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Characterization of a new β(1-3)glucan branching activity of *Aspergillus fumigatus*

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A new HPLC method was developed to separate linear from β(1-6) branched β(1-3) gluco-oligosaccharides. This methodology has permitted the isolation of the first fungal β(1-6)/β(1-3)glucan branching transglycosidase using a cell wall autolysate of *Aspergillus fumigatus*. The encoding gene, Af*BGT2* is an ortholog of Af*BGT1*, another transglycosidase of *A. fumigatus* previously analyzed (Mouyna et al., 1998). Both enzymes release laminaribiose from the reducing end of a β(1-3)-linked oligosaccharide and transfer the remaining chain to another molecule of the original substrate. The AfBgt1p transfer occurs at C-6 of the non-reducing end group of the acceptor, creating a kinked β(1-3;1-6) linear molecule. The AfBgt2p transfer takes place at the C-6 of an internal group of the acceptor, resulting in a β(1-3)-linked product with a β(1-6)-linked side branch.

The single Af*bgt2* mutant and the double Af*bgt1*/Af*bgt2* mutant in *A. fumigatus* did not display any cell wall phenotype showing that these activities were not responsible for the construction of the branched β(1-3)glucans of the cell wall.

Introduction

The fungal cell wall is a highly dynamic essential organelle that protects the organism from external stress and that accounts for 20 to 40% of the cellular dry weight (1). It is a rigid but permeable structure mainly composed of fibrillar (β–glucans, chitin), or amorphous (α–glucans, mannans) polysaccharides. The central core of the *Aspergillus fumigatus* cell wall is composed of β(1-3)/β(1-6) branched glucan chains on which other polysaccharides (chitin and galactomannan, β(1-3)/(1-4)glucan) are cross-linked (2). The chitin-β(1-6) branched β(1-3)glucan complex is found in almost all yeast and filamentous fungi and is thought to be responsible for cell wall stability.

β(1-3)glucans are synthesized by a plasma membrane-bound glucan synthase complex, which uses UDP-glucose as a substrate and extrudes linear chains through the membrane (3). Once these glucans reach the cell wall space, they have to become β(1-6) branched before they can serve as an anchor for other polysaccharides.

The first known β(1-3)glucanosyltransferase family of *A. fumigatus* (Gelp) splits a β(1-3)glucan chain internally and transfers the newly generated reducing end to the non-reducing end of another β(1-3)glucan molecule through a β(1-3)linkage, resulting in an elongation of the glucan chain (4). Gelps are glycosylphosphatidylinositol (GPI) anchored proteins that have orthologs in yeasts (Gasp in *Saccharomyces cerevisiae* or *Schizosaccharomyces pombe*, Phrp in *Candida albicans* (5-7)) and filamentous fungi to form the family GH72 of glycoside hydrolase in the carbohydrate active enzymes database (CAZy http://www.cazy.org/). These specific β(1-3)glucanosyltransferase activities play an important role in the biosynthesis of the fungal cell wall (5,7-9), showing that the remodelling of β(1-3)glucan chains are essential in the cell wall organisation.

The second family of β(1-3)glucanosyltransferases contains AfBgt1p of *A. fumigatus* and Bgl2p of *S. cerevisiae*. These glucanosyltransferases catalyze the release of a laminaribiose unit from the reducing end of a substrate and transfer the newly generated reducing end to the non reducing end of another β(1-3)glucan molecule, generating a new β(1-6) linkage (10,11). Both null mutants display a phenotype identical to the parental strain.

None of these transglycosidase activities described to date is responsible for the synthesis of a branched β(1-3)glucan. Here we report a joint molecular and biochemical approach that has led to the discovery of the first β(1-3)glucan branching activity in fungi.

Materials and Methods

Strains, plasmids and growth conditions

The *A. fumigatus Afbgt1* mutant, obtained from CBS144.89 parental strain (11) was used for enzyme purification and the CEA17*ku80Δ* strain (12) was used for gene deletion. They were maintained in 2% Malt agar slants at room temperature. Malt agar slants were supplemented with 150 µg/ml hygromycin B (Sigma) for *Afbgt1* mutant and *Afbgt2* mutant and supplemented with 150 µg/ml hygromycin B and 20 µg/ml of phleomycine (Invivogen) for *Afbgt2*A*fbgt1* mutant. Cultures grown in Sabouraud liquid medium (2% glucose + 1% mycopeptone, Biokar Diagnostics, Pantin, France) were used for DNA extraction. For transformation experiments, minimal medium (glucose, 10 g/l; ammonium tartrate, 0,92 g/l; KCl, 0.52 g/l; $MgSO_4.7H_2O$, 0.52 g/l; KH_2PO_4, 1.52 g/l; trace element solution (13), 1ml/l; pH adjusted to 6.8) was used. Drug inhibition assays were performed in 1% yeast extract medium (9). *Escherichia coli* strain that was used to produce the recombinant endoβ(1-3)glucanase (laminarinase A) from *Thermotoga neapolitana*, is a kind gift from Dr Vladimir V. Zverlov, Institute of Molecular Genetics, Russian Academy of Science, Moscow, Russian Federation) (14).

Preparation of cell walls and autolysis

The *Afbgt1* mutant was grown in a 4-L fermenter in 2% glucose and 1% mycopeptone for 24 h at 25°C as described previously (4). The mycelium cell wall was prepared as described (4). The cell wall was resuspended in 100 mM sodium acetate pH 6.2 containing 5 mM sodium azide (around 250 g wet weight/liter) and incubated at 37°C under agitation for 3h. The suspension was centrifuged (10,000g, 10 min) and the supernatant was dialyzed against 20 mM TrisHCl pH 7.5.

Protein purification

The dialyzed autolysate was applied to a DEAE-Sepharose column (10 x 3.2 cm, Sigma) equilibrated in 20mM TrisHCl pH 7.5 and eluted with a linear gradient of 0-0.5 M NaCl (400 ml) at a flow rate of 25 ml/h. Fractions containing branching activity were pooled, dialyzed against 20mM TrisHCl pH 8.0, applied to a DEAE-5PW column (7.5 x 0.8 cm, TosoHaas) and eluted with a NaCl gradient (0-250 mM in 50 min and 250-500 mM in 10 min) at the flow rate of 0.75 ml/min. Fractions containing branching activity were pooled, dialyzed against 20 mM sodium acetate pH 4.0, applied to a Mono-S column (HR 5/5, Pharmacia) and eluted with a NaCl gradient (0-250 mM in 50 min and 250-500 mM in 10 min) at the flow rate of 0.75 ml/min. Fractions containing branching activity were pooled and dialyzed against 20 mM NaOAc pH 5.0 and stored at -20°C.

SDS-PAGE and Western blot

Protein amount was estimated by the Bradford colorimetric assay (Biorad) using BSA as standard. Protein samples were analyzed by SDS-PAGE using 10 or 12% acrylamide separating gels (15). Protein bands were visualized by silver nitrate or Coomassie blue staining. Galactofuranose containing glycoproteins were detected by western blot with a monoclonal antibody (E2b, (16)).

Deglycosylation methods

Enzymatic N-deglycosylation of purified protein was carried out using a recombinant PNGase F (Roche) according to the manufacturer's instructions. Total chemical deglycosylation was carried out using the trifluoromethanesulfonic acid (TFMS) reagent (17). 10 µg of purified protein was freeze-dried and kept under vacuum in presence of P_2O_5. Sample was treated with 50 µl of TFMS/anisol solution (2/1, v/v) in a water-ice bath under argon atmosphere for 3h. The reaction was stopped by addition of 60% pyridine in an ethanol/dry-ice bath until pH 6 was achieved. Deglycosylated protein samples were dialyzed against water and analyzed by SDS-PAGE.

Peptide Sequencing

Internal peptides were obtained after in-gel endolysin C-digestion of the proteins that had been separated by SDS-PAGE. N-terminal and internal peptide sequencing was performed by J. d'Alayer (Laboratoire de microséquençage des protéines, Institut Pasteur, Paris) on an Applied Biosystems 470 gas-phase sequencer, as previously described (18).

Production and characterization of linear and branched β(1-3)glucan oligosaccharides

Branching activity was based on the detection of endo β(1-3)glucanase-resistant branch point by HPLC. A mixture of linear β(1-3)glucan oligosaccharides, used as substrate for branching activity, was obtained by acetolysis of curdlan (a kind gift from Hidemitsu Kobayashi) as previously described without BH_4Na reduction step (19). MALDI-TOF analysis showed that the oligosaccharide fraction used is a mixture of DP5 to DP20 with an estimated average DP of 10. Soluble β(1-3)glucans were freeze-dried and stored at room temperature.

Individual reduced laminarioligosaccharides were obtained by acetolysis of curdlan and reduced by 10 mg/ml BH_4Na in NaOH 0.1M overnight at room temperature (19), then purified by HPAEC as described below.

