Characterization of the family GH54 α-l-arabinofuranosidases in Penicillium funiculosum, including a novel protein bearing a cellulose-binding domain

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Characterization of the family GH54 α-L-arabinofuranosidases in *Penicillium funiculosum*, including a novel protein bearing a cellulose-binding domain

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Abstract The soil deuteromycete *Penicillium funiculosum* is characterized by its remarkable capacity to produce a wide variety of cellulolytic and hemicellulolytic enzymes. In the course of the genome sequencing of this industrial fungus, four different genes encoding glycosyl hydrolase family 54 (GH54)22 α-L-arabinofuranosidases were identified. Three of them termed *PfabfB1*, *PfabfB3*, and *PfabfB4* were highly similar, encoding proteins of 507, 508, and 505 amino acids, respectively. They exhibited structural features typical of GH54 enzymes, including an N-terminal catalytic domain connected to a C-terminal arabinose-binding domain (ABD). The fourth gene termed *PfafbB2* codes for an unusual 400 amino acid length GH54 α-L-arabinofuranosidase, in which the ABD was replaced by a fungal cellulose-binding domain (fCBD). This domain was shown to be functional since it allowed this protein to be retained onto microcrystalline cellulose, and the fusion of this CBD to the C-terminal end of *Pf*AbfB1 allowed this protein to bind to cellulose. Expression analysis of the four *PfabfB* genes during an industrial-like process fermentation on complex carbohydrates revealed that *PfafB2* was expressed more than 20,000-fold, while *PfabfB3* and *PfabfB4* were increased moderately at the end of the fermentation. In contrast, the transcript levels of *PfabfB1* remained unchanged throughout the process. This new type of GH54 α-arabinofuranosidase encoded by *PfabfB2* showed enzymatic properties slightly different to those of other GH54 enzymes characterized so far, including a higher thermostability, an optimum pH, and temperature of 2.6 and 50 °C, instead of 3.5 and 60 °C as found for *Pf*AbfB1. Nonetheless, like other GH54 α-arabinofuranosidases, *Pf*AbfB2 was able to release arabinose from various sources of branched arabinoxylan and arabinan.

Keywords Arabinofuranosidase · Hemicelluloses · Carbohydrate module · Protein secretion · Filamentous fungi

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Introduction

Hemicelluloses are heterogeneous polymers of pentoses (D-xylose and L-arabinose), hexoses (D-mannose, D-glucose and D-galactose), and sugar acids. Unlike cellulose, they are not chemically homogeneous, and one of their major polymeric structures is made of xylan. Xylans are heteropolysaccharides with homopolymeric backbone

chains of 1→4-linked β-D-xylopyranose units, and besides xylose, they contain arabinose, glucuronic acids, ferulic, and *p*-coumaric acid. Total enzymatic hydrolysis of xylan molecules requires endo-β-1,4-xylanase, β-xylosidase, and a bunch of accessory enzymes such as α-L-arabinofuranosidase, α-glucuronidase, acetyl xylan esterase, ferulic acid esterase, and *p*-coumaric acid esterase. The α-L-arabinofuranoside arabinofuranohydrolase (α-L-AFases; EC 3.2.1.55) are receiving much attention because they represent potential rate limiting enzymes in xylan degradation (Margolles-Clark et al. 1996; Gielkens et al. 1999; Saha 2000). They are exo-type enzymes that can hydrolyze (1→2), (1→3), and (1→5) α-L-arabinofuranosidic bonds in L-arabinose-containing hemicelluloses such as arabinoxylan and L-arabinan (reviewed in Saha (2000)). A former classification based on the mode of action and substrate specificity classified these α-L-AFases into three types (Beldman et al. 1997). Type A AFases (AbfA) preferentially degrade (1→5) α-L-arabinofurano-oligosaccharides to monomeric arabinose, while type B AFases (AbfB) cleave L-arabinose residues from side chains of arabinan or arabinoxylan. These two types of AFases are active on the synthetic *p*-nitrophenyl-α-arabinofuranoside (*p*NPAra). A third type is an arabinoxylan arabinofuranohydrolase (AXH) that is active on arabinosidic linkage in arabinoxylan from oat spelts, wheat, and barley (Kormelink et al. 1993) but cannot cleave the arabinofuranoside from *p*NPAra. Another useful classification that allows evolutionary and structural analyses of the protein is based on amino acid sequence similarities. This classification regroups the α-L-AFases into glycosylhydrolase (GH) families GH43, GH51, GH54, and GH62 (Cantarel et al. 2009). The link between the two classification methods is that AbfA, AbfB, and AXH correspond to family GH51, GH54, and GH62, respectively. The α-L-arabinofuranosidase from the GH43 family has been also denoted arabinoxylan arabinofuranohydrolase due to its activity on arabinoxylan. This α-AFase presents the originality to catalyze the release of (1→3) α-L-linked arabinofuranosyls of doubly substituted xylopyranosyl residues in wheat arabinoxylan (Van Laere et al. 1997; Sorensen et al. 2006). One should notice, however, that this linkage between enzyme activity and amino acid sequence classification is an oversimplification due to the complexity that exists within the various α-L-AFases, as for instance two enzymes of the same family can exhibit different substrate specificities.

Among the various α-L-AFases isolated from bacteria and fungi, proteins of the GH54 family have been well studied at the structural and enzymatic levels. The type B α-L-AFase exhibits a relative broad substrate specificity acting on arabinoside moieties at O-5, O-2, and/or O-3 as a single substituent in linear and branched arabinose-containing hemicelluloses (arabinoxylan, L-arabinan, and arabinogalactan) as well as on the synthetic *p*NPAra (Saha 2000). Genes encoding GH54 α-L-AFase have been cloned or identified in several filamentous fungi, including *Aspergillus niger*, *Aspergillus nidulans*, *Aspergillus kawachii*, *Aspergillus oryzae*, *Penicillium purpurogenum*, *Phanaerochaete chrysosporium*, *Trichoderma reesei* (see CAZY at http://www.cazy.org/fam/GH54.html), and more recently in *Penicillium chrysogenum* (van den Berg et al. 2008). The crystal structure of the GH54 enzyme from *A. kawachii* has been determined (Miyanaga et al. 2004a). A general feature shared by all GH54 α-L-AFases is to comprise a catalytic domain at the N-terminus and an arabinose-binding domain (ABD) at their C-terminal end. This ABD domain has a strict specificity for α-L-arabinofuranose and is classified as a type C carbohydrate-binding module (CBM42), which allows binding of the enzyme to arabinofuranosyl moieties of insoluble polysaccharides such as hemicelluloses (Miyanaga et al. 2004b; Miyanaga et al. 2006; de Wet et al. 2008).

The soil deuteromycete *P. funiculosum* is an industrially important fungus, currently exploited for the production of a commercial cocktail termed "Rovabio Excel™". This product is used as feed additive for enhancing digestibility of complex agricultural residues (cellulose, hemicellulose, arabinoxylan, arabinogalactan, etc.) in animal nutrition. A recent proteomic study revealed the presence of more than 50 different proteins in this commercial cocktail. Sequence similarity search in sequenced fungal genomes showed that the majority of these secreted proteins corresponded to glycosylhydrolytic, hemicellulolytic, and proteolytic enzymes (Guais et al. 2008). Considering α-L-AFases as the rate limiting enzyme in xylan degradation (Saha 2000) and that enzymes usage in animal feed formulation is increasing, cloning and functional analysis of genes encoding this glycosyl hydrolase in *P. funiculosum* represent an important issue to improve this enzymatic cocktail for animal feed digestibility. We here report that this filamentous fungus is unique with respect to gene encoding four different GH54-α-L-arabinofuranosidases, including a new type of protein bearing at its C-terminus a cellulose-binding module (CBM1) instead of the classical arabinose-binding domain. Moreover, we showed that the gene encoding this new GH54 protein is strongly expressed under industrial-process fermentation and that the encoded protein exhibits enzymatic properties slightly different from those of the classical GH54 α-L-AFase.

