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GH16 and GH81 family β-(1,3)-glucanases in *Aspergillus fumigatus* are essential for conidial cell wall morphogenesis

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Summary

The fungal cell wall is a rigid structure because of fibrillar and branched β-(1,3)-glucan linked to chitin. Softening of the cell wall is an essential phenomenon during fungal morphogenesis, wherein rigid cell wall structures are cleaved by glycosylhydrolases. During the search for glycosylhydrolases acting on β-(1,3)-glucan, we identified seven genes in the *Aspergillus fumigatus* genome coding for potential endo-β-(1,3)-glucanase. *ENG1* (previously characterized and named *ENGL1*, Mouyna *et al.,* 2002), belongs to the Glycoside-Hydrolase 81 (GH81) family, while *ENG2* to *ENG7*, to GH16 family. *ENG1* and four GH16 genes (*ENG2–5*) were expressed in the resting conidia as well as during germination, suggesting an essential role during *A. fumigatus* morphogenesis. Here, we report the effect of sequential deletion of *AfENG2–5* (GH16) followed by Af*ENG1* (GH81) deletion in the *Δeng2,3,4,5* mutant. The *Δeng1,2,3,4,5* mutant showed conidial defects, with linear chains of conidia unable to separate while the germination rate was not affected. These results show, for the first time in a filamentous fungus, that endo β-(1,3)-glucanases are essential for proper conidial cell wall assembly and thus segregation of conidia during conidiation.

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Introduction

The *Aspergillus fumigatus* cell wall is predominantly composed of polysaccharides. The central fibrillar core of the cell wall is composed of branched β-(1,3)-glucan to which chitin, galactomannan and β-(1,3-1,4)-glucan are covalently bound (Fontaine *et al.,* 2000). In *A. fumigatus*, β-(1,3)-glucan is synthesized by a plasma membrane-bound glucan synthase complex, which uses UDP-glucose as the substrate and extrudes linear β-(1,3)-glucan chains through the membrane into the periplasmic space (Beauvais *et al.,* 1993; Beauvais *et al.,* 2001). Upon arrival in the cell wall space, linear β-(1,3)-glucan is remodelled in order to be incorporated with the pre-existing cell wall components. The remodelling processes are mainly elongation and branching, catalysed by glucan-elongases and transglycosidases (Gastebois *et al.,* 2009). On the other hand, morphogenetic events like conidial swelling, germ tube emergence and the production of lateral hypha during mycelial growth require softening of the complex and rigid β-(1,3)-glucan structure by β-(1,3)-glucan hydrolyzing enzymes.

β-(1,3)-Glucan hydrolysing enzymes can be divided into exo-β-(1,3)-glucanases (EC 3.2.1.58) and endo-β-(1,3)-glucanases (EC 3.2.1.6 and EC 3.2.1.39). Endo-β-(1,3)-glucanases cleave inside a glucan chain in a less random fashion, while exo-β-(1,3)-glucanases release single glucose residues from the non-reducing end. Several exo-β-(1,3)-glucanases have been described in *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe* and *Candida albicans* (Vazquez de Aldana *et al.,* 1991; Chambers *et al.,* 1993; Suzuki *et al.,* 2001; Dueñas-Santero *et al.,* 2010) but the phenotype of the Δ*exg1exg2ssg1* mutant in *S. cerevisiae* is similar to that of the wild type strain. Considering the essential structural role of β-(1,3)-glucan in the fungal cell wall (Gastebois *et al.,* 2009), it would be logical that endo-acting β-(1,3)-glucanases would play a significant role in morphogenetic events during fungal life. Indeed, it was shown that the endo-β-(1,3)-glucanase, *ENG1*, of both *S. cerevisiae* and *S. pombe* (which belong to the GH81 family) were essential for cell separation (Baladrón *et al.,* 2002; Martín-Cuadrado *et al.,* 2003; Esteban *et al.,* 2005). *ENG2* of *S. pombe*, on the other hand, is involved in ascus wall degradation after sporulation (Encinar del Dedo *et al.,* 2009).

In the *A. fumigatus* genome, two endo-β-(1,3)-endoglucanase genes, *ENG1* (previously named *ENGL1*) belonging to GH81 family and *ENG2* of the GH16 family, have been previously characterized (CaZy database: http://www.cazy.org/, (Lombard *et al.,* 2014)) (Mouyna *et al.,* 2002; Hartl *et al.,* 2011). Deletion of the only member of GH81 family, *ENG1* did not lead to any phenotypic defect. While the family GH81 features only one known activity (endo-β-(1,3)-glucanase; EC 3.2.1.58), the specificity of the members of the family GH16 seems wider because the members of this GH-family can act on different glycan polysaccharides and belong to various Enzyme Classification (EC) families. The GH16 superfamily has been divided into nine sub-families based on a phylogenetic analysis of conserved domains of members (http://www.ncbi.nlm.nih.gov/Structure/cdd/cddsrv.cgi?uid=29534) (Mertz *et al.,* 2009). One of the subfamilies contain putative endo-β-(1,3)-glucanases encoded by genes *ENG2* to *ENG7* in *A. fumigatus*, of which *ENG2* has been studied by our group. Like *ENG1*, deletion of *ENG2* did not lead to any morphological defect (Hartl *et al.,* 2011). Functional redundancy of the endo-(1,3)-β-glucanases belonging to different families could be the reason that single *A. fumigatus* endo-(1,3)-β-glucanase gene deletion mutants (Δ*eng1* or Δ*eng2*) did not alter phenotypes. Accordingly, in order to analyse exhaustively the function of endo-β-(1,3)-glucanases from both GH16 and GH81 families in *A. fumigatus*, we undertook a successive sequential deletion of members of these families expressed during conidial germination.

