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Subashini Nagendran
DOE Plant Research Laboratory, East Lansing, MI

Heather E. Hallen-Adams
University of Nebraska-Lincoln, hhallen-adams2@unl.edu

Janet M. Paper
DOE Plant Research Laboratory, East Lansing, MI

Nighat Aslam
DOE Plant Research Laboratory, East Lansing, MI

Jonathan D. Walton
DOE Plant Research Lab, East Lansing, MI, walton@msu.edu

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Reduced genomic potential for secreted plant cell-wall-degrading enzymes in the ectomycorrhizal fungus *Amanita bisporigera*, based on the secretome of *Trichoderma reesei*

Subashini Nagendran [a,b], Heather E. Hallen-Adams [a], Janet M. Paper [a], Nighat Aslam [a,b], Jonathan D. Walton [a,b,*]

[a] DOE Plant Research Laboratory, Michigan State University, E. Lansing MI 48824, USA
[b] Great Lakes Bioenergy Research Center, Michigan State University, E. Lansing MI 48824, USA

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ABSTRACT

Based on the analysis of its genome sequence, the ectomycorrhizal (ECM) basidiomycetous fungus *Laccaria bicolor* was shown to be lacking many of the major classes of secreted enzymes that depolymerize plant cell wall polysaccharides. To test whether this is also a feature of other ECM fungi, we searched a survey genome database of *Amanita bisporigera* with the proteins found in the secretome of *Trichoderma reesei* (syn. *Hypocrea jecorina*), a biochemically well-characterized industrial fungus. Additional proteins were also used as queries to compensate for major groups of cell-wall-degrading enzymes lacking in the secretome of *T. reesei* and to substantiate conclusions drawn from the *T. reesei* collection. By MS/MS-based "shotgun" proteomics, 80 proteins were identified in culture filtrates of *T. reesei* strain RUTC30 grown on corn cell walls and in a commercial "cellulase" preparation, Spezyme CP. The two *T. reesei* enzyme preparations were qualitatively and quantitatively similar, the most striking difference being the lack of at least five major peptidases from the commercial enzyme mixture. Based on our analysis of *A. bisporigera*, this ECM fungus is deficient in many major classes of cell-wall-degrading enzymes, including both glycosyl hydrolases and carbohydrate esterases. By comparison, the genomes of the saprophytic basidiomycetes *Coprinopsis cinerea* and *Galerina marginata* (using a genome survey sequence approximately equivalent in depth to that of *A. bisporigera*) have, like *T. reesei*, a much more complete complement of cell-wall-degrading enzymes.

1. Introduction

The secretion of enzymes to degrade biological polymers is a defining feature of the Kingdom Mycota. Plant cell walls constitute the largest single source of available reduced carbon in terrestrial environments, and fungi secrete a large number and variety of enzymes that can act on the linkages found in plant cell walls, including cellulases, hemicellulases, pectinases, esterases, oxidoreductases, and proteases. Some filamentous fungi secrete more than two hundred glycosyl hydrolases alone (Cuomo et al., 2007; Dean et al., 2005).

Based on the analysis of the full genome sequence of *Laccaria bicolor*, Martin et al. (2008) concluded that this ectomycorrhizal (ECM) fungus is deficient in the capacity to produce extracellular enzymes active on plant cell wall polysaccharides, compared to ascomycetes and both saprophytic and pathogenic basidiomycetes. This observation has potentially major implications for understanding ECM symbiotic interactions, because cell-wall-degrading enzymes are well-documented to play an important role in modulating the interactions between fungi and plants. Many fungal enzymes are directly or indirectly detrimental to plant cells. They can kill plant cells by weakening the essential cell wall, leading to reduced growth and ultimately death by osmotic shock, and can also trigger a number of defense responses such as phytoalexin biosynthesis, reactive oxygen species, callose deposition, and the hypersensitive response (Walton, 1994). Induction of defense responses in some cases is caused by the enzymes themselves and sometimes by the oligosaccharide products of their enzymatic action (Beliën et al., 2006; D'Ovidio et al., 2004). Plants respond in multiple ways to degradative enzymes such as pectinases and xylanases of both pathogens and saprophytes, and plants contain a number of proteins that inhibit microbial enzymes, including polygalacturonase, pectin lyase, xylanase, and pectin methylesterase (Enkerli et al., 1999; Lionetti et al., 2007). Whether an ECM fungus does or does not secrete enzymes (and other proteins) that interact with plant cell walls could therefore have a major impact on its ability to form a viable ECM symbiotic relationship.

* Corresponding author. Address: DOE Plant Research Lab, Michigan State University, E. Lansing MI 48824, USA. Fax: +1 517 353 9168.
E-mail address: walton@msu.edu (J.D. Walton).

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Of 12 families of glycosyl hydrolases active on plant cell wall polysaccharides, the genome of *L. bicolor* lacks nine (Martin et al., 2008). *L. bicolor* does have six members in family GH28, which contains all known fungal polygalacturonases (PGs). PGs are among the best-studied plant-cell-wall degradative enzymes. They induce defense responses via the release of elicitor-active oligogalacturonides and in some pathosystems contribute to virulence (D'Ovidio et al., 2004; Scott-Craig et al., 1998; ten Have et al., 1998). *L. bicolor* also has genes encoding enzymes in GH families 5 and 9, as well as members of carbohydrate esterase (CE) family 8 (Martin et al., 2008).

The results from studies on plant-active degradative enzymes in other ECM fungi are mixed. Colpaert and van Tichelin (1996) found that ECM fungi had no or limited ability to decompose beech leaf litter, and Hutchison (1990) found no cellulase or pectinase producers among 96 species of ECM fungi. On the other hand, *Amanita regalis*, *Paxillus involutus*, and *Suillus bovinus* produce cellulase activity (Maijala et al., 1991), and *Thelephora terrestris* and *S. bovinus* produce β-xylosidase and β-glucosidase activities (Colpaert and van Laere, 1996). *Pisolithus tinctorius* has endoglucanase and exoglucanase activities (Cao and Crawford, 1993), and *Tricholoma matsutake* has glucosidase, cellulase, xylanase, and amylase activities (Kusuda et al., 2008; Terashita et al., 1995). However, apparently none of these reports of enzyme activities in ECM fungi have been confirmed by enzyme purification or genetic analysis. It therefore remains an open question whether a general deficiency of such enzymes is a general attribute of ECM fungi, or whether it might be peculiar to *L. bicolor*. Rather than being a general property of ECM fungi, a deficiency in enzymes might instead be negatively correlated with the capacity to grow saprophytically (at least facultatively), or positively with the ability to form a symbiotic association with a particular host or hosts, or with ecological biome (Read and Perez-Moreno, 2003).