Material and methods

Strains, media, and culture conditions

Escherichia coli ElectroMax DH10B™ (Invitrogen) was used as a host for cloning experiments. The bacterial cells

were cultivated in LB liquid or solid (2% agar) media supplemented with 150-µg/ml ampicillin. The *Saccharomyces cerevisiae* CEN.PK113-5D strain (MATa *MAL2-8^c ura3-52*) (van Dijken et al. 2000) was used as host for heterologous protein production. Yeast cells were cultured in minimal medium (0.17% yeast nitrogen base without amino acids and ammonium) containing 2% glucose and 0.5% ammonium sulfate. The industrial Pf 8/403 *P. funiculosum* used in this work is an ADISSEO proprietary strain (patent no. W0 99/57325). It originated from the *Pf IMI123457* (International Mycological Institut, UK) by UV and gamma X mutagenesis. To inoculate liquid media, *P. funiculosum* spores were obtained by growing the strains on LYMM agar plates, which contained (in w/v): 1% malt extract; 0.1% yeast extract; 0.6% Na_2HPO_4; 0.4% KH_2PO_4; 0.2% $(NH_4)_2SO_4$; 0.2% $MgSO_4$, 0;001% $CaCl_2$, 0.0001% H_3BO_4; 0.0001% $FeSO_4$; 0.0001% $MnSO_4$; 0.001% $ZnSO_4$; 0.001% $CuSO_4$; 0.0001% (NH_4) $M0_7O_{24}$; and 1.5% agar. The fungal spores were inoculated in either one of the two following liquid media. The M0 is a minimal glucose non-inducing medium containing for 1 l: 1.9 g KH_2P0_4, 0.65 g KCl, 0.65 g $MgSO_4$, 12.5 mg $ZnSO_4$, 12.5 mg $MnCl_2$, 12.5 mg $FeSO_4$, 5 g NH_4Cl, and 10 g glucose. The pH was adjusted to 6.0 with 50-mM KH_2P0_4. The M2 medium is closely similar to the industrial-fermentation medium that is used for the production of the enzymatic cocktail Rovabio Excel™. It contained for 1 l: 20 g Fibrim (fiber of soya at 75% carbohydrates moisture purchased from Protein Technologies International, Inc. St. Louis, USA), 20 g corn steep liquor, 20 g provasoy (soya flour at 55% protein moisture purchased from Roquette SA, France), 10 g $(NH_4)_2SO_4$, 8 g CaCl2, and 0.6 g Clerol (from Société PMC, France). Unless otherwise stated, the liquid media were inoculated with 2×10^5 spores/ml in Belco™ Erlenmeyer. The cultures were carried out at 30 °C and agitated at 180 rpm up to 56 h.

DNA preparation

DNA was obtained from 200 mg of *P. funiculosum* cultivated in M0 medium. The mycelia were collected by filtration through two sheets of Whatman no. 1 filter, washed two times with cold water, and flash frozen in liquid nitrogen. The frozen mycelia was pulverized using a pestle in a mortar continuously filled with liquid nitrogen, lysed by the addition of 400 µl of TE buffer pH 8.0 complemented with 100-mM NaCl, 1% (w/v) SDS, 2% Triton X-100, and 2 U of RNAase A, which was followed by the addition of 400 µl of phenol/$CHCl_3$. After vigorous shaking on a vortex, two times for 1 min, the mixture was centrifuged at 10,000×*g* in a microcentrifuge. The supernatant was recovered and mixed with 2 vol of 100% ice-cold ethanol. After 5 min on ice, the tube was centrifuged 5 min at 10,000×*g*, and the pellet was dissolved in pure distilled water. It was then treated with RNAse A (2 U) for 1 h 30 min at 37 °C, followed by phenol/chloroform extraction and ethanol precipitation as above. After resuspension of the pellet in 50-µl TE, the amount and the purity of the DNA were checked using Nanodrop spectrometer at 260 and 280 nm.

Construction of an arrayed *P. funiculosum* genomic DNA library

A partial DNA library of *P. funiculosum* was created on membrane arrays as follows. 500 ng of DNA was digested in the presence of 0.5 U of *Sau*3A for 15 min at 37 °C. This mild digestion generated DNA fragments in the range of 0.5 to 3.5 kbp, which were cleaned by phenol/$CHCl_3$/isoamylalcool solution, dried, and resuspended in 30 µl of water. The DNA fragments were end-modified by Taq polymerase (5 U) at 70 °C for 30 min in a 20-mM Tris-buffer solution pH 8.0 containing 10-mM KCl, 2-mM $MgSO_4$, 0.1% Triton X-100, and 300-µM dNTPs. They were then purified by the addition of 1 vol. of phenol/$CHCl_3$/isoamylalcool solution, dried, and the pellet was dissolved in 12 µl of sterile water. The modified DNA fragments present in this mixture were cloned into the pGEM-T easy vector following the manufacture's recommendations for ligation reaction, which was used to transform ultra-competent DH10B™ bacterial cells. The transformed cells were spread on QTray™ plates (24×24 cm, Genetix) containing 300 ml of LB medium supplemented with 150-µg/ml ampicillin and 1 ml of 2% X-Gal. White colonies were picked from the QTray™ plates by an automat (QPix™ from Biorobotic) programmed with the Q-Pixing software and transferred into 384-well microplates containing 60-µl LB with 150-µg/ml ampicillin and 10% glycerol. The clones were spotted onto 22×22 cm nylon N^+ membranes using the Microgrid II robot spotter. This set of membranes resulted in approximately 40,000 independent bacterial clones bearing a *P. funiculosum* DNA insert of 1 kbp±650 bp. Based on the Clarke and Carbon formula (Clarke and Carbon 1976), we estimated that the DNA library covered about 65% of the ~35-Mbp-sized *P. funiculosum* genome.

Isolation of the four *PfabfB* genes

The first gene termed *PfabfB1* was obtained by screening our nylon macroarrays, using the full-length coding DNA sequence of *abfB* gene from *A. niger* as a probe. This 1,503 pb probe was PCR-amplified using the genomic DNA from *A. niger* N-400 strain as template (kind gift of Dr. A Ram, Leiden University, The Netherlands) and ABFB-S/ABFB-AS primers (see Table 1). The probe was labeled with [α-^{33}P]dCTP with the Random Primers DNA

Table 1 List of primers used in this work

Name	Sequence	Function
ABFB-S	CATGTTCTCCCGCCGAAAC	Amplification of *abfB* from *A. niger*
ABFB-AS	CCTTACGAAGCAAACGCCGT	
BlockA_S	CCGCCGGCCCTTGYGAYATHTA	Amplification of *pfabfB2* (CODEHOP PCR)
BlockB_S	ACCTGCCTGATCACCATCATHTAYGAYCA	
BlockD-AS	TCGGCGTTGCCGTAGTCRAARCARCA	
BlockG_AS	AGGATGATAGCGCCCTCCTTGCTCAT	
BlockK_AS	AGGCGACGTACAGGACGTTBTYRWARTG	
SON-1	TGAAGCGGGCTTTATACTGCT	Promoter sequence of *abfb2* by SON-PCR
SON-2	AGAATGGAAGAGGGGTATGGTG	
SON-3	GGAGGCTTTCACCATGCAGTAG	
SON-A	TCAGAACTCCGTGTCACTTGGT	
SON-B	AGCGGGCCAGTATAAGCATC	
SON-C1	TGGTGTGCCACCTGAAGAGTAG	
SON-C2	CAACAAGAGAGCTCGTAGCAATAAG	
SON-C3	CGTCATGTTGAATCGTTTTGTGAG	
SON-1_abfB3	TGGCAGTTCCAGGGCAG	5′ sequence and promoter of *abfB3* by SON-PCR
SON-2_abfB3	GCCGGTGAACGGTGAGATG	
SON-3_abfb3	TGCGCCAATAGCGCTGGC	
Xba1-abfB1	TCTAGA ATG TTTCCAAGAATAAAACCAG	*abfB*1 cloning in pJL52
HindIII-abfB1	AAGCTT TCA TGCAAAGGCAGTCT	
Xba1-abfB2	TCTAGA ATG ACGTCCAAACATAGTTTCGAACGAGCC	*abfB2* cloning in pJL52
HindIII-abfB2	AAGCTT TCA GAGACATTGAGCGTAGTAAGGG	
ABFB2_AS_short	AAGCTT CTA TGTCTGGCCAGCTCCAG	*abfB2*ΔCBD in pJL52
abfB3_S	GGATCC ATG TTGTTCTCTCGACCGGTC	*abfB3* cloning in pJL52
abfB3_AS	AAGCTT TTA AGCAAAGCCGACGCTGAC	
abfB4-S	TCTAGA ATG CTATTCTCGAAACCGGTG	*abfB4* cloning in pJL52
abfB4_AS	AAGCTT CTA CGCAAACGCCGGCGCT	
AbfB1_AS_Q3	ATCGGCGTTGAAGGAAGC	qPCR on *abfB1*
AbfB1_S_Q3	TGATGGCACCAAGCAGTTC	
AbfB2_AS_Q3	TCCGCTACCTCCACACTGACC	qPCR on *abfB2*
AbfB2_S_Q3	CACACCACTTCCACCACCCTTAC	
2-AbfB3_S_Q3	CCAACGACGGCAGCAAGC	qPCR on *abfB3*
2-AbfB3_AS_Q3	CCAGTAACGGGCAGGGTAGC	
AbfB4_S_Q3	CGCCACTCCAACAGTCAAC	qPCR on *abfB4*
AbfB4_AS_Q3	GCTCCATGATCGTATAGAATTGC	
Tub_AS_Q	TGATGGCCGCTTCTGACTTCC	qPCR on *β-tub*
Tub_S_Q	GTTCTGGACGTTGCGCATCTG	

