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Functional Analysis of the Degradation of Cellulosic Substrates by a *Chaetomium globosum* Endophytic Isolate

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Most photosynthetically fixed carbon is contained in cell wall polymers present in plant biomasses, the largest organic carbon source in the biosphere. The degradation of these polymers for biotechnological purposes requires the combined action of several enzymes. To identify new activities, we examined which enzymes are activated by an endophytic strain of *Chaetomium globosum* to degrade cellulose-containing substrates. After biochemical analyses of the secretome of the fungus grown on cellulose or woody substrates, we took advantage of the available genomic data to identify potentially involved genes. After *in silico* identification of putative genes encoding either proteins able to bind to cellulose or glycohydrolases (GHs) of family 7, we investigated their transcript levels by reverse transcription-quantitative PCR (RT-qPCR). Our data suggest that eight genes compose the core of the cellulose-degrading system of *C. globosum*. Notably, the related enzymes belong structurally to the well-described GH families 5, 6, 7, 16, and 45, which are known to be the core of the cellulose degradation systems of several ascomycetes. The high expression levels of cellobiose dehydrogenase and two GH 61 enzymes suggest the involvement of this oxidoreductive synergic system in *C. globosum*. Transcript analysis along with relevant coding sequence (CDS) isolation and expression of recombinant proteins proved to be a key strategy for the determination of the features of two endoglucanases used by *C. globosum* for the first attack of crystalline cellulose. Finally, the possible involvement of transcriptional regulators described for other ascomycetes is discussed.

Plant biomass is the most abundant organic carbon source in the biosphere, and a large part of this carbon is contained in cell wall polymers (38). Cellulose, hemicelluloses, and lignin, in association with pectins and structural proteins, make a very complex and hardly accessible structure; the industrial degradation of this material to monosaccharides requires extreme physicochemical conditions and/or a number of different enzymes. Of great interest, especially for biotechnological purposes, is the degradation of crystalline cellulose fibers performed by three different classes of enzymes: endoglucanases (EGs) (EC 3.2.1.4), which break the intrachain beta bonds; cellobiohydrolases (CBHs) (EC 3.2.1.91), which hydrolyze 1,4-beta-D-glycosidic bonds, releasing cellobiose from both ends of the chain; and β-glucosidases (BGs) (EC 3.2.1.21), which hydrolyze cellobiose and terminal nonreducing β-D-glucosyl residues, releasing glucose (37). Moreover, oxidoreductive enzymes such as cellobiose dehydrogenase (CDH) (EC 1.1.99.18) may act synergically with canonical glycohydrolases (GHs), accelerating the enzymatic conversion of polysaccharides (24, 30, 35). In most fungi, the degradation of crystalline cellulose is carried out by a set of cellulases that contain a GH domain that may be connected by a flexible linker peptide to the fungal carbohydrate binding domain 1 (CBM1), which belongs to the family 1 carbohydrate binding modules (62). All studied CBM1s are characterized by high amino acid sequence conservation, independent of their position at the C or N terminus of the enzyme (44). Nevertheless, this domain is not limited to cellulases, since it was also described to increase the activity of enzymes involved in the degradation of hemicelluloses and other cell wall components (29).

Within the extremely biologically and ecologically diverse kingdom of fungi, the endophytes represent a new source of potentially useful microorganisms, mainly in the field of fungal biotechnology (5). Many plants harbor endosymbiotic fungi within their tissues as a result of symptomless intercellular infections, and since fungal endophytes interact biochemically with host tissues for the survival of the symbiotism, their physiological adaptation and metabolic activity deserve a careful examination (5). During a mycological survey focused on the investigation of endophytic fungi of *Vitis vinifera* L. (46, 47), the recovery of quiescent filamentous fungi from nonsymptomatic leaves of the "Cabernet Sauvignon" and "Chardonnay" cultivars showed the prevalence of a strain of *Chaetomium globosum* Kunze (Chaetomiaceae, Ascomycota), which belongs to a genus whose species are frequently reported to be cellulase producers with the ability to degrade cellulosic materials (15, 34, 52). Biochemical studies performed on related, nonendophytic species, i.e., *Chaetomium cellulolyticum* Chahal and Hawksworth and *Chaetomium erraticum* Ames, revealed that a small number of enzymes are part of the core of the cellulase complex, namely, three endoglucanases and one β-glucosidase (4, 52). The production of said enzymes was found to depend on the growth substrate (54); however, no transcriptional studies were available for *Chaetomium* species. Conversely, studies on other cellulolytic fungi reported that transcriptional regulation is central for the expression of cellulases (19, 31, 41, 59), and several transcription regulators have been characterized, especially for ascomycetes described as cellulase producers. XlnR and Xyr1 are two orthologous transcriptional regulators, found in

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Aspergillus species and *Hypocrea jecorina* (*Trichoderma reesei* Simmons), respectively, which are considered to be crucial for transcriptional activation of cellulase and hemicellulase genes in response to the available carbon source (3, 22, 56). Additionally, two other cellulase-specific transcriptional regulators were described for *H. jecorina*, namely, Ace1 and Ace2 (reviewed in reference 57). *H. jecorina ace1* deletion mutants showed an increased level of expression of both cellulases and hemicellulases, suggesting that Ace1 may act as a transcriptional repressor. However, the same factor acts as an activator in *Saccharomyces cerevisiae* (6). Recent investigations on the role of Ace2 in *H. jecorina* elucidated its role in the transcriptional activation of xylanase and *xyr1* genes (58). Nonetheless, orthologues of *ace2* were not found in the genome of an *Aspergillus* sp. (57). Moreover, broad-range regulators such as the carbon catabolite repressor (CreA in *Aspergillus* sp. or Cre1 in *H. jecorina*) may modulate cellulase gene transcription. The latter is present in a large number of fungal species, including yeast, and is involved in transcriptional repression in response to a readily available carbon source (7, 18, 31). Additionally, the Hap2/3/5 complex, which binds to the *cis* element (known as the "CCAAT motif"), is found in regulative elements of many cellulases and hemicellulases (reviewed in reference 7).

The availability of genomic data for *C. globosum* (http://www.broad.mit.edu) prompted us to use a postgenomic transcriptional approach to identify those genes actually involved in the degradation of crystalline cellulose contained in lignocellulosic substrates and to verify if they share a regulation system with other ascomycetes. This information will allow us to functionally characterize the *C. globosum* genes involved in crystalline cellulose breakdown. Moreover, these analyses will possibly contribute to the isolation of new fungal enzymes necessary to degrade the cellulose present in wood biomasses for use in industrial applications such as bioethanol production, textile biopreparation, and extraction of peculiar sugars (23, 43, 51).

MATERIALS AND METHODS

Biological material and experimental plan. *Chaetomium globosum* was detected endophytically in nonsymptomatic foliar tissues of *Vitis vinifera* plants of both the "Cabernet Sauvignon" and "Chardonnay" cultivars, collected in northern Italy during two vegetative seasons (2007 to 2008). The strain was isolated following the procedure of Cardinali et al. (13), which was already tested in a previous survey similarly focused on the study of the endophytic community of grapes (46). The protocol was based on the fragmentation of leaves in correspondence with foliar veins, superficial sterilization of single tissue pieces (10 × 10 mm) by a 3-step immersion process (100% ethanol-3% sodium hypochlorite-100% ethanol), and deposition onto the rich medium malt extract agar (2%). Samples were incubated at 25°C under 12-h daylight conditions and constantly observed at low magnification (×50) for 2 months. Assignation to the *C. globosum* species was confirmed by sequencing of the rRNA spacers (see Fig. S1 in the supplemental material). *Chaetomium globosum* developed an aerial mycelium after 2 to 3 weeks of incubation, while ascocarp production required an additional 7 to 10 days. The stock culture was maintained on slants of potato dextrose agar, while strain sporulation was stimulated by means of soaked paper disks as growth substrates. A *C. globosum* inoculum of 40 mature ascocarps was drawn aseptically from paper suspended in 1 ml of sterile distilled water and finally added to different lignocellulosic substrates. A modified *Aspergillus niger* medium [6 g liter^{-1} KNO_3, 0.52 g liter^{-1} NaCl, 0.52 g liter^{-1} $MgSO_4 \cdot 7H_2O$, 1.52 g liter^{-1} NaH_2PO_4, and trace elements, i.e., 100 mg liter^{-1} EDTA, 22.8 mg liter^{-1} H_3BO_3, 44 mg liter^{-1} $ZnSO_4 \cdot 7H_2O$, 10.12 mg liter^{-1} $MnC_2 \cdot 4H_2O$, 9.98 mg liter^{-1} $FeSO_4 \cdot 7H_2O$, 3.22 mg liter^{-1} $CoCl_2 \cdot 6H_2O$, 3.14 mg liter^{-1} $CuSO_4 \cdot 5H_2O$, 2.2 mg liter^{-1} $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, pH 5] containing 10 g liter^{-1} of a carbon source, namely, glucose, milled poplar wood (MPW), or microcrystalline cellulose (MCC), was used. MPW was obtained from poplar branch wood that was ball milled for 10 min with a model MM301 miller (Retsch). Fungal growth was controlled macro- and microscopically in order to check *C. globosum* colonization and maintenance of axenic conditions.

