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# Characterization of the GPI-anchored endo β-1,3-glucanase Eng2 of *Aspergillus fumigatus*

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ABSTRACT

A GPI-anchored endo β-1,3-glucanase of *Aspergillus fumigatus* was characterized. The enzyme encoded by *ENG2* (AFUA_2g14360) belongs to the glycoside hydrolase family 16 (GH16). The activity was characterized using a recombinant protein produced by *Pichia pastoris*. The recombinant enzyme preferentially acts on soluble β-1,3-glucans. Enzymatic analysis of the endoglucanase activity using Carboxymethyl-Curdlan-Remazol Brilliant Blue (CM-Curdlan-RBB) as a substrate revealed a wide temperature optimum of 24–40 °C, a pH optimum of 5.0–6.5 and a $K_m$ of 0.8 mg ml$^{-1}$. HPAEC analysis of the products formed by Eng2 when acting on different oligo-β-1,3-glucans confirmed the predicted endoglucanase activity and also revealed a transferase activity for oligosaccharides of a low degree of polymerization. The growth phenotype of the *Afeng2* mutant was identical to that of the wt strain.



## 1. Introduction

β-1,3-Glucan is the most abundant polysaccharide of the fungal cell wall and together with chitin it is responsible for cell wall rigidity (Gastebois et al., 2009). When facing morphological changes, for example during germination and branching in filamentous fungi or the separation of mother and daughter cells in yeasts, the cell wall needs to undergo partial lysis in order to obtain the required plasticity. Because of their mode of action endo β-1,3-glucanases should play an essential role during such morphogenetic events. Indeed the endoglucanase Eng1p has been shown to be essential for cell separation in yeasts (Baladron et al., 2002; Martin-Cuadrado et al., 2003; Esteban et al., 2005). A role of endo β-1,3-glucanases in cell wall remodeling of filamentous fungi on the other hand has not been shown to date. For example the deletion of the respective homolog *ENG1* in *Aspergillus fumigatus* did not lead to a phenotype different from the parental strain (Mouyna et al., 2002).

Glycosylphosphatidylinositol (GPI)-anchored proteins have been repeatedly shown to play a role in cell wall polysaccharide remodeling. For example it was found that GPI-anchored proteins belonging to the Gas (Gel) and Crh (Crf) families are able to modify cell wall β-glucans (Mouyna et al., 2000; Cabib et al., 2007). This makes GPI-anchored carbohydrate-active enzymes a promising target when studying the formation of the cell wall of *A. fumigatus*. Here we report the characterization of Eng2 (XP_755769) which was selected because it is a new endo β-1,3-glucanase belonging to the glycoside hydrolase family 16 (GH16), it features a GPI-anchor and it is unique to filamentous fungi.

## 2. Materials and methods

### 2.1. Strains and growth conditions

The *Afeng2* mutant and its parental strain CEA17*ku80Δ* (da Silva Ferreira et al., 2006) were maintained on 2% malt agar slants at room temperature. In the case of the *Afeng2* strain the medium was supplemented with 150 μg ml$^{-1}$ hygromycin B (Sigma). For DNA extraction cultures were grown in Sabouraud's liquid medium (2% glucose + 1% mycopeptone). Transformations were performed on minimal medium (10 g l$^{-1}$ glucose, 0.92 g l$^{-1}$ ammonium tartrate, 0.52 g l$^{-1}$ KCl, 0.52 g l$^{-1}$ $MgSO_4 \cdot 7H_2O$, 1.52 g l$^{-1}$ $KH_2PO_4$, 1 ml l$^{-1}$ trace element solution (Cove, 1966), pH adjusted to 7.0). The *Pichia pastoris* strain GS115 was used to produce recombinant Eng2. All media were prepared according to the supplier's manual (Invitrogen). The *Escherichia coli* strain One Shot TOP10 (Invitrogen) was used for plasmid propagation following the manufacturer's protocol.

*Abbreviations:* AI, alkali insoluble cell wall fraction; BSA, bovine serum albumin; CD, conserved domain; CM, carboxymethyl; DP, degree of polymerization; GC, gas liquid chromatography; GH, glycoside hydrolase; Gn, laminarioligosaccharide containing *n* glucose residues; GPI, glycosylphosphatidylinositol; HPAEC, high performance anion exchange chromatography; RBB, Remazol Brilliant Blue; RBV, Remazol Brilliant Violet; rGn, borohydride reduced laminarioligosaccharide containing *n* glucose residues.

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### 2.2. Nucleic acid extraction and RT-PCR

DNA was isolated as described by Girardin et al. (1993). RNA was isolated using phenol–chloroform extraction followed by ethanol precipitation. After an additional precipitation step using 5 M lithium chloride, RNA samples were loaded on RNeasy columns (QIAGEN) and subjected to on column DNase treatment using recombinant RNase-free DNase I from Roche. After elution from the RNeasy columns, a second DNase treatment was performed using the Turbo DNA-free kit from Ambion. RT-PCRs were performed using SuperScript II Reverse Transcriptase from Invitrogen following the manufacturer's instructions.