Based on the phenotypic analyses of the mutants constructed, endo-(1,3)-β-glucanases of the GH16 and GH81 together were found to be important during conidiogenesis as their deletion resulted in a phenotype wherein there were inseparable conidial chains; however, they did not seem to play a role during germination and hyphal branching.

Results

Characterization of endo-β-(1,3)-glucanases in A. fumigatus

In the GH16 family, the subfamily GH16_MLG1_glucanases contained four members: *ENG2* (AFUA_2G14360) (Hartl *et al.,* 2011), *ENG3* (AFUA_1G05290), *ENG4* (AFUA_5G02280) and *ENG5* (AFUA_4G13360), and the GH16_laminarinases sub-family contained two members *ENG6* (AFUA_6G14540) and *ENG7* (AFUA_3G03080) (Mouyna *et al.,* 2013). The characteristics of the gene expression products are described in the Supplementary Table 1. Sequence alignment showed many conserved motifs and especially two potential catalytic sites (G**E**X**D**XX**E**) (Supplementary Fig. 1) which is a characteristic of GH16 family as described in the bacteria *Clostridium thermocellum* and *Cellulosimicrobium cellulans* (Tanabe and Oda, 2011; Chen *et al.,* 2015). The percent identity among GH16 and GH81 family members of *A. fumigatus* varied between 7 and 82%. Using bioinformatics software, it was predicted that Eng2p, Eng5p, Eng6p and Eng7p exhibited a *N*-terminal signal peptide but only Eng2p and Eng7p holds a putative GPI signal sequence (Supplementary Tables 1 and 2). Eng4p and Eng5p did not show the presence of a signal peptide but both featured a transmembrane helix.

Expression of the GH16 and GH81 family members during growth

The expression of each gene in these families as well as the *ENG1* gene expression was investigated by RNAseq analysis on dormant (T0), swollen (T4h) and germinating conidia (T8h). As shown in Fig. 1, *ENG1* to *ENG5* were expressed constitutively, *ENG1* and *ENG2* being the most expressed especially in dormant conidia. In contrast, *ENG6* and *ENG7* were not expressed in any growth condition tested.

Construction of a Δeng1,2,3,4,5 deletion mutant

Because *ENG6* and *ENG7* were not expressed, we undertook successive deletions of all expressed members of the subfamily GH16_MLG1_glucanases (*ENG2* to *ENG5*) followed by the deletion of *ENG1* in the Δ*eng2,3,4,5* mutant in order to understand the role of all putative endo-β-(1,3)-glucanases in *A. fumigatus*. The multiple deletions were undertaken with three different markers pyrithiamine, phleomycin and finally hygromycin using β-rec/six system (Hartmann *et al.,* 2010). Strategies and validation of the gene deletions are shown in the Supplementary Fig. 2 and all the mutants generated are listed in the Supplementary Table 3. Because, we did not observe expression of the *ENG6 a*nd *ENG7* gene by RT-PCR in the Δ*eng1,2,3,4,5*

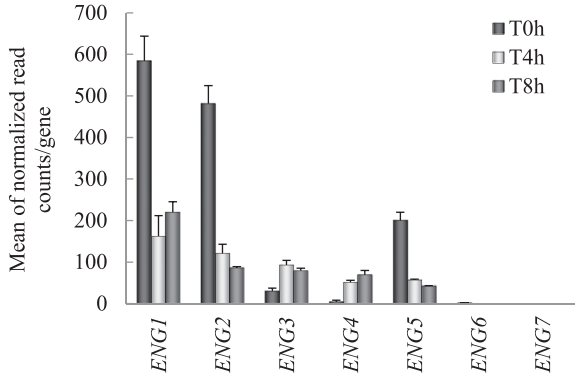

Fig. 1. Gene expression profile of the GH16 and GH81 endo β-(1,3)-glucanase genes in dormant (T0), swollen (T4h) and germinated conidia (T8h) in Glucose 3%/YE 1% media, in the parental strain. The data are extracted from an RNAseq analysis data. The error bars represent standard deviation from the mean values of the three different experiments.

mutant, we did not delete these two genes (Supplementary Fig. 3).

Phenotypic analysis of the multiple mutants generated

The phenotypes of single as well as the sequentially deleted multiple endo-β-(1,3)-glucanase mutants were analysed. Conidiation was not affected quantitatively and the hyphal morphology and hyphal branching were similar to that of the parental strain (data not shown). Growth of these mutants in different media (1% yeast extract with 3% glucose, Sabouraud and Minimal Medium), both on agar and in the liquid cultures at different temperatures and pH was similar to that of the parental strain. The mutants did not show increased sensitivity to cell wall perturbing agents (Congo red and Calcofluor White) and glucan synthase inhibitors (Caspofungin and Micafungin) (data not shown).