Experiments were performed at least in triplicate during the whole duration of the growth cycle of *C. globosum*, from the inoculum until the formation of new ascocarps. Specifically, the mycelium of *Chaetomium globosum* was harvested and 100-mg aliquots collected from each sample, frozen in liquid nitrogen, and stored at −80°C for gene expression analyses, while the remaining biomass was used for the determination of fungal growth. The spent growth medium was collected by filtration through a 0.20-μm nylon filter (Sartorius Stedim Biotech S.A.) and then concentrated using a Vivaspin 2 minifilter (Sartorius Stedim Biotech S.A.) (10-kDa molecular mass cutoff). In order to remove soluble sugars, a second spin was performed with a 0.7 volume of 0.05 M sodium acetate buffer (pH 5.5), and the retained fraction, containing proteins secreted from the fungus, was recovered. The protein concentration was determined with the standard Bradford method (7). Growth kinetic measurements and analyses of enzymatic activities were performed every 2 days, while gene expression analyses were done on day 7 after inoculation.

Growth kinetics measurement. Growth kinetics evaluations were carried out by determining the amount of NaOH-soluble protein in the biomass-carbonium source mixture, according to the method of Mach-Aigner and coworkers (37), for mycelia collected 3, 5, 7, and 11 days after inoculation. Harvested mycelia were weighed, suspended in an equal volume of 0.1 M NaOH in a reaction tube, and then homogenized by means of an Ultra Turrax T10 homogenizer (IKA). After incubation at room temperature for 3 h, the samples were centrifuged at 18,000 × *g* and 4°C for 5 min. Protein concentration in the supernatant was determined by the standard Bradford method (9), and the results were used for estimation of the total amount of mycelium (37).

Determination of enzyme activities. To assay the glycolytic activity, 1 μg of soluble protein was added to 500 μl of 0.05 M sodium acetate buffer (pH 5.5) containing substrate, namely, 1% carboxymethyl cellulose (CMC), 1% birch wood xylan (Sigma), 1% MCC, or 0.1% acid-swollen cellulose (ASC). The last substrate was prepared in accordance with the work of Zhang and coworkers (63), with minor modifications. In order to discriminate between the degradative EG and CBH activities of recombinant enzymes, commercial BG (NS22118; Novozymes) was added as specified by the manufacturer, and the incubation was performed at 40°C for 1 or 16 h. The amount of released reducing sugars was determined by employment of dinitrosalicylic acid reagent according to the method of Ghose (21), with the following minor modifications. A 500-μl aliquot of the incubated extract was diluted 1:1 with water and added to 500 μl of dinitrosalicylic acid reagent in an Eppendorf tube. The mixture was boiled for 5 min and then cooled to room temperature. Absorption was recorded at 540 nm (reducing sugars) and 700 nm (turbidity) against an enzyme blank, and sugar concentrations were determined using a standard curve prepared with increasing amounts of glucose (0.2 to 0.5 mg ml^{-1}). One unit (U) of enzyme activity was defined as the amount of enzyme producing 1 μmol of glucose equivalents per minute (43).

CDH activity was assayed according to the method of Baminger and coworkers (8), by measuring the decrease in absorbance of the electron acceptor 2,6-dichlorophenol-indophenol (DCPIP) at 520 nm ($\varepsilon = 6.8 \times 10^3$ M^{-1} cm^{-1}), pH 4.0, and 37°C. One microgram of soluble protein was added to 1 ml of sodium acetate buffer, pH 4.0, containing 0.3 mM DCPIP, 30 mM lactose, and 4 mM NaF, and the decrease in absorbance was recorded during the first 5 min. One unit (U) of enzyme activity was defined as the amount of enzyme reducing 1 μmol of DCPIP per min (8).

***In silico* sequence analysis.** Sequence analysis was performed using the BLAST method offered by the NCBI website (http://blast.ncbi.nlm.nih.gov/Blast.cgi). A protein-protein alignment was performed using the

NCBI protein database and predicted protein products from the Broad Institute *C. globosum* site (http://www.broadinstitute.org/annotation/genome/chaetomium_globosum.2/Home.html; B. Birren et al., unpublished data).

Investigation settings were as follows: matrix, BLOSUM62; gap cost existence, 11; and extension, 1. Domain identification was performed using InterProScan (http://www.ebi.ac.uk/Tools/InterProScan/) set with default parameters and queried with the predicted protein product (36) and using NCBI CD-Search with default parameters (39), and GH function was inferred on the basis of the CAZy database (http://www.cazy.org; 12). The promoter binding sites were analyzed using the SCOPE motif finder (http://genie.dartmouth.edu/scope/; 14).

RNA extraction and cDNA synthesis. RNA extraction was performed using freshly collected mycelia. Samples were ground with liquid nitrogen and then processed using an Aurum RNA Fatty and Fibrous kit (Bio-Rad). RNA integrity was evaluated by 1% agarose gel electrophoresis. A 9-μl sample of the extracted RNA was retro-transcribed using the ImProm-II reverse transcription system (Promega) with oligo(dT) as the primer, following the manufacturer's instructions.

Real-time RT-PCR. Real-time reverse transcription-PCR (RT-PCR) was performed with GoTaq qPCR master mix (Promega) following the manufacturer's instructions in a Rotorgene 2000 thermal cycler (Corbett), using two-step cycling conditions (95°C for 2 min, followed by 40 cycles of 95°C for 30 s and 60°C for 60 s).

Reaction mixtures were set up in duplicate, using 0.5 μl of cDNA diluted 1:4 and 0.5 μl of each primer (0.5 μM final concentration for each) in a 20-μl final volume. For each couple of primers (see Table S1 in the supplemental material), a no-template control (NTC) reaction was performed. For each reaction, the amplicon melting curve was calculated using software provided in a bundle with the Rotorgene thermal cycler, by raising the temperature from 50 to 95°C (in steps of 1°C) and plotting the fluorescence as a function of temperature. In order to assess the specificity of amplification, we looked for the presence of a single peak on the plot of the first derivative of the melting curve with respect to temperature (45). The reference genes were evaluated by means of NormFinder software (2), using the values obtained for three biological replicates and setting each growth condition as a distinct group for stability analysis purposes. Comparative quantification of each transcript was then carried out using LRE (48, 49) with respect to the histone H4.2 transcript.

Cloning of endoglucanase coding sequences. The coding sequences of CHGG_01188 and CHGG_08509 of *C. globosum* were amplified from the total cDNA by using specific primers (see Table S1 in the supplemental material). The obtained fragments were recombined by means of Gateway technology (Invitrogen) into the entry vector pDONR221 and sequenced with primers M13 and SP6 by an external company (Macrogen Inc.). The sequences were then recombined into a destination vector (pDEST17) that allows protein expression under the control of the T7 promoter, with an N-terminal fusion with a 6×His tag.

Protein expression in a bacterial system. *Escherichia coli* strain BL21(DE3) was transformed by heat shock with the pDEST17 (Invitrogen) vector containing the cloned sequence. Single-colony cultures were grown overnight and then reinoculated and grown to an optical density at 600 nm (OD_{600}) of 0.6 to 0.8. Cultures were induced with three different concentrations of IPTG (isopropyl-β-D-thiogalactopyranoside) (0.25, 0.5, and 1 mM) at three temperatures (24, 28, and 37°C) with agitation (200 rpm on an orbital shaker) for 4 h. After this, the cultures were collected, centrifuged at 12,000 × *g*, and resuspended in 50 mM acetate buffer, pH 5.5. After sonication (10 s, 3 times), the bacterial lysates were centrifuged again, the supernatant fraction was recovered, and the pellet was resuspended in 50 mM acetate buffer, pH 5.5. The cellulolytic activity was measured both in the recovered supernatant and in the resuspended pellet as described for the protein secreted from the fungus.

