, cutinase activity was also reported in *M. fructicola* and *M. laxa* cultures (Garcia-Benitez et al. 2018). In *M. fructicola*, a negative role on virulence has been reported for the *MfPG1* endoPG upregulated expression pattern under different culture conditions (Chou et al. 2015). Recently, Baró-Montel et al. (2019) have highlighted the role of one pectin methylesterase and two rhamnogalacturonan hydrolases genes on *M. laxa* virulence. Despite this knowledge, a specific study on the content and regulation of pectinolytic machinery of *M. laxa* was required. The advent of genomic data from *M. laxa* 8L and other strains made this aim possible (Landi et al. 2020; Naranjo-Ortíz et al. 2018).

In this work, we evaluate the ability of *M. laxa* to use pectin and its byproducts as carbon sources in vitro. Through *in silico* analysis, we describe the CAZymes arsenal in the *M. laxa* genome and determine experimentally the transcriptional pattern of some potential pectinolytic genes in response to glucose and pectin. Furthermore, we have characterized the extracellular proteins produced in both nutritional conditions by proteomic analyses.

RESULTS

Ability of *M. laxa* to utilize pectin and its structural components as carbon source.

The general structure of pectin and its components, some of which are obtained by partial degradation by microbial activities is shown schematically in Figure 1A. Colonial growth of *M. laxa* 8L was evaluated on minimal medium supplemented with pectin or a selection of products of its degradation as sole carbon source. After 7 days of incubation, significant differences in growth rate and mycelium density between carbon sources were found (Fig. 1B) and measured (Fig. 1C). The highest growth rate was observed in medium containing apple pectin (6.52 mm day⁻¹) and the lowest in medium supplemented with L-rhamnose (2.34 mm day⁻¹). The highest density of mycelium was observed in D-glucose and L-arabinose cultures, while the lowest density was observed in medium with L-rhamnose monosaccharide and, as expected, on agar plates without an added carbon source. Considering both parameters, growth rate and mycelium densities, different clusters of utilization were observed (Fig. 1C). Apple pectin and D-glucose clustered at the same ratio, followed by structural pectic elements galactan, arabinan, polygalacturonic acid sodium salt, and the monosaccharide L-arabinose. In contrast, L-rhamnose monosaccharide showed an even smaller ratio than without added carbon source.

Predicted CAZymes and identification of pectinolytic enzymes.

Searches for carbohydrate-active enzymes using the dbCAN meta server predicted 403 CAZymes in the *M. laxa* 8L

proteome (Supplementary Table S1). Among them, 221 glycoside hydrolases (GHs), 88 glycosyltransferases (GTs), 55 auxiliary activities (AAs), 30 carbohydrate esterases (CEs), seven PLs, and two carbohydrate-binding modules (CBMs). These 403 CAZymes represent 4.21% of *M. laxa* 8L total predicted proteins. According to their expected substrate specificity, 38 putative pectinolytic enzymes that target the homogalacturonan (HG) and rhamnogalacturonan I major structural components of pectin were identified. All known pectinase families GH28, GH78, CE8, CE12, PL1, and PL3 are represented in the CAZyme prediction (Supplementary Table S1). Blast searches were conducted using annotated pectinolytic enzymes of *B. cinerea* and the *M. laxa* genome (Landi et al. 2020) to confirm or improve the automatic annotation of the selected genes (Table 1). Among the pectinase families, most members have not been studied. Expression analysis of 17 selected genes coding for pectin and pectate lyases, α -L-rhamnosidases, RGAEs, and PGs was performed (Table 1).

Exoproteome profiles in glucose and pectin as sole carbon source.

Differences in the extracellular protein profiles of *M. laxa* 8L were analyzed after growing mycelium for 6 h in medium containing pectin or glucose as the sole carbon source (Fig. 2). Of a total of 9,567 predicted proteins in *M. laxa* 8L (Naranjo-Ortíz et al. 2018), liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis identified 527 proteins in glucose and 107 proteins in pectin culture (Fig. 2A). A total of 79 proteins were common in both conditions, 28 proteins specifically identified in pectin cultures, and 448 proteins were only present in glucose cultures (Fig. 2A). The presence of N-terminal signal peptide was analyzed by SignalP to identify which extracellular proteins might be secreted by the classical pathway. Seventy-six proteins found in glucose cultures carried a signal peptide (14.42%) (Supplementary Table S2); however, in pectin cultures, the percentage of putative secreted proteins was higher (64.48%), corresponding to 69 of the identified proteins (Supplementary Table S2). To provide a biological context and give a comprehensive interpretation, they were classified by gene ontology (GO) terms according to their predicted function. The GO analysis indicated that the most represented molecular functions on both media were associated with the general hydrolase activity category and several different subcategories related to transferase, oxidoreductase, and binding (Fig. 2B and C). However, the most represented molecular functions on pectin are associated with hydrolase activities and protein binding among others, hydrolysis of O-glycosyl compounds, exopeptidase, and serine-type peptidase activities (Fig. 2C). The presence of CAZymes among the proteins present in both carbon sources was also analyzed. A total of 42 CAZymes were detected in pectin and 38 in glucose medium (Table 2; Fig. 2D). Fourteen putative CAZymes were only found in pectin cultures, among them the PG family member MIPG1 (Table 2). Twenty-eight CAZymes were found in both media; among them were the pectin degradation-related proteins MIPME2, MIPME3, MIPNL2, M α RHA2, and MIRGAE1 (Table 2). Finally, ten CAZymes were only found in glucose cultures, although neither of the pectinase family members was found uniquely (Table 2).

Timecourse analysis of gene expression with pectin as a sole carbon source.

The set of 17 genes of *M. laxa* 8L chosen for transcription analysis in response to pectin is shown in Table 1. This collection of genes included PG genes *MIPG1*, *MIPG2*, and *MIPG3*, pectin lyase genes *MIPNL1*, *MIPNL2*, and *MIPNL3*, pectate lyase genes *MIPEL1*, *MIPEL2*, and *MIPEL3* that target HG. Moreover, those that target rhamnogalacturonan pectin

backbone include the six genes coding for α -L-rhamnosidases (*Ml α RHA1*, *Ml α RHA2*, *Ml α RHA3*, *Ml α RHA4*, *Ml α RHA5*, and *Ml α RHA6*) and the two genes coding for RGAEs (*MIRGAE1* and *MIRGAE2*).