We recently sequenced 84.3 Mb (final assembled size) of genomic DNA of *Amanita bisporigera* (constituting ~2× coverage, assuming a genome size of ~40 Mb) (Hallen et al., 2007) and 46.1 Mb of *Galerina marginata* (~1.2×) (unpublished results). Our original interest in both of these fungi is their ability to make cyclic peptide toxins such as amatoxins and phallotoxins (Hallen et al., 2007), but the availability of these genome survey sequences are a useful resource for other purposes as well. In the present case, we have used these survey genome sequences to address the question of whether *A. bisporigera*, an ECM fungus, has, like *L. bicolor*, a reduction in its number of secreted cell-wall-degrading enzymes.

A. bisporigera is an obligate ectomycorrhizal symbiont – notably of oaks and pines – and is native to North and Central America. Some but not all specimens possess two-spored basidia, and the current consensus is that North American specimens called by the European name *A. virosa* are really four-spored *A. bisporigera*. *G. marginata* is a saprophytic (wood-rotting) mushroom found circumboreally (Gulden et al., 2001). *L. bicolor* and *Coprinopsis cinerea* (which is sequenced and used in the present study) grow well and readily form fruiting bodies in culture (Davis and Jong, 1976), whereas *A. bisporigera* and *G. marginata* grow slowly in culture. On rich nutrient agar, *A. bisporigera* grows ~1 cm/month, and *G. marginata* ~3 cm/month (Zhang et al., 2005; unpublished results from this lab).

The goal of the present investigation was to test whether *A. bisporigera* more closely resembles *L. bicolor* in having a reduced genomic capacity to synthesize and secrete enzymes that break linkages found in plant cell wall polysaccharides and other polymers, or whether *A. bisporigera* more closely resembles most other fungi in making a large number of such enzymes. In order to address this question, it is first necessary to define a suitable set of enzymes/genes to use as queries. For the analysis of *L. bicolor*, Martin et al. (2008) relied on the CAZy "Carbohydrate-Active EnZYme" database, (Cantarel et al., in press; Henrissat and Davies, 1997), which contains >140 families of enzymes that degrade linkages found in natural polysaccharides. However, many of these families, as well as some specific members within each family, are known or predicted to be involved in processes other than extracellular degradation of the wall components of higher plants, such as endogenous remodeling of fungal walls, modification of glycoproteins, degradation of small saccharides such as sucrose and trehalose, degradation of storage polysaccharides such as starch and glycogen, and degradation of insect and animal-derived polymers. In addition, many of these classified genes/enzymes are biochemically uncharacterized (as are the vast majority of GenBank entries). As an alternative, but also complementary, approach, we have used as query proteins a set of relevant proteins, namely the secreted proteins of the ascomycete *Trichoderma reesei* (*Hypocrea jecorina*).

T. reesei is a well-studied degrader of lignocellulose and its enzymes are used industrially on a large scale (Merino and Cherry, 2007). It secretes a large quantity and diversity of enzymes that are active on linkages found in plant cell polysaccharides (Stricker et al., 2008). Many of the extracellular enzymes of *T. reesei* have been biochemically characterized and therefore their use to search for orthologs in other organisms provides more robust information and avoids potentially misleading results associated with using proteins of only hypothetical function or function deduced by amino acid similarity. Many of the classes of cell-wall-degrading enzymes of *T. reesei* are widespread in the fungal kingdom and therefore they constitute an archetypal set of proteins to use as queries against other fungi.

Compared to degradative enzymes secreted by ascomycetes, those of basidiomycetes are collectively less well characterized. Nonetheless, it has been shown that basidiomycetes secrete many of the same enzyme activities and classes of carbohydrate-active enzymes as ascomycetes, such as cellulases, pectinases, and xylanases (Ding et al., 2006; Baldrian and Gabriel, 2003). Therefore, it appears justified to use the *T. reesei* secretome as a starting point to explore the secreted, degradative potential of another fungus such as *A. bisporigera*.

2. Materials and methods

2.1. Fungal growth and protein production

T. reesei (*Hypocrea jecorina*) was grown in medium containing 4 mM $Ca(NO_3)_2{\cdot}4H_2O$, 1 mM K_2HPO_4, 1 mM $MgSO_4{\cdot}7H_2O$, and 2.5 mM NaCl, supplemented with 1% (w/v) corn stover pretreated by ammonia fiber explosion (AFEX) (Teymouri et al., 2005). Each 1-l flask contained 125 ml medium. The flasks were inoculated with five 1-cm^2 agar plugs of actively growing *T. reesei* RUTC30 (NRRL 11460; Agricultural Research Service Culture Collection, Peoria, Illinois) growing on V8-juice agar. The cultures were maintained without shaking at 21 °C for 14 days. Culture filtrates were concentrated 100-fold by rotary evaporation under vacuum at 35 °C and lyophilized to dryness, then redissolved in 5 ml water and buffer-exchanged into 0.1 M citrate buffer (pH 4.8). Spezyme CP was obtained from Genencor (Batch # 301-05330-206). Protein concentration was determined by the BCA method (Pierce, Rockford, Illinois), using bovine immunoglobulin as standard (Smith et al., 1985).

2.2. Proteomics

For proteomics analysis, the protein preparations (200 μg protein each) were fractionated in two lanes on a BioRad (Richmond, CA) 4–20% gradient SDS polyacrylamide gel. The gel lanes were each divided into 10 slices and each slice was individually processed. The proteins were alkylated with iodoacetamide and di-

gested with sequencing-grade modified porcine trypsin (Promega). The digested peptides were injected with a ThermoElectron Micro-Autosampler onto an Agilent Zorbax 300 SB-C18 5 × 0.3 mm peptide trap column and desalted for 10 min. The bound peptides were then eluted onto a 10 cm × 75 μm New Objectives Picofrit column packed with Microm Magic C18 AQ and eluted over 120 min with a gradient of 5% B to 50% B in 75 min, then 50% B to 90% B from 75 min to 79 min using a ThemoElectron Surveyor high-pressure liquid chromatograph. Buffer A was 99.9% water + 0.1% formic acid, and buffer B was 99.9% acetonitrile + 0.1% formic acid. The peptides were eluted into a ThermoElectron LTQ/FT mass spectrometer at a flow rate of 250 nl/min. Survey scans were taken at a resolution of 100,000 and the top ten ions in each survey scan were subjected to automatic low-energy collision-induced dissociation (CID) in the linear trap (LTQ). Fragment tolerance was 0.80 Da (monoisotopic), and the parent was tolerance 200 ppm (monoisotopic). Complete and partial residue modifications were allowed as well as point mutations and two missed cleavage sites.