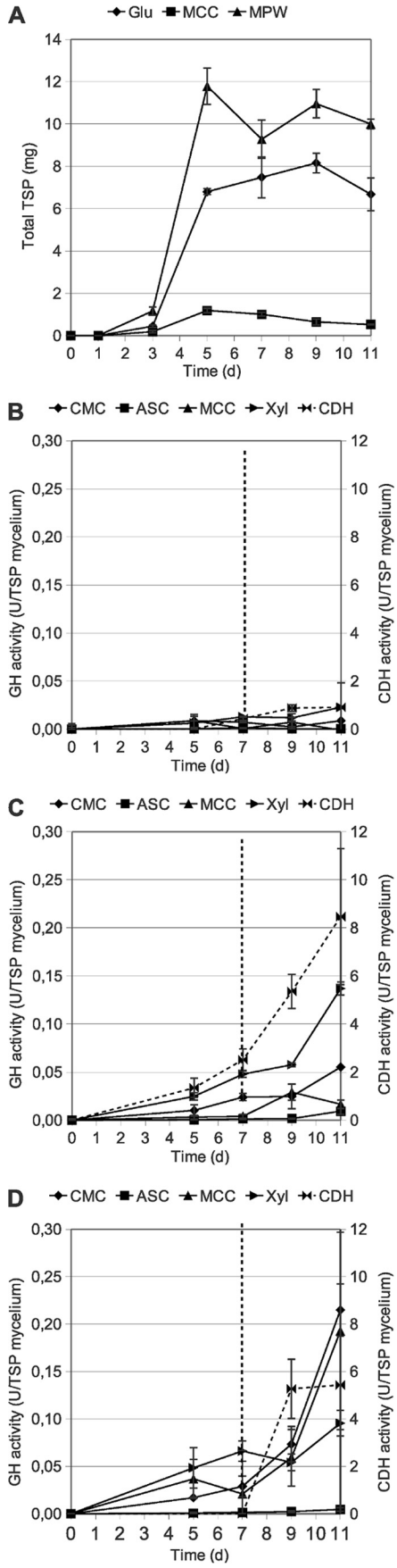
Protein electrophoresis. Protein quantification was carried out by the standard Bradford method (9). For loading into SDS-PAGE gels, the samples were solubilized on ice for 10 min in a 1/6 volume of protein loading buffer (120 mM Tris-HCl, pH 6.8, 6% [wt/vol] SDS, 20% [wt/vol] glycerol, and 0.1% bromophenol blue) and then centrifuged at 18,000 × *g* at 4°C for 5 min. Ten micrograms of protein was loaded into each well and separated by SDS-PAGE (12% acrylamide, 0.05% SDS) in accordance with the work of Laemmli (33), with minor modifications. After electrophoresis, the proteins were visualized by Coomassie blue staining or electroblotted onto a nitrocellulose membrane.

Immunoblot analysis. After electrophoresis, the proteins were electroblotted onto a nitrocellulose membrane (Protran BA 85; Whatman) according to the method of Towbin et al. (60) and blocked with 0.5% bovine serum albumin (BSA) (fatty acid free; Sigma-Aldrich). Western blotting with chemiluminescence detection was performed by standard techniques, using a mouse monoclonal anti-polyhistidine antibody (H1029; Sigma-Aldrich) and an anti-mouse IgG (whole molecule)–peroxidase antibody produced in goats (A4416; Sigma-Aldrich). Molecular weight was determined using Gel Analyzer 1.6 (http://www.gelanalyzer.com/), with reference to an unstained protein molecular weight marker (Fermentas).

RESULTS

Growth kinetics and degradative activity of *Chaetomium globosum* cultured on different carbon sources. Soluble protein determination showed that growth of *Chaetomium globosum* was strongly dependent on the carbon source available (Fig. 1A). In the presence of glucose or MPW, the maximum biomass accumulation was obtained within the first 5 to 9 days, with a higher level of growth with MPW. In the latter case, biomass accumulation at stationary phase was 45% higher than that with glucose. Conversely, MCC cultures were characterized by significantly less growth: under this condition, biomass accumulation was only about 12% of that in glucose cultures (Fig. 1A). However, under all of the aforementioned conditions, the growth cycle of *C. globosum* was completed within 11 days, with the formation of new ascocarps.

In order to determine to what extent the type of available carbon source triggers the production of cellulose and hemicellulose degradative enzymes, including oxidoreductive CDH (30), spent medium of *C. globosum* mycelia grown on glucose, MPW, or MCC was analyzed using different polysaccharidic substrates (ASC, CMC, MCC, or xylan). Due to growth differences observed on different substrates during the time course experiment, enzyme activity was expressed on the basis of biomass production in order to exclude growth effects dependent on the carbon source (Fig. 1B to D). A very low enzymatic activity was detected in the spent medium of *C. globosum* grown on glucose, indicating that when the available carbon source was this sugar monomer, the secretion of hydrolases and related enzymes was limited; therefore, this basic level was used for comparison (Fig. 1B). Conversely, the presence of a complex carbon source such as MPW or MCC stimulated the secretion of enzymes able to degrade the tested substrates (Fig. 1B to D). It is worth noting that the level of activity and specificity of the secreted enzymes were found to depend on the carbon source present in the growth medium. With respect to GH enzymes, in the case of spent MPW medium, the highest enzymatic activity was observed against xylan, while the production of cellulolytic enzymes was less stimulated (Fig. 1C). Conversely, in spent MCC medium, the highest activity levels were detected against the CMC and MCC substrates, while xylanase activity was significantly lower than that present in spent MPW medium (about 30% less at day 11) (Fig. 1D). Production of CDH was also stimulated by the presence of a complex carbon source, with slight differences in the activity levels: the accumulation of CDH in



spent MPW medium began at an early growth stage and remained constant thereafter, while in spent MCC medium this activity was detectable only at a later growth stage, reaching its maximum level at day 9 (Fig. 1C and D).

These data clearly show that the pattern of secreted enzymes depends on the carbon source available in the growth medium. Enzyme activity generally showed an increase during the course of the experiment, with some differences depending on the composition of the growth medium. The availability of genomic data for *C. globosum* prompted us to investigate at the transcriptional level the strategy used by this ascomycete to degrade cellulose-containing substrates. Since the maximum enzyme activity referred to the mycelium biomass was observed in the late growth phase of *C. globosum* (from day 9), a slightly earlier time (day 7) was chosen for determination of the related transcripts.

***In silico* identification of putative genes involved in cellulose degradation.** In order to extract the relevant information, several queries were made to mine *C. globosum* genomic data. Since the CBM1 domain is highly conserved among known processive cellulases, the first interrogation was made by using the wuBLASTP algorithm, using the consensus sequence of the canonical CBM1 reported in the NCBI CD database (accession no. Cdd: smart00236 [PLYGQCGGSGYSGPTCCASGYTCQKLNDWYSQCL]) as input (40). The results of this query led to the identification of 30 putative coding sequences (CDSs) (Table 1), one of which was previously reported to be similar only to an endoglucanase V gene (CHGG_08509) (Birren et al., unpublished data). Among the retrieved sequences, 11 CDSs encode GHs that belong to fungal families known to be involved in the degradation of cellulose or hemicelluloses (44). According to family classification based on protein structure (25–28), 6 of these 11 CDSs encode enzymes with putative EG activity. One belongs to family GH 45 (CHGG_08509), which is predicted to have only EG activity, while the remaining 5 are members of families that in addition to EG activity may display other activities, such as mannase (GH 5 family) (CHGG_01188), xylanase (GH 10 family) (CHGG_00030 and CHGG_00304) (16, 50), or exoglucanase (GH 6 family) (CHGG_08591 and CHGG_10762) activity, although the actual role of the last family is still debated. The remaining 5 retrieved genes encode GHs belonging to families that do not include EGs: CHGG_06870 belongs to the GH 11 family, whose members act as xylanases (EC 3.2.1.8); CHGG_09551 is a member of the GH 16 family, which comprises enzymes involved in the cleavage and assembly of β-glucosides in branched polysaccharides; and the CHGG_04264, CHGG_04265, and CHGG_06075 enzymes (GH 62 family) are involved in the cleavage of arabinosyl side chains of arabinoxylans and arabinans (23). On the whole, the polymers degraded by these GHs (i.e., xylans, mannans, arabinans, and arabinoxylans) are the main components of hemicelluloses, though they are also present in the pectin fraction of the cell wall (43).