Expression of all analyzed pectinase genes was detected in both short- and long-term studies, although at different levels depending on the carbon source used, glucose or pectin (two pectins from different batches were used, labeled under assay A

Fig. 1. Ability of *Monilinia laxa* to use pectin and its derivatives as carbon sources. **A**, Schematic representation of pectin and its structural elements. **B**, Growth test showing the ability of *M. laxa* 8L to grow on minimal medium supplemented with different pectin sources and pectin structural elements. Rhamnogalacturonan I (RGI), homogalacturonan (HG), arabinan, arabinogalactan, galactan, polygalacturonic acid (PgA), polygalacturonic acid sodium salt (PgA ss), galacturonic acid (GalA), glucose (Glc), arabinose (LArA), galactose (LGal), rhamnose (LRha), and noncarbon source (NCS). **C**, Effect of different pectin sources and its structural elements on *M. laxa* 8L rate (colony growth × mycelium density). Data represent the average of two independent experiments ($n = 6$). Different letters indicate significant differences ($P \leq 0.05$) according to Student-Newman-Keuls range test.

and assay B). The expression levels differed among members of each pectinase family without a general pattern for the whole family.

Quantitative PCR (qPCR) data allowed their classification into different groups. There was only an immediate pectin-induced gene (>5 fold), *MIRGAE1*, at 0.5 h in both assays (Fig. 3), while *MIPG2* and *MlaRHA3* were only induced in one of them (Figs. 4 and 5). However, the expression levels of *MIPG1*, *MIPG2*, *MIRGAE1*, *MlaRHA2*, *MlaRHA3*, and *MlaRHA6* increased at short incubation times (2 to 6 h) in both assays (Figs. 3, 4, and 5). Overall, the expression levels of short-term pectin-induced genes reached their maximum transcript levels at 6 h in pectin cultures, including *MIPG1* and *MlaRHA6*. Expression of *MIPG2* was also upregulated at long incubation times (24 h) in both assays (Fig. 4). In addition to *MIPG2*, *MIPNL3* and *MlaRHA5* displayed a long-term pectin-induction pattern in only one assay (Figs. 4, 5, and 6). Pectin as sole carbon source did not trigger a transcriptional response in any member of the pectate lyase family (Supplementary Fig. S1).

Expression of *MIPG2*, *MIRGAE1*, *MlaRHA3*, and *MlaRHA6* were detected throughout the assays with the greatest upregulation among PGs, RGAEs, and α -L-rhamnosidases, respectively (Fig. 3, 4, and 5).

Putative recognition sites for CreA transcription factor were identified in the promoters supporting possible carbon catabolite repression (CCR) effects in pectinase genes (Table 1). Sequence analysis of the 1,500-bp upstream promoter region of pectinase genes revealed, in most of them, the existence of putative CreA binding motifs (Table 1). To determine whether

the pectinase genes were regulated by CCR, the fold change in the expression of the pectinase genes in minimal medium with glucose as a carbon source was calculated (Figs. 3C, 4C, 5C, 6C; Supplementary Fig. S1C). Glucose repressed expression of *MIRGAE1*, *MIRGAE2*, *MIPNL1*, *MIPNL3*, *MIPEL1*, *MIPEL3*, *MlaRHA2*, *MlaRHA3*, and *MlaRHA6*. However, glucose did not affect the expression of the PG family and some rhamnosidase and lyase genes.

DISCUSSION

Knowledge about pathogenesis-related genes in *Monilinia* spp. is sparse, mostly limited to several pectinases (Baró-Montel et al. 2019; De Cal et al. 2013; Chou et al. 2015), a few CWDEs such as cutinase (Lee and Bostock 2006; Lee et al. 2010; Wang et al. 2002), and the redox-responsive transcription factor *MfAPI* (Yu et al. 2016). However, several studies using *B. cinerea* and *Sclerotinia sclerotiorum* (Blanco-Ulate et al. 2014; Kubicek et al. 2014; Reignault et al. 2008; ten Have et al. 2002) have pointed to CWDEs and CAZymes as significant virulence factors during the fruit infection process because they target the major plant cell-wall components such as cellulose, hemicellulose, and pectin.

This work is a detailed in-vitro study of pectin utilization by the necrotroph *M. laxa* defining the pectinolytic arsenal, which pectinolytic enzymes are present in media, and the regulation of a subset of genes whose products are closely related to pectin degradation. Searches in the *M. laxa* 8L genome showed the presence of 403 putative CAZymes genes (4.21% of its total predicted genes), i.e., 221 GHs, 88 GTs, 55 AAs, 30 CEs, seven