Analysis of the laminarinase resistant product (G3)

β(1-6) branched β(1-3)glucans oligosaccharides were previously purified as the SNQz I-B fraction (2). 15 mg of SNQz I-B fraction were digested by 500 µl of

recombinant laminarinase A (Lam-A, specific activity: 10 µmol eq. glucose/ml/min) in 1 ml of 100 mM sodium acetate pH 6.2 at 45°C for 24h (14). Resulting products were purified by gel filtration on a Biogel P2 column (Biorad, 1.4 x 90 cm) equilibrated in 0.2% acetic acid at the flow rate of 5 ml/h. Sugars were detected by refractometry and the laminarinase A resistant product (G3) was identified by HPAEC as described below. Size of G3 was determined by MALDI-TOF. Methylation analysis was done on the borodeuteride reduced G3 using the NaOH procedure (20). Permethylated sample was analyzed by GC-MS after trifluoroacetic acid hydrolysis, reduction and peracetylation.

Enzymatic assays

β(1-3)glucanase activity was measured by reducing sugar assay using the *p*-hydroxybenzoic acid hydrazide reagent with borohydride-reduced laminarin as previously described (4).

The presence of branching activity was assayed by incubating enzyme fractions in 50 mM sodium acetate, pH5 at 37°C with soluble non-reduced linear β(1-3)glucans (4 mg/ml final concentration) in a final volume of 40 µl for 2 h. Reaction was stopped by addition of 80 µl of a solution of chloroform/methanol (1/1, v/v). After drying under vacuum, the material was solubilized in 30 µl of 50 mM sodium acetate pH 6.2 and 10 µl of a Lam-A (specific activity: 10 µmol eq. glucose/ml/min) (14). After an overnight incubation at 45°C, digested products were analyzed by HPAEC as described below.

Transfer product analysis

To characterize transfer products, reduced laminarioligosaccharide (rG5, rG6, rG7, rG8, rG10, rG12) were incubated at the final concentration of 3mM with 0.05 µg of recombinant enzyme in 30 µl of 50 mM sodium acetate pH 5.5 at 37°C. Enzymatic products were analyzed by HPAEC. For a complete characterization of products, 6 mg of reduced laminari-hexaose (rG6) were incubated in the same condition for 2 h. Transfer products were purified by gel filtration on a TSK-HW40S column, (TosoHaas, 90 x 1.6 cm) equilibrated in 0.25% acetic acid at the flow rate of 0.4 ml/min. Oligosaccharides were detected by a refractometric detector. Separated fractions were freeze-dried and analyzed by MALDI-TOF, methylation and NMR. Methylation was performed with the NaOH procedure (20).

High performance anion exchange chromatography (HPAEC, Dionex, model ISC3000) of oligosaccharides

The Lam-A digests were analyzed on a CarboPAC-PA1 column (4.6 x 250 mm) using NaOH (50 mM) and sodium acetate 500 mM in 50 mM NaOH as eluent A and B respectively. The column was pre-equilibrated for 20 min in 98%A+2%B. Following sample injection, a gradient run (flow rate 1 ml/min) was performed as follows: 0-2 min, isocratic step (95%A + 2%B), 2-15 min 98%A + 2%B – 80%A + 20%B, 15-22 min 80%A + 20%B -57%A + 43%B, 22-23 min 57%A + 43%B-100%B, and 23-25 min 100%B. Samples were detected on a pulsed electrochemical detector. Transfer products obtained after enzymatic reaction were analyzed by HPAEC on a CarboPAC-PA200 column (3.2 x 250 mm). Same eluents are used in the following condition, a flow rate of 0.35 ml/min and a gradient run: 0-2 min, isocratic step (95%A + 2%B), 2-15 min 98%A + 2%B – 65%A + 35%B, 15-35 min 65%A + 35%B - 40%A + 60%B, 35-37 min 40%A + 60%B- 100%B, and 37 - 40 min 100%B. Reduced laminarioligosaccharides were purified on a semi-preparative CarboPAC-PA-1 column (9 x 250 mm). Same eluents are used in the following conditions: flow rate of 4 ml/min and a gradient run: 0-2 min, isocratic step (90%A + 10%B), 2-20 min 90%A + 10%B – 60%A + 40%B, 20-45 min 60%A + 40%B - 40%A + 60%B, 45-50 min 40%A + 60%B- 100%B, and 50 - 51 min 100%B.

Gas liquid chromatography (GC) and mass spectrometry (MS).

GC and GC-MS was performed on a Perichrom PR2100 instrument and on an Automass II apparatus (Finigan) coupled to a CarloErba gas chromatograph (model 8000top) as previously described (21). Matrix-assisted desorption ionisation/time of flight (MALDI-TOF) mass spectra were acquired on a Voyager Elite DE-STR mass spectrometer (Perspective Biosystems, Framingham, MA, USA) equipped with a pulsed nitrogen laser (337 nm) and a gridless delayed extraction ion source. The spectrometer was operated in positive reflectron mode as previously described (22).

NMR Spectroscopy

NMR spectra were acquired at 288K on a Varian Inova 600 spectrometer equipped with a cryogenically-cooled triple resonance $^{1}H\{^{13}C/^{15}N\}$ PFG probe. Samples were dissolved in 420 µl D_2O (99.97% ^{2}H atoms, Euriso-top, CEA, Saclay, France) and transferred in a 5 mm Shigemi tube (Shigemi Inc., Alison Park, United States). ^{1}H and ^{13}C chemical shifts were referenced to external DSS (23). Intra-glycosidic residue nuclei were first assigned as previously described (2). Then, $^{1}H,^{1}H$ coupling constants were extracted from 1D and/or 2D spectra (^{1}H resolution of 0.1 Hz and 0.6 Hz respectively). The anomeric configuration was established from the knowledge of $^{3}J_{1,2}$ value. Finally, interglycosidic linkages were established *via* through-space dipolar interactions using ^{1}H-^{1}H ROESY experiment (mixing time of 250 ms) (24) and/or via multiple-bond ^{1}H-^{13}C correlations using ^{1}H-^{13}C gHMBC experiment (long range delay of 60 ms) (25).

Enzymatic Kinetic analysis

The amount of transfer product was measured by the integration of the branched glucan area analyzed by HPAEC after Lam-A digestion of the Bgt2p reaction products. pH optimum was studied by the incubation of 0.2 µg of recombinant protein with 20 mg/ml of laminarioligosaccharides in 40 µl of 125 mM sodium citrate buffer in the pH range of 3 to 9 at 37°C for 30 min. Temperature optimum was estimated by the incubation of 0.2 µg of recombinant protein with 20 mg/ml of laminarioligosaccharides in 40 µl of 0.2 M sodium acetate buffer at pH 5.5 in the temperature range of 4 to 65°C for 15 min.
Specific activities and *K*m were estimated by the incubation of 0.075 µg of recombinant protein in 0.2 M sodium acetate buffer pH 5.5 at 37°C for 30 min with a range of substrate concentration of 1 to 12 mg/ml.

Construction of the AfBGT2 deletion cassette
The cassettes for deleting Af*BGT2* and Af*BGT1* genes were constructed by PCR fusion with the method described by Lamarre *et al*. (26). The resistance marker used to replace Af*BGT2* was the *HPH* gene of *E. coli* coding for hygromycin B phosphotransferase obtained from pAN7-1 plasmid (27). Site specific recombination was ensured by association of an approximately 1.4 kb upstream and 1.3 kb downstream Af*BGT2* flanking fragments. In a first PCR round (Suppl Fig. 1) flanking region 1 (amplicon 1, primers Bgt2A-Bgt2B), flanking region 2 (amplicon 2, primers Bgt2E-Bgt2F) and *HPH* gene (amplicon 3, primers Bgt2C-Bgt2D) were amplified respectively from wild type DNA template and pAN7,1 with primers cited in the table 1 of supplementary data. Thirty cycles consisting of a 30 s 95°C melting step and a 3 min 68°C annealing/extension step were performed (Advantage 2 polymerase, Clonetech). Primers B, C, D and E were 60 bp chimeric oligonucleotides, containing at the 5'- end a reverse complement sequence (B with C and D with E) for fusion PCR. The obtained PCR products were gel-purified and used for a second PCR step that allowed the fusion of these three separate fragments by using Bgt2A and Bgt2F primers. The PCR conditions were the same as described above except a 6 min elongation step. The resulting PCR was purified and used to transform *A. fumigatus* conidia.
The *BLE* gene, coding for phleomycin resistance, was used as a resistance marker and amplified from the pAN8-1 to delete the Af*BGT1* gene (28). The site specific recombination was ensured by association of an approximately 1 kb upstream and 1.1 kb downstream *BGT1* flanking fragment. Primers Bgt1A and Bgt1B were used to amplify the first flanking region, primers Bgt1E and Bgt1F to amplify the second flanking region and primers Bgt1C and Bgt1D to amplify *BLE* gene. To perform the PCR fusion step primers Bgt1A and Bgt1F were used.