Composition of degenerated primers: Y (C or T); B (G, C or T); R (A or G); W (A or T). Restriction sites underlined

labeling system (Promega). Pre-hybridization, hybridization, and washing conditions for membrane macroarrays were carried out according to protocols available at http://biopuce.insa-toulouse/fr. We retrieved from this genomic DNA library a single bacterial clone that contained the full-length coding DNA sequence of an *abfB* gene (1,524 bp), which presented ~75% identity with *A. niger abfB*. The second gene was obtained starting with the CoDeHOP PCR (COnsensus-DEgenerate Hybrid Oligonucleotide Primers (Rose et al. 1998; Rose et al. 2003)) that has been specifically developed to isolate unknown, distantly related sequences from known homologous sequences. Specific primers (see Table 1) were first designed from highly conserved regions between fungal GH54 AbfB proteins found in databases. The amplification was performed starting from 200 ng of purified *P. funiculosum* genomic DNA as template and applying a touchdown PCR procedure (Don et al. 1991), i.e. starting from 58 °C as annealing temperature and applying a stepwise reduction of this parameter each new cycle down to 48 °C. A 502-bp PCR product, whose sequence was different with the previously cloned *PfabfB1* gene, was obtained using the BlockA-S/BlockD-AS primers. The remaining sequence was then obtained by screening the *P. funiculosum* DNA library

made on nylon membranes with this 502-bp fragment as a probe. We identified three clones that yielded to a 1800-bp contig, which contained an open reading frame of 1203 bp (*PfabfB2*) exhibiting only ~70% sequence identity with *pfabfB1* over the most conserved 900 nucleotides (catalytic core, see Results section). Due to the small size of the *PfabfB2* promoter sequence in this contig (241 bases) and the absence of further overlapping clones in our arrayed genomic DNA library, we performed a SON-PCR (Single Oligonucleotide Nested-PCR (Antal et al. 2004)), which is specifically designed for the isolation of genomic fragments that flank known DNA sequences. We carried out three consecutive single primer-based amplifications (see Table 1 for primers), the PCR product of the first (second) reaction being used as DNA template in the second (third) reaction that uses a single, although nested primer, to increase specificity of the reaction. Practically, the first SON-PCR was carried out in 50 µl of reaction mix, containing a primer at 2 µM, 200-µM dNTP, 2 U Taq polymerase and its buffer (1×final), and 20 ng of purified *P. funiculosum* genomic DNA. The second and third reactions used as template 2 µl of the first and second SON-PCR products, respectively, in a 50-µl reaction mix. The PCR protocol was as follows: 5-min denaturation at 94 °C; five elongation cycles (30 s at 94 °C; 45 s at 58 °C; 2.5 min at 72 °C); one ramping step (30 s at 94 °C; 3 min at 29 °C; 3-min ramp to 72 °C; 2.5 min at 72 °C); and finally 60 (for first reaction) or 30 (second and third reactions) new amplification cycles (30 s at 94 °C; 45 s at 58 °C; 2.5 min at 72 °C). The amplified products were gel purified, cloned, and sequenced, and allowed increasing *PfabfB2* contig size for a total of 1,080 bases of the promoter sequence.

Two additional *PfabfB* genes were later disclosed from the genome sequencing of this filamentous fungus that we have done recently (Guais O, Parrou JL, Maestracci M, Neugnot-Roux V and François JM, unpublished), which was performed using the Genome Sequencer FLX System (454 Life Sciences, Roche). Generated data were treated to keep only highly accurate sequences based on the Phred score, and reads were then assembled with the Newbler software to yield high quality contigs used in this study. The sequence of the *abfB4* gene was completely retrieved from a single contig, in contrast to *abfB3* gene, for which we only recovered the 3′-end of coding DNA sequence and its terminator. The complementary 5′-end of the gene was therefore isolated using the SON-PCR methodology, as presented above for *PfabfB2* (specific primers listed in Table 1).

Recombinant DNA techniques

The 2 µm, *URA3* plasmid YEplac195 (Gietz and Sugino 1988) in which we cloned the *PGK1*/*CYC1* promoter–terminator cassette from pYPGE2 (Brunelli and Pall 1993) to yield the Yeplac195-PGK/CYC1 plasmid (pJL52), was used to express the coding DNA sequence of the four *PfabfB* genes under the control of the strong *PGK1* yeast promoter. The PCR products (primers listed in Table 1) were inserted into the *Hin*dIII/*Xba*I sites of pJL52. The plasmids bearing the *abfB* genes were introduced into the CEN.PK 113-7D yeast strain according to Schiestl and Gietz (1989). Transformants were cultured overnight in 10 ml of 2% glucose minimal medium and the supernatant of these cultures was checked for the presence of α-L-arabinofuranosidase activity. For production of high amount of AbfB proteins, yeast transformants bearing either one of the four *pfabfB* genes in pYEplac195-*PGK*/*CYC1* plasmid were cultured in 250 ml of the same medium up to stationary phase. The supernatant was then collected by centrifugation (10 min at 5,000×*g*) and concentrated 40- to 100-fold using AMICON 10 kDa porous membrane.

Different chimeras between the AbfB1 protein, with or without its ABD domain and the CBD domain from AbfB2 (see Fig. 8A for detailed structure), were obtained using recombinant PCR for easy fusion of gene sequences (recombinant primers listed in Table 2). As for the native AbfB1 and AbfB2 proteins, these chimeras were expressed from the strong *PGK1* yeast promoter after cloning of the gene fusions in the *Bam*HI/*Hin*dIII sites of pJL52 (see above).

RNA isolation and transcript analysis by qPCR

Extraction of total RNA from M2-cultivated mycelium was performed with the plant RNA-easy mini kit from Qiagen, following the manufacturer's instructions except that the mycelia was pulverized in a mortar as described above and the powder was lysed with 450 µl of lysis buffer from the Qiagen kit containing 1% (v/v) β-mercaptoethanol and 100-mM guanidine isothiocyanate. The quantity and the quality of the extracted RNA were determined by micro-capillary electrophoresis (Bioanalyzer 2100, Agilent).