Table 1. Potential pectinolytic enzymes of *Monilinia laxa* 8L

Gene name	Gene ID ^a	GenBank accession	Putative protein function ^b	CreA ^c	PacC ^c	<i>B. cinerea</i> B05.10 ^d	% Identity	% Coverage	<i>M. laxa</i> Mlax316 ^e	% Identity	% Coverage
Homogalacturonan (HG) (pectin structural element target)											
<i>MIPG1</i>	Monilinia__000380	BK012078	Polygalacturonase 1	3	1	BCIN_14g00850	94.52	100.00	EYC80_008482	100.00	100.00
<i>MIPG2</i>	Monilinia__000560	BK012079	Polygalacturonase 2	5	0	BCIN_14g00610	92.70	100.00	EYC80_008466	100.00	100.00
<i>MIPG3</i>	Monilinia__061300	BK012080	Polygalacturonase 3	1	1	BCIN_04g04930	90.61	98.63	EYC80_002929	100.00	100.00
<i>MIPNL1</i>	Monilinia__061280	BK012081	Probable pectin lyase precursor	1	2	BCIN_14g03430	71.07	99.49	EYC80_002931	89.47	99.13
<i>MIPNL2</i>	Monilinia__069050	BK012082	Putative pectin lyase A protein	1	3	BCIN_03g00280	80.10	99.48	EYC80_000707	100.00	82.68
<i>MIPNL3</i>	Monilinia__089870	BK012083	Polysaccharide lyase family 1 protein	4	3	BCIN_03g07360	87.79	99.48	EYC80_000031	100.00	100.00
<i>MIPEL1</i>	Monilinia__028400	BK012075	Pectate lyase A	0	6	BCIN_03g05820	83.89	100.00	EYC80_000171	100.00	100.00
<i>MIPEL2</i>	Monilinia__054970	BK012076	Pectate lyase B	6	1	BCIN_14g01630	73.13	100.00	EYC80_008399	100.00	100.00
<i>MIPEL3</i>	Monilinia__090060	BK012077	Pectate lyase	1	1	BCIN_10g05620	58.57	70.82	EYC80_002299	100.00	100.00
Rhamnogalacturonan I (RGI) (pectin structural element target)											
<i>MlaRHA1</i>	Monilinia__018280	BK012069	α -L-rhamnosidase C protein	1	0	BCIN_10g03550	72.81	98.73	EYC80_002452	100.00	83.13
<i>MlaRHA2</i>	Monilinia__020670	BK012070	Glycoside hydrolase family 78 protein	4	3	BCIN_06g06580	81.50	99.85	EYC80_001371 ^f	99.26	34.53
<i>MlaRHA3</i>	Monilinia__022450	BK012071	Putative α -L-rhamnosidase protein	5	1	BCIN_14g00510	84.71	100.00	EYC80_008457	99.99	99.88
<i>MlaRHA4</i>	Monilinia__053260	BK012072	Glycoside hydrolase family 78 protein	7	1	BCIN_09g02720	82.68	100.00	EYC80_010799	99.88	100.00
<i>MlaRHA5</i>	Monilinia__066350	BK012073	Glycoside hydrolase family 78 protein	2	1	BCIN_01g11040	75.23	99.19	EYC80_005597	99.58	97.82
<i>MlaRHA6</i>	Monilinia__076710	BK012074	Glycoside hydrolase family 78 protein	5	0	BCIN_13g04850	88.71	99.85	EYC80_009641 ^g	99.85	56.72
<i>MIRGAE1</i>	Monilinia__001430	BK012067	Rhamnogalacturonan acetyltransferase protein	8	4	BCIN_02g07100	85.49	99.61	EYC80_007451	100.00	100.00
<i>MIRGAE2</i>	Monilinia__006160	BK012068	Carbohydrate esterase family 12 protein	1	2	BCIN_02g07100	62.18	91.41	EYC80_006143	100.00	100.00

^a Gene unique identifier in the 8L *M. laxa* genome.

^b Putative protein function based on functional annotation.

^c Number of putative recognition sites for CreA transcription factor (carbon catabolite repression) and pH-response PacC transcription identified in the promoters.

^d Gene unique identifier in *Botrytis cinerea* genome; BLAST coverage and BLAST identity.

^e Gene unique identifier in the Mlax316 *M. laxa* genome; BLAST coverage and BLAST identity.

^f *M. laxa* entry EYC80_001371 corresponds to Monilinia__020670 and Monilinia__020660, with the former representing the rhamnosidase ortholog.

^g *M. laxa* entry EYC80_009641 corresponds to Monilinia__076710 and Monilinia__076700, with the former representing the rhamnosidase ortholog.

PLs, and two CBMs. This catalog of CAZyme genes is similar to other well-known Sclerotiniaceae genomes (367 CAZyme genes in *B. cinerea* and 346 in *S. sclerotiorum* [Amselem et al. 2011]). Figure 1 shows the ability to degrade pectin by *M. laxa* 8L, which could reside in the presence of 38 putative pectinolytic genes (24 GH, eight CE, six PL) in its genome, whose predicted proteins target the HG and rhamnogalacturonan pectin backbones. A positive correlation between the capacity of pectin degradation and the number of pectinolytic genes present in fungal genomes had been reported (Benoit et al. 2012). Thus, pectin breakdown capacity in *M. laxa* 8L must be comparable

to *S. sclerotiorum* and *B. cinerea*, with 33 and 44 pectin-specific genes, respectively (Amselem et al. 2011). Since production and secretion of pectinases is a mechanism to increase the porosity of the wall and facilitate the degradation of other polysaccharides (Blanco-Ulate et al. 2016), the ability of *M. laxa* to utilize pectin may follow a common necrotrophic growth strategy with other Sclerotiniaceae species.

In agreement with the capacity to use pectin as a carbon source in in-vitro tests, exoproteome studies identified a number of pectin-degrading enzymes in the presence of either glucose or pectin in the medium. Of nine proteins identified,

Fig. 2. The comparative analysis of *Monilinia laxa* 8L proteins identified in cultures containing pectin or glucose as the sole carbon source. **A**, Number of total proteins identified from *M. laxa* in minimal medium supplemented with glucose or pectin as carbon source. Venn diagram shows the shared proteins and specific proteins in glucose (blue) and pectin (orange). **B**, Classification of molecular functions of the identified proteins in glucose. **C**, Classification of molecular functions of the identified proteins in pectin. **D**, Identified CAZyme family members in exoproteomes from glucose (blue) and pectin (orange) supplemented medium. CBM = carbohydrate-binding module, AA = auxiliary activity, GT = glycosyltransferase, GH = glycoside hydrolase, CE = carbohydrate esterase, and PL = polysaccharide lyase.

there were two pectin methyl esterases (PMEs) (MIPME2 and MIPME3), one pectin lyase (MIPNL2), one RGAE (MIRGAE1), one α -L-rhamnosidase (MIRHA2), and five accessory enzymes with related galactan and arabinan side chains (GH43, GH53, GH78, and GH93). In fact, we identified 420 more proteins when *M. laxa* 8L was cultured with glucose than pectin as a carbon source for a 6-h incubation. However, 69 of 107 carry a secretory

signal peptide, representing the total extracellular proteins produced in pectin cultures, while only 14.42% of those found in glucose cultures were categorized as potentially secreted. These results are consistent with previous studies in *B. cinerea* reporting that, in non-nutritional media or sucrose, the relative amount of secreted proteins decrease as compared with nutritionally rich media or pectin (Espino et al. 2010; Shah et al. 2009a and b).