Conidial morphology

Multiple deletion of the *ENG* gene affected the capacity of the conidia to separate from the conidial chain (Fig. 2). For each single and multiple mutants, the number of conidia per conidial chains was counted. Figure 3 shows that the number of conidia per conidial chain increased with the number of genes deleted, with up to 25 for the *Δeng1,2,3,4,5* mutant. These results show that this phenotype is not associated to a specific gene deletion but to the number of genes deleted. Some of the Δ*eng2,3,4,5* as well as the Δ*eng1,2,3,4,5* mutant swollen conidia (pre-formed during the germination process) showed abnormal shapes (Supplementary Fig. 4). However, they germinated normally (data not shown).

Characterization of the conidial linkage

Transmission electron microscopy studies confirmed that in the conidial chains of the *Δeng1,2,3,4,5* mutant, conidia remained attached to one another in a linear manner (Fig. 4). In order to identify the component(s) involved in the linkage, after removing the surface melanin layer, conidia were treated with an endo-β-(1,3)-glucanase (LamA) (Zverlov *et al.,* 1997), a chitinase (Vorgias *et al.,* 1993) or an endo-α-(1,3)-glucanase (mutanase) (Fuglsang *et al.,* 2000). There was separation of conidia only upon LamA treatment, suggesting that β-(1,3)-glucans were present in the linear chain conidial junctions

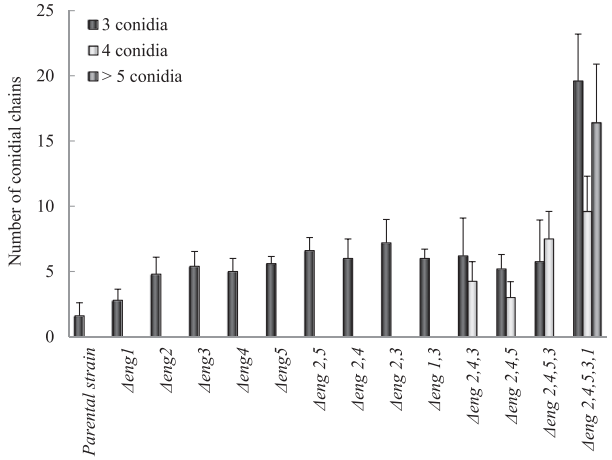

Fig. 3. Quantification of the conidial chains (containing three to five or more conidia) in the parental strain, single deletion mutants 1–5, double, triple, quadruple and the quintuple deletion mutants. Counting was performed with three different conidial cultures, each time using a conidial suspension of 8.4×10^7/ml, mounting 15 μl of this suspension and observing at five different areas on the slide.

(Supplementary Fig. 5). Addition of recombinant Eng1p, a 74 kDa protein encoded by *ENG1*, one of the endo-β-(1,3)-glucanase deleted (Mouyna *et al.,* 2002), also resulted in the partial conidial separation from the linear chains (data not shown). Further, immune-electron microscopy with GNBP3 (Fig. 5A), a receptor protein that binds specifically to β-(1,3)-glucan (Mishima *et al.,* 2009) and immune-labelling with the β-(1,3)-glucan specific dectin-1 (Fig. 5B) confirmed that β-(1,3)-glucan was present in the conidial junctions of the linear conidial chain.

Endoglucanase activity

The endo-β-(1,3)-glucanase activity of the Δ*eng1,2,3,4,5* mutant was investigated using CM-Curdlan-RBB as the substrate (Brisset *et al.,* 2000). There was a significant decrease in the endo-β-(1,3)-glucanase activity in all the three conidial morphotypes (dormant, swollen and germinating conidia); the decrease was ~51% in dormant conidia, ~74% for swollen morphotype and was less pronounced in germinating conidia (~32%) compared to that of the parental strain. However, all the conidial morphotypes showed persisting endo-β-(1,3)-glucanase activity (Fig. 6).

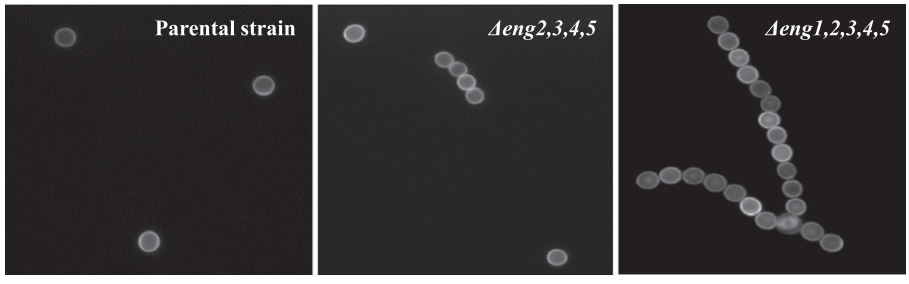

Fig. 2. Morphology of the dormant conidia of the parental strain, the Δ*eng2,3,4,5* and the Δ*eng1,2,3,45* mutant stained with Calcofluor White (X63).