### 2.3. Production of recombinant Eng2 using P. pastoris

The *Pichia* Expression Kit from Invitrogen was used for recombinant protein production. A 1.9 kb cDNA fragment of *ENG2* was amplified using the primers Eng2p-FW and Eng2p-RV (Table 2) introducing an *Xho* I site at the 5′-end and a 6×His-tag followed by a stop-codon and an *Eco* RI site at the 3′-end. This fragment, coding for a truncated protein not featuring the 21 N-terminal (signal peptide) and 23 C-terminal (GPI anchoring sequence) amino acids, was subcloned into pCR-Blunt (Invitrogen) and sent for sequencing. After verification of the sequence, the two inserted restriction sites were used to clone the fragment into the expression vector pHIL-S1 resulting in pEng2. Correct integration was verified by digesting with *Sal* I (resulting in a 3.6 and a 6.5 kb fragment) and *Xho* I + *Eco* RI (resulting in a 1.9 and an 8.2 kb fragment). The plasmid was linearized using *Pst* I and used to transform the *P. pastoris* strain GS115 using the lithium chloride method according to the manufacturer. Transformants were selected on a histidin-depleted medium. The clones carrying integration at the alcohol oxidase gene (*AOX1*) were selected for their poor growth on methanol medium. The protein was produced following the manufacturer's instructions (Invitrogen) using an induction time of 56 h. Eng2 was concentrated and purified from culture supernatants using ProBond Resin (Invitrogen), eluted from the resin using 50 mM histidine and dialyzed against deionized water to remove histidine. The purity of the protein was checked by SDS–PAGE using silver nitrate staining. N-terminal peptide sequencing was performed as described previously (Beauvais et al., 1997).

### 2.4. SDS–PAGE and Western blot

Protein concentrations were measured using the Bradford Protein Assay (Bio-Rad) with BSA as a standard. Protein samples were mixed 3:1 with 4× protein loading buffer (50 mM Tris–HCl pH 6.8, 2% SDS, 10% glycerol, 1% β-mercaptoethanol, 0.02% bromophenol blue), boiled for 5 min and analyzed by SDS–PAGE using 10–15% acrylamide separating gels (Laemmli, 1970). Protein bands were visualized by silver nitrate or Coomassie blue staining. Recombinant Eng2 was detected by western blot with a monoclonal anti-polyhistidine antibody (peroxidase conjugated monoclonal anti-polyhistidine clone HIS-1, Sigma) using the Pierce ECL Western Blotting Substrate (Thermo Scientific). To test the functionality of recombinant Eng2 as an antigen, sera from a previous study were used (Sarfati et al., 2006). Sera from four aspergilloma patients and from four healthy persons as a negative control were pooled and detected with an anti-human IgG secondary antibody (peroxidase conjugated anti-human IgG antibody developed in goat, Sigma) using the same ECL substrate as mentioned above.

### 2.5. Enzymatic characterization

The recombinant protein Eng2 was characterized using the substrate Carboxymethyl-Curdlan-Remazol Brilliant Blue (CM-Curdlan-RBB, Loewe Biochemica GmbH) (Brisset et al., 2000). Enzyme assays comprised 0.1 ml of substrate, 0.2 ml 0.1 M sodium acetate buffer pH 5.5 and 0.1 ml enzyme solution containing 0.8 μg of protein (Bradford Protein Assay, Bio-Rad). Reactions were performed at 37 °C for 2 h and stopped by adding 0.1 ml 0.5 M HCl following incubation on ice for 10 min and centrifugation at 10,000*g* for 5 min. OD of the supernatants was measured at 600 nm.

To test Eng2 activity with different oligoglucan substrates, the amount of reducing sugars formed was estimated as described previously (Fontaine et al., 1997). Curdlan (insoluble β-1,3-glucan) was purchased from Serva, laminarin (rarely branched β-1,3-glucan), lichenan (β-1,3-1,4-glucan) and mannan were from Sigma, pustulan (linear β-1,6-glucan) from Chalbiochem, schizophyllan (highly β-1,6-branched β-1,3-glucan) from Kaken (Japan), and mutan (insoluble β-1,3-glucan) from Novozymes. The AI cell wall fraction and galactomannan were prepared in the lab as described previously (Beauvais et al., 2005; Costachel et al., 2005). Reactions were performed in 50 mM acetate buffer pH 6.2 containing 2 mg $ml^{-1}$ of the respective substrate and 8 μg $ml^{-1}$ of recombinant protein. After incubation at 37 °C for 15–120 min, 20 μl aliquots of the assays were added to 980 μl of a 50 mM sodium sulfite, 250 mM NaOH, 25 mM sodium citrate, 10 mM $CaCl_2$ solution containing 10 mg $ml^{-1}$ ρ-amino-hydroxybenzoic acid. After boiling for 10 min, OD was taken at 405 nm using glucose as a standard. Hydrolysis of chitin was tested using the substrate Carboxymethyl-Chitin-Remazol Brilliant Violet (CM-Chitin-RBV, Loewe Biochemica GmbH) in an assay similar to that of CM-Curdlan-RBB.