Scaffold version 01_07_00 (Proteome Software, Portland, Oregon) was used to probabilistically validate protein identifications in the *T. reesei* proteome (v. 2.0, DOE Joint Genome Institute) using the X!Tandem and ProteinProphet computer algorithms (Craig and Beavis, 2003; Nesvizhskii et al., 2003). Minimum criteria for positive identification were at least two peptides and >95% probability in at least one of the two samples as determined by Scaffold.

2.3. Genome sequencing of A. bisporigera and G. marginata

The genome of *A. bisporigera* was sequenced randomly by a combination of Sanger and first and second-generation 454 pyrosequencing. (First-generation 454 sequencing produced read lengths of ~110 bp and the second generation FLX machine produces read lengths of ~250 bp). The resulting sequences were assembled using the 454 *de novo* assembler Newbler and put into a BLAST searchable database (Hallen et al., 2007). The assembled database of *A. bisporigera* contains 84.2 Mb in 620,627 contigs. *G. marginata* was sequenced using the 454 FLX for a total of 198,877 contigs in 46.1 Mb.

2.4. Database searching

Each *T. reesei* protein was used as query in TBLASTN against the *L. bicolor* database (v. 1, DOE Joint Genome Institute, http:// genome.jgi-psf.org/euk_cur1.html), the *A. bisporigera* and *G. marginata* databases (http://genomics.msu.edu/Amanita/blast/blast.html), and the *C. cinerea* (*Coprinus cinereus*) database at the Broad Institute (http://www.broad.mit.edu/annotation/genome/coprinus_cinereus.2/Blast.html). For all searches, the word size was three and the matrix was BLOSUM62. For *L. bicolor* and *C. cinerea*, the databases used were "Best Models (transcripts)" or "transcripts", respectively. Expect cutoff scores greater than 1.00E−05 were tabulated as "No hit". Hits to Carbohydrate Binding Modules (CBM) alone were analyzed separately.

Because the genome of *A. bisporigera* is not complete, some genes may be absent or underrepresented. To control for this, we did two experiments. One was to search all four fungi with a representative group of housekeeping proteins. The second was to search the genome survey sequences of the saprophytic mushroom *G. marginata*, whose incompleteness is comparable to that of *A. bisporigera*. Most of the *A. bisporigera* and *G. marginata* database contigs are shorter than full-length transcripts, resulting in the best hit scores being typically less significant than those obtained from searching the *C. cinerea* or *L. bicolor* genomes. For *A. bisporigera* and *G. marginata*, the best hits were saved no matter how poor their scores were. All hit sequences from *A. bisporigera* and *G. marginata* were re-checked using BLASTX against the GenBank NR database. In every case in which the best expect score was <1.00E−03, the *A. bisporigera* or *G. marginata* sequence identified at least one fungal gene annotated as related to the function of the original query protein. In every case in which the best score was >1.00E−03, the best hit by BLASTX was to a clearly unrelated gene.

3. Results

3.1. The secretome of T. reesei

A total of 82 proteins were identified at high reliability in the secretome of either *T. reesei* RUTC30 grown on maize cell walls or Spezyme CP. For subsequent analysis, proteins that could be identified as housekeeping proteins (e.g., ferredoxin, topoisomerase, aldolase, transcription factor, ubiquitin, etc.) were discarded, reducing the number to 60 (Table 1). Fifty-one of these were found in culture filtrates of RUTC30 and 41 in Spezyme CP. A number of proteins near the bottom of the list (i.e., those of lowest abundance based on spectral counts) (Table 1) also appear to be only in one or the other preparation, but some of these qualitative differences might be spurious, i.e., due to the greater technical difficulty of detecting lower abundance proteins. The mass spectrometry data were also analyzed using the entire GenBank NR protein database instead of only the *T. reesei* proteome. No evidence for proteins from other organisms was found in Spezyme CP, that is, this commercial cellulase preparation is derived from *T. reesei* alone.

Thirty-one of the identified proteins were glycosyl hydrolases, ten were peptidases, six were esterases, two were nucleases, one was an oxidoreductase, and ten were non-enzymatic or unknown (Table 1). Altogether, 21 CAZy Glycosyl Hydrolase (GH) families, 10 peptidase families, and three carbohydrate esterase (CE) families are represented in the *T. reesei* secretome (Table 1). Based on our results, *T. reesei* does not secrete any significant quantity of any pectin lyase (PL). BlastP searching of the *T. reesei* database supported this conclusion; representatives of PL1, PL3, and PL4 (see Table 2) did not find any orthologous proteins in *T. reesei* at an expect score of <1.00E−5.

The proteins in Table 1 are listed in order of descending abundance based on total spectral counts in RUTC30. The number of spectral counts (i.e., the number of times the mass spectrometer detected a peptide corresponding to a particular protein) and the percent of the full-length protein sequence that was covered by the peptides (percent amino acid coverage) are shown. Spectral counts and sequence coverage are strongly correlated with protein abundance, although exceptions due to, e.g., heavy glycosylation, can occur (Chen et al., 2005). Most proteins have approximately the same relative abundance in Spezyme CP and RUTC30 grown on corn stover (Fig. 1). Xylanase 3, β-xylosidase, and an unknown predicted glycosyl hydrolase (GH30A) appear to be significantly more abundant in one or the other. Unknown protein 70840 was present at >95% probability in RUTC30 but 80–94% probability in Spezyme CP. Of the qualitative differences among the more abundant proteins, the most striking difference is the absence in Spezyme CP of at least six extracellular proteases (proteins 123234, 73897, 107142, 110220, 70800, and 22459) (Fig. 1, Table 1). The presence of all of these in RUTC30 is strongly supported by percent coverage and spectral counts (Table 1). A probable explanation for this observation is that the strain of *T. reesei* used to make Spezyme CP has had its major protease genes mutated or down-regulated. Intentional genetic modification might also explain the absence of chitinase (80833) from Spezyme CP (Fig. 1).