AfBgt2 and double Afbgt1/Afbgt2 mutants
The fusion PCR product (1 to 2 µg) was used to transform *A. fumigatus* CEA17*ku80Δ* conidia by the electroporation method described by Sanchez *et al*. (29) with subsequent modifications. Conidia from CEA17*ku80Δ* strain were washed five times with water and 10^9 conidia were inoculated in 125 ml of YG medium (0,5% yeast extract, 2% glucose) and incubated at 37°C at 300 rpm for 4 hours. Conidia were recovered by centrifugation, washed with water, inoculated in 12.5 ml of YG medium (1% yeast extract, 1% glucose, 20mM Hepes pH 8.0) and incubated for 1 hour at 30°C at 100 rpm. Conidia were centrifuged and resuspended in 1 ml of cold 1 M sorbitol. One to 2 µg of DNA was added to 50 µl of conidial suspension, incubated for 15 min on ice and transferred to 0.1 cm electroporation cuvettes. Electroporation was performed using the Bio-Rad gene pulser (Gene Pulser Xcell) with the following parameters: voltage 1 kV, capacitance 25 µF and resistance 400 Ω. After transformation, 1 ml of cold YG medium was added to the cuvette, conidia were transferred to a 10 ml sterile tube and incubated on ice for 15 min. Tubes were incubated at 30°C at 100 rpm for 1h30. Conidia were plated on minimal medium (500 µl/9 cm diameter Petridish) and incubated at 20°C overnight. Hygromycin (150 µg/ml) or phleomycine (20 µg/ml) was added in 10 ml overlay of minimal medium + 0.7% agarose, to allow transformants selection, and plates were incubated at 37°C for two days.
Genomic DNAs from hygromycin or phleomycin resistant transformants and parental strain were isolated as described by Girardin et al (30). To check the integration of the *HPH* cassette at the Af*BGT2* locus, DNA of the CEA17*ku80*Δ and Af*bgt2* mutant strains were digested with *Xba*I and *Hind*III restriction enzymes (Roche) and verified by Southern blot analysis with probe corresponding to the second flanking region.
To analyze the integration of the *BLE* cassette at the Af*BGT1* locus in the Af*bgt2* mutant, DNA of CEA17*ku80*Δ and the transformants were digested by *Xba*I and *Hind*III to check the Af*BGT2* interruption and by *Hind*III / *Eco*RI to check Af*BGT1* interruption and verified by Southern blot analysis with a PCR product corresponding to the second flanking region as probe.

Phenotypic analysis
Conidial germination and mycelial growth was analyzed on media of different composition (1% yeast extract+3% glucose medium, Sabouraud, minimal medium and RPMI medium) in 2% agar or liquid medium, at 20°C, 30°C, 37°C or 50°C, in absence or presence of sorbitol (0.5 M to 2 M), at different pH (5, 7 and 9). The effect of drugs was tested as described previously (9).

Cell wall analysis

After 24 h growth at 37°C in Sabouraud medium, mycelium of wild type and *Afbgt2* mutant strain were filtered on a Buchner funnel and extensively washed with water. Mycelium was broken during 2 min with 1mm diameter glass beads and TrisHCl 200 mM pH 8 in a 50 ml Falcon vial with a Fast-prep apparatus (MP). After protein extraction (2% SDS, 40 mM-βmercaptoethanol 50 mM TrisHCl, 5 mM EDTA, pH 7.4, 100°C for 10 min), alkali-soluble and insoluble fractions of cell wall were prepared as previously described (11). Neutral hexoses and hexosamines from cell wall were estimated by phenol/sulfuric acid and dimethylaminobenzaldehyde (DMAB) colorimetric assays respectively (31,32). Monosaccharides were identified by GC as alditol acetates obtained after hydrolysis (4N trifluoroacetic acid, 100°C, 4 h, or 8 N HCl, 3 h, 100°C) (33). Branching of cell wall β(1-3)glucan was estimated by HPAEC after laminarinase A digestion of the alkali-insoluble fraction. 50 µg of alkali-insoluble fraction were digested by Lam-A (10 µl at 10 µmol/min/ml) in 100 µl of 50 mM sodium acetate pH 6.2 and incubated for 24h at 45°C. β(1-3)glucan amount was estimated by Lam-A digestion and the *p*-hydroxybenzoic acid hydrazide reagent for the detection (4).

Recombinant protein expression and purification

The yeast *Pichia pastoris* GS115 and the pHILS1 plasmid (Invitrogen) were used to produce the recombinant protein, Bgt2p. A truncated form of Bgt2p lacking 23 amino acid at the C-terminal part (GPI anchoring sequence) was obtained by PCR amplification of Af*BGT2* cDNA with the primers Bgt2p1 and Bgt2p2 (Suppl. Table 1). The Bgt2p1 primer, complementary to nucleotide 56 to 73, contains an *Xho*I restriction site used for the cloning procedure in the pHILS1 plasmid, and the Bgt2p2 primer, complementary to 1315 to 1333 carry a *BamH*I site and an in frame TGA stop codon (Suppl. Table 1). The product of 1286 bp was cloned in the pHilS1 plasmid, carrying the *HIS* auxotrophy marker, at *Xho*I *Bam*HI site and used to transform GS115 strain with the lithium chloride method (Invitrogen). Transformants were selected on a histidine-depleted medium. The clones carrying integration in the alcohol oxidase gene (*AOX1)* are selected for their inability to grow in methanol medium.

P. pastoris strain was inoculated in BMMY medium (Invitrogen) for 72 h at 30°C and the induction was done by addition of 1% of methanol to the culture medium every 24 hours (Invitrogen manufacturer instructions). Five ml of culture supernatant of *P. pastoris* producing Bgt2p were dialyzed against 20 mM TrisHCl pH 7.5, then applied to a DEAE 5PW column and eluted with a NaCl gradient (0-250 mM in 50 min and 250-500 mM in 10 min) at the flow rate of 0.75 ml/min. Proteins were detected with a UV detector (λ=280 nm). The purity of the protein was checked by SDS-PAGE. The protein was dialyzed against sodium acetate 10 mM pH 6 and kept frozen.

Results

An HPLC method to detect β(1-6)branching of β(1-3)glucan chains

The digestion of linear β(1-3)glucan oligosaccharides by a recombinant endoβ(1-3)glucanase (14) produced glucose and laminaribiose (G2). In contrast, the enzymatic digestion of a β(1-6)branched β(1-3)glucan fraction from the alkali-insoluble cell wall extract produced an additional peak (G3), eluted after the laminaribiose by HPAEC (Fig. 1). MALDI-TOF spectra revealed one $[M + Na]^+$ pseudomolecular ion at m/z = 527, corresponding to a trisaccharide. Methylation analysis done on the borodeuteride reduced G3, revealed the presence of three equimolar methyl-ethers: 1,2,4,5,6-penta-O-methyl-glucitol, 2,3,4,6-tetra-O-methyl-glucitol and 2,3,4-tri-O-methyl-glucitol corresponding to a glucose at the reducing end substituted at the position 3, a terminal non-reducing end glucose and a glucose substituted in the position 6 respectively (Fig. 1C). Based on these MALDI-TOF and methylation data, the G3 structure was deduced as the following linear sequence βGlc1-6βGlc1-3GlcOH. This HPAEC detection of the β(1-3)/(1-6) glucan was used to develop an HPLC assay to detect β(1-3)glucan branching activity.

Purification of a β(1-3)glucan branching enzyme

A cell wall autolysate of a Af*bgt1* mutant (11) was incubated with a curdlan acetolysate containing a mixture of linear soluble β(1-3)glucan chains with DP 5-20. The presence of G3 after Laminarinase A digestion and HPLC step indicated the presence of a β(1-3)glucan branching enzyme in the cell wall autolysate.

Two fractions containing a β(1-3)glucan-branching activity were eluted at 40 and 100 mM NaCl respectively in a DEAE-5PW column (Fig.2). After the chromatographic step on MonoS column, SDS-PAGE showed that the first fraction migrated as a wide band with a Mr of 25 to 50 kDa typical of glycosylated protein, and the second fraction as a discrete band with a Mr of 21 kDa (Fig. 2). Fraction I was digested with N-glycosidase F or treated with TFMS reagent. N-glycosidase F treated enzyme did not modify the SDS-PAGE pattern. In contrast, TFMS treatment released a protein with a Mr of 21 kDa, indicating that the native protein of fraction I was highly O-glycosylated. The presence of mannose and galactose residues identified by GC in the respective ratio 1/1.2, (not shown) and the positive reactivity in western blot of fraction I with anti-galactofuran MAb indicated

the presence of O-glycan with galactofuranose residues (Fig 2). This was in agreement with the presence of O-glycan previously characterized in glycoproteins isolated from *A. fumigatus* mycelium (34).