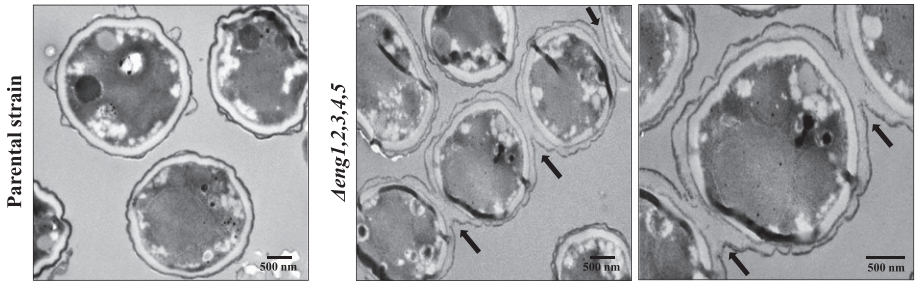

Fig. 4. Transmission electron microscopic observations of 1-week-old conidia of the parental strain and the *⊿eng1,2,3,4,5* mutant conidia (scale bars are representing in the figures).

Discussion

To date, in *A. fumigatus*, only Eng1p and Eng2p have been biochemically characterized as endo-β-(1,3)-glucanase (Fontaine *et al.,* 1997; Hartl *et al.,* 2011). It has been previously demonstrated that Engl1p is present in the cell wall and accounts for 10–15% of the total endo-β-(1,3)-glucanase activity (Fontaine *et al.,* 1997) and the active site recognizes at least five glucose residues. It can cleave both soluble as well as insoluble β-(1,3)-glucans and can act directly on β-(1,3)-glucans polymers in the entire cell wall. In contrast, Eng2p preferentially acts on the soluble β-(1,3)-glucan oligomers and the minimum substrate is a laminaritetraose (Hartl *et al.,* 2011).

In the present study, we focused on the two subfamilies of GH16 in *A. fumigatus,* GH16_MLG1_glucanases (*ENG2–5*) and GH16 laminarinase-like (*ENG6* and *ENG7*), together containing seven members. These two sub-families are characteristics of filamentous fungi and no orthologues are present in the yeasts, *S. cerevisiae* and *C. albicans*. The only endo-β-(1,3)-glucanase belonging to GH16 in *S. pombe* (emb|CAB57923.1) has not been characterized so far. The alignment of these proteins showed a very conserved motif, G**E**X**D**XX**E**, the two glutamic acid residues (E) representing the nucleophile and acid/base amino acids characteristic of an enzyme active site. It was demonstrated in *C. cellulans* (Tanabe and Oda, 2011) that the mutation of these two amino acid residues induced the loss of endo-β-(1,3)-glucanase activity.

The single *⊿eng1* or *⊿eng2* deletion mutants did not show phenotypic aberrations. However the multiple deletion mutant lacking the MGL1-glucanases (*ENG2–5*) showed a defect in conidial separation as well as abnormal morphology during conidial swelling. As there was no expression of laminarinase-like (*ENG6* and *ENG7*) GH16-subfamily members, we deleted the other endo-β-(1,3)-glucanase, *ENG1*, in the *⊿eng2,3,4,5* background and we showed an increased defect of conidial separation. Further, enzymatic assay, immune-labelling and immuno-electron microscopic analyses confirmed that the defect in conidial separation is correlated to the presence of β-(1,3)-glucan at the conidial junction, suggesting a role played by the endo-β-(1,3)-glucanases in separating conidia from one another during conidiation. Moreover, we show that the defect of conidial separation is not because of the major contribution of a single gene but to the cumulative effect of successive deletion of all these genes.

In *A. fumigatus*, the presence of multigenic families with redundant activity but different biological function has been reported many times. For the *GEL* family (seven members) encoding for enzyme involved in the elongation of β-(1,3)-glucans, it was shown that despite the fact that they display the same enzymatic activity, the single mutants have distinct phenotypes: Δ*gel1* behaves like wild type, Δ*gel2* shows reduced growth, while *GEL4* is essential (Mouyna *et al.,* 2000; Mouyna *et al.,* 2005; Gastebois *et al.,* 2010). For the chitin synthase family (eight members), two sub-families are present. Among the sub-family 1 *CHS*, Δ*chsA*, Δ*chsB* and Δ*chsC* mutants showed limited impact on chitin synthesis. In contrast, there were reduced conidiation, altered mycelial morphotype and reduced growth and Chs-activity in the single Δ*chsG* and the multiple Δ*chsA/C/B/G* mutants. Among sub-family 2 of the *CHS* genes phenotypic defects mainly resulted from the *CSMA* deletion. Despite significant morphological mycelial and conidial growth phenotypes in the quadruple Δ*csmA/csmB/F/D* mutant, the chitin content