β-Galactosidase was found in Spezyme CP but not in RUTC30. A plausible explanation for this is the use of lactose, the substrate of β-galactosidase, for induction of cellulase gene expression in

Table 1
Summary of secreted proteins in culture filtrates of *T. reesei* RUTC30 and Spezyme CP. "Spectral counts" are across all gel slices (10 total), whereas "percent coverage" is for the best slice. Names indicate known or predicted function; "glycosyl hydrolase" is a general prediction based on GenBank/pfam conserved domains or annotation of orthologs.

	Trichoderma protein number	Function, alternate names, and GenBank accession number	RUTC30				Spezyme CP		
			CAZy family	Probability (%)	% Coverage	Spectral counts	Probability (%)	% Coverage	Spectral counts
1	123989	CBH I (Cel7A); CAA49596	GH7	>95	31.5	1755	>95	31.5	969
2	49081	Xyloglucanase (Cel74A); AAP57752	GH74	>95	38.8	623	>95	43.2	341
3	72567	CBH II (Cel6A); P07987	GH6	>95	27.8	506	>95	27.8	548
4	120229	Xylanase 3; BAA89465	GH10	>95	46.7	285	>95	44.7	61
5	123992	Swollenin; CAB92328		>95	30.6	210	>95	21.5	125
6	73638	Cip1; AAP57751		>95	28.8	199	>95	12.0	309
7	123818	Xylanase 2; AAB29346	GH11	>95	39.0	198	>95	39.0	81
8	123234	Peptidase S8, subtilisin		>95	46.6	121	0	0.0	0
9	73643	Endoglucanase (Cel61A); CAA71999	GH61	>95	5.8	117	>95	5.8	74
10	120312	Endoglucanase (Cel5A or Egl3), AAA34213	GH5	>95	37.1	115	>95	31.8	245
11	80833	Chitinase; BAD44715	GH18	>95	22.4	81	0	0.0	0
12	122081	Endoglucanase (Cel7B or Egl1); AAA34212	GH7	>95	17.4	76	>95	25.5	196
13	73897	Peptidase S1; chymotrypsin		>95	24.3	76	0	0.0	0
14	123232	Endoglucanase (Cel12A); BAA20140	GH12	>95	17.9	69	>95	19.2	156
15	123940	Glucuronoyl esterase (Cip2); AAP57749	CE15	>95	24.1	66	>95	24.4	218
16	70840	Unknown		>95	45.3	65	80–94	45.0	12
17	120961	Endoglucanase (Cel61B); AAP57753	GH61	>95	27.7	58	>95	27.7	25
18	76672	β-Glucosidase; AAA18473	GH3	>95	17.7	54	>95	36.6	78
19	107142	Peptidase M35, deuterolysin		>95	25.1	54	0	0.0	0
20	56996	β-Mannase; AAA34208	GH5	>95	15.5	42	>95	15.1	63
21	76210	Arabinosidase; AAP57750	GH62	>95	36.3	39	>95	19.3	70
22	70800	Peptidase M36		>95	20.2	38	0	0.0	0
23	69276	GH 30A	GH30	>95	21.4	37	>95	31.8	130
24	121127	β-Xylosidase; CAA93248	GH3	>95	15.1	33	>95	37.3	94
25	103049	Polygalacturonase	GH28	>95	17.0	26	80-94	17.0	6
26	110894	Endo beta 1,6 galactanase	GH5	>95	15.5	20	>95	14.6	17
27	110220	Peptidase M6		>95	10.7	19	0	0.0	0
28	124282	Copper radical oxidase		>95	5.6	18	>95	4.9	7
29	123039	Unknown cell wall protein		>95	13.1	16	0	0.0	0
30	22459	Carboxypeptidase A		>95	16.5	15	0	0.0	0
31	123283	Arabinosidase; CAA93243	GH54	>95	15.2	14	>95	18.6	10
32	55319	Arabinosidase B	GH54	>95	26.7	14	0	0.0	0
33	105279	Peptidase M28		>95	17.1	12	0	0.0	0
34	81004	Peptidase A1, pepsin		>95	12.9	12	0	0.0	0
35	45717	α-Mannosidase; AAF34579	GH47	>95	4.4	11	0	0.0	0
36	44214	Acetylxylan esterase (Axe2); AAP57757	CE5	>95	18.7	9	>95	9.7	2
37	121746	β 1,3-glucanase	GH55	>95	9.0	9	0	0.0	0
38	45085	Unknown		>95	25.4	8	0	0.0	0
39	122127	Unknown		>95	24.3	6	>95	28.0	14
40	109345	Unknown		80–95	3.0	6	>95	7.0	18
41	66792	Membrane-bound β-glycosidase	GH17	>95	6.7	6	50–79	3.0	1
42	54219	Acetylxylan esterase	CE5	>95	14.6	6	80–94	4.0	9
43	123226	α-Trehalase	GH37	>95	3.2	5	0	0.0	0
44	72379	Unknown		>95	4.1	4	50–79	1.0	1
45	70919	Endoribonuclease		>95	26.3	4	0	0.0	0
46	73632	Acetylxylan esterase (Axe1); CA93247	CE5	>95	16.9	3	80–94	17.0	9
47	81070	Peptidase M28		80–94	12.9	2	>95	5.7	7
48	66041	GH 18A	GH18	>95	5.5	2	0	0.0	0
49	65483	Unknown		>95	8.4	2	0	0.0	0
50	49274	GH 16A	GH16	>95	7.8	2	0	0.0	0
51	4213	Ribonuclease T2		80–94	7.0	1	>95	16.7	4
52	80240	β-Galactosidase; CAD70669	GH35	0	0.0	0	>95	15.7	32
53	72526	α-Glucuronidase; CAA92949	GH67	0	0.0	0	>95	9.5	27
54	65406	GH 16B	GH16	0	0.0	0	>95	35.3	25
55	121418	Acetyl esterase (Aes1); ABI34466	CE16	0	0.0	0	>95	13.0	7
56	111849	GH 95	GH95	0	0.0	0	>95	11.8	12
57	76155	Acid phosphatase		0	0.0	0	>95	7.4	7
58	104797	GH 3A	GH3	0	0.0	0	>95	4.9	3
59	112018	Unknown		0	0.0	0	>95	18.1	3
60	103039	Peptidase S51		0	0.0	0	>95	11.2	2

commercial fermentations (Fekete et al., 2008). Corn stover contains little if any β-linked galactose.