FIG 1 Growth of *C. globosum* in terms of estimated biomass production (A) and activities measured in fungal media collected from cultures grown on glucose (B), MPW (C), and MCC (D). Glycohydrolytic activity on different substrates (CMC, MCC, ASC, and Xyl), as well as CDH activity, was measured. ASC, acid-swollen cellulose; CMC, carboxymethyl cellulose; GH, glycohydrolases; Glu, glucose; MCC, microcrystalline cellulose; MPW, milled poplar wood; TSP, total soluble proteins; Xyl, xylan.

TABLE 1 List of *C. globosum* genes retrieved by NCBI-BLAST queried with CBM1 (Cdd:smart00236) or GH 7 family CD (Cdd:cd07999)[a]

C. globosum gene ID	CD[b]	Predicted activity or activities[c]
EG genes		
CHGG_00030	GH 10	EC 3.2.1.8 xylanase/EC 3.2.1.4 endoglucanase
CHGG_00304	GH 10	EC 3.2.1.8 xylanase/EC 3.2.1.4 endoglucanase
CHGG_01188	GH 5	EC 3.2.1.78 mannase/EC 3.2.1.4 endoglucanase
CHGG_08509	GH 45	EC 3.2.1.4 endoglucanase
CHGG_08591	GH 6	EC 3.2.1.4 endoglucanase/EC 3.2.1.91 exoglucanase
CHGG_10762	GH 6	EC 3.2.1.4 endoglucanase/EC 3.2.1.91 exoglucanase
Other GH genes		
CHGG_04264	GH 62	EC 3.2.1.55 arabinosidase
CHGG_04265	GH 62	EC 3.2.1.55 arabinosidase
CHGG_06075	GH 62	EC 3.2.1.55 arabinosidase
CHGG_06870	GH 11	EC 3.2.1.8 xylanase
CHGG_09551	GH 16	EC 3.2.1.73 lichenase/EC 3.2.1.39 laminarase
Non-GH enzyme genes		
CHGG_00024	Cutinase	EC 3.1.1.74 cutinase
CHGG_01312	Esterase lipase superfamily	EC 3.1.1.72 acetylxylan esterase (uncertain)
CHGG_03380	CDH	EC 1.1.99.78 cellobiose dehydrogenase
CHGG_03415	GH 61	EC 3.2.1.4 endoglucanase
CHGG_04384	GH 61	EC 3.2.1.4 endoglucanase
CHGG_06059	GH 61	EC 3.2.1.4 endoglucanase
CHGG_06702	GH 61	EC 3.2.1.4 endoglucanase
CHGG_07530	Endoglucanase E-like	EC 3.2.1.4 endoglucanase
CHGG_07593	GH 61	EC 3.2.1.4 endoglucanase
CHGG_07756	GH 61	EC 3.2.1.4 endoglucanase
CHGG_08481	GH 61	EC 3.2.1.4 endoglucanase
CHGG_08545	Alpha/beta hydrolase 5	Not deducible
CHGG_09279	XynB	EC 3.2.1.8 xylanase
CHGG_09380	GH 61	EC 3.2.1.4 endoglucanase
CHGG_10137	Fatty acyltransferase	Not deducible
CHGG_10485	Alpha/beta hydrolase lipase	EC 3.1.1.- carboxylic esterase
Genes with unknown products		
CHGG_09061	VPS10 domain	Not found
CHGG_10528	Not found	Not deducible
CHGG_10774	AXE1 (part)	Uncertain (may be EC 3.1.1.72 acetylxylan esterase)
GH 7 genes		
CHGG_08330	GH 7	EC 3.2.1.4 endoglucanase/EC 3.2.1.91 exoglucanase
CHGG_01505	GH 7	EC 3.2.1.4 endoglucanase/EC 3.2.1.91 exoglucanase
CHGG_03452	GH 7	EC 3.2.1.4 endoglucanase/EC 3.2.1.91 exoglucanase
CHGG_04958	GH 7	EC 3.2.1.4 endoglucanase/EC 3.2.1.91 exoglucanase
CHGG_05657	GH 7	EC 3.2.1.4 endoglucanase/EC 3.2.1.91 exoglucanase
CHGG_08475	GH 7	EC 3.2.1.4 endoglucanase/EC 3.2.1.91 exoglucanase
CHGG_09175	GH 7	EC 3.2.1.4 endoglucanase/EC 3.2.1.91 exoglucanase

[a] CBM1, fungal carbohydrate binding domain; CD, catalytic domain; EG, endoglucanase; GH, glycohydrolase.
[b] The presence of the CD was verified by InterPro Scan (EBI) (2).
[c] Enzymatic activities were inferred according to literature data (10, 25, 37).

Among the 30 predicted CBM1-containing proteins, 19 lack a GH catalytic domain and thus were analyzed further using NCBI CD-Search in order to identify their functions (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) (39, 40). Only 8 genes encode proteins with a recognizable catalytic domain involved in degradative processes, and these were included in a "non-GH" group. This comprises an endoglucanase E-like (endo E-like) protein (CHGG_07530), a xylanase-like (XynB-like) protein (CHGG_09279), and a fatty acyltransferase-like protein (CHGG_10137), all belonging to the family of SGNH hydrolases, reported to act mainly as esterases and lipases (1). Other genes encode proteins containing an acetylxylan esterase domain (CHGG_01312), a cutinase domain (CHGG_00024), an alpha/beta hydrolase domain (CHGG_08545 and CHGG_10485), and a CDH domain (CHGG_03380). The last domain is a feature present in oxidoreductases acting on cellulose and lactose in *Chaetomium* sp. INBI 2-26(−) (11, 32). We include in this "non-GH" group 8 additional genes encoding GH 61 proteins (CHGG_03415, CHGG_04384, CHGG_06059, CHGG_06702, CHGG_07593, CHGG_07756, CHGG_08481, and CHGG_09380). In fact, in spite of the GH designation, the purified GH 61 proteins were not shown to have a direct hydrolytic activity on model cellulosic substrates (24). Remarkably, GH 61 and CDH enzymes were

TABLE 2 Evaluation of the stability of reference genes with respect to different growth conditions

Reference gene-gene product	Stability value[a]
CHGG_10298-histone H4.2	0.117
CHGG_06544-elongation factor 1 alpha	0.293
CHGG_07158-actin	0.451
CHGG_09039-tubulin beta chain	0.461
CHGG_07980-GPDH[b]	0.647

[a] Evaluated with Normfinder (2) on the basis of three biological replicates and division of the three growth conditions (glucose, MPW, and MCC) into distinct groups.
[b] GPDH, glyceraldehyde-3-phosphate dehydrogenase.

recently shown to act synergically with canonical cellulose hydrolases for cellulose degradation (30, 35).

Overall, only 3 of the 30 identified CDSs encode proteins whose function remains elusive: one encodes a vacuolar protein sorting 10-like (VPS10-like) domain (CHGG_09061), while in the remaining two CDS products (CHGG_10528 and CHGG_10774) a complete catalytic domain was not identified, although part of an acetylxylan esterase domain was recognized in the CHGG_10774 gene product.

Considering all the genes whose products may be involved in degradative processes, we assumed that 90% of the retrieved genes were potentially active in cell wall degradation or deconstruction. This strongly supports the notion that CBM1 is common to enzymes active on different substrates present in lignocellulosic biomasses in order to achieve a synergic effect.

Several studies assign a fundamental role in cellulose degradation by most fungi, including other ascomycetes such as *Neurospora crassa* and *Hypocrea jecorina* (59, 61), to members of GH family 7. In these two organisms, there are GH 7 enzymes characterized by the presence of a CBM1, even though this motif is not essential for CBH activity. Our first search for CBM1 did not retrieve any predicted GH 7 enzymes. Therefore, an additional query was made by aligning the *C. globosum* database protein sequences with the conserved motif of the GH 7 catalytic domain reported in the NCBI CD database (accession no. Cdd:cd07999). This led to the identification of 7 additional putative genes, none of which, as expected, was found to contain a CBM1 (Table 1). Considering the central role played by GH 7 enzymes in fungal cellulose degradation, we included them in the following analyses even though they did not contain a CBM1.

Gene expression pattern in response to available carbon source. All 37 gene sequences identified *in silico* were used to analyze the transcriptional response of *C. globosum* to complex carbon sources, for instance, MPW, by comparing it to the basal level on glucose, since cellulase gene transcription is reported to be induced by several plant cell wall components (19, 57). Transcript levels were measured by semiquantitative (data not shown) as well as quantitative RT-PCR (RT-qPCR), using total RNA extracted from *C. globosum* grown under the above-mentioned conditions.