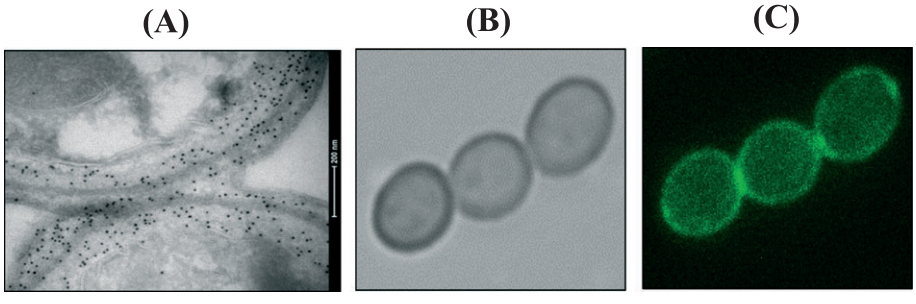

Fig. 5. (A) Electron microscopic images of the Δ*eng1,2,3,4,5* mutant conidial cryosections labelled with β-(1,3)-glucan specific receptor (GNBP3)/IgG-gold particles and (B and C) bright field and fluorescence microscopy of the conidia upon permeabilization and labelling with human Fc-conjugated Dectin-1/ human Fc-specific IgG-FITC respectively.

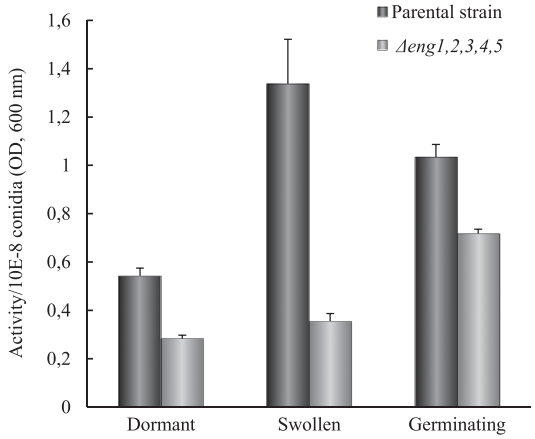

Fig. 6. β-(1,3)-Glucanase (intracellular + cell wall bound) activity in the parental strain and the Δ*eng1,2,3,4,5* mutant. Glucanase activity was determined using CM-Curdlan-RBB as the substrate and the activity in the different morphotypes (dormant, swollen and germinating) was expressed per 10^8 conidia. The error bars represent standard deviation from the mean values of the three different experiments.

was poorly affected by gene deletions in this family (Muszkieta *et al.,* 2014).

The phenotype observed for the *Δeng1,2,3,4,5* mutant is reminiscent of the phenotype observed for the Δ*eng1* and Δ*eng2* mutants in *S. cerevisiae* and *S. pombe*. In *S. cerevisiae*, Eng1p is expressed and secreted in vegetative cells (Baladrón *et al.,* 2002) and the gene expression level decreased during sporulation. In contrast, Eng2p is intracellular and this gene expression level increased during the sporulation process. The *ScENG2* deletion mutant showed no defect in terms of morphology in contrast to *ScΔeng1* which had a defect in cell separation. The double *ScΔeng1eng2* mutant behaved like the single *ScΔeng1* mutant. In *S. pombe*, Δ*eng1* did not show growth defect but cells failed to complete septum dissolution during cell separation (Martín-Cuadrado *et al.,* 2003). This failure to separate was because of the inability to degrade primary septa rich in β-(1,3)-glucan. Mutants lacking this gene form short chains of cells that do not separate completely. In the present study, we have shown that conidial separation requires enzymatic hydrolysis of β-(1,3)-glucan in a process that is mediated by the endo-β-(1,3)-glucanase belonging to the GH16 and GH81 families. Such multiple deletions of endo-β-(1,3)-glucanases has never been undertaken in filamentous fungi before and highlights the role of endo-β-(1,3)-glucanase in conidial morphogenesis.

The *Δeng1,2,3,4,5* mutant still showed a remaining endo-β-(1,3)-glucanase activity in all the growth stages tested (dormant, swollen and germinating), more predominantly in the germinating condition. Although, the *Δeng1,2,3,4,5* mutant conidia showed altered shapes during swelling, their ability to germinate normally suggests that other endo-β-(1,3)-glucanase family members may be involved in the cell wall remodelling during conidiation and conidial germination. *ENG6* and *ENG7* are unlikely to be involved in this process because they were not expressed in the *Δeng1,2,3,4,5* mutant. In the CaZy database, another GH-family, GH55, is reported to contain putative endo-β-(1,3)-glucanases. Several members of this GH family have been identified in the *A. fumigatus* genome and the role of these putative endo-β-(1,3)-glucanases in *A. fumigatus* is currently being investigated.

Experimental procedures

Strains and growth conditions

A. fumigatus strains used in this work are listed in Supplementary Table 3. The *A. fumigatus* parental strain KU80Δ*pyrG* (auxotrophic to uridine and uracil) (da Silva Ferreira *et al.,* 2006) was maintained on 2% malt-agar slants at room temperature. For DNA extraction, cultures were grown in Sabouraud liquid medium (2% glucose + 1% mycopeptone). Transformations using the *pyrG* marker gene 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). When using the markers phleomycin (ble) (Mattern *et al.,* 1988) or hygromycin B (hph) (Sigma) (Punt *et al.,* 1987), they were added to transformation plates in an overlay after one night of incubation at room temperature resulting in a final concentration of 30 and 150 μg ml^{-1} respectively. Transformations using pyrithiamine (*ptrA*) (Kubodera *et al.,* 2000) were done in Brian's medium (2.4 g l^{-1} NH_4NO_3, 10 g l^{-1} KH_2PO_4, 10 g l^{-1}asparagine, 2 g l^{-1} $MgSO_4 \cdot 7H_2O$, 50 g l^{-1} glucose, 1.3 ml l^{-1} solution A [20 g l^{-1} $ZnSO_4 \cdot 7H_2O$, 2 g l^{-1} $CuSO_4 \cdot 5H_2O$, 1 g l^{-1} $Co(NO_3) \cdot 6H_2O$], 1.3 ml l^{-1} solution B [50 g l^{-1} $CaCl_2$], pH adjusted to 5.4) with pyrithiamin added in an overlay after one night of incubation at 4°C to a final concentration of 10 μg ml^{-1}. Conidia were collected from agar slants/plates after seven days of growth at room temperature using 0.05% Tween 80 solution.