Table 2. Carbohydrate-active enzymes (CAZymes) identified from *Monilinia laxa* 8L in glucose and pectin cultures

Carbon source ^a	Protein ID ^b	Putative function ^c	SignalP ^d	Predicted CAZy class	Score ^e		Coverage ^f		MW ^g	pI
					G	P	G	P		
B	Monilinia__000370	<i>M. laxa</i> pectin methyl esterase 3 (MIPME3)	0.986	CE8	6.58	26.28	8.56	37.61	34.3	7.37
P	Monilinia__000380	Polygalacturonase 1 (MIPG1)	0.976	GH28		3.53		2.47	36.5	9.11
B	Monilinia__001430	Rhamnogalacturonan acetyltransferase protein (MIRGAE1)	0.870	CE12	4.96	23.31	6.25	38.28	26.6	6.99
P	Monilinia__001890	Cellulase	0.999	GH45		5.60		4.81	31.5	4.83
P	Monilinia__005200	Cell wall glucanase	0.998	GH16		3.50		2.41	49.6	4.87
B	Monilinia__015060	Putative GPI-anchored cell wall β -endoglucanase protein	0.950	GH17	17.44	29.66	11.21	18.76	43.1	4.75
B	Monilinia__019140	Glycoside hydrolase	0.995	GH5	7.87	31.56	4.98	18.72	46.5	5.67
B	Monilinia__020670	Putative α -L-rhamnosidase protein (MIRHA2)	0.895	GH78	3.77	3.78	1.59	1.59	74.4	5.43
P	Monilinia__024990	Endo-14- β -xylanase A protein	0.978	GH11		4.86		6.61	24.0	9.26
P	Monilinia__025670	Putative acetyl xylan esterase protein	0.892	CE1		3.20		2.87	33.9	6.52
G	Monilinia__025740	Cutinase	0.984	CE5	2.05		3.11		26.4	5.72
B	Monilinia__028960	Glucan endo-1,6- β -glucosidase; β -glucosidase; glucan 1,3- β -glucosidase	0.903	GH5	2.88	17.12	2.41	15.66	45.9	4.72
G	Monilinia__030910	Glycoside hydrolase family 1 protein	—	GH1	1.87		1.43		55.3	5.39
B	Monilinia__032820	Glycoside hydrolase family 16 protein	0.998	GH16	14.73	17.05	14.03	11.69	40.3	4.93
B	Monilinia__033030	Multicopper oxidase, laccase	0.630	AA1	10.97		6.21		65.1	6.00
B	Monilinia__034890	Glucosyltransferase family 8 protein	0.937	AA7	8.75	12.21	4.64	4.64	66.1	5.68
B	Monilinia__034900	GMC oxidoreductase	0.991	AA3	22.53		10.15		66.3	5.47
B	Monilinia__035620	1,3- β -Glucanotransferase activity	0.740	GH72	5.49		2.40		47.8	5.03
B	Monilinia__038270	Sun domain-containing protein	0.964	GH132	12.15	7.12	5.96	5.08	46.8	5.67
B	Monilinia__038540	MIPME2	0.993	CE8	17.29	81.69	15.65	24.35	37.1	5.27
B	Monilinia__039580	Cellobiose dehydrogenase	0.933	AA3	19.90	11.72	6.56	4.66	61.2	5.34
P	Monilinia__039760	Glycoside hydrolase family 39 protein	0.989	GH39		4.04		2.38	50.4	6.34
B	Monilinia__042800	Cutinase protein (MICUT1)	0.989	CE5	8.23	18.80	10.40	15.84	20.3	7.83
P	Monilinia__042880	Cellobiohydrolase	0.990	GH7		3.65		2.12	53.8	5.55
P	Monilinia__044130	Arabinogalactan endo- β -1,4-galactanase	0.982	GH53		18.90		15.71	42.8	6.37
B	Monilinia__046180	Arabinogalactan endo- β -1,4-galactanase	0.865	GH53	3.47	18.64	6.32	15.23	37.6	5.20
G	Monilinia__046830	Multicopper oxidase	—	AA1	21.42		11.50		73.3	6.39
P	Monilinia__048210	α -Glucosidase; β -fructofuranosidase; sucrose α -glucosidase	0.971	GH32		3.80		1.60	75.0	4.87
B	Monilinia__048390	β -N-acetylhexosaminidase	0.888	GH20	9.48	12.76	5.52	10.88	70.4	5.52
B	Monilinia__048950	Xyloglucan-specific endo- β -1,4-glucanase A	0.999	GH12	16.88	11.13	15.79	20.24	25.9	9.23
P	Monilinia__049630	Glucan 1,4- α -glucosidase	—	GH15+CBM20		5.57		3.55	71.2	5.24
B	Monilinia__053590	Glycosyl hydrolase family 43 protein	0.954	GH43	9.91	67.55	6.67	28.44	47.6	5.97
B	Monilinia__053610	Glycoside hydrolase family 79 protein	0.835	GH79	4.07	9.06	2.51	5.85	49.9	6.13
G	Monilinia__054990	Glycosyltransferase family 8 protein	—	GT8	2.60		3.48		35.4	5.96
B	Monilinia__057050	Cutinase; carboxylesterase	0.990	CE5	14.98	29.58	19.83	19.83	23.4	5.99
P	Monilinia__061730	Probable 1,4- β -D-glucan cellobiohydrolase A	0.992	GH7		11.83		4.27	61.6	4.74
G	Monilinia__064990	Glycoside hydrolase family 128 protein	0.954	GH128	2.96		2.85		40.4	4.93
B	Monilinia__069050	Putative pectin lyase A protein (MIPNL2)	0.982	PL1	24.40	28.91	18.85	17.28	39.4	8.00
P	Monilinia__072740	α -Galactosidase	0.994	CBM35		5.05		2.40	70.6	6.18
G	Monilinia__074330	Glycoside hydrolase family 17 protein	0.968	GH17	8.96		6.07		47.6	4.89
B	Monilinia__074360	Probable GAS1 Glycophospholipid-anchored surface glycoprotein	0.978	GH72+CBM43	9.15	14.27	2.59	2.59	56.6	4.81
B	Monilinia__074640	Glycoside hydrolase family 72 protein	0.953	GH72	4.45		2.71		54.1	4.81
G	Monilinia__076140	Glucan 1,4- α -glucosidase	0.971	GH15+CBM20	4.01		2.11		67.7	5.71
P	Monilinia__076810	Cellulase	0.988	GH12		3.57		3.16	38.9	5.50
G	Monilinia__078580	β -Glucosidase	—	GH5	2.47		0.97		92.9	7.55
B	Monilinia__080070	Glycoside hydrolase family 93 protein	0.998	GH93	14.44	50.75	13.54	25.78	41.8	4.94
G	Monilinia__082630	Glycoside hydrolase family 55 protein	0.983	GH55	4.83		1.91		83.3	5.25
P	Monilinia__083030	Glycoside hydrolase family 125 protein	0.995	GH125		2.61		1.95	56.7	4.91
B	Monilinia__087200	Putative glucan endo-1,3- β -glucosidase	0.996	GH17	10.98	26.16	15.59	25.08	31.9	4.60
B	Monilinia__088190	Carbohydrate-binding module family 18 protein	0.997	AA5+CBM18	61.19	10.03	15.96	3.05	85.1	5.34
B	Monilinia__088910	Fungal α -L-arabinofuranosidase, putative	0.850	GH54+CBM42	50.70	301.24	32.15	57.00	52.5	6.44
G	Monilinia__091650	Carbohydrate esterase family 4 protein	0.981	CE4+CBM18	4.53		2.38		76.7	5.57