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

To characterize Eng2 glucanase and transferase activity reaction mixtures were prepared as follows: 500 μg $ml^{-1}$ substrate and 6 μg $ml^{-1}$ protein (Bradford Protein Assay, Bio-Rad) in 50 mM sodium acetate buffer (pH 6.2). Borohydride reduced laminarioligos prepared in the lab and reducing laminarioligos (G2–G7) purchased from Seikagaku Biobusiness (Japan) served as substrates. After incubation at 37 °C for the desired time, 10 μl aliquots were used for analysis. To test for branching activity, 25 μl aliquots of the reaction mixtures described above were boiled for 10 min to deactivate Eng2 and incubated over night at 37 °C with 0.2 U (μmol of reducing equivalents released $h^{-1}$ $mg^{-1}$ of protein) of recombinant LamA of *Thermotoga neapolitana* expressed in *E. coli* (a kind gift from Dr. Vladimir V. Zverlov, Institute of Molecular Genetics, Russian Academy of Science, Moscow, Russia; Zverlov et al., 1997). Again, 10 μl aliquots were used for analysis. The resulting oligosaccharides were analyzed by HPAEC on a CarboPAC-PA200 column (3.2 × 250 mm, Dionex) using NaOH (50 mM) and NaOAc (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 0.35 ml/min) was performed as follows: 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. Samples were detected on a pulsed electrochemical detector.

### 2.7. Analysis of the carbohydrate moiety of Eng2 using GC

Protein concentration was measured using the BCA protein assay (Pierce). Aliquots of the protein were mixed with meso-inositol serving as an internal standard. Hexoses were hydrolyzed (4 N trifluoroacetic acid, 100 °C, 2.5 h), reduced and peracetylated. The resulting alditol acetates were analyzed by gas–liquid chromatography using a Perichrom PR2100 Instrument equipped with flame

ionization detector (FID) and fused silica capillary column (30 m × 0.32 mm id) filled with BP1. Sugar concentrations were calculated using a standard mix containing meso-inositol, mannose, glucose and galactose which was treated in parallel with the samples.

### 2.8. Construction of strain Afeng2

Construction of the *ENG2* deletion cassette by fusion PCR and subsequent transformation of *A. fumigatus* conidia was performed as described by Gastebois et al. (2010). The gene *HPH* of *E. coli* coding for hygromycin B phosphotransferase was used as a marker gene to replace *ENG2*. Site specific recombination was ensured by association of approximately 1.4 kb up- and downstream flanking regions of *ENG2*. In a first PCR round the upstream flanking region, the downstream flanking region and the *HPH* gene were amplified from wild type DNA and pAN7-1 (Punt et al., 1987) using the primer pairs Eng2A + Eng2B, Eng2E + Eng2F, and Eng2C + Eng2D respectively. Primers Eng2B, Eng2C, Eng2D and Eng2E were 60 bp chimeric oligonucleotides, containing a reverse complement sequence (Eng2B with Eng2C and Eng2D with Eng2E) for fusion PCR at the 5′-end. The obtained PCR products were gel-purified and used for a second round of PCR with the primers Eng2A and Eng2F to join the three fragments. The resulting amplicon was gel-purified and used to transform *A. fumigatus* conidia by electroporation. For Southern analysis DNAs of the wt and the *Afeng2* mutant were digested with *Xba* I and *Eco* RV and hybridized with a 459 bp fragment of the upstream flanking region of *ENG2*. The fragment was amplified using the primers Pr-FW and Pr-RV and labelled with [α-$^{32}$P]dCTP using the Rediprime random prime labelling system from Amersham Biosciences. See Table 2 for primer sequences and Fig. 5 for construction of the deletion cassette, gene deletion and Southern analysis.

### 2.9. Phenotypic analysis

Mycelial growth on different media (on 1% yeast extract with 3% glucose, Sabouraud's medium, minimal medium and RPMI medium in solid and liquid cultures), growth at different temperatures (20, 30, 37 and 50 °C), conidial germination, growth in presence of different concentrations of sorbitol (0.5, 1, 1.5 and 2 M), at different pH (5, 7 and 9) and growth inhibition tests on SDS, Nikkomycin Z, Caspofungin, Mycafungin, Congo red and Calcofluor white were performed as described previously (Mouyna et al., 2005). To test growth on β-1,3-glucan minimal medium containing 1% curdlan (Wako, Japan) as the sole carbon source was prepared. Curdlan plates and liquid cultures were incubated at 37 °C.