The transcripts levels of *PfabfB* genes were determined by quantitative RT-PCR using the BioRad MyIQ Real-time PCR system using primers (Table 1) that were designed using Beacon Designer 2.0 software (PREMIER Biosoft International). One microgram of total RNA was reverse transcribed into cDNA in a 20-µl reaction mix using the iScript cDNA synthesis kit (BioRad). Each sample was tested in duplicate in a 96-well plate (BioRad, CA, USA). The PCR reaction mix consisted of 12.5 µl of iQ SYBR Green Supermix (BioRad), 2.5 µl of each primer (250 nM final concentration), 2.5 µl H_2O, and 5 µl of 1/10 dilution of cDNA preparation. The thermocycling program consisted of a hold at 95°C for 4 min, followed

Table 2 List of primers used to construct fusions between *abfB1* and *abfB2* CBD domain

Final chimera	Rec-1 primer (*abfB1*)	Rec-2 primer (*abfb2*)
AbfB1+CBD	**CCCCAGTG** TGCAAAGGCAGTCTCG	**GCCTTTGCA** ACACTGGGGTCAGTGTGGAG
AbfB1+LCR+CBD	**GGCGAAGT** TGCAAAGGCAGTCTCG	**GCCTTTGCA** CACTTCGCCTGGTAGCCAC
AbfB1ΔABD+CBD	**CCCCAGTGT** ATAGCGGAGCCGACTGTG	**CTCCGCTAT** ACACTGGGGTCAGTGTGGAG
AbfB1ΔABD+LCR+CBD	**GGCGAAGTG** ATAGCGGAGCCGACTGTG	**CTCCGCTAT** CACTTCGCCTGGTAGCCAC

In the first step of the recombinant PCR, the primary PCR products were obtained using Up/Rec-1 and Rec-2/Dw primers for the amplification of *abfB1* and *abfB2* regions, respectively (see Fig. 8A for structure of these chimeras). In *bold*: 5′ extensions of the primers that will allow overlap of primary products. In the second step, the amplification was carried out using Up/Dw primers and aliquots of overlapping primary PCR products as template. Up primer: GGATCCATGTTTCCAAGAATAAA (*Bam*H1 site underlined); Dw primer AAGCTTTCAGAGACATTGAGCGTAG (*Hin*dIII site underlined). The *Bam*H1 and *Hin*dIII sites were used for final cloning of the gene fusions in pJL52

by 40 cycles of 10 s/95 °C and 45 s/56 °C. Melting curve data were then collected to verify PCR specificity and absence of primer dimers. The PCR efficiency of each primer pair was evaluated by the dilution series method using a mix of sample cDNA as template. Relative expression levels were determined using the ΔCt method, which takes into account differences in primer pair amplification efficiencies, the reference gene being the genome-encoded *Pfβ-tub*.

Assay of α-L-AFase activity

Unless otherwise stated, the α-L-AFase activity was determined in 250 µl of a reaction mixture containing 0.2-M glycine pH 2.5 and 4-mM *p*-nitrophenyl α-L-arabinofuranoside (*p*NPAra), for 15 min at 40 °C. The reaction was stopped by the addition of 0.5 ml of 1-M Na_2CO_3. The absorbance was measured at 405 nm, and the activity was calculated using an extinction molar coefficient of 17,000 M^{-1} cm^{-1}. Activity against natural polymeric substrates including wheat arabinoxylan (low viscosity, arabinose/xylose (41/59), oat spelt xylan (10% arabinose, Sigma), sugar beet arabinan, and debranched arabinan) was also assayed in 50-mM acetate buffer at pH 5.0 and 40 °C for 1 h. These substrates, purchased from Megazyme Inc (Ireland), were added as up to 8.5-mg/ml final concentration polymeric solution from a stock solution of 1.1% made in acetate buffer, pH 5.0, and the reaction was terminated by boiling at 80 °C for 4 min. The released arabinose was quantified by HPIC (Dionex).

Binding assay

Binding of *Pf*AbfB on complex carbohydrates was carried out following the procedure of Nogawa et al. (1999), except that sodium acetate 50 mM pH 5.5 was used as the buffer. The binding was determined in two different ways. One method relied on the measurement of residual α-L-AFase activity in the supernatant after centrifugation of the insoluble carbohydrates as described in Nogawa et al. (1999). The other method was to load the pellet on a 12.5% (w/v) SDS-gel electrophoresis. Briefly, xylan birchwood (Sigma) and crystalline cellulose (Arbocell B600) were washed three times with sodium acetate, 50 mM pH 5.5. The pellets were resuspended in the same buffer to give suspensions at 30 mg/ml. A concentrated preparation of heterologous AbfB1 or AbfB2 preparation obtained from yeast as described above was mixed with 450 µl of the substrate suspension and kept at 4 °C for 10 min, with regular agitation. The solutions were centrifuged at 13,000×*g* for 2 min, the supernatants containing unbound proteins were removed and each pellet was washed twice with 200 µl of 50-mM sodium acetate buffer pH 5.5 to completely remove unbound protein. Each pellet was boiled for 10 min in 15 µl of loading buffer and then subjected to SDS-PAGE 12.5% (w/v). The supernatant was used to measure the unbound α-L-AFase activity.

Other methods

Soluble proteins were determined by the Coomassie-dye binding assay (Bradford 1976) using BSA as the standard. SDS-PAGE was carried out using a MINI-Protean III electrophoresis Cell (BioRad) according to the manufacturer's manual and using SeeBlue Plus 2 pre-stained proteins (Invitrogen) as protein markers. Proteins on the gels were revealed using either Blue Coomassie staining or the nitrate silver staining procedures (Chevalier et al. 2007). Sugars were analyzed by high performance ion exchange chromatography (HPIC) on a DX 500 chromatography workstation (Dionex, Sunnyvale, CA, USA) equipped with a CarboPac PA10 column (250×4 mm) and a Dionex PED detector in the pulsed amperometric detection (PAD) mode according to Kormelink et al. (1993).

Nucleotide sequence accession numbers

The nucleotide sequences of *PfabfB1*, *PfabfB2*, *PfabfB3*, and *PfabfB4* reported in this paper have been submitted to GenBank under the accession number CAL81213

(*PfabfB1*), CAL85369 (*PfabfB2*), GU363149 (*PfabfB3*), and GU363150 (*PfabfB4*). The DNA sequences can be uploaded from the supplementary data, and they can be also retrieved at http://www.cazy.org/GH54_eukaryota.html.

Results

The *P. funiculosum* genome bears four different genes encoding family GH54 α-L-arabinofuranosidase

In the course of the genome analysis of the industrial *P. funiculosum* 8/403, we were specifically interested to characterize genes encoding α-L-arabinofuranosidase. The first gene termed *PfabfB1* was obtained by screening our nylon macroarrays using the *abfB* gene from *A. niger* as a probe. Following this strategy, we retrieved a genomic fragment that contained a CDS of 1,524 bp, with 73% identity to *abfB* from *A. niger* (data not shown). The second *PfabfB2* gene was obtained by application of a degenerated PCR amplification termed CODEHOP PCR (Rose et al. 1998; Rose et al. 2003) that has been developed to optimize amplification of unknown homologous sequences. Two additional *PfabfB* genes exhibiting strong homology with *PfabfB1* were later disclosed from our genome sequencing of this filamentous fungus. Interestingly, none of these four genes contained any intron in their DNA sequence. Three of them, namely *PfabfB1*, *PfabfB3*, and *PfabfB4* showed more than 70% similarity at both the nucleic and the amino acid sequences with the *abfB* genes isolated from other filamentous fungi including *A. niger*, *A. nidulans*, *A. kawachi*, and *A. oryzae* as well as *Aspergillus awamori* and *Fusarium oxysporum* (Flipphi et al. 1993; Miyanaga et al. 2004b; Gielkens et al. 1999; Margolles-Clark et al. 1996), whereas *PfabfB2* exhibited only 50% similarity over the whole sequence with the three other *PfabfBs* (Fig. 1).

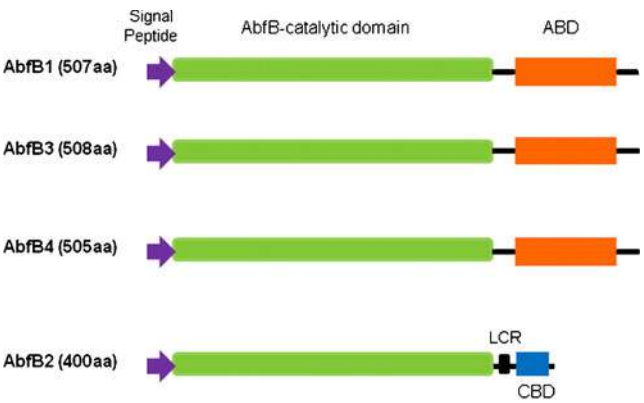

Fig. 1 Structure of the GH54 α-L-arabinofuranosidases from *P. funiculosum*. *ABD* arabinose-binding domain; *CBD* (*fCBD*) fungal cellulose-binding domain; *LCR* low-complexity region