^a Carbon source: P = pectin, G = glucose, and B = both carbon sources.

^b Protein identification number from *M. laxa* 8L genome.

^c Function was assigned based on sequence similarity when blasted using the National Center for Biotechnology Information nonredundant database and predicted CAZyme motifs.

^d SignalP prediction value using the online version of SignalP 5.0.

^e Protein scores reported by liquid chromatography-tandem mass spectrometry (LC-MS/MS) in each carbon source.

^f Percentage of the protein sequence that has been identified by LC-MS/MS in each carbon source.

^g MW = molecular weight, in kiloDaltons; pI = calculated isoelectric point.

Baró-Montel et al. (2019) suggested a role of the pectin methyl esterase *MIPME2* gene in virulence of *M. laxa* on peach and confirmed that expression of PME was independent of the carbon source, as also reported in *B. cinerea* secretion patterns (Espino et al. 2010; Shah et al. 2009b). However, secretion of PLs by *B. cinerea* was host-dependent (Espino et al. 2010; Shah et al. 2009b, 2009a, 2012), finding the MIPNL2 homolog (BCIN_03g00280) on tomato, kiwi, and commercial pectins with different esterification grades. The RGAE gene *MIRGAE1* was expressed by *M. laxa* 8L along 48 h of incubation and was present in the exoproteome after 6 h. Besides the de-esterification of PMEs, RGAEs remove the acetyl groups from the RGI backbone as a prerequisite, for the action of rhamnogalacturonan hydrolases (de Vries et al. 2000). We did not identify any RG hydrolase in the proteomic analysis, but, according to Baró-Montel et al. (2019), *RG-HYD1* and *RG-HYD2* may be potential virulence factors in later phases of *M. laxa* infection. Other enzymes that act on RGI were α -L-rhamnosidases, releasing α -L-rhamnose from the nonreducing end. In agreement with our results, Shah et al. (2009a) identified M α RHA2 *B. cinerea* homologous (BCIN_06g06580) as a constitutively expressed gene in sucrose and two different pectins.

M. laxa 8L also produced PG (MIPG1) and another accessory arabinogalactan degrading enzyme (GH53) in pectin media. Among CWDEs, many functional studies have tried to elucidate the role of PG genes in pathogenicity. Some of them, including *PG1*, may be part of a common toolbox based on necrotrophy of Sclerotiniaceae lineage (Andrew et al. 2012). Regardless of the possible virulence role, PG1 was the most abundant PG detected in *B. cinerea* proteomic studies, such as those comparative studies between *B. cinerea* strains (González-Fernández et al. 2014), when medium was supplemented with plant extracts (Espino et al. 2010; Shah et al. 2009a and b) or on tomato (Shah et al. 2012). A previous proteomic report on *M. laxa* found no pectin-related enzymes when grown on different

strains in malt extract broth (Bregar et al. 2012). Despite the absence of cell-wall components in culture, we identified, in *M. laxa* 8L, exoproteome enzymes involved in cellulose, hemicellulose, and lignin degradation, as has been described in other phytopathogens (Ismail and Able 2016; Shah et al. 2009b).

In addition to proteomic analyses, 17 genes of *M. laxa* 8L were selected for transcriptional studies. The HG backbone represents 65% of the total content of pectin and forms the so-called smooth region of pectin (Mohnen 2008). HG is a target of PG and PL activities. We detected that *MIPG1* (6 h) and *MIPG2* (24 h) showed upregulation on pectin compared with glucose, whereas *MIPG3* expression was not modified. The maximal expression of *MIPG1* at 6 h in pectin cultures correlates with the detection of MIPG1 in the exoproteome. Consistent with these results, Chou et al. (2015) described *PG1* as predominant in vitro and pectin-inducible in *M. fructicola*. In contrast, overexpression of *PG1* in *M. fructicola* (*MfPG1*) produces smaller brown rot lesions; however, *BcPG2* was essential for *B. cinerea* virulence in pectin-rich plants (Kars et al. 2005). Nonetheless, expression of *MIPG2* was delayed for up to 24 h, which coincided with the acidification of ambient pH (value of 3.50) (data not shown). In agreement with findings by De Cal et al. (2013), acidification has been correlated with the secretion of gluconic acid in *M. fructicola* and resulted in higher expression of *MfPG2* when the pH decreased to 3.6. Although the promoters of the three endoPG genes contain at least one binding motif for CreA, any member of the family showed carbon catabolite repression, a result consistent with previous studies in *M. fructicola* (Chou et al. 2015) and *B. cinerea* (Wubben et al. 2000).