## 3. Results

### 3.1. ENG2 sequence analysis

BLAST analysis identified 10 potential endo β-1,3-glucanases in the *A. fumigatus* genome based on homologies with different fungal and bacterial glucanases (Gastebois et al., 2009), and one of them has already been characterized (Mouyna et al., 2002). Two of the remaining proteins feature a GPI modification site predicted by the big-PI Fungal Predictor (Eisenhaber et al., 2004): AFUA_3g03080 and AFUA_2g14360 (*ENG2*). We were unable to detect expression of AFUA_3g03080 using two different primer pairs for RT-PCR. *ENG2* however was constitutively expressed in all tested growth conditions and expression was not stimulated by the presence of β-1,3-glucan (laminarin) in the culture medium (Fig. 1), suggesting that it is not involved in the acquisition of nutrients. Additionally recombinant Eng2 was highly recognized by sera pooled from aspergilloma patients while the negative control from

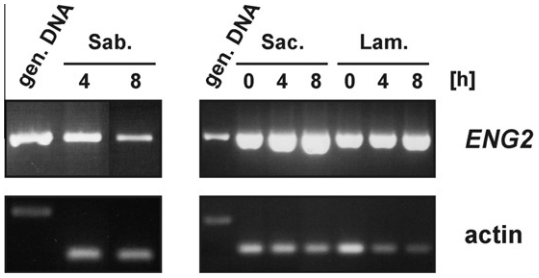


**Fig. 1.** The expression profile of *ENG2*. RT-PCR expression of a 358 bp fragment of *ENG2* after 4 and 8 h of growth on Sabouraud's medium (Sab. 4 and Sab. 8) and 0, 4 and 8 h of growth after addition of 0.5% saccharose (Sac. 0, Sac. 4, and Sac. 8) and 0.5% laminarin (Lam. 0, Lam. 4, and Lam. 8) to cultures pregrown for 16 h on minimal medium containing 0.5% saccharose. Primer sequences (Eng2-RT-FW, Eng2-RT-RV, Actin-RT-FW, and Actin-RT-RV) are listed in Table 2.

healthy persons only showed weak recognition (Fig. 2). This indicates that the protein is also formed in vivo. A BLASTP search based on the Eng2 sequence followed by the prediction of a signal peptide cleavage site (Emanuelsson et al., 2007) as well as a GPI modification site only yielded orthologs in other filamentous fungi. No *ENG2* orthologs were found in yeasts, only *Schizosaccharomyces pombe* features one glucanase of the GH16 family (CAB57923) which is however not GPI-anchored. The *ENG2* sequence was retrieved from the Central Aspergillus Data Repository site (http://www.cadre-genomes.org.uk/). The gene features an ORF of 1959 bp without introns. The encoded protein (XP_755769.1) of 652 amino acids has a predicted molecular mass of 66 kDa. Sequence analysis revealed a signal peptide cleavage site (SignalP) at position VSA$^{21}$-T$^{22}$A, a GPI modification site (big-PI) at LSN$^{630}$GA and two potential N-glycosylation sites (NetNGlyc) at positions N$^{200}$GSS and N$^{453}$GTS. A conserved domain search (CD search; Marchler-Bauer et al., 2007) has placed Eng2 in the family GH16 (cd00413). This family is divided into nine sub-families. Most of the sub-families have been characterized as pure glycoside hydrolases like GH16_lichenase (cd02175) or GH16_laminarinase (cd02180). The sub-family GH16_GPI_glucanosyltransferase (cd02183) however contains glycosyltransferases like Crh1p and Crh2p (Cabib et al., 2007). Eng2 features a GH16_MLG1_glucanase domain (cd02181) between amino acids 31 and 328.

### 3.2. Recombinant production of A. fumigatus Eng2 by P. pastoris

To characterize the enzymatic activity of Eng2 it was decided to construct a recombinant protein with conserved activity in *P. pastoris*. In the recombinant protein the original signal peptide sequence was replaced by that of *PHO1* for secretion by *P. pastoris*. The C-terminal GPI signal sequence was removed and a 6×His-tag was added resulting in a protein with a calculated protein mass of 63 kDa. A protein with an apparent protein size of approximately 150 kDa was produced by *P. pastoris* at a concentration between 10 and 50 ng ml$^{-1}$ and its identity was verified by Western

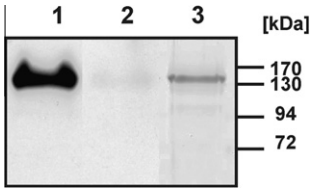


**Fig. 2.** The detection of Eng2 by sera from aspergilloma patients. Aliquots of recombinant Eng2 (0.62 μg per lane) analyzed by Western blot using sera pooled from four patients suffering from aspergilloma (1) and from four healthy persons (2) as a negative control. Lane 3 shows a parallel gel stained with Coomassie blue.

blot using a monoclonal anti-polyhistidine antibody (Fig. 3A). N-terminal sequencing showed that the eight first amino acids of the recombinant protein were QSQTYQLA instead of RETALEAR meaning that the N-terminus has been processed further than expected and that the first six amino acids of the original Eng2 sequence were lost (Fig. 3B). However the conserved domain identified by CD search was not altered by this deletion and suggested that the absence of these six amino acids at the N-terminus should not have an effect on the activity of the enzyme. Analysis of the carbohydrate content of recombinant Eng2 showed that the mass of the mature recombinant protein is composed to 56% of mannose and 2% of glucose. This result indicates that the considerable increase in apparent protein mass was due to hypermannosylation of this protein. Hyperglycosylation of recombinant proteins produced by *P. pastoris* has already been reported but did not influence enzymatic activity of the respective protein (Gastebois et al., 2010).