Protein sequence analysis

Protein sequences were analysed using CD search (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi, (Marchler-Bauer *et al.,* 2015)), SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP/) (Emanuelsson *et al.,* 2007), big-PI fungal predictor (http://mendel.imp.ac.at/gpi/fungi_server.html) (Eisenhaber *et al.,* 2004), and the TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM/). Protein sequence and AFUA number were retrieved from the CADRE Genome Browser (http://fungi.ensembl.org/Aspergillus_fumigatus/Info/Index, (Gilsenan *et al.,* 2012)). Protein sequences were aligned using ClustalX 2.0.12 (Larkin *et al.,* 2007).

Construction of the single and multiple deletion mutants

For the single deletion, the four deletion cassettes for *ENG2, ENG3, ENG4* and *ENG5* were constructed by fusion PCR as described and illustrated previously (Hartl *et al.,* 2011) because this work has been initiated before the publication of the re-usable β-rec cassette (Hartmann *et al.,* 2010). Primer sequences (Eng2A-F, Eng3A-F,

Eng4A-F and Eng5A-F) are listed in Supplementary Table S4. All PCRs for the construction of deletion cassettes were performed with Phusion high-fidelity DNA polymerase from FINNZYMES (Finland). To construct the *Δeng1,2,3,4,5* mutant, first *ENG2* was replaced by the *Aspergillus niger* gene *pyrG* to complement uridine and uracil auxotrophy of the strain KU80Δ*pyrG* (da Silva Ferreira *et al.,* 2006). Next *ENG4* was replaced by the *Aspergillus oryzae* gene *ptrA* conferring resistance to pyrithiamine (Kubodera *et al.,* 2000). Then the *Streptococcus hindustanus phleo*^{R} gene for phleomycin resistance was used to replace *ENG5* (Mattern *et al.,* 1988). Next *ENG3* was replaced by the *Escherichia coli* gene *hph* for selection on hygromycin using the β-rec cassette (Hartmann *et al.,* 2010); the construction of this *ENG3* cassette has been done using the GENEART seamless cloning and assembly kit (Life Technologies, A13288). This cassette can be reused after excision of the selection marker hygromycin. Then the Δ*eng2,3,4,5* mutant was cultivated in the presence of 2% xylose-containing minimal medium that allows the excision of the selection marker, by recombination of the six recognition regions. Finally, *ENG1* was replaced by the *E. coli* gene *hph* for selection on hygromycin using the β-rec cassette (Hartmann *et al.,* 2010) in the excised Δ*eng2,3,4,5* strain. Transformations were performed by electroporation of conidia as described previously (Gastebois *et al.,* 2010). After each deletion step, proper integration of the deletion cassette was verified by Southern.

RNA seq and RT-PCR analysis

Parental strain conidia (10^8) were inoculated in Glucose (3%) + Yeast Extract (1%) at 37°C for 4–8 h at 150 r.p.m. Dormant (0 h), swollen (4 h) and germinated (8 h) conidia thus obtained were disrupted with 0.5-mm diameter glass beads in a volume of 500 μl and then RNA was isolated using the QIAGEN RNA/DNA kit. A first DNase treatment was carried out on RNeasy column (Qiagen, Courtaboeuf, France) using DnaseI (Roche, France) and a second one was performed after elution of the RNA from the column with Turbo DNA-free DNAse (Ambion, Courtaboeuf, France). For RNAseq analysis, we used 2 to 5 μg of total RNA to purify polyadenylated mRNAs and construct the sequencing libraries using the TruSeq RNA Sample Prep Kit v2 (Illumina, RS-930-1021, San Diego, CA) as recommended by the manufacturer. The nondirectional libraries were controlled on Bioanalyzer DNA1000 Chips (Agilent Technologies, #5067–1504, Santa Clara, CA). They were sequenced on an Illumina Hiseq 2000 sequencer using a TruSeq SR cluster kit v3 cBot HS (Illumina, # GD-401-3002) and a TruSeq SBS kit v3 HS 50 cycles (Illumina, # FC-401-3002) in order to have around 50 million single end reads of 50 bases per sample. Reads were cleaned of adapter sequences and low-quality sequences using an in-house program (https://github.com/baj12/clean_ngs). Only sequences at least 25 nt in length were considered for further analysis. TopHat (version 1.4.1.1, default parameters, http://ccb.jhu.edu/software/tophat) was used for alignment on the reference genome. Genes were counted using HTseq-count (parameters: -m intersection-nonempty, -s yes, -t exon) (Trapnell *et al.,* 2009; Anders and Huber, 2010). RNA-seq count data were analysed with R (Core Team, R, 2013) version 2.15.1 and the Bioconductor (Gentleman *et al.,* 2004) DESeq package version 1.10.1. Ribosomal RNAs were first excluded from the count table, as well as RNAs that had no reads in the nine samples. The remaining counts were normalized according to the DESeq method with default parameters (size factors were computed for each sample). The size factor obtained for a given sample was used to divide all the counts associated with this sample. Dispersion estimates for each gene were computed with the *estimateDispersions* function with parameters *method=pooled*, *fitType=parametric* and *sharingMode=maximum*. The data have been normalized/size of the gene.