Besides the hydrolysis by PGs, the HG backbone breakdown depends on pectin and pectate lyase activities. *MIPNL3* seems to be the only member of those protein families having a late response (24 h) to the presence of pectin in only one assay; however, at 6 h, we identified MIPNL2 independently of carbon

Fig. 3. Differential expression of two rhamnogalacturonan acetyl esterase (RGAE) coding genes. **A** and **B**, Relative amounts of transcripts for three individual RGAE genes in *Monilinia laxa* 8L cultured in minimal medium with two different pectin batches. The amounts of transcripts for each RGAE gene at different timepoints were compared with glucose at the same incubation time (hours postinoculation [hpi]), using the comparative cycle threshold (C_T) method (orange gradients). Indicated values are the means of triplicates and standard deviations. **C**, Relative amounts of transcripts of RGAE genes in *Monilinia laxa* 8L on minimal medium with glucose for carbon catabolite repression analysis (blue). The amounts of transcripts for each gene at different timepoints were compared with that at 0.5 hpi, using the comparative C_T method. Each timepoint represents the mean and vertical bars indicate the standard deviation of the mean ($n = 2$).

source in the extracellular medium (Table 2). But differences between both expression analyses could be, in part, related to the use of apple pectin of two different batches that could differ in their esterification grade. PLs contribute to virulence in early stages of infection in *B. cinerea* (Chilosi and Magro 1997) and *Alternaria brassicola* (Cho et al. 2015) and also induce host cell death in *Veticillium dahliae* (Yang et al. 2018) and *Phytophthora capsici* (Fu et al. 2015) host interactions. Pectin lyases act preferentially in higher esterified pectin Ca^{2+} dependent at acid pH, while the anionic pectate form is the target of pectate lyases (Yadav et al. 2009), where acts the pH-response PacC transcription factor that has been described as a regulator of virulence in different plant pathogens (Caracul et al. 2003; Huertas-González et al. 1999; Zhang et al. 2013).

To complete the degradation of pectin, the elimination of hairy regions or rhamnogalacturonan I is necessary. The *MIRGAE1* gene was expressed within the first hours of pectin-induction with a significant rise at 6 h. According to its expression and presence in exoproteome, *MIRGAE1* could be in charge of the deacetylation of RGI. The *M. laxa* genome also includes α -L-rhamnosidases (GH78) that cleave terminal nonreducing α -L-rhamnose from RGI and various secondary metabolites (Yadav et al. 2010). Three genes of the α -L-rhamnosidase family had an

early response in *M. laxa* 8L, two extracellular (*MlaRHA2*, *MlaRHA6*) and one intracellular (*MlaRHA3*), while in one of the assays, a late response was detected in *MlaRHA5*. Despite these results and the presence of MIRHA2 in the exoproteome, growth tests identified L-rhamnose as an extremely poor carbon source to *M. laxa*. The presence of α -L-rhamnosidases are frequent in filamentous fungi as well as in phytopathogens, however, most research has been focused on their biotechnology applications. This work envisages a secondary role for α -L-rhamnosidases during *M. laxa* infection, mainly at “cleaning” rhamnose tails from other polysaccharides.

M. laxa 8L was able to utilize apple pectin in vitro and byproducts clearly indicated the presence of functional pectinolytic machinery in this fungus that may play an essential role in its pathogenicity to pectin-rich fruit. We show that *M. laxa* 8L expresses and produces, in vitro, a set of enzymes necessary for the degradation of pectin into simpler molecules to be assimilated as carbon sources, i.e., polygalacturonic acid sodium salt, galactan, arabinan, and L-arabinose, D-galactose, and D-glucose monosaccharides. However, L-rhamnose monosaccharide has a negative effect on colony density and growth, which suggests L-rhamnose catabolism weakness. In fungi, four enzymatic steps are described in the nonphosphorylating pathway that converts

Fig. 4. Differential expression of three polygalacturonase (PG) coding genes. **A** and **B**, Relative amounts of transcripts for three individual PGs genes in *Monilinia laxa* 8L cultured in minimal medium with two different pectin batches. The amounts of transcripts for each PG gene at different timepoints were compared with glucose at the same incubation time (hours postinoculation [hpi]), using the comparative cycle threshold (C_T) method (orange gradients). Indicated values are the means of triplicates and standard deviations. **C**, Relative amounts of transcripts of PGs genes in *Monilinia laxa* 8L on minimal medium with glucose for carbon catabolite repression analysis (blue). The amounts of transcripts for each gene at different timepoints were compared with that at 0.5 hpi, using the comparative C_T method. Each timepoint represents the mean and vertical bars indicate the standard deviation of the mean ($n = 2$).

L-rhamnose to pyruvate and L-lactaldehyde (Aguilar-Pontes et al. 2018). At least one putative orthologous gene was found in *M. laxa* 8L for each gene described in *Aspergillus niger*; in order, L-rhamnose-1-dehydrogenase (*Monilinia_035750*), L-rhamnonic

acid lactonase (*Monilinia_025920*), L-rhamnonate dehydratase (*Monilinia_035760*), and 2-keto-3-deoxy-L-rhamnonate aldolase remain unknown in *A. niger* (Aguilar-Pontes et al. 2018; Khosravi et al. 2017). Therefore, we propose that rhamnose catabolism

Fig. 5. Differential expression of six α -L-rhamnosidase coding genes. **A** and **B**, Relative amounts of transcripts for three individual α -L-rhamnosidase genes in *Monilinia laxa* 8L cultured in minimal medium with two different pectin batches. The amounts of transcripts for each α -L-rhamnosidase gene at different timepoints were compared with glucose at the same incubation time (hours postinoculation[hpi]), using the comparative cycle threshold (C_T) method (orange gradients). Indicated values are the means of triplicates and standard deviations. **C**, Relative amounts of transcripts of α -L-rhamnosidase genes in *Monilinia laxa* 8L on minimal medium with glucose for carbon catabolite repression analysis (blue). The amounts of transcripts for each gene at different timepoints were compared with that at 0.5 hpi, using the comparative C_T method. Each timepoint represents the mean and vertical bars indicate the standard deviation of the mean ($n = 2$).

metabolism could be functional in *M. laxa* 8L despite not being clustered as in *Scheffersomyces stipites* and *A. niger* (Khosravi et al. 2017; Koivistoinen et al. 2012).