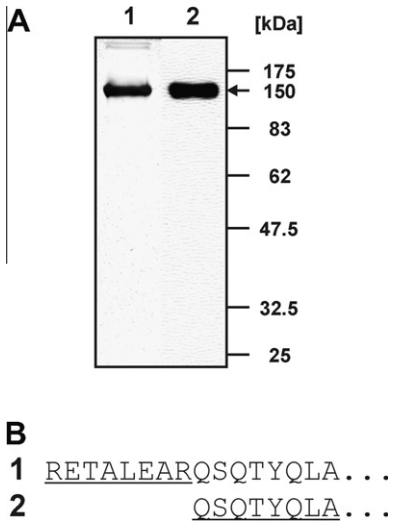


**Fig. 3.** Purification of Eng2, detection of the C-terminal 6×His-tag, and N-terminal sequencing. (A) Silver nitrate staining of an aliquot of recombinant Eng2 after purification and concentration using ProBond Resin (lane 1) and detection of Eng2 via Western blotting using a monoclonal anti-polyhistidine antibody (lane 2). (B) The expected N-terminal protein sequence (1) predicted by SignalP and the real N-terminal protein sequence (2) determined by sequencing.

**Table 1**
Endoglucanase activity of recombinant Eng2 with different β-1,3-glucans.

| Substrate | Activity (μmol $min^{-1}$ $mg^{-1}$) |
|---|---|
| rG6[a] | 1.16 ± 0.03 |
| rG19[a] | 0.86 ± 0.03 |
| Laminarin | 0.71 ± 0.01 |
| Curdlan | 0.18 ± 0.01 |
| Alkali insoluble (AI) cell wall fraction | Not detected |
| Lichenan | 0.23 ± 0.01 |

[a] rGn: reduced laminarioligosaccharide of *n* glucose units.

### 3.3. Enzymatic characterization of Eng2

The glycosylhydrolase activity of Eng2 was investigated by incubating the enzyme with different carbohydrate polymers. Among all polymers tested, only β-1,3-glucans and lichenan were degraded (Table 1) whereas no activities were found with pustulan, schizophyllan, mutan, galactomannan, mannan and chitin. Enzyme assays with different β-1,3-glucan substrates showed that Eng2 preferentially acts on soluble polymers like laminarin and shorter linear oligo β-1,3-glucans (Table 1). Hydrolysis of curdlan, an insoluble β-1,3-glucan, was low. No degradation of the β-1,3-glucan containing alkali insoluble fraction of *A. fumigatus* cell wall (AI), which is also insoluble in water, was found. In contrast, we found high glucanase activity of Eng2 when using the commercially available substrate CM-Curdlan-RBB that is water-soluble due to carboxymethylation, making it accessible for the enzyme. Enzyme kinetics were determined with CM-Curdlan-RBB that is easily detectable because of its labelling with Remazol Brilliant Blue. Using this substrate a wide temperature optimum of 24–40 °C, a pH optimum of 5.0–6.5 and a $K_m$ value of 0.8 mg $ml^{-1}$ for the hydrolytic activity of Eng2 were determined. To further characterize the mode of action of Eng2, the products formed with linear laminarioligosaccharide substrates featuring different degrees of polymerization (DP) were analyzed using HPAEC. The smallest substrate for Eng2 was laminaritetraose and the enzyme acted on both the reduced (rG4) and non-reduced (G4) forms of the oligosaccharide (Fig. 4A and B). By comparing the retention times to a standard of glucose and reducing linear laminarioligos (G1–G7) we could clearly identify products resulting from hydrolytic activity (G2 and G3) as well as transferase activity (G5–G7, Fig. 4A). No glucose monomers were found. All products formed within 1 h could be completely degraded by LamA (laminarinase) from *Thermotoga neapolitana* (Zverlov et al., 1997) showing that all transferase products were linear β-1,3-glucans. When acting on oligo β-1,3-glucans with a higher DP like rG16, the formed products all featured a lower DP than the original substrate indicating only hydrolytic activity to be present (Fig. 4C).