RNAseq data have been deposited at the ncbi (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=stkrkomwxteldqz&acc=GSE81956) number GSE81956.

For RT-PCR analysis, 1 μg of total RNA was reverse-transcribed using reverse transcriptase Biorad kit (Iscript cDNA synthesis kit) following the instructions of the manufacturer. Primers used to do RT-PCR are listed in Supplementary Table 4.

Growth, sporulation, germination and morphology of the mutant strains

Mycelial growth was tested on different agar media [Sabouraud, minimal and Glucose 3%-Yeast Extract 1% (YG)] at 37°C and 50°C as well as in liquid Sabouraud and minimal media. Conidial germination was followed microscopically and quantified on agar Sabouraud medium. Growth inhibition tests with Congo Red (CR) (50–240 μg/ml) and Calcofluor White (CFW) (50–200 μg/ml) were performed on microtiter plates containing 100 μl of 2× YG medium with different concentrations of CR/CFW. Wells were inoculated with 100 μl of conidial suspension containing 2×10^5 CFUml^{-1}, incubated at 37°C and growth was compared after 24 and 48 h of incubation. Susceptibility testing to Caspofungin (Merck & Co.; 0.07–38 μg/ml) and Micafungin (Astellas Pharma Inc.; 0.07–10 μg/ml) were performed in RPMI liquid medium by resazurin method (Clavaud *et al.,* 2012).

Transmission electronic microscopy (TEM)

Conidia were prepared as already published (Muszkieta *et al.,* 2014) and then observed at 120 kV accelerating voltage under Tecnai T12 (FEI company). Images were recorded using Eagle camera and Tia software.

Immuno-electron microscopy

A conidial suspension was fixed for 15 min at room temperature by the addition of an equal volume of 2× fixative [4% formaldehyde/0.4% glutaraldehyde in PHEM buffer (60 mM Pipes, 25 mM Hepes, 10 mM EGTA and 2 mM $MgCl_2$, pH 6.9)]. Conidia were collected by centrifugation for 5 min at 2000 ***g*** and fixation was continued for 45 min at room temperature with fresh fixative [2% (w/v) formaldehyde/0.2% (v/v) glutaraldehyde in PHEM buffer]. Then the cells were washed with PHEM buffer, and resuspended in 1% (w/v) formaldehyde in PHEM buffer and stored at 4°C overnight. The cells were washed three times with PHEM buffer and incubated for 1 h at room temperature in 1% (v/v) periodic acid in PHEM buffer.

Subsequently, the cells were washed three times with PHEM buffer and embedded in 12% (w/v) gelatin in PHEM buffer at 37°C. Cells were centrifuged for 2 min at 20 000 ***g*** and gelatin was solidified on ice. Cubes (1 mm^3) were cut, which were impregnated overnight on a turning wheel with 2.3 M sucrose at 4°C (Tokuyasu, 1973). Sections (50–55 nm) were cut for electron microscopy with cryo-ultramicrotome (UC6/FC6; Leica Microsystems, Vienna, Austria). Sections were collected with 1.15 M sucrose/0.1% (w/v) methylcellulose in half-concentrated PHEM buffer and dipped on to Formvar/carbon-coated Cu grids (Stork Veco B.V., Eerbeek, The Netherlands) for electron microscopy.

Mounted sections of JBY20 were washed five times with 0.02 M NH_4Cl 50 mM. The sections were blocked by one incubation in PBS/1% BSA (Sigma, Zwijndrecht, The Netherlands) and subsequently incubated with GNBP3 protein (king gift of A. Roussel, (Mishima *et al.,* 2009)) dilution 1/250 washed six times with PBS/0.1% BSA then incubated with anti-GNBP3 dilution 1/200, washed six times with PBS/0.1% BSA and incubated with anti-mouse IgG/IgM-Gold (10 nm) at 1/25 dilution (BioCell, batch 16070). The sections were then rinsed briefly three times and then seven times for 2 min with PBS. The labelled sections were fixed for 5 min in PBS-1% glutaraldehyde and subsequently washed 10 times with distilled water. This was followed by an incubation for 5 min in 2% uranyl oxalate, pH 7.4 (Tokuyasu, 1978), and then two washes with distilled water. The sections were then washed twice with 0.4% (w/v) aqueous uranyl acetate/1.8% (w/v) methylcellulose on ice and the incubation was continued with fresh solution for 5 min on ice. Excess of fluid was then drained off and the sections were dried at room temperature. The sections were observed 80 kV accelerating voltage under Tecnai T12 (FEI company) at 120 kV accelerating voltage. Images were recorded using Eagle camera and Tia software.