Analysis of the Af*BGT2 gene sequence*

The sequence of three peptides from the fraction I (AAIAQYGDDLAK, NLVGTSGFTSAR and ANAGIGTNPDEI) and the N-terminal peptide (AHQGFN) from the fraction II were obtained. Based on these sequences, the homology search in the *A. fumigatus* JCVI genome sequence (http://www.tigr.org/tdb/e2k1/afu1/) showed that all four peptides belong to the same protein encoded by a gene we named Af*BGT2* (AFUA_3G00270) (Suppl. Fig. 2). The 1341 bp long open reading frame predicted a 447 amino-acid protein with a predicted molecular weight of 44.6 kDa. This gene contains one intron, four putative N-glycosylation sites, a region rich in serine at the C terminus (14%), a hydrophobic N-terminus, and a C-terminus characteristic of GPI anchored proteins with a carboxy peptidase cleavage site consensus sequence (Ω site: N423) (Suppl. Fig. 2). Comparative genomic analysis revealed that AfBgt2p orthologs only shared homology with members of the Glycosyl Hydrolases family 17 (CAZy: http://www.cazy.org/) and was homologous to AfBgt1p (17% identity) and ScBgl2p (18% identity). The GH17 family is constituted of enzymes that have been found in plant and fungi only, and are characterized by a catalytic site composed of two glutamic acids identified in plant enzymes (35,36). Peptide sequence alignment between AfBgt1p and *S. cerevisiae* Bgl2p suggested that the two glutamic acid of the catalytic site were conserved and were E128 and E239 in Bgt2p (Suppl. Fig. 2). A recombinant protein, lacking GPI signal sequence, was produced in *Pichia pastoris*. The recombinant protein, rBgt2p, migrated with a Mr of 175 kDa (Suppl. Fig. 3). After N-deglycosylation, the protein had an apparent Mr of 43 kDa (not shown), corresponding to the sequence predictions. The glycosylation of the rBgt2p was higher than the native *A. fumigatus* protein. In contrast to the recombinant rBgt2p, the deglycosylated form of the native protein was smaller than the predicted Mr (Fig. 2), suggesting that the enzyme was partially degraded by proteolysis during its secretion or during the cell wall autolysis.

Characterization of the transglycosidase activity

The incubation of proteins, purified from the cell wall autolysate and the recombinant protein rBgt2p with a mixture of linear laminarioligosaccharides followed by the LamA digestion, produced G1, G2 and the β(1-6)-β(1-3) trisaccharide (G3). This result confirmed that the branching activity of Bgt2p was not influenced by its glycosylation. The enzymatic characterization of the activity was performed with the recombinant protein. Figure 3 shows the products of the enzymatic reaction obtained after incubation of linear reduced β(1-3)glucan with rBgt2p. For each reduced laminarioligosaccharide, two types of products were obtained. Based on their retention time, one was the reduced laminaribiose (rG2) and the other one had an elution time depending on the substrate size. Using the rG6 as a substrate, MALDI-TOF mass spectra indicated that the higher molecular weight peak corresponded to rG10 $[M + Na]^+$ pseudomolecular ions at m/z = 2311. This result indicated a two-step transglycosidase reaction:

$$rG6 + E \rightarrow E\text{-}G4 + rG2$$
$$E\text{-}G4 + rG6 \rightarrow E + rG10$$

In the first step of reaction, the enzyme cleaved between the glucose unit 2 and 3 from the reducing end releasing reduced laminaribiose (rG2) from the reduced substrate. In the second step of reaction the enzyme transferred the remainder of the substrate to a second molecule of substrate, used as an acceptor, to give a reduced G10 (rG10).

The methylation analysis of rG10 transfer product showed the presence of several substitutions of glucose residue (Table I). In addition to glucose substituted at position 3 and glucose residues from each chain extremity, two other substitutions were observed: substitution at position 6 and bi-substitution at positions 3 and 6. A ratio of 6 between these two types of substitution indicated that the enzyme has transferred the glucan donor mainly inside the acceptor chain and for a minor part at the non-reducing end through a 1-6 linkage (Table I).

The NMR data (chemical shifts and coupling constants) of the rG10 transfer product are summarized in Table II. The 1D ^1H and 2D ^1H, ^{13}C gHSQC spectra exhibited five major signals (4.78/105.2 ppm, 4.75/105.3 ppm, 4.74/105.4 ppm, 4.66/105.7 ppm and 4.53/105.3 ppm) and one minor signal (4.71/105.5 ppm) in the sugar anomeric region. The ^1H and ^{13}C chemical shift analysis of the corresponding sugars spin system and the examination of coupling constants values permitted the identification of β-glucose residues. The integration of these 5 major signals in the 1D spectrum showed that they were in the ratio 3/2/2/1/1 (corresponding respectively to A'+2A, B'+B, 2C, E and G in table II and suppl Fig 4). Other ^1H signals in the 1D spectrum (4.00 and 4.03 ppm) were not associated with a signal in the anomeric region and were thus assigned to one glucitol residue (suppl Fig 4). Downfield shifts were observed for H3 and C3 resonances of A', A, B', B, G and E glucose residues and for the glucitol indicating a 3-O substitution for these residues (in bold in Table II). Furthermore, in edited gHSQC experiment, four distinct methylene

carbons have been identified (3.91-3.73/63.3 ppm, 3.77-3.66/64.6 ppm, 3.84-3.63/65.4 ppm and 4.20-3.86/71.5 ppm) (data not shown). The downfield shifted C6 at 71.5 ppm assigned to B and minor D glucose residues indicated that both glucose residues were 6-O-substituted (in bold in Table II). The methylene carbons resonating at 64.6 and 65.4 ppm were assigned to the glucitol residue. In agreement with MALDI-TOF analysis, ten major residues were identified among which were identified: one glucitol residue, one 3,6-O-disubstituted β-Glc (B), six 3-O-monosubstituted β-Glc (A', 2A, B', E and G) and two unsubstituted β-Glc residues corresponding to non reducing ends (2C). These data are indicative of a major 6-O-branched β-(1-3)-Glc decasaccharide. In agreement with the two-step enzymatic reaction, it corresponds to a reduced laminarihexaose substituted by a laminaritetraose side chain. The ROESY and gHMBC experiments allowed to establish the following sequence of the laminaritetraose chain: C1-3A1-3B'1-3G that is branched to the B residue through a β(1-6)linkage. However, the position of B in the main hexaose chain could not be exactly determined. Indeed, due to strong overlap, it was not possible to distinguish between the two following sequences for the main chain: C1-3A1-3B1-3A'1-3E1-3Glcol or C1-3A1-3A'1-3B1-3E1-3Glcol. From these results, the branching point on the main chain occurred on the third or fourth residue from the non reducing end. A minor 6-O-monosubstituted β-Glc residue was identified (D) meaning a branching chain on the non reducing end of the main chain. A total branching degree of 1.0 was determined from the ratio of -6)-β-Glc- and -3,6)-β-Glc H6' protons area to glucitol H2 and H3 protons half area in the 1D spectrum. From NMR and methylation data, the transglucosidase reaction was estimated at 85% inside of the acceptor chain and 15% at the non reducing end using the laminari-hexaositol as substrate (Fig. 4). With larger substrate such as rG8 or rG10, transfer products were eluted as doublet or triplet peaks in the HPAEC analysis, indicating a multiple site of transfer on the acceptor (Fig. 3).

In addition to the rG10 transfer product, a rG14 transfer product has been observed after 1h of reaction (Fig. 3). The 1D ^{1}H spectrum of this rG14 was similar to the rG10 one except for the intensities of signals, showing an increase in -3,6-O-disubstituted and 6-O-monosubstituted β-Glc residues relative to glucitol (Suppl. Fig 4). A total branching degree of 1.9 was determined from the ratio of -6)-β-Glc- and -3,6)-β-Glc H6' protons area to glucitol H2 and H3 protons half area in the 1D spectrum, indicating that the rG10 transfer products was used again as a substrate. This enzyme is a new β(1-3)glucanosyl transferase producing a cross-linking between β(1-3)glucan chains through a β(1-6) linkage as described below.

The $G_{(2n-2)}$ transfer product has been observed with all substrates tested with a DP≥5 (Figs 3 and 5). After 60 min of incubation with the DP12 (rG12), the disproportionate amount of rG2, compared to rG18 and rG26, indicated that transfer products were too large to be eluted from the column. The percentage of substrate, first, second and third transfer products, estimated from pulsed amperometric detection, showed that the kinetic rate of transferase reaction and the recycling of transfer products increased with the substrate size (Fig. 5). The HPLC analysis of the lam-A digest of the incubation of rBgt2p with curdlan revealed that rBgt2p was able to branch insoluble β(1-3)glucan (data not shown).

In addition to transfer products, other smaller peaks were observed suggesting that a hydrolytic activity occurred at the same time. The estimation of these peaks in comparison with transfer products showed that lowering the concentration of substrate promoted hydrolysis (Fig. 6). At a concentration lower than 0.1 mM of rG10, less than 50% of transfer activity was observed whereas at 1mM of rG10 the transfer activity reaches 90%, indicating that the enzymatic reaction is an equilibrium between hydrolysis and transglucosylation depending on the substrate concentration.