T. reesei is known to lack significant capacity to degrade lignin, which process is catalyzed by oxidative enzymes in white rot fungi such as *Phanerochaete chrysosporium* (Kersten and Cullen, 2007). Consistent with this, only a single putative oxidoreductase (124282) was detected in the secretome of *T. reesei* (Table 1).

3.2. Analysis of plant-wall-degrading enzymes in two saprophytic and two ECM basidiomycetes

Two approaches were used to validate the use of the incomplete genome of *A. bisporigera* for analyzing its complement of secreted degradative enzymes. First, all searches were also performed against the *G. marginata* partial genome database. Because the sequences of this basidiomycete fungus were also generated by 454

Table 2
Summary of presence (Y) or absence (N), and overall distribution, of genes encoding the indicated proteins in the genomes of *C. cinerea* (C), *L. bicolor* (L), *G. marginata* (G), or *A. bisporigera* (A).

	Trichoderma reesei protein number	Name; known or predicted function	CaZy family	*Coprinopsis cinerea*	*Laccaria bicolor*	*Galerina marginata*	*Amanita bisporigera*	Distribution
1	123989	Cel7A (CBH I)	GH7	Y	N	Y	N	CG
2	49081	Cel74A (xyloglucanase)	GH74	Y	N	Y	N	CG
3	72567	Cel6A (CBH II)	GH6	Y	N	Y	N	CG
4	120229	Xylanase 3	GH10	Y	N	Y	N	CG
5	123992	Swollenin		N	N	N	N	None
6	73638	Cip1		N	N	N	N	None
7	123818	Xylanase 2	GH11	Y	N	Y	N	CG
8	123234	Peptidase S8		Y	Y	Y	Y	All
9	73643	Cel61A	GH61	Y	Y	Y	Y	All
10	120312	Cel5A	GH5	Y	Y	Y	N	CLG
11	80833	Chitinase	GH18	Y	Y	Y	Y	All
12	122081	Cel7B	GH7	Y	N	Y	N	CG
13	73897	Peptidase S1		N	N	N	N	None
14	123232	Cel12A	GH12	Y	N	Y	N	CG
15	123940	Cip2	CE15	Y	N	Y	N	CG
17	120961	Cel61B	GH61	Y	Y	Y	Y	All
18	76672	β-Glucosidase	GH3	Y	Y	Y	Y	All
19	107142	Peptidase M35		Y	N	N	N	C
20	56996	β-Mannanase	GH5	Y	Y	Y	Y	All
21	76210	Arabinosidase	GH62	Y	N	Y	N	CG
22	70800	Peptidase M36		Y	Y	Y	Y	All
23	69276	GH 30A	GH30	Y	N	N	N	C
24	121127	β-Xylosidase	GH3	Y	N	Y	N	CG
25	103049	Polygalacturonase	GH28	Y	Y	Y	N	CLG
26	110894	Endo-galactanase	GH5	N	N	Y	N	G
27	110220	Peptidase M6		N	N	N	N	None
28	124282	Copper radical oxidase		Y	Y	Y	Y	All
30	22459	Carboxypeptidase A		N	N	N	N	None
31	123283	Arabinosidase	GH54	N	N	Y	N	G
32	55319	Arabinosidase B	GH54	N	N	Y	N	G
33	105279	Peptidase M28		Y	Y	Y	N	CLG
34	81004	Peptidase A1		Y	Y	N	Y	CLA
35	45717	α-Mannosidase	GH47	Y	Y	Y	Y	All
36	44214	Acetylxylan esterase (Axe2)	CE5	Y	Y	Y	N	CLG
37	121746	β 1,3-glucanase	GH55	Y	Y	Y	Y	All
42	54219	Acetylxylan esterase	CE5	Y	Y	Y	N	CLG
43	123226	α-Trehalase	GH37	Y	Y	Y	Y	All
45	70919	Endoribonuclease		N	N	N	Y	A
46	73632	Acetylxylan esterase (Axe1)	CE5	Y	Y	Y	N	CLG
47	81070	Peptidase M28		Y	N	Y	N	CG
48	65162	GH 18A (chitinase)	GH18	Y	Y	Y	Y	All
50	49274	GH 16A	GH16	Y	Y	Y	Y	All
51	4213	Ribonuclease T2		N	Y	Y	Y	LGA
52	80240	β-Galactosidase	GH35	N	Y	Y	Y	LGA
53	75526	α-Glucuronidase	GH67	N	N	Y	Y	GA
54	65406	GH 16B	GH16	Y	Y	Y	N	CLG
55	121418	Acetyl esterase (Aes1)	CE16	Y	N	Y	N	CG
56	111894	GH 95A	GH95	N	Y	Y	Y	LGA
57	71092	Acid phosphatase		N	Y	Y	Y	LGA
58	104797	GH 3A	GH3	Y	Y	Y	Y	All
60	103039	Peptidase S51		N	N	N	N	None
	Other genes: organism and accession number							
61	*Cochliobolus carbonum* (AF095243)	β-Xylosidase (XYP1)	GH43	Y	N	Y	N	CG
62	*C. carbonum* (L48982)	Exo-polygalacturonase (PGX1)	GH28	Y	Y	Y	N	CLG
63	*C. carbonum* (M55979)	Endo-polygalacturonase (PGN1)	GH28	Y	Y	Y	N	CLG
64	*T. reesei* (CAA83846)	Endoglucanase V	GH45	N	N	Y	N	G
65	*Aspergillus kawachii* (BAB96815)	Arabinosidase	GH51	Y	N	Y	N	CG
66	*Aspergillus niger* (CAA43130)	Pectin lyase	PL1	Y	N	Y	N	CG
67	*Nectria haematococca* (AAA33338)	Pectin lyase	PL3	Y	N	N	N	C
68	*Aspergillus aculeatus* (AAA64368)	Pectin lyase	PL4	Y	N	N	N	C
69	*Aspergillus awamori* (BAA13434)	Acetyl xylan esterase	CE1	Y	N	Y	N	CG
70	*Neocallimastix patriciarum* (AAB69091)	Acetyl xylan esterase	CE2	N	N	N	N	None
71	*N. patriciarum* (AAB69092)	Acetyl xylan esterase	CE3	Y	Y	N	N	CL
72	*Glomerella lindemuthiana* (AAT68493)	Chitin deacetylase	CE4	Y	Y	Y	Y	All
73	*N. patriciarum* (AAB69090)	Acetyl xylan esterase	CE6	N	N	N	N	None
74	*C. carbonum* (AF159252)	Pectin methylesterase (PME1)	CE8	N	Y	Y	N	LG
75	*Candida albicans* (AAF04335)	N-Acetylglucosamine 6-phosphate deacetylase	CE9	Y	N	Y	N	CG
76	*Aspergillus niger* (CAC41360)	Rhamnogalacturonan acetyl esterase	CE12	Y	N	Y	N	CG
	Totals			48	31	52	22	