Characterization of the conidial linkage by enzymatic assay immunolabelling

(A) Enzymatic assay: Parental strain as well as mutant conidia was treated with 10% H_2O_2 for 2 h at 65°C to remove the surface melanin layer and separated in three batches. Further, each batch was treated either with recombinant 0.2 μg of LamA (endo-β-(1,3)-glucanase) (Zverlov *et al.,* 1997), 0.2 μg of chitinase A (Vorgias *et al.,* 1993) or 0.2 μg of mutanase (endo-α-(1,3)-glucanase) (Fuglsang *et al.,* 2000) or Engl1p (Mouyna *et al.,* 2002) for 24–48 h at 37°C. After glycosyl-hydrolase treatment, conidia were observed under light microscope. **(B) Immuno-labelling:** Conidia (10^5) were fixed with 2.5% *p*-formaldehyde as described earlier (Aimanianda *et al.,* 2009). Further, fixed conidia were permeabilized as follows: conidia were treated with glucanex (50 mg in PEM buffer; (50 mM PIPES, 5 mM $MgSO_4$ and 25 mM EGTA pH 7.4)) at room temperature (RT) for 20 min, washed with PEM buffer thrice, incubated with 0.1% NP40 in PEM for 5 min, washed thrice with PEM, incubated with methanol at −20°C for 10 min, washed again with PEM buffer thrice and incubated with PBS-BSA (1%) at RT for 1 h. For immune-labelling, permeabilized conidia were incubated with PBS-BSA containing 5 μg/ml Fc-conjugated human dectin-1 at RT for 1 h (kind gift of G. Brown, Aberdeen, UK), washed thrice with PBS-BSA, incubated further with Fc-specific FITC conjugated human IgG at RT for 1 h, washed with PBS-BSA and subjected to fluorescent microscopy.

Endoglucanase activity measurement

The endo-β-(1,3)-glucanase activity was measured using Carboxymethyl-Curdlan-Remazol Brilliant Blue (CM-Curdlan-RBB, Loewe Biochemica GmbH) as the substrate (Brisset *et al.,* 2000). Swollen and germinating conidial morphotypes were obtained upon incubating dormant conidia in Sabouraud liquid medium for 5 h and 7.5 h respectively. Dormant, swollen and germinating conidia (suspended in aqueous solution) were disrupted using 0.5 mm glass beads (three times, each time at 6.0 speed for 60 s) in a Fast prep®24 instrument (Mp-Bio). After centrifugation (4500 r.p.m., 10 min), the supernatant was collected (intracellular fraction) and kept at 4°C while the cell wall was washed twice with water and then incubated in a 50 mM sodium acetate pH 5.5 containing 5 mM sodium azide at 37°C for two-days as described by (Fontaine *et al.,* 1997). The soluble cell wall autolysate was collected by centrifugation (4500 r.p.m., 10 min). Endoglucanase assays comprised 0.1 ml of CM-Curdlan-RBB, 0.2 ml 0.1 M sodium acetate buffer pH 5.5 and 0.1 ml of intracellular fraction or cell wall autolyzate. Reactions were performed at 37°C for 2 h and stopped by the addition of 0.1 ml 0.5 M HCl and incubated on ice for 10 min. The mix was centrifuged at 10 000 r.p.m. for 5 min and the OD of the supernatants was measured at 600 nm.

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Supporting information

Additional supporting information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Sequence alignment of the GH16 Endo-β-(1,3)-glucanases family of *A. fumigatus* using ClustalW program. The asterisk indicated the two glutamic acid residues which are conserved in this family.
Fig. S2. Construction of the deletion strain. Subsequent deletion of the four endo β-(1,3)-glucanase genes from strain KU80Δ*pyrG* (da Silva Ferreira *et al*., 2006) using the marker genes *pyrG* of *A. niger* (Mattern *et al*., 1987), *ptrA* of *A. oryzae* (Kubodera *et al*., 2000), *phleo*^{R} of *Streptococcus hindustanus* (Mattern *et al*., 1988), and *hph* of *E. coli* (Punt *et al*., 1987) and the β-rec/six cassette (Hartmann *et al*., 2010).
Fig. S3. RT-PCR analysis of the gene expression level of the *ENG6* and *ENG7* gene using the primers *ENG6RT* F and R and *ENG7RT* F and R in the Δ*eng1,2,3,4,5* mutant at 8 h or 24 h. Control PCR of genomic DNA is included as well as the TEF gene as a control.
Fig. S4. Morphology of the swollen conidia of the parental, the Δ*eng2,3,4,5* and Δ*eng1,2,3,4,5* mutant strains labelled with Calcofluor White (× 63 magnifications).
Fig. S5. 10^6 conidia/ml of Δ*eng1,2,3,4,5* mutant was treated with 10% H_2O_2, separated in three batches and treated either with 0.2 μg mutanase (endo-α-(1,3)-glucanase), 0.2 μg LamA (endo-β-(1,3)-glucanase) or 0.2 μg chitinase.