In order to establish the reliability of a reference gene, a comparison among potential genes was performed. Genes encoding actin, beta tubulin, elongation factor 1α, glyceraldehyde-3-phosphate dehydrogenase, and histone H4.2 were considered (10, 20, 42, 53). When analyzed with Normfinder (2), the histone H4.2 transcript level was the most stable under the considered conditions (Table 2), and therefore it was used as a standard in subsequent experiments.

Among the 30 tested CBM1-encoding genes, only 6 showed a transcript accumulation of >0.05-fold compared to the level of histone H4.2 transcripts. Of these 6 genes, CHGG_01188 and CHGG_08509 were both predicted to encode EG enzymes and were also characterized by significant levels of expression under glucose growth conditions. The remaining 4 genes with high transcript levels were also induced in the presence of MPW but were very poorly expressed in the presence of glucose. In fact, the relative transcript accumulation increased from 15-fold (CDH CHGG_03380) to 400-fold (GH 61 CHGG_03415). The latter was the highest increase observed among all tested genes (Fig. 2). The remaining two genes, characterized by high transcript levels and highly induced in MPW culture, encode putative GH 61 (CHGG_07593) and GH 6 (CHGG_10762) proteins. With respect to the 24 genes with lower transcript levels, only 10 were induced >10-fold under MPW growth conditions. More variation was observed in the case of the GH 61 CHGG_06059 protein and the GH 10 CHGG_00304 protein, whose transcriptional increases were around 70-fold. Additionally, a second GH 10 protein (CHGG_00030) and a GH 6 protein (CHGG_08591) were both found to be induced compared to the basal level on glucose. The upregulation of the remaining 14 retrieved genes in the presence of MPW ranged from none to <10-fold (Fig. 2). It is worth noting that all induced genes encode a recognizable catalytic domain, while those predicted to encode proteins lacking a functional domain associated with CBM1 displayed not only a low transcript accumulation but also a negligible induction level under all tested conditions.

Since GH 7 EGs and CBHs are considered to be crucial for cellulose degradation in other ascomycetes (44, 59), we focused upon the expression levels of their genes in *C. globosum* even if they lacked the CBM1 domain in order to ascertain their actual involvement in these processes. Interestingly, two of them (CHGG_08475 and CHGG_08330) showed a transcript accumulation of >0.05-fold relative to histone H4.2. Remarkably, these genes were both highly induced, especially CHGG_08475, whose transcript level increased >140-fold over the basal level on glucose, thus showing the highest increase recorded among retrieved GH 7 genes, while CHGG_08330 showed a lower but significant increase (22-fold) with respect to the glucose control. The remaining 5 genes were characterized by both low transcript accumulation and low induction and thus were not considered primarily involved in complex lignocellulosic substrate degradation (Fig. 2).

Since MPW is a complex substrate which comprises cellulose and hemicelluloses along with lignin and other minor components, we have shown that it induces the transcription of a large set of degradative enzymes that may be involved differentially in depolymerization of various substrates. In order to identify if these enzymes are also induced specifically in the presence of MCC, a second expression analysis was performed, but supplying this carbon source instead of MPW. We focused on genes encoding GHs that, by sequence analysis, are able to degrade both cellulose and hemicelluloses, i.e., those genes that are included in the "EG," "other GH," and "GH 7" groups. All genes belonging to the last group showed lower transcript levels in the presence of MCC, indicating that they are not strongly induced under this condition (Fig. 3). As for the "EG" group, two genes which were highly expressed on MPW (CHGG_01188 and CHGG_10762) showed the same expression levels on MCC (93% ± 13% and 96% ± 2%, respectively). Transcription of the other genes of this group was

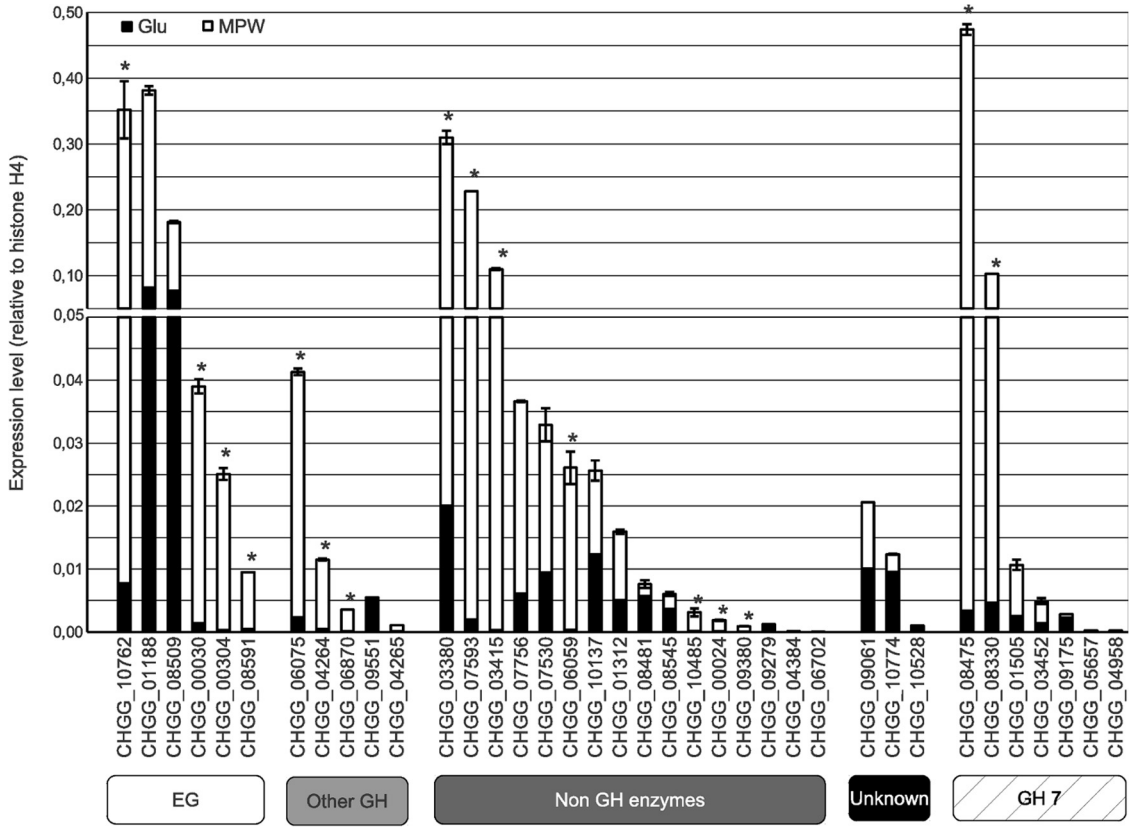


FIG 2 Expression levels of analyzed genes compared to histone H4 (expression level = 1), measured by RT-qPCR using total RNA. Black bars report the expression levels under glucose growth conditions, and white bars report the expression levels of the fungus grown on MPW. *, genes induced >10-fold under MPW growth conditions compared to glucose growth conditions. Data are from at least two independent experiments, and error bars show standard deviations. EG, endoglucanases; GH, glycohydrolases; MPW, milled poplar wood.

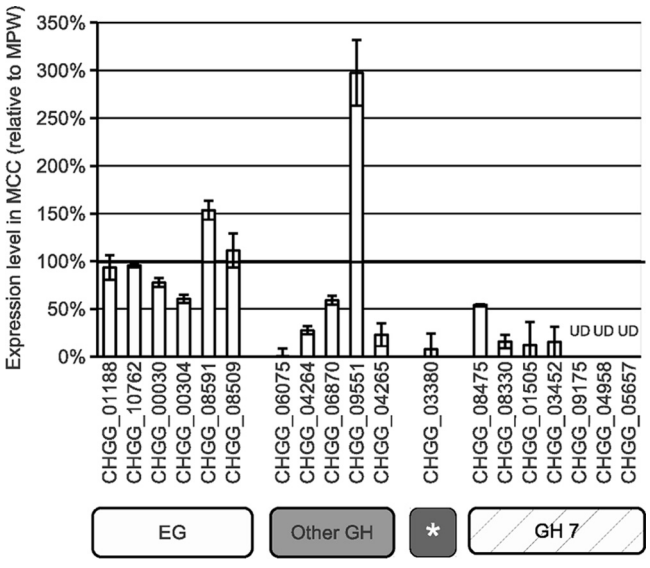


FIG 3 Expression levels in *C. globosum* grown on MCC with respect to those on MPW, measured by RT-qPCR. Data are from at least two independent experiments, and error bars show standard deviations. *, non-GH enzyme. EG, endoglucanases; GH, glycohydrolases; MCC, microcrystalline cellulose; MPW, milled poplar wood; UD, undetectable.

slightly modulated depending on the growth conditions: the putative GH 6 gene (CHGG_08591) showed a little (154% ± 10%) transcript increase in the presence of MCC compared to MPW, while the two GH 10 genes showed slightly lower transcript levels on MCC (78% ± 5% and 60% ± 5% for CHGG_00030 and CHGG_00304, respectively). As for the GH 45 gene, the relative transcript accumulation levels were very similar under these two growing conditions (111% ± 18%). The genes included in the "other GH" group were characterized by a lower expression level with MCC than with MPW, with the notable exception of the unique GH 16 gene (CHGG_09551), whose expression increased nearly 3-fold (297% ± 35%). With regard to CDH, a "non-GH" enzyme, the accumulation of relevant transcripts was much lower with MCC than with MPW (7% ± 17%). Interestingly, transcript accumulation paralleled the measured CDH activity.