Optimum pH and temperature were estimated to be 5.5 and 45°C respectively (data not shown). Using a range of substrate (mixture of laminari-oligosacccharides) concentration of 1 to 12 mg/ml showed that this enzyme activity followed a Michaelis-Mentens kinetic with a *K*m of 3 mM and a specific activity estimated at 1.2 µmol G3/min/µg of protein (Suppl. Fig. 5). At substrate concentration of the *K*m value the hydrolysis represented less than 10% of transfer activity. Even at concentrations far from the *K*m such as 0.1 mM there was still 40% transfer (Fig. 6) showing that the activity of Bgt2p was a true transferase activity.

Phenotype of Afbgt2 and Afbgt1/Afbgt2 mutants

Deletion of the Af*BGT2* gene and deletion of the Af*BGT1* gene in the Af*bgt2* mutant were undertaken, as shown in supplementary figure 1. The PCR and Southern blot analysis confirmed the replacement of Af*BGT2* gene by *HPH* gene at the gene locus. Also, PCR and Southern blot analysis confirmed the gene replacement of Af*BGT1* locus by *BLE* gene at the gene locus in the Af*bgt2* mutant (Suppl. Fig. 1).

Phenotypic analysis revealed that Af*bgt2* and Af*bgt1*Af*bgt2* mutants did not display a phenotype different from the wild type strain. Mycelium morphology and germination rate were the same for both strains. Growth rates on different media (RPMI, Sabouraud, 1% yeast extract + 3% glucose, minimal medium) and at different temperature (20°C, 30°C, 37°C, 50°C) were identical for all strains. Growth on solid Sabouraud medium with increasing concentration of

sorbitol up to 2 M or at different pH values (pH5, pH7 and pH9) were similar for both wild type and the 2 mutant strains. After 48 h at 37°C of growth in presence of different inhibitors (0.5 mg/ml Congo red, 1 mg/ml calcofluor white, 0.6 µg/ml caspofungin, 0.06 µg/ml micafungin, 0.6 mg/ml SDS) MIC values for each molecule were the same for the wild type strain and mutant strains (data not shown).

Cell wall analysis of both mutants and wild type strain showed no difference in terms of hexose and hexosamine content (data not shown). The branching level of β(1-3)glucan from both strains was measured by Laminarinase A digestion of alkali-insoluble fraction and HPAEC analysis. The profiles were the same for the wild type and mutants with a branched signal corresponding to 5% of the released glucose and laminaribiose (data not shown), showing that the branching of the cell wall β(1-3)glucans was not modified by the Af*BGT1*/Af*BGT2* deletion.

Discussion

We report the biochemical and molecular analysis of the first fungal β(1-3)glucanosyltransferase with a β(1-6)branching activity, AfBgt2p. Bgt2p cleaves laminaribiose from the reducing end of a β(1-3)glucan chain and transfers the remaining part mainly inside another β(1-3)glucan chain by a β(1-6)glucan linkage.

Orthologous proteins, AfBgt1p in *A. fumigatus* and ScBgl2p in yeast, have also a transglycosidase activity. The first enzymatic reaction step is the same for all of these enzymes with the release of the laminaribiose from the reducing end of the donor substrate. However, in contrast to AfBgt2p, the transfer is exclusively performed on the non-reducing end of the acceptor substrate during the second step of the enzymatic reaction. The reasons for having a transfer of β(1-3)glucan both at the non-reducing end of the donor β(1-3)glucan and inside the chain are unknown. It could only indicate that the environment (substrate, physicochemical conditions) are not adequate to permit enzymatic reactions *in vitro* that would totally mimick the activity of this protein *in vivo* or to an intrinsic property of the enzyme conformation. Sequence analysis showed that AfBgt1p and AfBgt2p belong to the GH17 family. Based on enzymatic, sequence and crystallography data, this family contains β-glucanase and transglucosidases (36). The active site of the GH17 family enzyme consists of two glutamate residues acting respectively as proton donor and nucleophile residues to cleave β-linked substrates at equatorial bonds and retaining the β-anomeric configuration. The transglycosidase activity of members of the GH17 family has only been demonstrated in the fungal kingdom. In bacteria, proteins belonging to the GH17 family are involved in the biosynthesis of cyclic β(1-3)β(1-6)glucans from *Bradyrhizobium japonicum* (37), but no enzyme activity has been characterized yet. Members of the GH17 family in plant are only endoglucanases cleaving homogeneous β(1-3) or heterogeneous β(1-3;1-4) linear glucans. Both enzymes have a similar eightfold β/α barrel 3D structure and substrate specificities are associated to minor differences in amino acid residues (35,38). In contrast to transglucosidase, β-glucanase does not recognize the reducing end of the β-glucan chain to cleave it, suggesting differences of substrate recognition between endoglucanase and transglycosidase activities belonging to the GH17 family.

A specific comparative genomic analysis of the GH17 family in fungi has been undertaken by BlastP (http://www.ncbi.nlm.nih.gov/sutils/). As shown in Figure 7, fungal members of the GH17 family can be assigned to five distinct groups: the first one including AfBgt1p (AFUA_1G11460) and ScBgl2p, the second one including AfBgt2p (AFUA_3G00270), groups III and IV including the Scw4p, Scw10p and Scw11p of *S. cerevisiae* and their homologs in *A. fumigatus* AfScw4p (AFUA_6G12380), Scw11p (AFUA_8G05610) and the fifth one composed only from filamentous fungus proteins including proteins encoded by AFUA_5G08780 from *A. fumigatus*. The transglucosidase activity has been identified in group I and II and it is interesting to note that the differences of transglucosidase activity are correlated with distinct groups. No enzymatic activity has been described for any members of the groups III, IV and V. In *S. cerevisiae*, the phenotypic analysis of the strains lacking *SCW4* and *SCW10* showed an increase in osmotic instability and in sensitivity to dye interfering with cell wall polysaccharides that was associated to a higher amount of chitin and alkali-soluble glucans, suggesting that three proteins have a role in cell wall morphogenesis (39,40).

The double Af*bgt1*/Af*bgt2* mutant has a wild type phenotype. This result suggested that at least one other branching activity is present in *A. fumigatus*. Other members of the GH17 family (AfScw4p and AfScw11p) are potential candidates for this new transglycosidase activity. This hypothesis is currently investigated. The biochemical methodology developed to identify branched β(1-3)glucans and the double Af*bgt1*/Af*bgt2* mutant will be essential tools to search for a new β(1-3)glucan branching enzyme.

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Abbreviations:

DEAE: Diethyl-amino-ethyl

DP: Degree of polymerization

DSS: 2.2-dimethyl-2-silapentane-5-sulfonate sodium salt

GC: Gas liquid chromatography

Gn: Oligosaccharide containing n glucose residues

gHMBC: Gradient-selected heteronuclear multiple bond correlation

gHSQC: Gradient-selected heteronuclear single-quantum correlation

HPAEC: High performance anion exchange chromatography

HPLC: High pressure liquid chromatography

MALDI-TOF: Matrix-assisted laser desorption ionization time-of-flight

MS: Mass spectrometry

PFG: Pulsed field gradient

rGn: Borohydride reduced oligosaccharide containing n glucose residues

ROESY: Rotating-frame Overhauser enhancement spectroscopy

TFMS: Trifluoromethanesulfonic acid

PMSF: Phenylmethylsulfonylfluoride

Legends to figures

Figure 1: Analysis of the Laminarinase A (Lam-A) digestion of β(1-3)glucans. HPAEC on a CarboPAC PA-1 of the Lam-A digest of linear laminari-oligosaccharides (A) and β(1-6) branched β(1-3)glucan (SNQz IB fraction) (B). GC analysis of partially methylated alditol acetates obtained after permethylation of borodeuteride reduced G3 (C). Glc, glucose; LB laminaribiose; 1,2,4,5,6-Glc, 1,2,4,5,6-penta-O-methyl-3-O-acetyl-glucitol; 2,3,4,6-Glc, 2,3,4,6-tetra-O-methyl-1,5-di-O-acetyl-glucitol; 2,3,4-Glc, 2,3,4-tri-O-methyl-1,5,6-tri-O-acetyl-glucitol.

Figure 2: SDS-PAGE and western blot analysis of Bgt2p from *A. fumigatus*. A, Chromatographic purification step on DEAE-5PW column of the β(1-3)glucan branching activity from the cell wall autolysate. B, SDS-PAGE analysis and silver nitrate staining. Lane 1, Purified fraction I, lane 2, PNGase treated fraction I, lane 3, TFMS treated fraction I, lane 4, purified fraction II; MW, molecular weight marker. C, Western blot and detection with anti-galactofuranose monoclonal antibody; lane 1, purified fraction I; lane 2, purified fraction II.

Figure 3: HPAEC on a CarboPAC PA-200 column of products obtained by incubation of reduced laminari-oligosaccharides with rBgt2p. rGn, reduced laminari-oligosaccharide containing n glucose residues

Figure 4: Scheme of the two steps of the enzymatic reaction of rBgt2p with the laminarihexaose as substrate.

Figure 5: Effect of substrate size on the transfer product formation. Subst, substrate; T1, first transfer product; T2, second transfer product; T3, third transfer product.