**Table 2**
Primers used in this study.

| Name | Sequence |
|---|---|
| Eng2A | TGAGATTAATCACTTTTCACCATCGCCATCT |
| Eng2B | TCGTGAATCTTTTACCAGATCGGAAGCAATCCAAGGACGAAGTAATGAGAGATCCTACGC |
| Eng2C | GCGTAGGATCTCTCATTACTTCGTCCTTGGATTGCTTCCGATCTGGTAAAAGATTCACGA |
| Eng2D | CTTCTTCATCCATTTGTCCTCTCCTCTCGTATCAGAGCAGATTGTACTGAGAGTGCACCA |
| Eng2E | TGGTGCACTCTCAGTACAATCTGCTCTGATACGAGAGGAGAGGACAAATGGATGAAGAAG |
| Eng2F | CTGATGATTGTTTGTTTGTTTGGGTCTGG |
| Pr-FW | GGGGCGCCAGGTCATTGACCATG |
| Pr-RV | CCAAGACTAAGGAGCGATGGTCGC |
| Eng2p-FW | GATCCTCGAGAAACAGCCCTGGAGGCTCGTCAATCACAAAC (*Xho* I)[a] |
| Eng2p-RV | GATCGAATTCCTAATGATGATGATGATGGCTGAGGCCAGGAGTGAAAAACACTTCAGAC (*Eco* RI)[a] |
| Eng2-RT-FW | CGGTACGGATCGAATCGAAA |
| Eng2-RT-RV | GTCCACTCCATAGCATACAC |
| Actin-RT-FW | ATCGGCGGTGGTATCCTC |
| Actin-RT-RV | TCTTCGTGCCATTCGTCTG |

[a] Restriction sites are underlined and the respective enzyme names are given in parentheses.

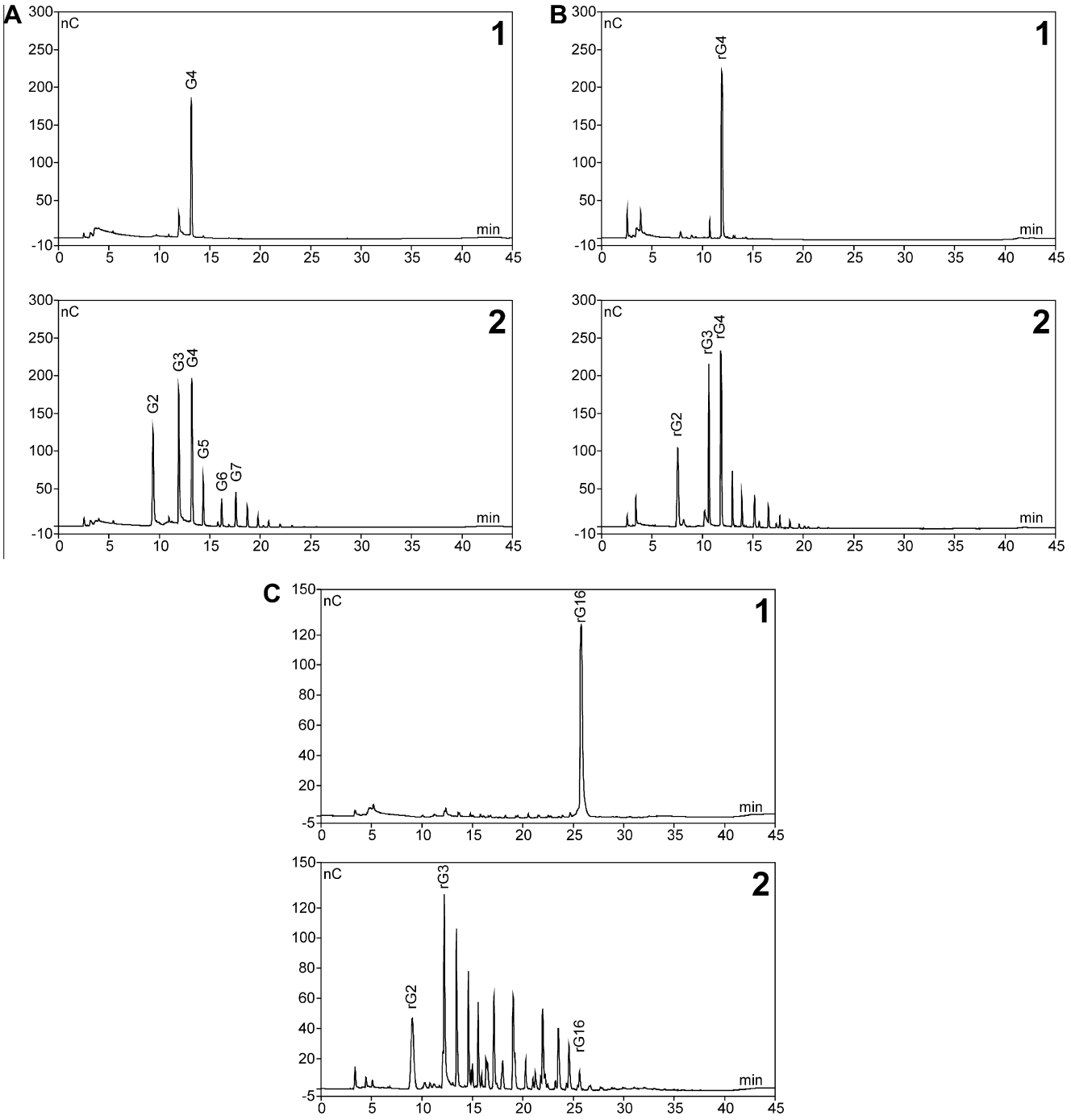


**Fig. 4.** Endoglucanase and transferase activity of Eng2. HPAEC profiles of Eng2 reaction mixtures using (A) non-reduced laminaritetraose (G4), (B) reduced laminaritetraose (rG4), and (C) a reduced laminarioligo of 16 glucose units (rG16) after 15 min of incubation at 37 °C (2). (1) shows a substrate blank without enzyme (rGn: reduced laminarioligosaccharide of *n* glucose units; Gn: laminarioligosaccharide of *n* glucose units; min: minutes, nC: nanocoulomb).