Production and activity of recombinant GH 5 and GH 45 endoglucanases. Among the 37 analyzed genes, only 2 (CHGG_01188 and CHGG_08509, encoding a GH 5 and a GH 45 enzyme, respectively) were also expressed at relevant levels under glucose growth conditions. Since the first cellulases involved in substrate degradation are usually processive EGs belonging to those families (7), we hypothesized that these two enzymes may be involved in the initial degradation of crystalline cellulose by *C. globosum*. An alternative hypothesis is that these genes are expressed constitutively by the fungus but their products are not able to degrade cellulose. In order to assess their actual

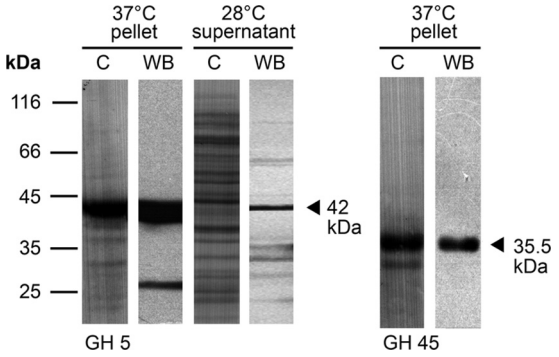


FIG 4 Electrophoretic analyses of recombinant GH 5 (CHGG_01188) and GH 45 (CHGG_08509) proteins. Expression in *E. coli* was induced with 1 mM IPTG for 4 h at different temperatures. C, polyacrylamide gel stained with Coomassie blue; WB, immunoblot detection of His-tagged proteins.

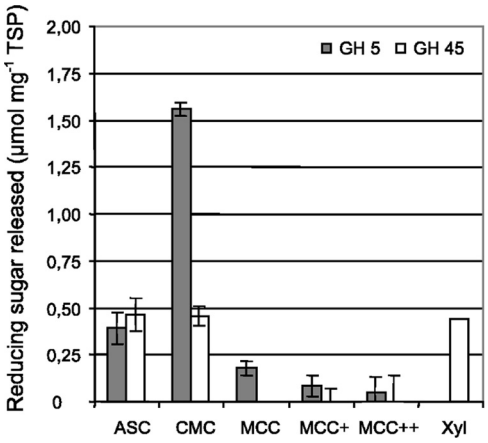


FIG 5 Specific activities of GH 5 and GH 45 enzymes in *E. coli* raw extracts toward cellulose- and hemicellulose-containing substrates after 16 h of incubation at 40°C. All substrates were present at 1% in 50 mM acetate buffer, pH 5.5. "MCC+" and "MCC++" indicate MCC plus 1 and 2 μl of BG (NS22118; Novozymes), respectively, added according to the manufacturer's instructions. Data are from at least three independent experiments, and error bars show standard deviations. ASC, acid-swollen cellulose; BG, β-glucosidases; CMC, carboxymethyl cellulose; MCC, microcrystalline cellulose; TSP, total soluble proteins; Xyl, xylan.

activity toward plant cell wall polysaccharides, we produced recombinant proteins in *E. coli* in order to assay their biochemical activity *in vitro*.

The relevant cDNAs were initially cloned into the vector pDONR221 for sequence control. The obtained sequence of the GH 5 gene was identical to those deposited in the *C. globosum* genomic database. In the case of the GH 45 gene (CHGG_08509), a unique difference was observed: the third predicted intron (*Chaetomium globosum* Sequencing Project [http://www.broad.mit.edu]) was retained in the amplified cDNA, while the first and second were removed as expected. Nonetheless, this variation did not affect the reading frame of the remaining portion of the CDS or the predicted protein's conserved domains. The two CDSs were then recombined in the vector pDEST17 (Invitrogen), and their expression was induced at different temperatures and IPTG concentrations. For each induction condition, the protein expression level was analyzed by means of immunoblotting with an anti-polyhistidine monoclonal antibody. The most satisfactory expression condition for both proteins was 4 h of induction with 1 mM IPTG. The GH 5 protein was detected in the insoluble fraction of *E. coli* cells grown at 37°C, with a differential band also clearly visible in Coomassie blue-stained gels, and in the soluble fraction at 28°C, although in smaller amounts. In both cases, the antibody reacted with a protein with an apparent molecular mass of 42 kDa, confirming the deduced molecular mass of 41.751 kDa. The GH 45 protein accumulated in the insoluble fraction of cells grown at 37°C (Fig. 4). The deduced molecular mass of the GH 45 protein was 35.5 kDa, corresponding to the calculated molecular mass of 35.428 kDa. The GH 45 protein was barely detected following induction at 28°C, and always in the insoluble fraction (data not shown).

In order to ascertain the enzymatic activity, the recombinant proteins were tested *in vitro* by assaying the reducing sugars released after overnight incubation with water-soluble and water-insoluble cellulose forms (Fig. 5). In soluble fractions obtained after induction, glycolytic activity toward ASC was detected for both GH 5 (0.39 ± 0.09 μmol mg^{-1} total soluble proteins [TSP]) and GH 45 (0.46 ± 0.20 μmol mg^{-1} TSP) enzymes (Fig. 5). The assay was also performed with amorphous CMC and pure crystalline MCC in order to discriminate between EG and CBH activities. Moreover, since the stimulatory effect of BGs on MCC hydrolysis performed by CBHs could be quite high, we tested the activity of GH 5 and GH 45 recombinant enzymes in the presence of increasing amounts of a commercial BG preparation (NS22118; Novozymes) (Fig. 5). Glycohydrolytic activity was detected for both recombinant enzymes: toward CMC, the activity levels were comparable, while toward ASC the GH 5 enzyme activity was higher. Conversely, in both cases, a very low activity was detected toward MCC, and the addition of BG did not have any significant stimulatory effects on hydrolysis within an incubation time of 2 to 120 h (data not shown). Enzyme activity was also measured using birch wood xylan as a substrate. The activity against this kind of hemicellulose was undetectable in the case of the GH 5 enzyme and relatively high in the case of the GH 45 enzyme (Fig. 5). A direct comparison of the differential levels of activity of the two enzymes was not possible, because the activity was related more to the amount of the recombinant protein present in the soluble fraction than to the absolute enzyme activity. However, these results show the contrasting behavior of these two enzymes. The GH 5 enzyme was more effective at releasing sugars from amorphous CMC than from crystalline cellulose-containing substrates (ASC or MCC), while the GH 45 enzyme was equally effective on ASC, CMC, and, interestingly, hemicellulose.

***In silico* analysis of transcriptional regulators.** In a preliminary attempt to evaluate whether the transcriptional regulation of cellulose-degrading genes of *C. globosum* shares common features with that of other studied ascomycetes, we used an *in silico* approach for the identification of known conserved regulators and related *cis* elements, searching for putative homologous transcription regulators (Table 3).