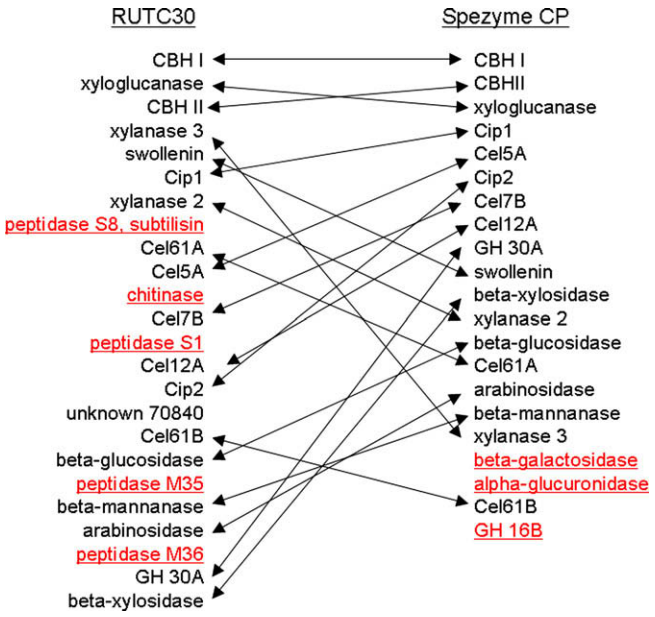

Fig. 1. Comparison of proteins found in the secretome of *Trichoderma reesei* RUTC30 grown on corn stover and Spezyme CP. Proteins are ordered by relative abundance in RUTC30 based on total spectral counts. Arrows connect the same proteins. Proteins found in one but not the other are underlined in red. Only the most abundant proteins are shown (see Table 1). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

pyrosequencing and are therefore also composed of hundreds of thousands, randomly spaced, short reads, observation of a gene in *G. marginata* that is unobservable in *A. bisporigera* strengthens the case that the gene is truly absent from *A. bisporigera*.

Second, five housekeeping proteins were used to test whether the *A. bisporigera* and *G. marginata* partial genomic sequences adequately sample their respective genomes. Enolase, β-tubulin, glyceraldehyde-3-phosphate dehydrogenase, triose phosphate isomerase, and actin from *C. cinerea* were used as queries. All of the corresponding genes were represented by multiple clear, strong hits in both *A. bisporigera* and *G. marginata* (Supplementary Table S1). We have also searched these databases with many other highly conserved proteins and have never failed to find multiple hits (unpublished results). From this we conclude that 454 pyrosequencing appears to cover the genome without significant bias. This was also the conclusion of an early 454 case study of *Neurospora crassa* (www.454.com/downloads/454_CASE_STUDY_genome_coverage.pdf).

The collection of *T. reesei* proteins (Table 1) was then used to search the genomes of *C. cinerea, L. bicolor*, *G. marginata*, and *A. bisporigera*. Proteins of completely unknown function (Table 1) were discarded as uninformative, although proteins of unknown function that could be assigned to a GH family were retained. A summary of the results are shown in Table 2. The protein numbering between Tables 1 and 2 was retained for ease of comparison. Details including expect scores, bit scores, and percent identities are provided in Supplementary Table S2. The sequences with the best hits against each search query in *A. bisporigera* and *G. marginata* are given in Supplementary Figure S1.

Not all of the CAZy GH families examined in *L. bicolor* by Martin et al. (2008) are present in the *T. reesei* secretome. Therefore, in order to make the comparative analysis of *A. bisporigera* more robust, we added some additional proteins as queries (Table 2). These included representatives of GH43 (β-xylosidase) and GH51 (arabinosidase), which are not present in the secretome of *T. reesei* (they are not in the genome, either), and GH45 (*T. reesei* endoglucanase V). *C. cinerea* and *G. marginata*, but not the two ECM fungi, have representatives of GH43 and GH51 (Table 2). Of the four fungi, only *G. marginata* has GH45 (Table 2).

T. reesei appears to lack any polysaccharide lyases (PL), including pectin lyases. *L. bicolor* has no members of PL families 1, 3, 4, or 9 (Martin et al., 2008). Biochemically characterized representatives of PL families 1, 3, and 4 were used as queries (PL9 was omitted because there are only two fungal members of PL9 in CAZy, and neither is biochemically characterized). One PL (PL1) is present only in the two saprophytic fungi, and the other two PL's (PL3 and 4) have orthologs only in *C. cinerea* (Table 1).

Two other classes of enzymes were investigated in more detail. The first was GH family 28, which contains fungal polygalacturonases (PG). PG's are the most extensively studied cell-wall-degrading enzymes from filamentous fungi, particularly from the point of view of their involvement in plant pathogenesis (Di Matteo et al., 2006). Many fungal PG's elicit strong plant defense responses, either directly or via the oligogalacturonide products that they release from pectins. Martin et al. (2008) reported that *L. bicolor* has six genes of GH family 28. The presence of at least two GH28 members in *L. bicolor* was confirmed by our results using the *T. reesei* PG (protein 103049) as query (Table 2 and Supplementary Table S2). *C. cinerea* and *G. marginata* also have orthologs of the *T. reesei* PG (Table 1). However, we could not find any evidence of any PG gene in the genome of *A. bisporigera* (Table 2, Supplementary Table S2). To confirm that this negative result was not due to reliance on a single PG as query, we analyzed the genomes of the four basidiomycetes with two additional PGs, endoPG (PGN1) and exoPG (PGX1) from the ascomycete *Cochliobolus carbonum*. Both of these enzymes are biochemically characterized (Scott-Craig et al., 1990, 1998). The results were the same as with the *T. reesei* PG, that is, at least one ortholog was found in *C. cinerea*, *L. bicolor*, and *G. marginata*, but none were found in *A. bisporigera* (Table 2). Furthermore, *A. bisporigera* does not contain orthologs of any of the six members of the putative (i.e., biochemically uncharacterized) PG family of the basidiomycete *Chondrostereum purpureum* (data not shown) (Williams et al., 2002).