### *3.4. Construction and characterization of the Afeng2 mutant strain*

To test a putative role of Eng2 for morphogenesis, *ENG2* was deleted using the strategy outlined in Fig. 5. Growing the deletion strain in various media (1% yeast extract with 3% glucose, Sabouraud's medium, RPMI, and minimal medium) in solid and liquid cultures at different temperatures did not show a growth phenotype. When growing on solid and liquid minimal medium containing curdlan as the sole carbon source *Afeng2* did not differ from the parental strain in morphology, growth and conidial germination. Conidial germination on Sabouraud's medium also showed no difference between the mutant and the parental strain. Subsequent growth tests in the presence of different concentrations of sorbitol (0.5, 1, 1.5 and 2 M), at different pH (5, 7 and 9) and in presence of various inhibitors such as SDS, Nikkomycin Z, Caspofungin, Mycafungin, Congo red, and Calcofluor white did not show a difference of growth between the wt and the deletion strain (data not shown). We conclude that Eng2 is not essential for mycelial growth under the tested conditions.

## 4. Discussion

Eng2 was selected as an endoglucanase potentially involved in the remodeling of the *A. fumigatus* cell wall for several reasons. A

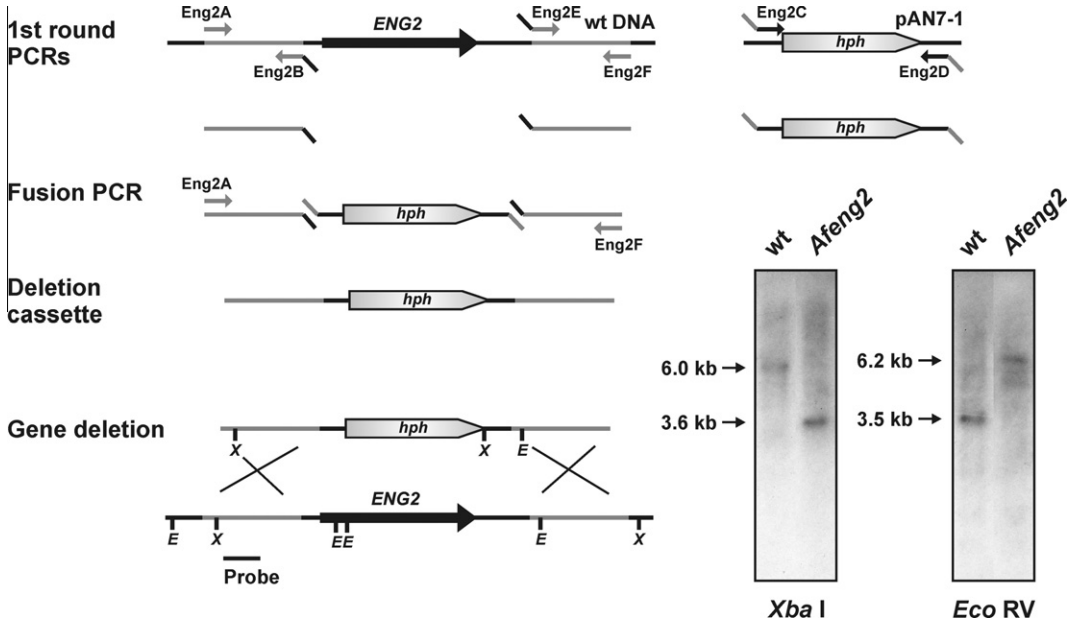


**Fig. 5.** Construction of the *Afeng2* mutant. Construction of the *ENG2* deletion cassette, gene deletion and Southern analysis of the strain *Afeng2*. Primers Eng2A to Eng2F can be found in Table 2. *Xba* I and *Eco* RV restriction sites which were used for Southern analysis are marked as *X* and *E* respectively. The probe (459 bp) was amplified from the 5′-flanking region using primers Pr-FW and PR-RV (Table 2).

CD search has predicted endo β-1,3-glucanase activity, which has already been shown to be essential for yeast morphogenesis (Baladron et al., 2002; Martin-Cuadrado et al., 2003; Esteban et al., 2005; Sipiczki, 2007). The presence of a GPI modification site suggested also a role in cell wall construction like reported for different other GPI-anchored enzymes (Mouyna et al., 2000; Cabib et al., 2007; Rodriguez-Pena et al., 2000; Kitagaki et al., 2002; Tougan et al., 2002). A BLASTP search based on the Eng2 sequence revealed that GPI-anchored endo β-1,3-glucanases belonging to the family GH16 are only found in filamentous fungi.