Figure 6: Effect of the substrate concentration.
Transfer rate with different concentration of reduced laminari-decaose (rG10) used as a substrate. Hydrolysed and transfer products were estimated by HPAEC on a CarboPAC PA200 column.

Figure 7: Dendrogram analysis of the GH17 protein family identified in yeast and filamentous fungus. The sequences of known and putative GH17 family proteins identified in *S.cerevisiae* (Sc), *Candida albicans* (Ca), *Magnaporthe grisea* (MGG), *Schizosacchromyces pombe* (Sp), *Aspergillus niger* (An), *Aspergillus nidulans* (AN), *Neurospora crassa* (NCU), *Yarrowia lipolytica* (YAL), *Nectria haematoccoca* (Nh), *Coccidioides immitis* (CIMG), *Penicillium marneffei* (PMAA or Pm), *Botryotinia fuckeliana* (BC or BfBC), *Paracoccidioides brasilensis* (Pb) were aligned and the dendrogram was generated using Clustaw X.

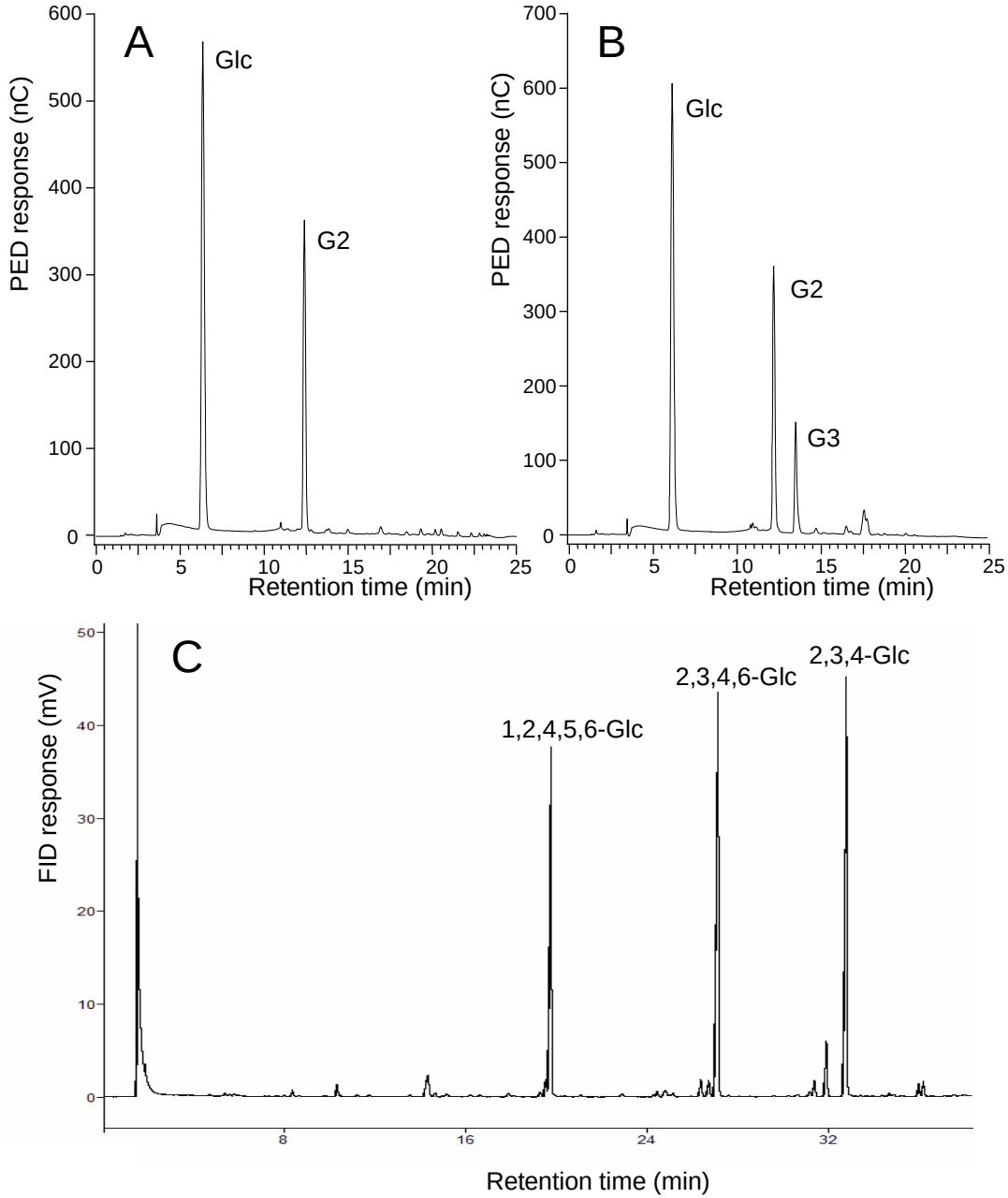
Table I : MALDI-TOF and methylation analyses of transfer product obtained after enzymatic reaction with reduced laminarihexaose. (methyl ethers were analysed as their itols).

Transfer product		
MALDI-TOF analysis		
m/z $[M + Na]^+$		1663
Corresponding dp		10
Methylation analysis		
Methyl ethers	Linkages	
1,2,4,5,6-Glc	-3Glucitol	0.5
2,3,4,6-Glc	Glc1-	1.6
2,4,6-Glc	-3Glc1-	6
2,3,4-Glc	-6Glc1-	0.2
2,3-Glc	-36Glc1-	1.2

Table II. ^{1}H and ^{13}C NMR chemical shifts (ppm) and coupling constants ($J_{H,H}$, Hz) for the rG10 transglycosidase product.The glucose residues were labelled A to G in order of decreasing chemical shift of their anomeric protons.

	H1 $^{3}J_{1,2}$ C1	H2 $^{3}J_{2,3}$ C2	H3 $^{3}J_{3,4}$ C3	H4 $^{3}J_{4,5}$ C4	H5 $^{3}J_{5,6}$ C5	H6 $^{2}J_{6,6'}$ C6	H6' $^{3}J_{5,6'}$
–3)–β-Glc-(1- A'	4.785 8.9 105.21	3.535 8.9 75.69	**3.757** #9 **87.38**	3.508 #9 78.28	#3.5 70.78	3.729 #11 63.34	3.914
–3)–β-Glc-(1- A	4.783 8.0 105.21	3.534 8.9 75.98	**3.767** 8.9 **86.73**	3.493 #8 78.28	3.493 70.78	3.727 #11 63.36	3.913
–3)–β-Glc-(1- B'	4.756 9.4 105.30	3.538 9.4 75.98or75.72	**3.775** **86.55**	3.506 78.20	3.506 70.70	3.727 #11 63.34	3.909
–3,6)–β-Glc-(1- B	4.751 8.7 105.30	3.558 8.7 75.97	**3.787** 8.9 **86.30**	3.598 8.7 70.66	3.675 77.06	3.881 10.7 **71.49**	4.211
β-Glc-(1- C non reducing enc	4.743 7.6 105.42	3.336 8.6-9.2 76.09	3.506 9.2 78.19	3.385 9.6 72.19	3.465 6.3 78.69	3.701 12.2 63.34	3.902
–6)–β-Glc-(1- D minor	4.712 7.62 105.52	3.358 8.6 75.91	3.516 8.3 78.01	3.475 8.7 72.13	3.653 77.35	3.859 10.6 **71.49**	4.202
-3)-β-Glc-(1- E	4.660 7.8 105.68	3.568 8.4 75.94	**3.768** 9.3 **86.63**	3.514 #8 77.97	3.492 70.95	3.724 11.5 63.33	3.920
–β-Glc-(1-6)- F minor	4.538						
–3)–β-Glc-(1-6)- G	4.530 7.6 105.27	3.493 8.6 75.67	**3.733** 9.1 **86.74**	3.489 #8 78.12	3.496 70.72	3.732 11.7 63.33	3.907
glucitol H reducing end	3.771-3.657 $^{3}J_{1,2}$ = 4.4 $^{3}J_{1',2}$ = 6.2 64.57	4.002 75.46	**4.034** **81.19**	3.627 9.1 72.66	3.884 73.35	3.835-3.627 11.1 65.35	 $^{2}J_{1,1'}$ = 12,1