The *C. globosum* database contains a putative protein whose sequence is similar to that of *A. niger* XlnR; however, the predicted protein (CHGG_03981) does not contain any DNA-binding domain, and thus it is unlikely that it is directly involved in transcriptional regulation (Table 3). Factors homologous to ACEII (58) were not found in *Aspergillus* species, and also in the case of *C. globosum*, alignment analysis did not identify any putative ACEII

TABLE 3 Results of BLASTP alignment of known transcription regulators with *C. globosum* database

Transcription regulator	Organism[a]	*C. globosum* gene ID	Protein alignment			DNA-BD alignment[b]	
			% Identity	% Positive results	% Query coverage	% Identity	% Positive results
ACE I	*H. jecorina*	CHGG_00776	56	69	95	93	96
ACE II	*H. jecorina*						
CreA	*A. nidulans*	CHGG_03907	61	69	100	82	87
XlnR	*A. niger*	CHGG_03981	55	67	89		
Hap2	*H. jecorina*	CHGG_05974	56	63	100	98	98
Hap3	*H. jecorina*	CHGG_01529	69	75	98	97	98
Hap5	*H. jecorina*	CHGG_03369	71	76	77	74	80

[a] Name of the organism whose transcription regulator was used as a BLASTP query.
[b] As reported in the GenPept database. Query coverage was 100%. DNA-BD, DNA-binding domain.

homologue. *Chaetomium globosum* contains a single putative factor similar to ACEI of *H. jecorina* (CHGG_00776) that features a 56% amino acid identity. Interestingly, the identity increases to 93% in considering only the DNA-binding domain (Table 3). A putative homologue with a 61% identity to the *Aspergillus nidulans* CreA protein was also found in the *C. globosum* database (CHGG_03907), and the DNA-binding domain proved to be particularly conserved (82% identity). Finally, predicted putative proteins with high similarity to *H. jecorina* Hap subunits were also found in *C. globosum* (Table 3).

Once the presence of putative homologues of known transcriptional regulators was ascertained, the 1-kb region upstream of the ATG translation start site of the 37 retrieved genes was examined in order to identify common *cis* elements possibly involved in transcriptional regulation. To this end, we used the following sequences as queries: 5′-AGGCA-3′, 5′-SYGGRG-3′, and 5′-

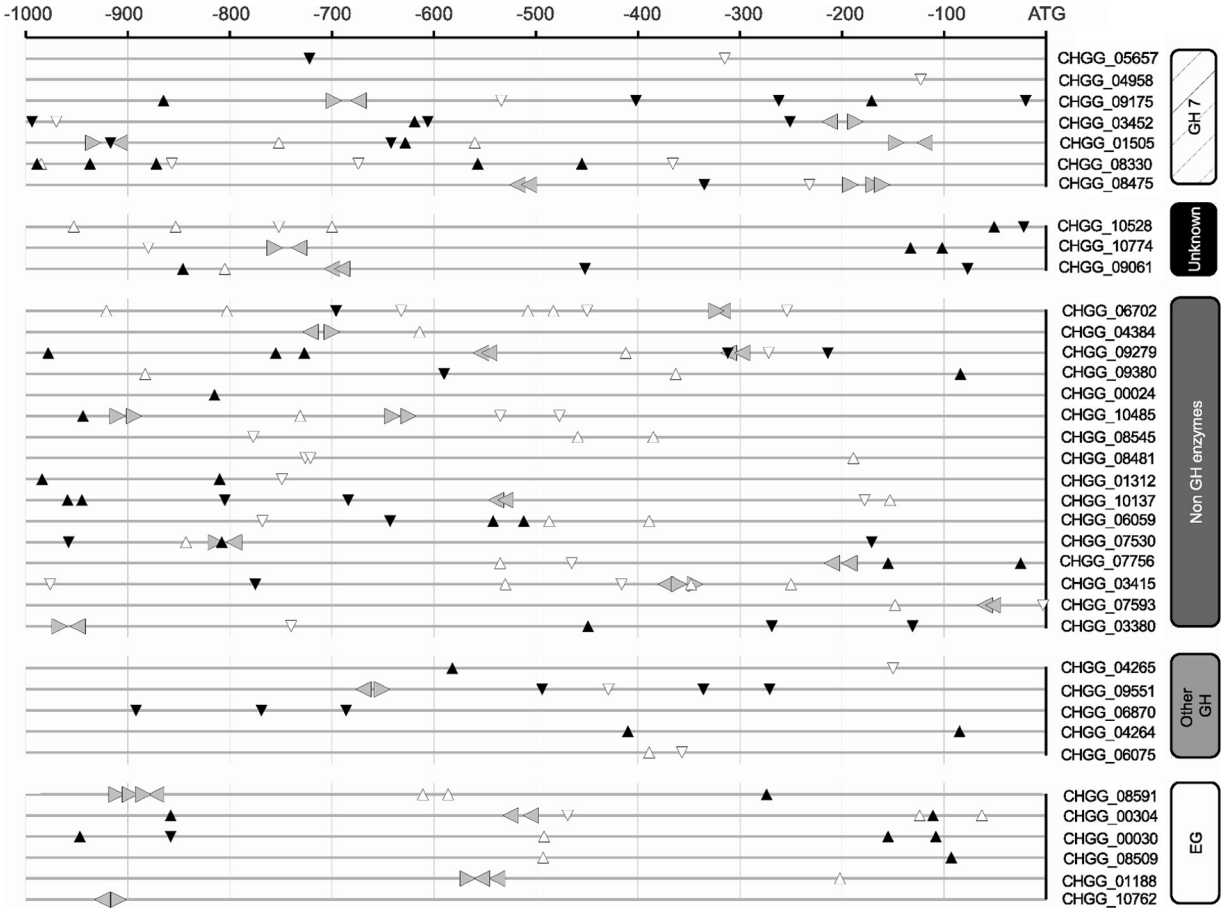


FIG 6 Schematic map of promoter regions with putative *cis* regulatory elements. The positions of putative binding sites for ACEI (black) and Hap2/3/5 (white) and of nonoverlapping neighbor consensus sequences for CreA (gray) identified by the SCOPE motif finder are indicated. EG, endoglucanases; GH, glycohydrolases.

CCAAT-3′ (the targets of ACEI, CreA, and Hap2/3/5, respectively) (Fig. 6). The repressor ACEI binding site was present in 23/37 analyzed promoters. Nevertheless, we did not observe any correlation between its presence and gene expression in response to glucose, which should activate the repression system. The sequence recognized by Hap2/3/5 was present in 33/37 analyzed promoters, but also in this case, a clear correlation between a gene transcriptional response and the presence of the putative binding sequence was lacking. The CreA degenerate binding motif appeared to be present in nearly all analyzed sequences (36/37 sequences), and moreover, this *cis* element was the only one found to be overrepresented in the analyzed promoters compared to the average distribution in *C. globosum* promoter sequences (significance value of 6.6). Nonetheless, there was no linear correlation between the number of sequences present in the promoter and the basal level of expression with either glucose (correlation coefficient = −0.104) or MPW (correlation coefficient = −0.098). Since closely replicated sites were found in functionally described CreA-regulated promoters (17), we paid particular attention to closely repeated sites (i.e., <20 bp) in the considered promoters. Excluding the overlapping sequences, neighboring binding sites were found in 20/37 queried sequences (Fig. 6). It is worth noting that in the three GH 61 genes with the highest transcript levels, a tandem repeat CreA site was present. Doubled sites were absent in the other GH 61 promoters, with the exception of the two genes that showed very low transcript accumulation. However, the double binding site is more than 300 bp from the translation start. Among GH 7 genes, a double CreA site was present in the highly activated CHGG_08475 gene and in CHGG_01505 and CHGG_03452, which were induced under MPW growth conditions compared to the remaining GH 7 genes, whose transcripts were less accumulated. As observed in the GH 61 gene family, we saw that the repeat CreA site was close (i.e., <400 bp) to the starting ATG in this case as well. On the other hand, CHGG_08330 was highly induced even though it does not contain a repeat CreA binding site. It is worth noting that the sole GH 16 gene (CHGG_09551), which proved to be induced more by MCC, contains two close CreA binding motifs, although they are located more than 600 bp from the ATG start site.

DISCUSSION

The deconstruction of cellulose contained in the plant cell wall requires the action of specific enzymes able to release degradation products from this recalcitrant substrate (20). The analysis performed in this study pointed to the identification of cellulolytic enzymes of the endophytic organism *Chaetomium globosum*. While genomic data are an invaluable source of information, they need to be investigated further using a postgenomic approach, and transcriptional analysis is a first step along this path. Our analysis initially focused on *C. globosum* genes encoding putative proteins containing a CBM1 domain and led to the identification of 30 genes, 27 of which are potentially involved in the degradation of different cell wall polymers. Another 7 genes, encoding GH 7 enzymes but lacking the CBM1, were considered because of the central role played by enzymes of this GH family in related ascomycetes (59, 61). In order to analyze the transcription of retrieved genes and confirm their actual involvement in cellulose degradation, *C. globosum* was grown under conditions known to promote (MPW and MCC) or inhibit (glucose) the production of extracellular enzymes, as reported by Soni and coworkers (54). All of the tested substrates were rapidly colonized by *C. globosum*; nonetheless, the most mycelial biomass formation and optimal ascocarp production were achieved on MPW, thus indicating a preference for this complex lignocellulosic substrate (Fig. 1A).