The second class that was studied in more detail was the carbohydrate esterase (CE) family. This was done because of the observations that two characterized acetylxylan esterases of *T. reesei* (Axe1 and Axe2, both in CE family 5) are present in all of the fungi except *A. bisporigera* (Table 2), and Aes1 (CE16) and glucuronyl esterase (also known as Cip2; CE15) are absent in both *A. bisporigera* and *L. bicolor*. Therefore, ECM fungi, especially *A. bisporigera*, appear to be deficient in carbohydrate esterases. To examine this further, a biochemically characterized representative of each of the other classes of CE were used as TBLASTN queries against all four fungi. There are 16 families of CE in CAZy, but only CE's 1–4, 6, 8, 9, and 12 contain any fungal members. The results support the conclusion that ECM fungi, especially *A. bisporigera*, are strongly deficient in CE's as well as GH's (Table 2).

T. reesei has 10–12 Carbohydrate Binding Modules (CBM) of family 1 based on BLASTP with the last 73 amino acids of CBHI from *T. reesei*. Martin et al. (2008) reported that *L. bicolor* has only one member of CBM family 1. We were not able to find any clear evidence for any CBMs in *A. bisporigera* by TBLASTN when using CBM1 of *T. reesei* CBHI as query. In contrast, *G. marginata* has eight to twelve CBMs of family 1, with expect scores ranging from 8.00E−10 to 6.00E−04 (data not shown). Therefore, at this level also, the ECM fungus *A. bisporigera* appears to be deficient in the capacity to interact with plant cell wall polysaccharides.

Collectively, our analysis indicates that *A. bisporigera*, like *L. bicolor*, is deficient in secreted enzymes that act on plant cell walls (Table 2). Orthologs of 18 of the 76 *T. reesei* proteins in Table 2 are present in the two saprophytic fungi but not the two ECM fungi. Of these, 11 are GH's, five are CE's, one is a PL, and one is a pep-

tidase. Fifteen are present in all four fungi, eight are in none, and the others show different patterns of distribution. Of the total number of analyzed proteins, 48 and 52 are present in the two saprophytes, *C. cinerea* and *G. marginata*, respectively, whereas only 31 and 22 are present in the two ECM fungi, *L. bicolor* and *A. bisporigera*, respectively. Based on presence or absence of carbohydrate-active families of GH, PL, and CE, the two saprophytes have at least one member of 38 and 41 families, respectively, and the two ECM fungi have 22 and 18, respectively. In other words, at the family level the ECM fungi have ~50% as many as the saprophytes.

If the analysis is restricted to GH's, 18 of the 35 GH's secreted by *T. reesei* (51%), representing 25 families, have no orthologs in either of the two ECM fungi, as opposed to 30 (86%) that are present in both of the two saprophytes. This might still underestimate the paucity of plant cell-wall-degrading enzymes in the ECM fungi, because some of the GH and CE enzymes of *T. reesei* are active on polysaccharides not found in plant cell walls, or might have functions other than supporting saprophytic growth on plant cell walls, such as remodeling fungal cell walls during growth and development. Such proteins include two GH18 proteins (encoding putative chitinases), β1,3-glucanase (fungal but not plant cell walls contain large amounts of β1,3-glucan), α-trehalase, two β-glucosidases (a large family that has many different substrates), and chitin deacetylase. All of these are present in all four fungi, which is consistent with them having an endogenous role in fungal growth and development and not saprophytic growth. β-Mannanase and α-mannosidase are also present in all four fungi, but because mannans and mannose are present in both plant and fungal cell walls, no conclusions can be deduced about their possible functions in either fungal growth or interaction with plants.

Of 10 peptidases in the *T. reesei* secretome, three have orthologs in all four fungi, three in none of the fungi, and the others have different patterns. An ortholog of one peptidase (81070) was present only in the two ECM fungi. Although plant cell walls contain structural and enzymatic proteins, secreted peptidases have many possible functions other than degradation of plant cell wall proteins (e.g., remodeling of fungal wall proteins or degradation of nonplant proteins). Therefore, it is not surprising that peptidases show no particular pattern of distribution among the four analyzed basidiomycetes.

Some encoding genes were found in only a single one of the four fungi. There were four genes specific to *G. marginata* (endo-galactanase, two arabinosidases, and endoglucanase V), four specific to *C. cinerea* (peptidase M35, GH30A, and two pectin lyases), and one specific to *A. bisporigera* (ribonuclease 70919). It is difficult to see any correlation between these proteins and the biology of the respective fungi.

4. Discussion

4.1. Proteomics of T. reesei

Proteomics has been used to evaluate the secreted proteins of a number of fungi grown *in vitro* and *in vivo* (Bouws et al., 2008; Paper et al., 2007). Despite its industrial importance, there have been few published proteomics studies of *T. reesei* (Vinzant et al., 2001; Bouws et al., 2008). With the completion of the genome sequence of *T. reesei*, it is now possible to obtain much more information from MS-based proteomics methods that do not rely on 2-D gels or orthology to proteins from other organisms (Martinez et al., 2008). Our results indicate that, as a conservative estimate, *T. reesei* secretes at least 80 proteins, many of which are biochemically and genetically characterized glycosyl hydrolases, peptidases, and carbohydrate esterases. Only one putative oxidoreductase was detected in the secretome of *T. reesei*, in contrast to some other fungi, such as *P. chrysosporium*, in which 13% of the secreted proteins are oxidoreductases such as lignin peroxidase (Vanden Wymelenberg et al., 2006).

A commercial "cellulase" preparation, Spezyme CP, known to be made by fermentation of *T. reesei*, is qualitatively similar to *T. reesei* RUTC30 grown on corn stover, except for the absence of multiple, abundant proteases in the commercial preparation. From this result, one can deduce that either the commercial strain of *T. reesei* used to make Spezyme CP has been engineered to delete or downregulate the protease genes, in order to enhance long term stability of the other enzymes, or that industrial growth conditions are adjusted to suppress protease production.