The putative *P. funiculosum* α-L-arabinofuranosidases encoded by these four *abfB* genes are schematically depicted in Fig. 1. They all harbor a signal peptide for secretion of 26 amino acids at their N-terminus, followed by a catalytic domain of approximately 320 residues that is highly conserved among the GH54-family protein. In particular, these four *PfAbfB* presented an average pairwise sequence identity of 79% over their catalytic domain. This catalytic domain is notably characterized by three disulphide bonds made of 6 cysteine residues (Miyanaga et al. 2004a), which are conserved among the four *P. funiculosum* α-L-AFases (data not shown). In addition, potential N-glycosylation sites were predicted on each *Pf*AbfB protein based on the Asn-Xaa-Ser/Thr consensus. As for instance, they were at position Asn^{92} and Asn^{376} for *Pf*AbfB1 and at position Asn^{123} and Asn^{127} for *Pf*AbfB2. Finally, the C-terminus of all fungal GH54 enzymes identified so far has been shown to correspond to an arabinose-binding domain (ABD), also termed CBM42 (for carbohydrate-binding module 42). An extensive structural analysis of the *A. kawachii* Abf54 (Miyanaga et al. 2006) revealed that this domain is composed of three subdomains α, β, and γ, which allow the α-L-AFase to bind the non-reducing end arabinofuranosidic moiety of hemicellulose (Miyanaga et al. 2006; de Wet et al. 2008). Interestingly, this domain is entirely conserved in *Pf*AbfB1, *Pf*AbfB3, and *Pf*AbfB4 (not shown), whereas *Pf*AbfB2 presented a much shorter C-terminal end (residues 367 to 400). The SMART program (http://smart.embl-heidelberg.de/) identified this C-terminal end as a putative fungal cellulose-binding domain (fCBD). It harbors the four conserved cysteine residues that are present in fCBD of cellulase-related proteins (cellobiohydrolases, xylanases, endo- and exo-glucanases) and are classified as carbohydrate domain module 1 (CBM1) in Cazy database (www.cazy.org) (Fig. 2). Moreover, this domain was also preceded by a short 23 amino acid sequence rich in proline and hydroxylated amino acids (aa 340 to 362) termed LCR

Fig. 2 Amino acid sequence alignment of fCBD of AbfB2 with (**a**) cellobiohydrolase I (*Cbh1*), (**b**) ferulic acid esterase A (*FaeA*), (**c**) cinnamoyl esterase (*FaeB*), (**d**) endo-β-1,4-xylanase D (*XynD*), and (**e**) endo-β-1,4-xylanase B (*XynB*) from *P. funiculosum*. The sequences of these proteins can be retrieved from the Cazy database (www.cazy.com)

(low-complexity region). This LCR or linker is widespread among carbohydrate-degrading enzymes, including cellulase-related proteins (Gilkes et al. 1991) and is known to be *O*-glycosylated (Tomme et al. 1988).

Transcript analysis of *PfabfB* genes

To get a first insight into the function of these four *abfB* genes in *P. funiculosum*, expression of these genes was determined by quantitative real time PCR (RT-qPCR) during the growth of the *P. funiculosum* in a mimicked industrial medium. The β-tubulin (*Pfβ-tub*) gene was used as the internal control for normalization. We firstly estimated transcript abundance of the four *PfabfB* genes relative to that of *β-tub* (value of this transcript set at 1.0 arbitrary unit) after 56 h of cultivation. As shown in Fig. 3A, the *PfafbB* genes were expressed at a different extent to each other, with the following clear ranking in transcript levels *abfB2*>*abfB4*>*abfB3*>*abfB1*. We then examined whether the expression of these genes could change along the fermentation process. Sampling was taken at T_0 (inoculation), 31 and 56 h after starting of the growth of *P. funiculosum* in M2 medium. Transcript levels of each gene were normalized at each time point to levels of *β-tub*, and then, expressed relative to the value taken at time T_0 (calibrator sample). As shown in Fig. 3B, *abfB2* showed the highest transcription increase, followed by *abfB3* and *abfB4*, whereas expression of *abfB1* did not vary during growth. While the transcript levels of *abfB3* and *abfB4* were maximal at 31 h and then dropped significantly at the end of the process (56 h), the expression of *PfabfB2* steadily increased up to 56 h, to reach transcript levels that were >20,000 more abundant than at time 0. Altogether, these results suggested that the main activity of GH54 α-L-AFase in the enzymatic cocktail produced by *P. funiculosum* is due to the expression of *PfabfB2*.

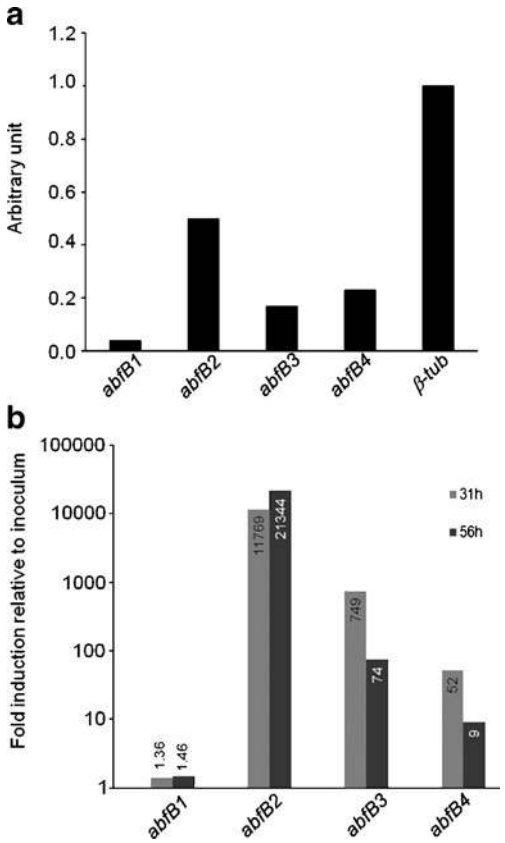

Fig. 3 Expression analysis of the four *abfB* genes during growth of *P. funiculosum* under industrial-fermentation process. The filamentous fungus was grown in M2 medium as described in Material and methods. At T_0 (inoculation) and after 31 and 56 h of growth, culture samples (about 100 mg dry mass) were harvested by centrifugation, washed once with water, and frozen in nitrogen liquid. RNA extraction and qRT-PCR were carried out as described in Material and methods. Shown in (**a**) are the transcript levels of *PfabfB* genes at 56 h expressed in arbitrary unit (*β-tub* set at 1). In (**b**), normalized expression of *abfB* genes to *β-tub*, with sample T_0 taken as the calibrator sample

Heterologous expression of *PfabfB* in yeast

In order to validate whether these four *abfB* genes encode a functional α-L-arabinofuranosidases, we cloned the coding sequence of these genes into a yeast 2-μm plasmid under the strong promoter of the *PGK1* gene and transformed the yeast CEN PK 113-7D strain with the recombinant plasmids. Heterologous proteins were isolated from the culture medium, following a similar work carried out with the *T. reesei abfB* gene (Margolles-Clark et al. 1996). We were able to detect a significant enzymatic hydrolysis of the chromogenic substrate *p*-nitrophenyl α-L-arabinofuranoside (*p*NPAra) from transformants expressing *PfabfB1* and *PfabfB2* genes. However, in the case of *PfabfB4* expression, the activity was only detectable after >50-fold concentration of the culture medium, whereas we failed to detect any activity corresponding to *PfabfB3* gene even after extensive concentration of the culture medium. The reason is unclear since we have verified by RT-PCR that this gene was actually expressed. In addition, the substitution of the signal peptide of *Pf*AbfB3 by that of *Pf*AbfB1 did not resolve this problem (data not shown), suggesting that the protein is either not produced or immediately destroyed as it is produced.

Since the production of these fungal enzymes in culture medium was relatively low after 40 h of cultivation in a glucose medium (less than 0.10 mg/L of culture), we concentrated 50- to 100-fold the culture medium and loaded an equivalent of 2–3-μg total proteins on a SDS-PAGE. A major band migrating at a size of 65 and 54 kDa was detected from yeast-culture sample that contained *PfabfB1* and *PfabfB2*, respectively (Fig. 4). Since these two bands were not seen in the control sample (Fig. 4, lane 1), we

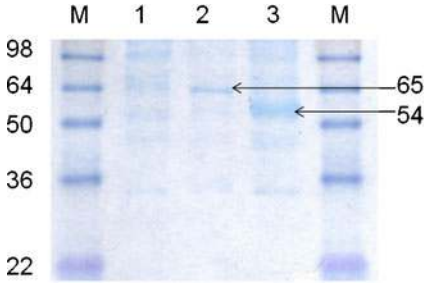

Fig. 4 Heterologous expression of *Pf*AbfB1 and *Pf*AbfB2 in yeast cells and detection of their presence in the culture medium. Coomassie blue staining of 10% SDS-PAGE loaded with concentrated culture supernatant of the *S. cerevisiae*. Control vector (YEplac195, *lane 1*), *Pf*AbfB1 (YEplac195-*abfB1*, *lane 2*), and *Pf*AbfB2 (YEplac195-*abfB2*, *lane 3*). About 2~3-μg total proteins were loaded on the gel before migration. *Mw* molecular marker

could conclude that they correspond to *Pf*AbfB1 and *Pf*AbfB2. The apparent molecular mass estimated on the SDS-gel was about 30 to 40% higher than that calculated from their amino acid sequence (52 and 38 kDa for *Pf*AbfB1 and *Pf*AbfB2, respectively), suggesting that the two heterologous proteins were produced in a highly glycosylated form.