Figure 1



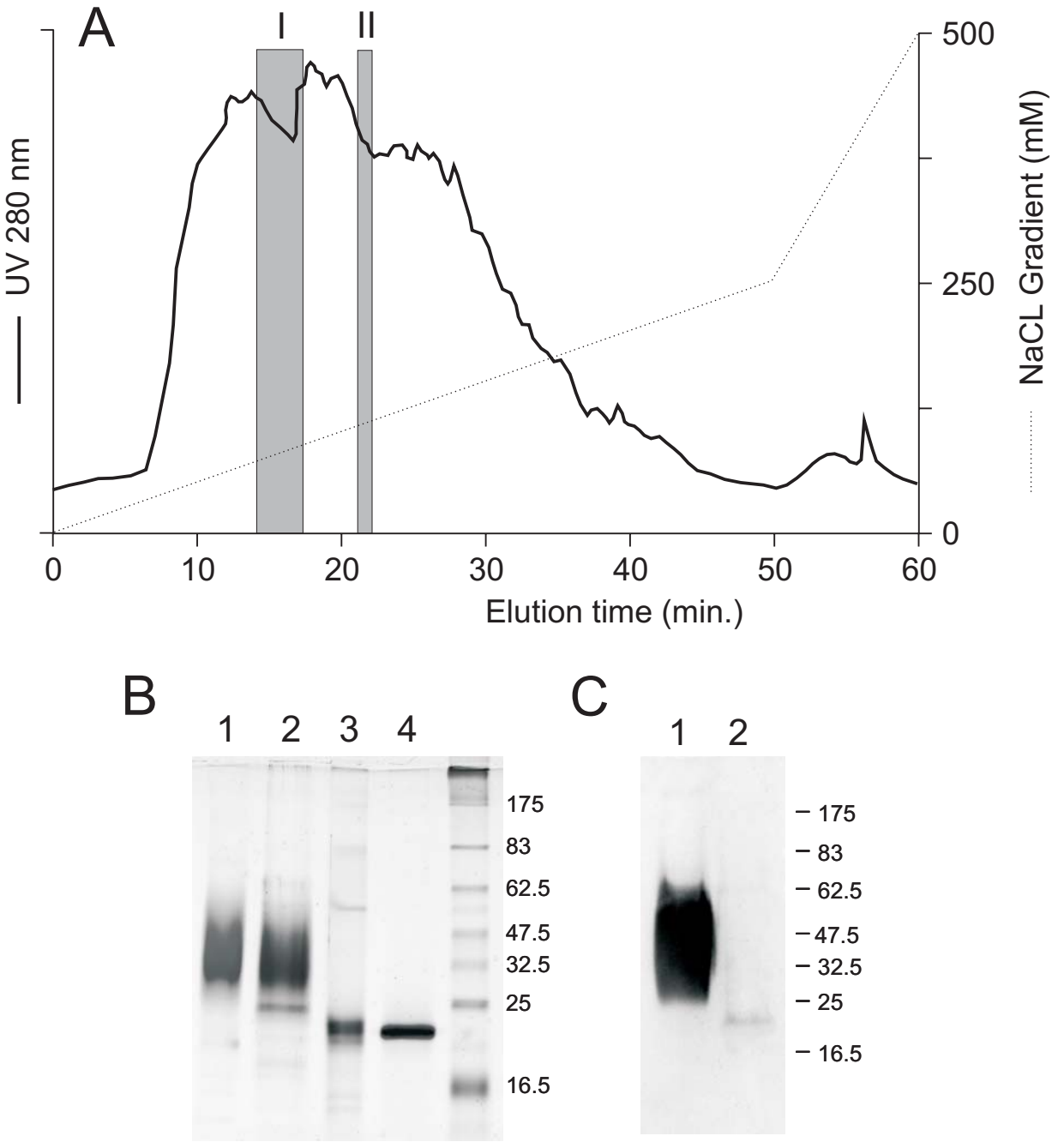
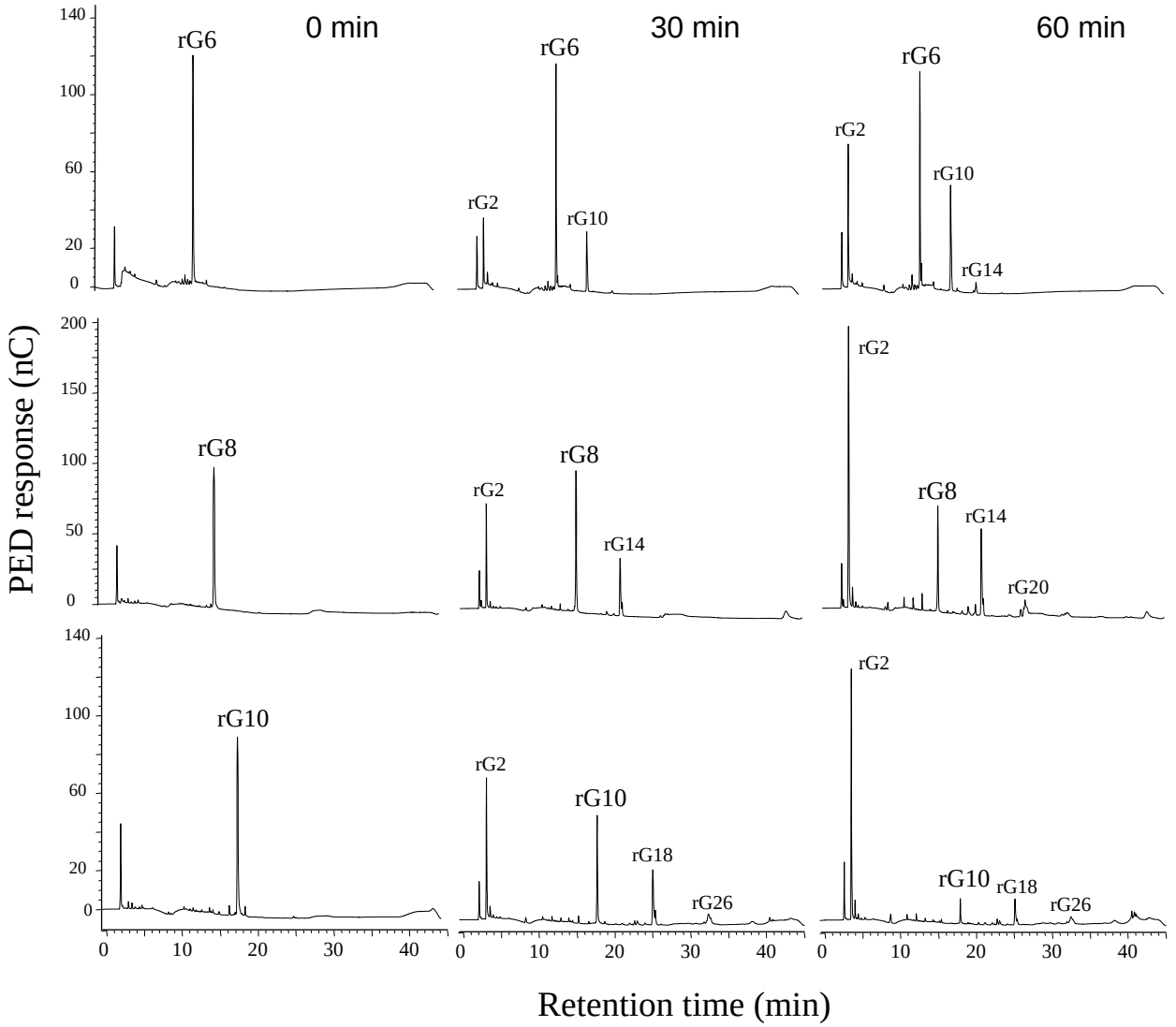