Recombinant Eng2 of *A. fumigatus* features all the characteristics of endo β-1,3(4)-glucanases belonging to the family GH16. The substrate specificity of this family was characterized following the establishment of the X-ray crystal structure of the laminarinase 16A of *Phanerochaete chrysosporium*. This multivalent β-1,3-glucanase can degrade substrates with single β-1,6 branches like laminarin but not a highly branched structure like lentinan or schizophyllan, which was also used in our study. In contrast β-1,4 linkages adjacent to β-1,3 linkages like in lichenan can be hydrolyzed (Vasur et al., 2009). Even though the endo β-1,3-glucanase activity of Eng2 was confirmed, a transferase activity was seen with substrates of low DP (e.g. DP4 and DP6) but not with substrates of higher DP (e.g. DP16). This result suggests that oligosaccharides of higher DP are the appropriate substrates for this enzyme, with the β-1,3-glucan filling the entire β-1,3-glucan groove of the catalytic site of the enzyme. A transglycosidase activity has often been found among glycoside hydrolases and especially β-1,3-glucanases (Dion et al., 2001; van der Kaaij et al., 2007; Neustroev et al., 2006; Chiffoleau-Giraud et al., 1999). The GH16 family also features enzymes with predominant transferase activities like the chitin β-1,6-glucanosyltransferases Crh1 and Crh2 of *S. cerevisiae* (Cabib et al., 2007) and plant xyloglucan endotransferases (Arrowsmith and de Silva, 1995; Johansson et al., 2004). This transglycosidase property of endoglucanases has even been taken into account to mutagenize key residues of the active site to promote transglycosylating activity (Neustroev et al., 2006; Feng et al., 2005; Vasur et al., 2010). With Eng2 like with the α-glucanosyltransferases AgtA and AgtB of *A. niger* (van der Kaaij et al., 2007), no glucose was found in reaction mixtures suggesting that it was either not formed or immediately transferred to another chain.

Here we report that the *Afeng2* mutant is similar to the wt suggesting that Eng2 alone does not have a morphogenetic role. In agreement Eng2 is unable to degrade β-1,3-glucan isolated from the cell wall of *A. fumigatus* (AI fraction), which would be essential for a direct role in the softening of the cell wall. The lack of phenotype in the *Afeng2* mutant can be due to the compensatory action of one or several of the nine other endo β-1,3-glucanases of *A. fumigatus*. In addition a simultaneous action of chitinases, α- and β-1,3-glucanases may be necessary to plasticize the cell wall. This has never been tested in filamentous fungi. It has to be mentioned that the two endo β-1,3-glucanases Eng1 and Eng2 described in yeasts (Baladron et al., 2002; Martin-Cuadrado et al., 2003, 2008; Esteban et al., 2005; Encinar del Dedo et al., 2009) belong to the family GH81 and not GH16 like the enzyme described in this study. *A. fumigatus* features only one ortholog belonging to the family GH81 (Eng1) which has been described previously. The *Afeng1* mutant like the *Afeng2* strain did not exhibit a growth phenotype (Mouyna et al., 2002). The endo β-1,3-glucanase of the family GH16 which is found in *S. pombe* on the contrary has not been characterized so far. In *Saccharomyces* and *Schizosaccharomyces* endo β-1,3-glucanases and chitinases are regulated by the Ace2 transcription factor. In *A. fumigatus*, no *ACE2* gene was identified in the genome. The direct control of genes involved in cell separation by the yeast Ace2p is in agreement with the role of these glycoside hydrolases during cell wall modification (Sipiczki, 2007). Consequently the absence of *ACE2* in *A. fumigatus* suggests a different role of endo β-1,3-glucanases present in this filamentous fungus. A function in the degradation of extracellular β-1,3-glucan seems unlikely because expression of *ENG2* was not stimulated by the addition of laminarin to the culture medium and the *Afeng2* mutant strain did not show reduced growth on curdlan as the sole carbon source. Consequently the cellular function of this endo β-1,3-glucanase remains unclear. Overexpressing *ENG2* could be a way to see if these endoglucanase is associated to cell wall remodeling since such overexpression of genes involved in cell wall

remodeling can lead to massive cell lysis (Garcia et al., 2005). Identification of proteins interacting with Eng2 may also identify a putative role of this protein in the fungal cell. A last explanation is that Eng2 has a function that is not directly associated to its enzymatic activity, like it was reported for several cell wall enzymes (Mouyna et al., 2002, 2005; Gastebois et al., 2010). A similar hypothesis was put forward with the class III chitinases that are active chitinases without an apparent role in the degradation of cell wall chitin. Taken together the role of endo β-1,3-glucanses in the morphogenesis of fungi growing in the filamentous form remains an open question.

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