Enzymatic properties of the AbfB1 and AbfB2

The source of the *Pf*AbfB1 and *Pf*AbfB2 used for enzymatic characterization was obtained from a 50- to 100-fold concentrated culture media of recombinant *S. cerevisiae*. The two enzymes were active on the synthetic *p*NPAra as substrate, which was therefore used for the determination of pH and temperature dependencies (Fig. 5A and B). The optimal pH was measured at 40 °C in the presence of 5-mM *p*NPAra. Fig. 5A shows that AbfB1 was active over a wide pH ranging from 2.5 to 4.5, with an optimum at pH 3.4 (note that the same pH dependency was found for *Pf*AbfB4, data not shown), whereas the pH range for AbfB2 activity was between 1.5 and 3.5, with an optimum at pH 2.6. The optimal temperature for activity of AbfB1 and AbfB2 was measured at their optimal pH. As shown in Fig. 6B, the temperature dependency of the two GH54 α-L-AFases was different, with an optimum at 50 °C for AbfB2 and 60 °C for AbfB1 (as well as for AbfB4, data not shown). Finally, we also investigated thermal stability of the two AbfB at their optimal pH for activity (Fig. 5C), after exposition of the two proteins for 12 min at temperatures ranging from 4 to 60 °C. It was found that the *Pf*AbfB1 activity dropped dramatically when the incubation temperature was above 40 °C, whereas *Pf*AbfB2 activity only dropped by 50% at 60 °C.

Apparent kinetic constants (K_m and V_m) were determined on the two heterologous *Pf*AbfB enzymes using *p*NPAra as the substrate and at their optimal pH and temperature. The

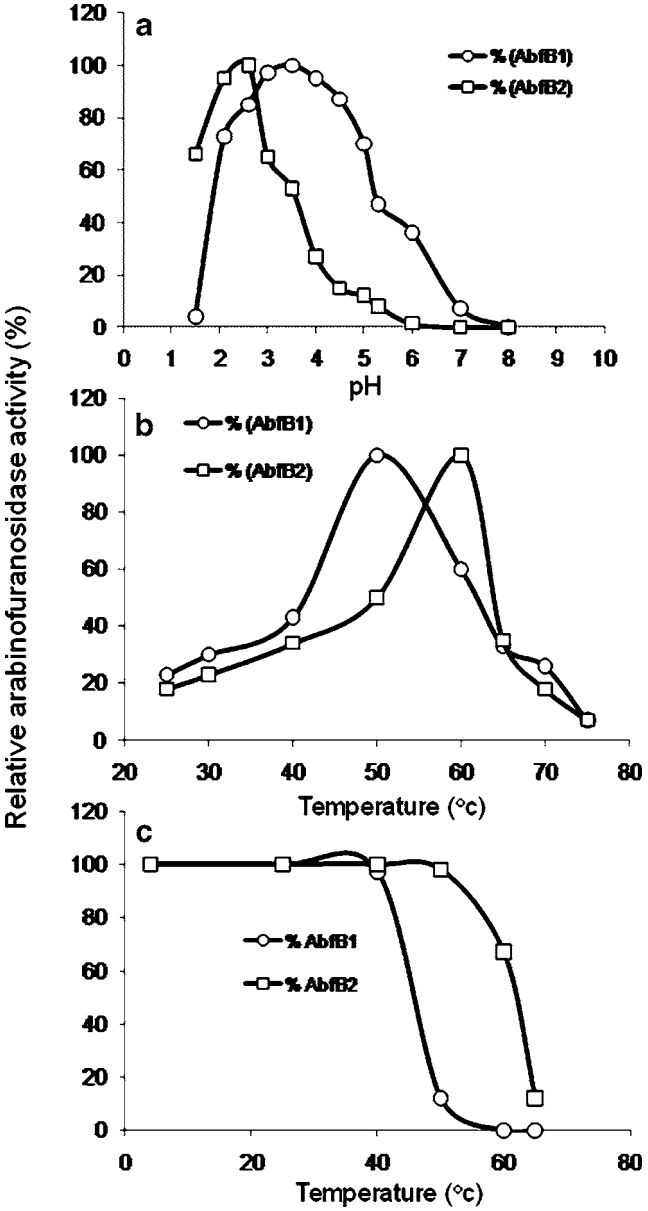

Fig. 5 Dependency to pH, temperature, and thermal inactivation of *Pf*AbfB1 and *Pf*AbfB2 enzymes. The two enzymes used for kinetic analysis were obtained from 50-fold concentrated supernatant of *S. cerevisiae* expressing Yplac195-*abfb1* and Yplac195-*abfB2*. (**a**) pH dependence. Assays were done at 40 °C for 5 min in the presence of 5-mM *p*NPAra using McIlvaine's phosphate–citrate buffer. (**b**) Temperature dependence. Assays were done at their respective optimal pH in the presence of 5-mM *p*NPAra. (**c**) Thermostability of the two GH54-α-L-AFases. Incubation for 12 min at temperature ranging from 4 to 65 °C was made in acetate buffer 50 mM pH 5.0. The remaining activity was measured in the presence of 5-mM *p*NPAra at their optimal pH. Data shown are from one typical experiment that was repeated three times with similar results

data were treated using the Lineweaver–Burk method. A K_m and V_m of 1 mM and 25 U/mg were determined for *Pf*AbfB1, and 0.7 mM and 16.5 U/mg for *Pf*AbfB2. Since *Pf*AbfB1 and *Pf*AbfB2 as expressed in the yeast culture

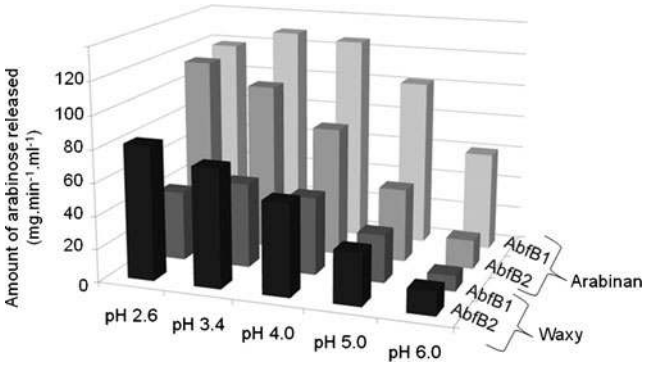

Fig. 6 Assay of *Pf*AbfB1 and *Pf*AbfB2 on different sources of xylan. About 5 μg of AbfB1 and AbfB2 prepared as in Fig. 5 was incubated in 1 ml 50-mM acetate buffer pH 5.0, containing 8.5 mg of arabinan or wheat arabinoxylan (waxy). The reaction was stopped after 10 min and the amount of arabinose released was determined by HPAEC