Figure 2

Figure 3



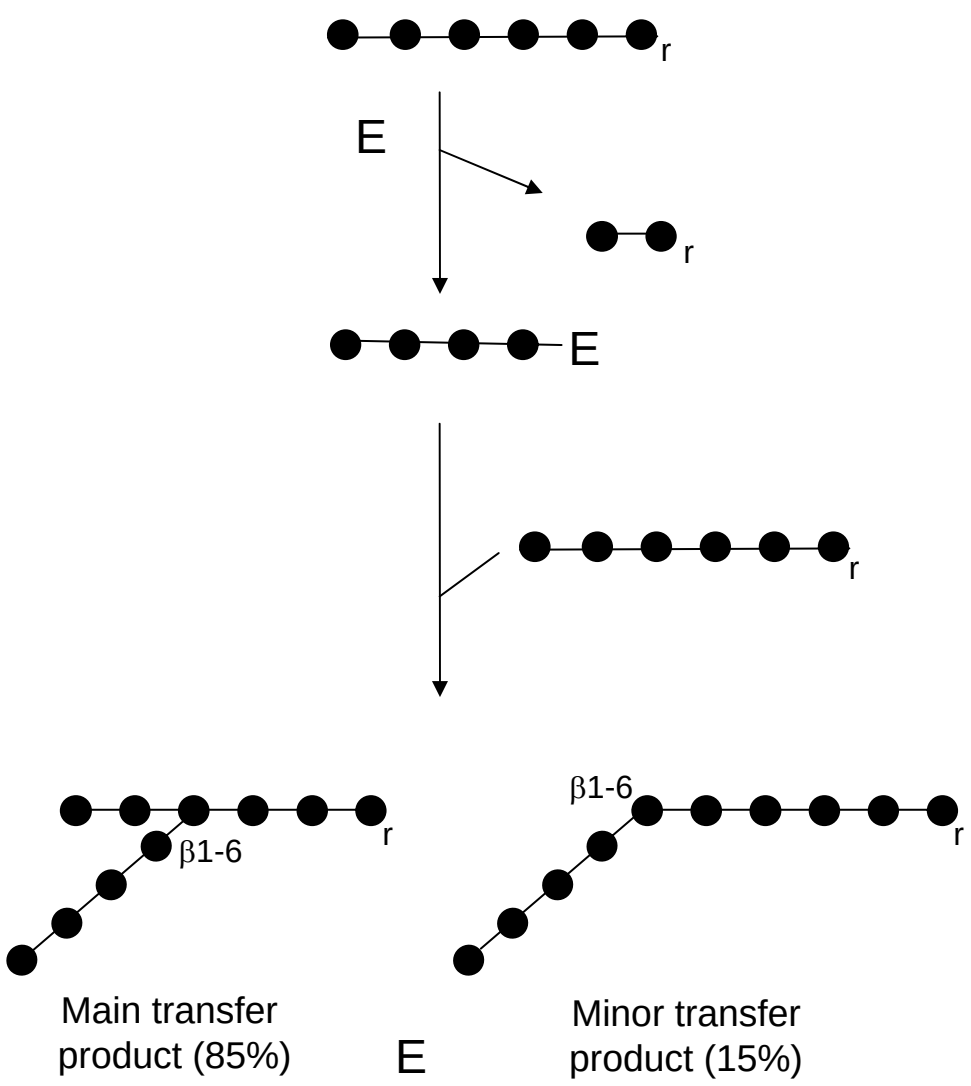
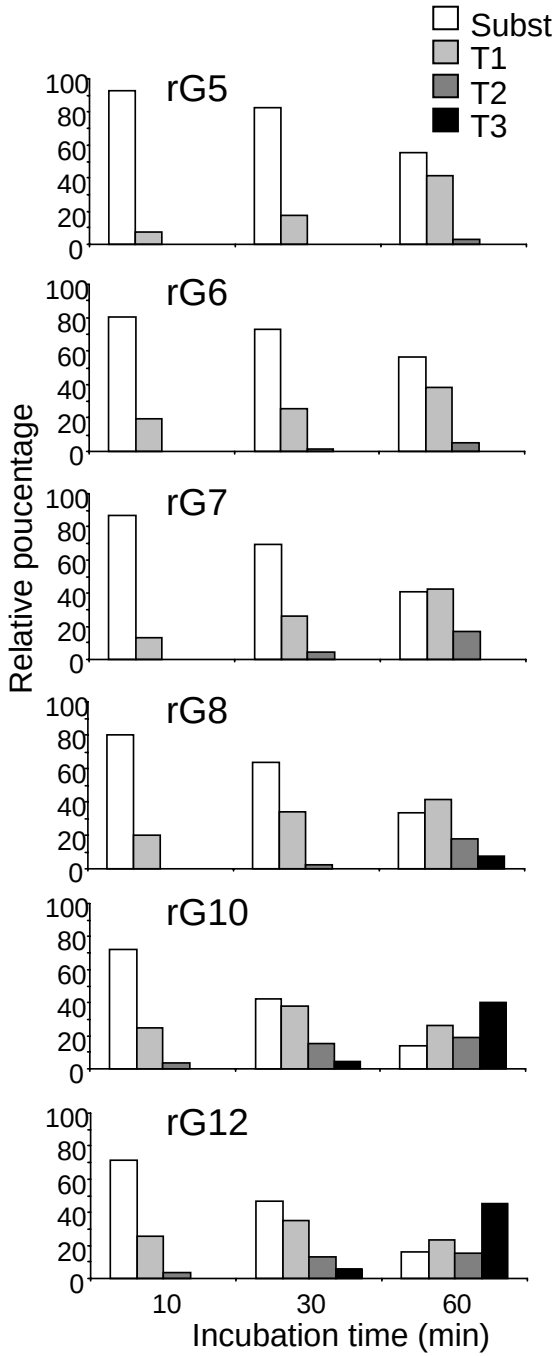


Figure 4

Figure 5



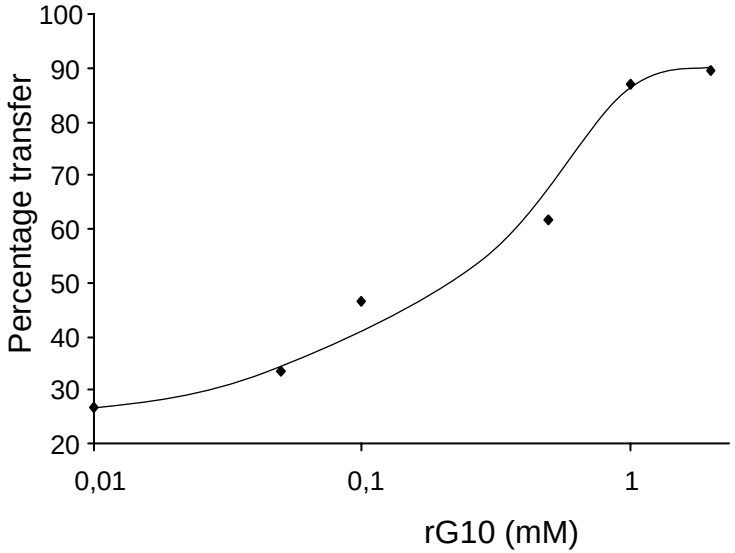


Figure 6

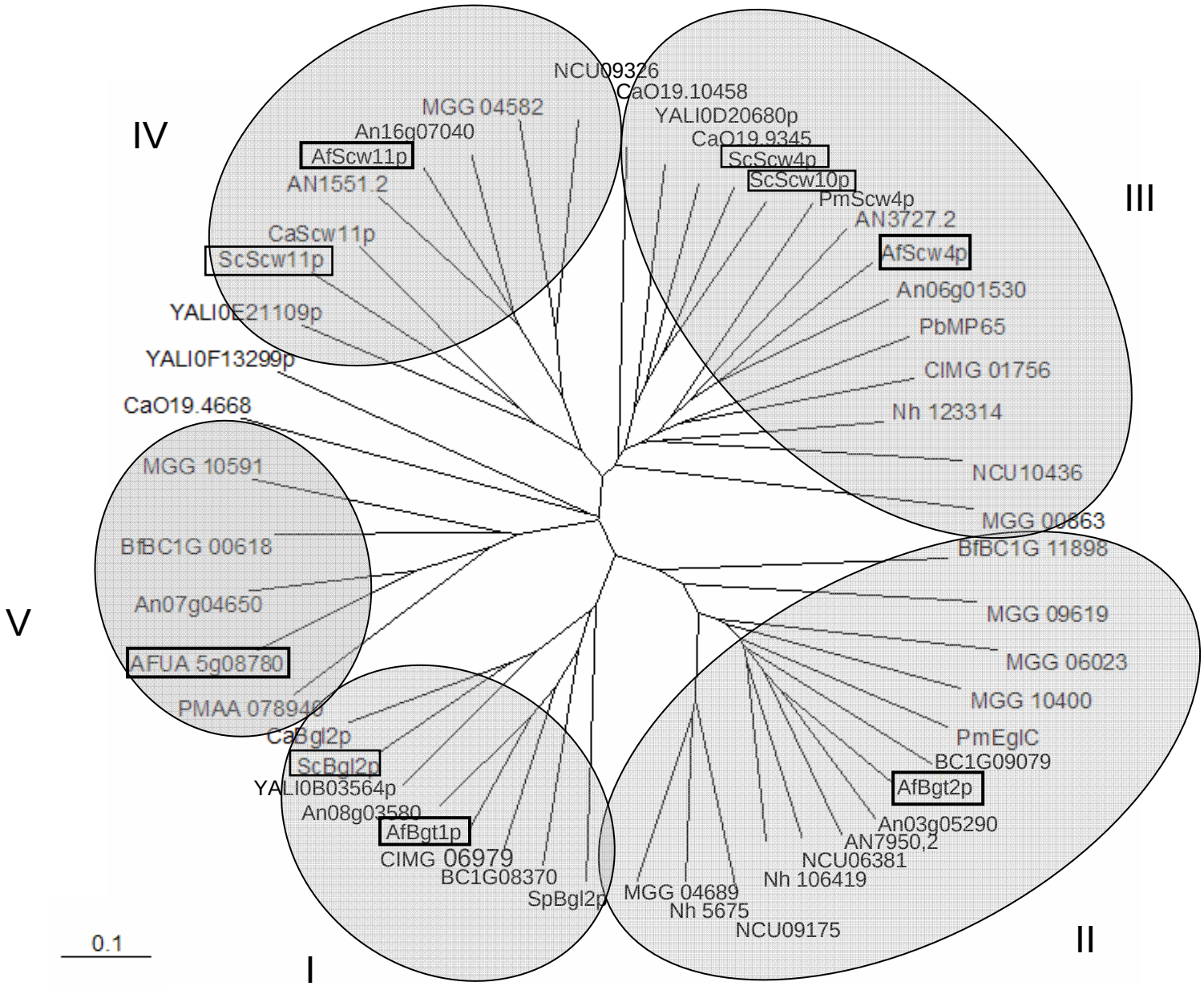


Figure 7

Characterization of a new β(1-3)glucan branching activity of Aspergillus fumigatus

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