Among the genes highly induced and expressed in the presence of MPW, we identified a GH 6 (CHGG_10762) and two GH 7 (CHGG_08475 and CHGG_08330) genes. Members of these two families have been described as acting in a synergic way as CBHs: GH 7 enzymes act at reducing ends, as CBHI enzymes, while GH 6 enzymes are supposed to act at nonreducing ends as CBHII enzymes, though the actual role of the latter family is still debated (37). Nevertheless, in *C. globosum*, the sole GH 6 gene (CHGG_10762) showed the same level of expression when MCC was supplied, while all GH 7 genes were less expressed (Fig. 3). This suggests involvement of a GH 7 enzyme in the degradation of other polymers, most likely hemicelluloses, rather than crystalline cellulose. This finding is also supported by two other observations: all putative *C. globosum* GH 7 proteins lack a CBM1, and a global activator similar to *Aspergillus* sp. XlnR, which may activate the transcription of genes encoding cellulases only in response to D-xylose, seems to be missing or not functional (22).

The three "non-GH" genes that were highly expressed and induced in the presence of MPW encode a CDH (CHGG_03380) and two GH 61 enzymes (CHGG_07593 and CHGG_03415). CDH may play an auxiliary role in cellulose degradation, as it is expected to degrade the cellobiose released by cellulases, thus limiting feedback inhibition of these enzymes. Interestingly, GH 61 enzymes and CDH from *Thielavia terrestris* were recently shown to cleave cellulose (35) via an oxidoreductive activity synergic with the activity of other GHs. On the other hand, CDH transcription was lower in the presence of cellulose (MCC) than with MPW, a result consistent with the cosecretion of GH 61 and CDH induced by xylan shown in *Phanerochaete chrysosporium* by Hori and coworkers (30). Taken together, these data suggest that these reactions may be a critical step for the degradation of cellulose.

Other enzymes induced only by MPW are most likely involved in the degradation of a minor component of the plant cell wall, e.g., cutin or pectins. Among them, there are highly induced but poorly expressed genes such as CHGG_00024 and CHGG_10485, whose products are predicted to act on the lipidic portion of the substrate, or CHGG_00030, CHGG_00304, and CHGG_06870, encoding GH 10 and GH 11 enzymes, both reported to act on xylan (16). These last data, along with the GH 7 expression pattern, are strongly consistent with the observation that xylanases are evidently more secreted by *C. globosum* when a complex lignocellulosic substrate is present in the medium, as in the case of MPW. This suggests that, especially for hemicellulases, the expression may be induced by degradation products deriving from other components of the plant cell wall rather than from cellulose. This observation is consistent with the induction of cellulolytic and hemicellulolytic genes in *H. jecorina* in response to degradation products such as xylose and simple sugars such as sophorose (31).

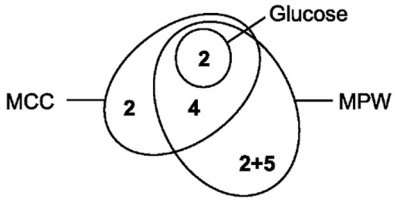
Only two genes were more highly expressed in the presence of MCC: the first encodes a GH 6 enzyme (CHGG_08591), and the second encodes a GH 16 enzyme (CHGG_09551). The higher expression level of the latter gene was unexpected, since members of the GH 16 family are generally reported to be active mainly as β-1-3-glucanases, although the biochemical characterization of this GH family in fungi is far from being exhaustive (http://www.cazy.org). However, the presence of a GH 16 enzyme containing a

CBM1 is quite uncommon among known ascomycetes, and this observation may account for the involvement of this enzyme in crystalline cellulose degradation. Although in the presence of MCC the transcription of these two genes was not very high, it was consistent with the detected increase in the secretion of endoglucanases (i.e., CMCases) in spent MCC medium. A similar increase of CMCase secretion on MCC was reported by Soni and Soni (55) in the case of *C. erraticum*.

Finally, genes encoding GH 5 and GH 45 enzymes (CHGG_01188 and CHGG_08509), which were expressed at appreciable levels even in the presence of glucose, showed high transcript levels in the presence of either MPW or MCC. Heterologous expression in bacteria allowed confirmation of the cellulose-degrading role of these two enzymes. Assay on cellulose revealed that they are both able to release reducing sugars at appreciable levels from a substrate containing amorphous cellulose (CMC and ASC), though at different levels. These experiments confirm the involvement of both GH 45 and GH 5 enzymes in cellulose degradation, and since they are secreted constitutively, they are thought to be involved in early stages of substrate degradation, thus acting as "detectors" of the presence of this polymer, as reported by Aro et al. (7). Moreover, the absence of any significant stimulatory effect on the hydrolytic activity when BGs were added to the reaction mix, together with the observed activity on the substituted cellulose CMC, suggests that GH 5 and GH 45 enzymes most probably act as EGs (55).

Despite the presence of putative proteins highly similar to the transcription factors ACEI and Hap2/3/5, the presence of binding sites for these factors does not correlate with their expression patterns on glucose and MPW. Moreover, these binding sites are widely distributed among all *C. globosum* regulative sequences. In fact, the *cis* elements of both factors have a negative significance value when analyzed by SCOPE (14). Most of the analyzed genes were repressed by glucose, and therefore a CreA-like factor was expected to be widely involved in their transcriptional regulation. Nevertheless, our data suggest that gene expression is not repressed by this factor only but that a multifactorial regulation is likely to occur, since transcription levels were different even for the considered gene set. This preliminary attempt to find a common regulatory system with other ascomycetes pointed out that the actual knowledge about the regulatory mechanisms of genes involved in cellulose degradation is far from being complete, even though our analysis indicates that the cellulose degradation strategy of *C. globosum* is similar to that of other ascomycetes. Although putative homologues of the transcriptional factors involved which have already been described for related ascomycetes are present in *C. globosum*, the distribution of consensus sequences in promoters of the considered genes was not different from that for unrelated ones. This preliminary analysis suggests that *C. globosum* may have a peculiar regulation of genes involved in cellulose degradation. This hypothesis is also supported by the observation that a functional global regulator similar to *Aspergillus* sp. XlnR and *H. jecorina* Xyr1, which regulate cellulase and hemicellulase expression in response to xylose and cellulosic substrates, respectively, is missing among annotated *C. globosum* genes.

In conclusion, the present report not only highlights the functional role of two cellulases of *C. globosum* involved in preliminary degradation of plant cell wall polymers but also points to the functional role of other enzymes involved in cellulose degradation.



C. globosum gene ID	Protein	Available carbon source		
		Glucose	MPW	MCC
CHGG_08509	GH 5	+	+	+
CHGG_01188	GH 45	+	+	+
CHGG_07593	GH 61	-	+	+
CHGG_03415	GH 61	-	+	+
CHGG_10762	GH 6	-	+	+
CHGG_03380	CDH	-	+	+
CHGG_08330	GH 7	-	+	-
CHGG_08475	GH 7	-	+	-
CHGG_ 00024	CUT	-	+	-
CHGG_ 10485	LIP	-	+	-
CHGG_ 00030	GH10	-	+	-
CHGG_ 00304	GH10	-	+	-
CHGG_ 06870	GH11	-	+	-
CHGG_08591	GH 6	-	-	+
CHGG_09551	GH 16	-	-	+

FIG 7 Venn diagram showing the distribution of differentially expressed genes of *C. globosum* in response to various carbon sources. MCC, microcrystalline cellulose; MPW, milled poplar wood.

The obtained data indicate that among the 37 identified genes, 7 may compose the core of the cellulose-degrading system of *C. globosum* (Fig. 7). In particular, the two constitutively expressed cellulases (GH 5 and GH 45 enzymes) are likely needed for substrate detection, while other induced GHs (two GH 6 enzymes and a GH 16 enzyme) are required for complete cellulose degradation. The present report also shows that in *C. globosum*, CDH and GH 61 proteins are involved in cellulose degradation, although it cannot be excluded that the oxidative reaction may be a critical step not only in this process but also for the degradation of other components of the plant cell wall, as recently suggested by Hori and coworkers (30). It is interesting that in the case of *C. globosum*, no GH 7 enzymes seem to be involved centrally in cellulose degradation, while they are possibly required for hemicellulose degradation. Additional genes encoding enzymes able to degrade minor components of the plant cell wall are expressed by this fungus for the degradation of MPW (Fig. 7).

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