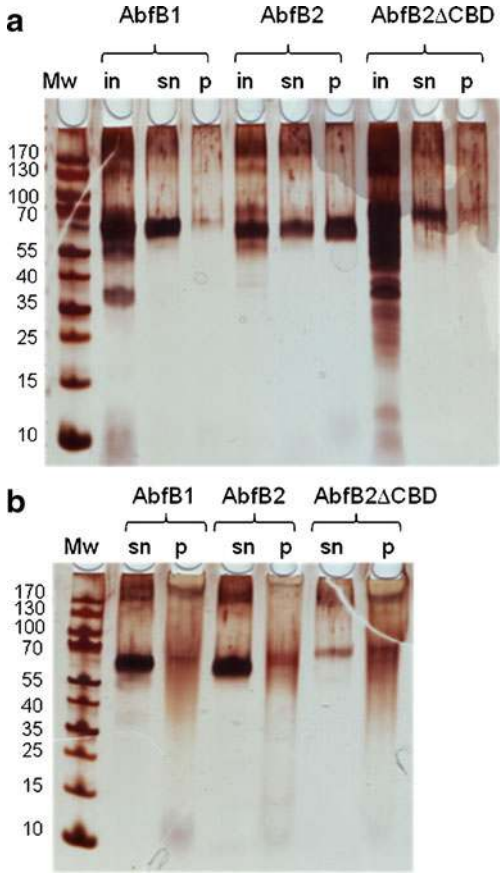

Fig. 7 SDS-PAGE analysis of *Pf*AbfB1 and *Pf*AbfB2 after incubation with cellulose or xylan. The supernatant of yeast culture (20-μg total proteins) containing the heterologous AbfB1, AbfB2, or AbfB2ΔCBD (lacking the cellulose-binding domain) was used for binding studies on 5 mg/ml of either cellulose (**a**) or xylan birchwood (**b**). After incubation at 4 °C for 1 h, the mixture was centrifuged and the supernatant (*sn*), resuspended pellet (*p*), or the sample before incubation with the polysaccharide (*in*) were loaded on the 12.5% SDS-PAGE. After migration, bands were revealed on the gel by the nitrate silver staining method

medium represented the major visible band on the gel after ~50-fold concentration of the supernatant, we made the assumption that the yeast supernatant was merely enriched of these recombinant proteins and therefore allowed approximating their specific molar activity. This k_{cat} was estimated to be about 283 s^{-1} for AbfB1 and 92 s^{-1} for AbfB2. Finally, we assayed the two α-L-AFases on natural arabinan and wheat arabinoxylan (waxy). They were active on these two natural substrates, exhibiting maximal activity at the optimal pH as it was determined with *p*NPAra (Fig. 6). In addition, various monosaccharides were assayed for their potential effect on *Pf*AbfB1 and *Pf*AbfB2. Activity of these two enzymes using *p*NPAra (2 mM) as substrate was inhibited by about 50% in the presence of 50-mM L-arabinose, whereas other sugars like D-xylose, D-glucose, D-mannose, and D-galactose had no effect, even at concentration up to 100 mM (data not shown). We also found that these two enzymes also expressed a very weak β-xylosidase activity (using *p*-nitrophenyl-β-xylopyranoside as the substrate), which represented less than 1% of their arabinofuranosidase activity using *p*NPAra as the substrate (data not shown).

Binding assay of *PfAbfB* on cellulose

As indicated above, the sequence analysis using SMART program suggested the existence of a fungal cellulose-binding domain (fCBD) (corresponding to CBM1 according to Cazy classification, see www.cazy.org) at the C-terminal end of *Pf*AbfB2, whereas the *Pf*AbfB1 displayed the typical carbohydrate-binding module (CBM42), also termed ABD for arabinose-binding domain, as found in other GH54 enzymes. To demonstrate the functionality and the specificity of the CBD in *Pf*AbfB2 and ABD in *Pf*AbfB1, the two proteins were incubated at 4 °C for 1 h with microcrystalline cellulose (Arbocell600) or insoluble arabinoxylan (xylan birchwood). The binding of the enzyme was evaluated by two methods. The first method, which is illustrated in Fig. 7, showed that AbfB1 protein was not retained onto cellulose (Fig. 7A) and a very weak retention was observed on arabinoxylan (Fig. 7B). In contrast, a great proportion of AbfB2 was retained onto cellulose (Fig. 7A), while a minor amount of this protein could also bind to xylan (Fig. 7B). The cellulose binding of AbfB2 was completely lost by removing the CBD domain of this protein (Fig. 7A). Curiously, it was noticed that the removal of the CBD resulted in a slightly slower mobility of the truncated AbfB2ΔCBD on the SDS-gel (see Fig. 7A). As reported above, this CBD domain was preceded by a linker of 23 amino acid sequence (LCR) that connects this domain to the catalytic domain of the AbfB2 protein. The fact that this short peptide sequence can be highly glycosylated may

explain the difference in mobility between the native and the CBD-truncated AbfB2. In addition, to further verify the role of LCR and CBD in the cellulose-binding properties, we fused either the CBD or the LCR alone, or both of them to the C-terminal end of AbfB1 (Fig. 8A). In this case, we used another strategy that consisted in measuring the residual activity of the arabinofuranosidase in the supernatant after incubation with the complex polysaccharides. Results reported in Fig. 8B indicated that the AbfB1 gained capacity to bind to cellulose when both the LCR and the CBD domain from AbfB2 were fused at its C-terminus, whatsoever the ABD was removed or not, whereas the presence of the CBD alone was not sufficient to retain the protein on cellulose.

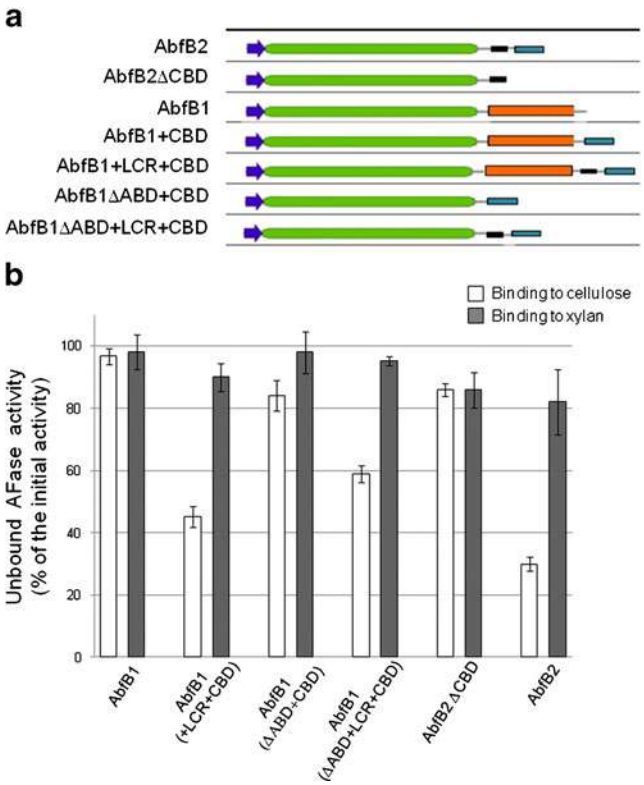

Fig. 8 Binding of AbfB on cellulose requires both the CBD and the low-complexity region (LCR). (**a**) Structure of the various AbfB2 and AbfB1 protein variants or chimeras that was obtained as described in Material and methods. Fusion of full-length AbfB1 (Met^{1}-Ala^{507}) or AbfB1 without ABD (Met^{1}-Ala^{358}), to CBD (His^{368}-Leu^{400}) or to LCR+CBD domain from AbfB2 (Thr^{341}-Leu^{400}), respectively. Primers used to construct the gene fusions encoding these chimeras are given in Table 3. (**b**) The experimental procedure for cellulose and xylan binding was as in Fig. 7, except that the retention of proteins on the complex carbohydrates was indirectly determined by measuring the α-arabinofuranosidase activity with *p*NPAra in the supernatant. This residual activity was expressed as % of the initial activity measured before incubation. White and gray histograms represented the residual α-arabinofuranosidase (*AFase*) activity after incubation with cellulose and xylan, respectively. Standard deviation of the three independent experiments is given by the bars on the histograms

Discussion

In a recent study, a proteomic approach was used to characterize the enzymatic cocktail produced by the industrial *P. funiculosum* 8/403 strain. This exoproteome, conditioned as "Rovabio Excel™" and exploited as feed additives to animal nutrition, was found to contain a large collection of hydrolytic enzymes capable to hydrolyze complex polysaccharides from plant (Guais et al. 2008). Towards characterizing further this exoproteome at the genome level, we were recently engaged into the genome sequencing of this industrial filamentous fungus using the next genome sequencing technology (Genome Sequencer FLX System). This sequencing, together with a strategy combining gene cloning by PCR and heterologous hybridization on membrane arrays bearing DNA fragments from this fungus, allowed us to identify the presence of four genes that belong to the GH54 family encoding α-L-arabinofuranosidase. These four proteins exhibit the conserved arabinofuran-catalytic domain (see CAZY at http://www.cazy.org) with its three disulphide bonds. The N-terminal catalytic domain of GH54 α-L-AFases is connected to a carbohydrate-binding module (CBM42 family, also called ABD) containing superficial pockets, which was found to provide binding properties to arabinofuranose side chains of hemicelluloses (Miyanaga et al. 2006). It is noteworthy that, contrary to expectation, the functionality of this ABD to bind to arabinoxylan was not strictly confirmed with *Pf*AbfB1, as only a weak retention of this protein was found on birchwood arabinoxylan. This lack of binding could be due to the type of xylan that was used in our study, since it was reported that GH54 α-L-AFases isolated from various fungal species show different binding selectivity towards various types of arabinoxylan. As for instance, *A. kawachii* GH54 enzyme only binds to wheat xylan (Miyanaga et al. 2006), whereas *Aureobasidium pullulans* AbfB can bind to wheat, oat, and birch xylan (de Wet et al. 2008).