The presence of multigene families in CWDEs is related to host adaptability in many phytopathogens, with different members of the same family being able to perform the same function under different conditions. A wide transcriptomic analysis and isoenzyme pattern studies using diverse isolates and growth conditions for the three *Monilinia* spp. showed differential gene expression among species. Some of these deregulated genes are important for pathogenicity, such as those involved in cell-wall degradation (De Miccolis Angelini et al. 2018; Pizzuolo et al. 2006). More understanding of pectinolytic enzymes and their transcriptional regulation will be obtained from fruit infected by *M. laxa*.

Our study provides a general in vitro estimate of how *M. laxa* 8L starts the sequential pectin disassembly with an early induction of HG and RGI de-esterification genes (PMEs and MIRGA1), accompanied by hydrolysis and beta elimination of HG (MIPG1 and MIPNL2), and removal of α -L-rhamnose residues from RGI (M α RHA2, M α RHA3, and M α RHA6), followed by a later response that continues targeting HG (MIPG2 and MIPNL3), and, finally, action of RGI hydrolases. Due to the complex expression pattern in cell wall-degrading

enzymes, further studies with individual gene disruption would be necessary to evaluate their function in pathogenicity, albeit the difficulty described in the closest *M. fructicola* (Chou et al. 2015) as well as in knowing its activity in different pectin, host, or environments for more in-depth knowledge about their regulation and virulence role.

MATERIALS AND METHODS

Fungal strain and culture conditions.

Monilinia laxa 8L (deposited in the Spanish Culture Type Collection, CECT 21100) is a single-spore isolate originally collected on mummified fruit from a commercial plum orchard (cv. Sungold) in Lagunilla, Salamanca, Spain, in 2015. *M. laxa* 8L was maintained as a culture on potato dextrose agar (PDA) (Difco) at 4°C in darkness for short-term storage and as a conidial suspension in 20% glycerol at -80°C for long-term storage. For conidia production, *M. laxa* 8L was grown on PDA amended with 20% tomato pulp (PDA-T) at 22°C for 7 to 9 days with a 12-h photoperiod.

Colony growth on pectin culture media.

In-vitro growth studies were conducted to test the capability of *M. laxa* 8L to use apple and citrus pectin, pectin structural

Fig. 6. Differential expression of three pectin lyase coding genes. **A** and **B**, Relative amounts of transcripts for three individual pectin lyase genes in *Monilinia laxa* 8L cultured in minimal medium with two different pectin batches. The amounts of transcripts for each pectin lyase gene at different timepoints were compared with glucose at the same incubation time (hours postinoculation [hpi]), using the comparative cycle threshold (C_T) method (orange gradients). Indicated values are the means of triplicates and standard deviations. **C**, Relative amounts of transcripts of pectin lyase genes in *Monilinia laxa* 8L on minimal medium with glucose for carbon catabolite repression analysis (blue). The amounts of transcripts for each gene at different timepoints were compared with that at 0.5 hpi, using the comparative C_T method. Each timepoint represents the mean and vertical bars indicate the standard deviation of the mean ($n = 2$).

elements, and three monosaccharides. A 10- μ l droplet of *M. laxa* 8L conidia suspension (10^6 conidia ml^{-1}) was placed on the center of Petri dishes filled with solid *Aspergillus* minimal medium (AMM) (Cove 1966) amended with 1% (wt/vol) of the carbohydrate compounds cited above as main carbon sources (Supplementary Table S3), pH was adjusted to 4.5, and the dishes were incubated for 7 days at 22°C in darkness. To determine the ability of *M. laxa* 8L to grow in the previous conditions, two perpendicular diameters were measured in each colony from 3-day to 7-day of incubation. Mycelium density was evaluated at the end of the assay by visual inspection (scored 1 to 5, with 5 for growth on medium containing glucose and no growth as 0). Growth on AMM agar without an added carbon source was used as a control. Four replicates were made of each carbon source, and the complete assay was done twice.

A rate with daily colony growth to mycelium density was analyzed by one-way analysis of variance, using a statistical program (Statgraphics-Centurion XVI version 16.1.03). Statistical significance was set at 5%. When the results of the F-test were significant ($P \leq 0.05$), means were compared using the Student-Newman-Keuls multiple range test (Snedecor and Cochran 1980).

Identification of pectin degradation related genes.

The complete genome sequence of *M. laxa* 8L (CECT 21100) is available in the National Center for Biotechnology Information (NCBI) database (Naranjo-Ortíz et al. 2018). Based on the *M. laxa* 8L genome and the annotated *Botrytis cinerea* B05.10 genome, the identification of pectinase-related genes was performed through two complementary approaches. First, annotated secreted proteins of *B. cinerea* B05.10 involved in cell-wall disassembly (Blanco-Ulate et al. 2014) and the recent Mlax316 *M. laxa* genome (Landi et al. 2020) were selected and used in BlastP searches using NCBI Genome Workbench against the *M. laxa* 8L genome. Second, CAZymes in *M. laxa* 8L were identified based on the predicted proteome (Naranjo-Ortíz et al. 2018), using dbCAN2 with default parameters and HMMER, DIAMOND, and Hotpep tools (Yin et al. 2012; Zhang et al. 2018). Classification into GO analysis of candidates was carried out using Blast2go v5.0. The 1,500-bp upstream of each putative pectinase gene was analyzed by DNA Pattern Find (Stothard 2000) to identify putative binding motifs for carbon catabolite repressor *CreA* and pH-response *PacC* transcription factors.

Inoculation with carbon-specific sources for proteomic and transcriptomic analyses.