4.2. Analysis of cell-wall-degrading enzymes in the ECM fungus A. bisporigera

The validity of using a partial genome sequence to study the presence and absence of plant cell wall-active enzymes in the ECM fungus *A. bisporigera* is supported by comparison to *G. marginata*, for which a similar survey genome sequence is available, and by the demonstration that genes for multiple housekeeping proteins are detectable in the genome sequences.

The *T. reesei* proteins were used as a representative set of extracellular plant cell wall-active enzymes to analyze the genomic potential of the ECM fungus *A. bisporigera* to make the same kind of enzymes. Several factors legitimize this approach. First, *T. reesei* is one of the best, if not the best, characterized fungi from the point of view of the combined genetic and biochemical analysis of its secreted degradative proteins, particularly ones active on plant cell wall polysaccharides (Martinez et al., 2008). Second, *T. reesei* makes a large number of such enzymes, giving good representation of the biochemical breadth of the Kingdom Mycota. Third, the analysis was strengthened by adding proteins from GH, PL, and CE families not present in *T. reesei*, as well as by adding additional representatives of GH28. The resulting set of proteins used in the analysis is thus comparable in scope to that used by Martin et al. (2008).

4.3. Comparison of A. bisporigera, L. bicolor, and other ECM fungi

Our analysis of the genome of *A. bisporigera* supports the conclusion of Martin et al. (2008) in regard to *L. bicolor* that at least these two ECM fungi lack the genomic potential to biosynthesize many extracellular enzymes that are active on linkages found in plant cell walls. Furthermore, although many fungi have multiple representatives of many GH families (i.e., there is high genetic redundancy), even single representatives of many of the major groups of glycosyl hydrolases and carbohydrate esterases are absent from the genomes of either of these two fungi. Of particular note, both *L. bicolor* and *A. bisporigera* lack any apparent enzymes related to CBHI or CBHII (exocellobiohydrolases), which are central fungal enzymes in the degradation of cellulose.

A. bisporigera appears to be somewhat more deficient than *L. bicolor* in extracellular enzymes active on plant cell walls. For example, *L. bicolor* but not *A. bisporigera* has genes encoding enzymes in GH family 28. In regard to carbohydrate esterases (CE's), whereas *L. bicolor* has genes encoding four CE families (3, 4, 5, 8), *A. bisporigera* has only one, CE4. Even this family is more likely to be involved in modification or degradation of fungal rather than plant walls because it contains chitin deacetylases.

A. bisporigera and *L. bicolor* are not completely devoid of extracellular degradative enzymes. Their genomes secrete multiple peptidases, as well as GH's in families 3, 5, 16, 18, 37, 47, 55, 61, 67, and 95 (Table 2; Martin et al., 2008). Of these, GH families 5, 18, 47, and 55 contain β-mannanases, chitinases, α-mannosidases, and β1,3-glucanases, respectively, whose substrates are major components of fungal cell walls. Therefore, these might be in-

volved in growth-related remodeling of the fungal cell wall or saprophytism on other fungi, and not in degradation of plant cell wall polymers. Family 37 contains α-trehalases, which are not plant cell-wall-degrading enzymes. GH3 and GH16 are both large families containing enzymes that act on many different types of substrates. Therefore, of the GH's that are potentially made by ECM fungi, most could have functions other than degradation of plant cell wall polymers.

Based on our results and those of Martin et al. (2008), it appears that the presence or absence of a large complement of extracellular enzymes is not correlated with ability to grow saprophytically. *L. bicolor* grows readily in culture and forms fruiting bodies on a variety of mycological media (Kropp and Fortin, 1988), but *A. bisporigera* grows extremely slowly (~1 cm/month) and does not form fruiting bodies in culture. *G. marginata*, which does have many enzymes, grows only slightly better than *A. bisporigera*. Therefore, the presence or absence of high plant-wall-degrading potential appears to correlate better with ecological niche, namely, being ectomycorrhizal, than with ability to grow saprophytically.

There are two plausible hypotheses to explain the association between loss of enzymes and the ECM ecological niche. One is that ECM fungi have lost these genes because they are no longer needed, i.e., once ECM fungi evolved to obtain their reduced carbon from their hosts and not, like many other fungi, from nonliving plant debris, their extracellular degradative enzymes no longer contributed to their fitness and were thus eventually lost by mutation. The second hypothesis is that the genes have been lost because the enzymes are unfavorable to the symbiotic interaction. Extracellular enzymes that degrade plant cell wall polymers can be detrimental to plants either by causing direct damage to potential host plants, or by releasing elicitor-active oligosaccharide fragments that induce plant defenses, including the hypersensitive response, which results in plant cell death (Samac and Graham, 2007).

Considering that there are >5000 species of ECM fungi, it is possible that the pattern of enzymes observed in *L. bicolor* and *A. bisporigera* is not universally true for ECM fungi. Previous studies have detected cellulases, xylanases, and other plant cell-wall-degrading enzymes in other ECM fungi, including *A. regalis* (syn. *A. muscaria* var. *umbrina*), although apparently in no case have these observations been confirmed by identification of the responsible enzymes or genes (Cairney and Burke, 1994; Cao and Crawford, 1993; Colpaert and van Laere, 1996; Hutchison, 1990; Maijala et al., 1991; Terashita et al., 1995). *L. bicolor* and *A. bisporigera* may form one end of a continuum of saprophytic capacity among ECM fungi (Read and Perez-Moreno, 2003). Because many ECM fungi, including *A. bisporigera*, grow prohibitively slowly in culture, genomic analysis is the only way to analyze their potential for biosynthesizing secreted degradative enzymes. The present work illustrates the value of even a partial genomic sequence for addressing such questions.

The genera *Coprinopsis*, *Laccaria*, and *Galerina* are all in the Agaricoid clade of the Agaricales, whereas *Amanita* is in the Pluteoid clade. There are at least 11 independent origins of the ECM habit in the Euagarics (Matheny et al., 2006), and this trait appears to have evolved independently in *Amanita* and *Laccaria*. Loss of cell-wall-degrading enzymes has therefore also evolved independently more than once.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.fgb.2009.02.001.

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