The GH54 α-L-arabinofuranosidase encoded by *PfabfB2* exhibits several divergences with other GH54 enzymes characterized so far, both at the structural and biochemical levels. The amino acid sequence of this novel GH54 protein is 100 aa shorter, and this shortening has occurred at the C-terminal end. Indeed, the ABD domain that features C-terminus of GH54 enzymes has been replaced by a shorter cellulose-binding domain (CBD), classified as CBM1 and conferring the ability of this protein to bind to cellulose. This new GH54 isoenzyme could therefore originate from genome rearrangement that created a hybrid gene from DNA encoding part of α-L-AFase and DNA encoding part of cellulose-binding proteins. Interestingly, we only identified a DNA sequence encoding this new type of GH54 enzyme in the genome of filamentous fungus

Talaromyces stipitatus ATCC 10500, which is still in progress at the J. Craig Venter Institute (http://gsc.jcvi.org/projects/msc/talaromyces_stipitatus/).

We confirmed the functionality of this CBD domain by showing that *Pf*AbfB2 can bind to microcrystalline cellulose. In addition, we demonstrated that the binding property required the presence of a LCR that links the catalytic domain to the CBD. This LCR, whose size is between 20 and 40 residues, is found in many cellulase-related enzymes (Gilkes et al. 1991). In the case of *T. reesei* cellobiohydrolase I, the linker located between aa 434 and 460 in the protein sequence was shown to participate in the rate of cellulose degradation, and its removal only reduced the binding of the enzyme to cellulose (Srisodsuk et al. 1993). This LCR is also known to be *O*-glycosylated on its Ser and Thr residues (Tomme et al. 1988). The finding that the removal of CBD from AbfB2 resulted in a truncated protein with an apparent higher molecular weight on SDS-PAGE could therefore be explained by a hyperglycosylation of this LCR. In addition, we found that LCR is needed for binding of AbfB2 to cellulose. Although the removal of the CBD and LCR from *Pf*AbfB2 did not affect its *in vitro* activity against the artificial substrate *p*NPAra, it remains to examine whether the binding to cellulose and the LCR is important for its catalytic activity on natural hemicellulose substrates.

The fact that *Pf*AbfB2 can release L-arabinose from various arabinoxylan like other α-L-AFase of the GH54 family is not sufficient to ascertain about its precise catalytic mechanism. The variability in substrate specificity of GH54 α-L-AFase is indeed quite remarkable; some of them being able to cleave arabinose side chains from internal xylopyranose units of oligosaccharides, while others cannot (Saha 2000). The kinetic properties using the heterologous AbfB1 produced in yeast were similar to those reported for other GH54 α-L-AFase purified from other filamentous fungi (de Wet et al. 2008; de Ioannes et al. 2000), indicating that the hyperglycosylation of AbfB1 in yeast has likely not altered its enzymatic properties. On the other hand, biochemical properties of the *Pf*AbfB2 enzyme were at variance to those from typical GH54 enzyme isolated so far. The optimal pH for activity was more acidic (i.e. 2.6 as compared to 3.4), the optimal temperature was lower (50 °C instead of 60 °C), and this enzyme was apparently more stable than *Pf*AbfB1 at 60 °C. It is possible that the weaker thermostability of *Pf*AbfB1 to high temperature was due to the absence of Asn at position 202 or 204 as found in other AbfB (Miyanaga et al. 2004a; de Wet et al. 2008) since $Asn^{202/204}$ has been shown to contribute to the thermostability of the *A. kawachii* enzyme (Koseki et al. 2006).

Table 3 Identification of GH54 family α-L-arabinofuranosidase from sequenced filamentous fungi

Species	Name	Protein size (aa length)	Reference
Aspergillus niger CBS513.88	AbfB	499	Flipphi et al. (1993)
	Abf2	499	Crous et al. (1996)
	An15g02300[a]	499	Pel et al. (2007)
Aspergillus nidulans	AbfB	510	Galagan et al. (2005)
Aspergillus fumigatus Af293	AFUA_6G14620	506	Nierman et al. (2005)
Aspergillus oryzae RIB40	AbfB	506	Machida et al. (2005)
Magnaporthe grisea	MGG_06009[a]	500	Dean et al. (2005)
Fusarium graminearum (*Gibberella zeae*)	XP_383989	499	Accessible at http://www.nrib.go.jp/ken/EST2.old/BLASTX/AoEST06876.htm
Neurospora crassa OR74A	NCU09775[a]	342	Galagan et al. (2003)
Phanerochaete chrysosporium RP78	Not present	Not present	Martinez et al. (2004)
Trichoderma reesei (sn hypocreae jecorina)	Abf1	500	Margolles-Clark et al. (1996)
	Abf3	nd	Akel et al. (2009)
Penicillium chrysogenum Wisconsin 54-1255	Pc22g19620[a]	506	van den Berg et al. (2008)
Penicillium funiculosum 8/403	AbfB1	507	This paper
	AbfB2	400	
	AbfB3	508	
	AbfB4	505	

[a] As given in CAZY (http://www.cazy.org/). Additional search was done using AbfB1 and AbfB2 sequences from *P. funiculosum* blasted against whole genome sequence of the other filamentous fungi. *nd* not determined

Expression analysis of the four *PfabfB* genes in *P. funiculosum* under growth conditions that mimicked the industrial-fermentation process showed that *PfabfB2* was the strongest expressed *abfB* gene at the end of the fermentation, whereas the expression of *PfabfB1* did not change during the fermentation process. This strong activation of *PfabfB2* could be due to the fact that the promoter of this gene, unlike the other three genes, possesses a binding site for the transcriptional activator XlnR/XYR1, which is known to be required for coordinated induction of cellulolytic and hemicellulolytic-related enzymes in *A. niger* (van Peij et al. 1998; Guillemette et al. 2007). This explanation is quite conceivable since the industrial growth medium contains cellulose as carbon source. Regulation of *abf* genes by the general cellulase/hemicellulase XYR1 has been reported also for *T. reesei abf2* (Akel et al. 2009), which encodes a protein that belongs to GH62 family and which does not harbor a cellulose-binding domain (see www.cazy.org).

Fungal species have been found to produce more than one type of α-L-AFases. In all cases, these isoenzymes belong to different classes, as for instance enzymes of the GH51 and GH54 categories that have been isolated and characterized in *P. purpurogenum* (de Ioannes et al. 2000; Fritz et al. 2008) or enzymes of the GH43 and GH51 categories in *H. insolens* (Sorensen et al. 2006). A reasonable explanation for this diversity of isoenzymes is to allow synergistic action on complex arabinoxylan. This hypothesis was recently supported by the finding that the combination of GH43 and GH51 α-L-AFases from *H. insolens* resulted in an increased release of arabinose from water soluble wheat arabinoxylan (Sorensen et al. 2006). In our work, we identified in the genome of *P. funiculosum* four genes encoding α-L-arabinofuranosidase that actually belong to the same protein family GH54. As it is summarized in Table 3, this gene redundancy is quite unique among filamentous fungi whose genomes have been sequenced, as in most cases, only one to two genes encoding GH54 α-L-AFase had been found. As notable exceptions, *P. chrysosporium* did not contain GH54 protein and *A. niger* is enriched with three genes encoding this type of arabinofuranosidase. The remarkable redundancy of GH54 enzymes in *P. funiculosum* could provide some synergistic action in the hydrolysis of complex polysaccharides, taking also into account the originality of AbfB2 to bind to cellulose and hence to offer better accessibility of these enzymes towards cellulose and hemicellulose. This hypothesis as well as the potential synergistic action on various arabino-containing polymers requires further investigation.

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