Freshly harvested conidia from *M. laxa* 8L (10^5 conidia ml^{-1}) were used to inoculate 200 ml of AMM with 1% D-glucose (wt/vol) and 5 mM ammonium tartrate as carbon and nitrogen sources, respectively, cultivated at 22°C. Mycelium was harvested by filtration through Miracloth, after 24 h of incubation in an orbital shaker at 150 rpm, and was washed with sterile distilled water. To deplete glucose reserves, washed mycelia were suspended in AMM without a carbon source and were incubated for 4 h at 22°C and 150 rpm. After carbon starvation, mycelium was newly harvested and washed as described above, and further, was equally divided and cultivated in AMM with two carbon sources (D-glucose or apple pectin [1% wt/vol]) and 5 mM ammonium tartrate as the main nitrogen source. Media were adjusted to pH 4.5, and cultures were incubated at 22°C with an orbital agitation of 150 rpm. Experimental approaches were carried out to determine, in both the short and long term, the effect of apple pectin on the expression levels of the codifying genes of the enzymes involved in its degradation. The mycelium was equally divided and was cultivated for 0.5, 2, 6, 24, and 48 h. Filtered mycelia were processed for quantitative reverse transcription PCR (qRT-PCR) studies, and the

filtered media were frozen at -20°C and were stored for secretome analyses. The complete assay was repeated from 0.5 to 48 h. The pH of the culture medium was measured at the time of collecting the mycelium with a Micro pH-meter 2000 (Crison).

Proteomic analyses.

Extracellular proteins produced by *M. laxa* 8L were identified after 6 h of culture in medium containing 1% D-glucose or pectin as the main source of carbon. Proteins present in filtered media were precipitated by adding trichloroacetic acid to a final concentration of 10%. Samples were incubated on ice for 20 min, were centrifuged at $12,880 \times g$ for 10 min at 4°C to collect precipitated proteins. The pellets were washed first with 1 ml of ethanol-ethyl ether (1:1), were centrifuged at $21,767 \times g$ for 10 min at 4°C , and then, secondly, with 1 ml of ethanol-ethyl ether (1:3) followed by centrifugation at $21,767 \times g$ for 10 min at 4°C . Protein pellets were dried at room temperature and were then suspended in Laemmli cracking buffer and were digested with trypsin (Cristobo et al. 2011) for LC-MS/MS.

Trypsin-digested samples were analyzed on a nano Easy nLC 1000 (Proxeon) coupled to an LTQ-Orbitrap Velos (Thermo Scientific). Peptides were loaded onto a Acclaim PepMap 100 (Thermo Scientific) trap column and were eluted onto Acclaim PepMap 100 C18 $3 \mu\text{m}$ (Thermo Scientific, $75 \mu\text{m} \times 25 \text{ cm}$). A 110-min gradient was run at a 250 nl/min flow rate, using gradients from 0 to 35% buffer B (0.1% formic acid in acetonitrile) for 90 min, from 45 to 95% buffer B for 10 min, 95% buffer B for 9 min, and 10% buffer B for 1 min. Mass spectra analyses were conducted on an LTQ-Orbitrap Velos (Thermo Scientific) in positive mode, full scan MS spectra (m/z 400 to 2,000) at a resolution of 60,000. The top 15 most-intense ions were selected and were fragmented using collision-induced dissociation in the ion trap, with 35% normalized collision energy, and a dynamic exclusion time of 45 s was applied. Pectin and glucose raw files were searched with Sequest through Proteome Discoverer version 1.4.1.14 against the *M. laxa* 8L proteome (Naranjo-Ortíz et al. 2018) with peptide tolerance of 10 ppm and fragment tolerance of 0.5 Da. Cysteine carbamidomethylation and methionine oxidation were considered fixed modifications. False discovery rate calculations were generated using Percolator at $q \leq 0.01$ (Käll et al. 2007). Functional annotation of extracellular proteins was carried out using Blast2GO v5.0, blastP (NCBI), and SignalP 5.0 (Almagro Armenteros et al. 2019).

Gene expression analysis of potential pectinolytic genes.

Total RNA was extracted from mycelia of each carbon source described above and time was assayed using TRI reagent (Sigma-Aldrich), adapted to the manufacturer indications. Briefly, frozen mycelia were ground in liquid nitrogen using a prechilled mortar and pestle. Approximately 100 mg of powdered mycelium were treated with 1 ml of TRI reagent for 5 min at room temperature, followed by incubation at room temperature for 3 min after the addition of 0.2 ml of chloroform. The aqueous phase was recovered after centrifugation at $13,709 \times g$ for 15 min at 4°C . The TRI reagent extraction protocol was repeated a second time. Nucleic acids, mainly RNA, were precipitated by adding 0.5 ml of 2-propanol to the second aqueous phase, followed by incubation at room temperature for 10 min. Total RNA was pelleted by centrifugation at $13,709 \times g$ for 15 min at 4°C and was subsequently washed with 75% ethanol. The dry RNA pellet was resuspended in RNase-free Milli-Q water, heated at 60°C for 10 min for complete dissolution, and was immediately frozen in liquid nitrogen and was maintained at -80°C until further use.

RNA concentration and purity were measured using a Nano-drop 2000 spectrophotometer, and RNA integrity was checked by 1.2% agarose gel electrophoresis. One microgram of RNA was treated with DNase I Amplification Grade (Invitrogen), according to manufacturer specifications, to remove any remaining genomic DNA. cDNA was synthesized from 1 µg of RNA, using the SuperScript First-Strand synthesis system for RT-PCR and oligo(dT) primer (Invitrogen). Real-time PCR was performed with a 7500 Fast Real-Time PCR (Applied Biosystems), using GoTaq qPCR Master Mix (Promega). Each reaction was carried out in triplicate in a total volume of 20 µl, containing 10 µl of 2× GoTaq qPCR Master Mix, 7.8 µl of nuclease-free water, 0.6 µl of each primer (10 µM), and 1 µl of cDNA. The cycling program was 2 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. After the amplification reaction, a melt curve analysis was performed to check the specificity of different reaction products and transcripts. The relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method (Schmittgen and Livak 2008). The *M. laxa* actin (BK012066) and histone H3 (BK012065) were used as endogenous reference genes. All the primers used in RT-qPCR were designed based on the *M. laxa* 8L genome, using vector NTI (Thermo Fisher), and are listed in Supplementary Table S4. Nucleotide sequence data reported are available in the Third Party Annotation Section of the DDBJ/ENA/GenBank database under accession numbers TPA: BK012065 to BK012083.

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AUTHOR-RECOMMENDED INTERNET RESOURCE

NCBI Genome Workbench: <https://www.ncbi.nlm.nih.gov/tools/gbench>

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