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Functional analysis of the fungal/plant class chitinase family in *Aspergillus fumigatus*

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ABSTRACT

A quintuple mutant was constructed to delete the entire family of the fungal/plant (class III) chitinases of *Aspergillus fumigatus*. Only a limited reduction in the total chitinolytic activity was seen for the different chitinase mutants including the quintuple mutant. In spite of this reduction in chitinolytic activity, no growth or germination defects were observed in these chitinase mutants. This result demonstrated that the fungal/plant chitinases do not have an essential role in the morphogenesis of *A. fumigatus*. A slight diminution of the growth during autolysis was seen for the quintuple mutant suggesting that class III chitinases may play only a nutritional role during this phase of the cycle, retarding fungal death.

1. Introduction

The fungal cell wall determines cellular shape and plays an essential role during fungal growth. Chitin, a homopolymer of *N*-acetyl-glucosamine (GlcNAc), along with β-(1,3)-glucan, represents the main structural component of the fungal cell wall, and contributes to its rigidity. However, this rigid layer must become somehow plastic and modifiable during yeast separation, conidial swelling or hyphal branching. Glycosylhydrolases, especially chitinase and endo-β-1,3-glucanases, are expected to play a major role during these modifications of the cell wall structure (Adams, 2004). Chitinases are the enzymes that can hydrolyze chitin by catalyzing the cleavage of β-(1-4)-linkage between GlcNAc residues.

In the CAZY database (http://www.cazy.org/), all the chitinases found in fungal cells to date belong to the glycosyl hydrolase (GH) family 18 and can be distributed basically in two subclasses: fungal/plant (class III) and fungal/bacterial (class V) families (Henrissat, 1991). Fungal/bacterial chitinases are secreted enzymes that have been shown to play a role in the digestion of exogenous chitin or utilization of fungal chitin during autolysis for energy. In contrast, fungal/plant chitinases have been shown to play a role during fungal morphogenesis. In *Saccharomyces cerevisiae, CTS1* is involved in separation of yeast cells during budding and its disruption results in cell clumping (Kuranda and Robbins, 1991) and promotes pseudo-hyphal growth (King and Butler, 1998). In *Candida albicans*, three fungal/plant chitinase encoding genes are found (*CHT1, CHT2* and *CHT3* (McCreath et al., 1996, 1995). Disruption of *CHT2* or *CHT3* suggested a role for encoded proteins similar to the role observed for Cts1p of *S. cerevisiae* in cell separation (Dunkler et al., 2005; McCreath et al., 1995). Filamentous fungi contain much higher numbers of chitinolytic enzymes than yeasts and phylogenetically they can be divided into three subgroups (A–C), which are further characterized by the presence of certain carbohydrate binding modules (CBMs) in the respective subgroups (Karlsson and Stenlid, 2008; Seidl, 2008). Subgroup A corresponds to fungal/bacterial chitinases, subgroup B to fungal/plant chitinases and subgroup C proteins are unique to filamentous fungi and distantly related to the fungal/bacterial group.

Functional analysis of chitinases in filamentous fungi has been very limited, probably because of the large number of chitinase orthologs per fungal species (Ihrmark et al., 2010; Karlsson and Stenlid, 2008; Seidl, 2008; Seidl et al., 2005). In *Aspergillus fumigatus*, the only chitinase gene deleted is the fungal/bacterial *CHIB1*. ChiB1p was responsible for the major extracellular chitinolytic activity during autolytic phase of the batch culture (Jaques et al., 2003). Accordingly, no growth or morphological phenotype was observed after disruption of this gene. Similarly, a *CHIB1* ortholog in *Aspergillus nidulans* was also responsible for the major chitinase activity during autolytic phase and strongly induced upon carbon source deprivation (Shin et al., 2009). The only fungal/plant chitinase encoding gene deleted in filamentous fungi is

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CHIA from *A. nidulans*. Although a first manuscript suggested a role in morphogenesis (Takaya et al., 1998), impossibility to complement the observed defect by *CHIA* lead these authors to conclude that their first observations were due to an additional cryptic mutation (Yamazaki et al., 2007).

To date, there is not a single demonstration that chitinases play a cell wall plasticizing role. The lack of effect of chitinase gene deletion on the growth or morphogenesis of filamentous fungi may be due to a redundancy effect in this multigene family (Jaques et al., 2003; Reichard et al., 2000; Yamazaki et al., 2007). However, it has been shown in filamentous fungi that chitinases are often closely associated with cell wall fractions and are present at germ tube and hyphal branching sites where cell wall remodeling activity takes place (Hearn et al., 1998; Iranzo et al., 2002; Rast et al., 1991; Yamazaki et al., 2008).

The aim of our study was to analyze the functional role of the fungal/plant chitinases in *A. fumigatus* following a step by step multiple gene family deletion strategy to knockout all the genes in the fungal/plant chitinase.

2. Material and methods

2.1. Strains and conidium production

A. fumigatus strains used in this study as well as the genetic backgrounds used to generate each mutant are listed in Table 1. Strains were routinely maintained on malt agar slants (2% (w/v) malt; 2% agar) at room temperature. Conidia were obtained from a mycelial culture incubated on malt agar slants at 37 °C for 3–7 days, harvested with 0.05% (v/v) Tween-20 aqueous solution, and filtered through a 40 μm nylon cell strainer (BD Falcon).

2.2. Construction of the deletion cassettes

The following five genes were targeted for sequential deletion in order to obtain a quintuple chitinase mutant strain: *CHI1* (=*CHIA1* (Jaques et al., 2003); AFU5G03760), *CHI2* (AFU8G00700), *CHI3* (AFU7G05140), *CHI4* (AFU5G03530) and *CHI5* (AFU3G07110). Deletion cassettes were constructed by PCR overlap as previously described (Lamarre et al., 2007), using the primers enumerated in Table 2. Each deletion cassette included two approximately 1 kb sequences flanking the targeted gene added to one of the four selection markers used in this study: the *Aspergillus niger* orotidine-5′-monophosphate decarboxylase gene (*PYRG*) carried by plasmid pAB4-1 (van Hartingsveldt et al., 1987), the *Escherichia coli* hygromycin B phosphotransferase gene (*HPH*) carried by plasmid pAN7-1 (Peng et al., 1992; Punt et al., 1987), the *Streptococcus hindustanus* phleomycin resistance gene (*BLE*) carried by plasmid pAN8-1 (Mattern et al., 1988) and finally the *Aspergillus oryzae* pyrithiamine resistance gene (*PTRA*) carried by plasmid pSK275 (Kubodera et al., 2000, 2002). As only four different selection markers were available for gene deletion in *A. fumigatus*, the *PYRG* marker used for the *CHI1* deletion was recycled by transforming the quadruple mutant with a pop-out cassette consisting of the fusion of the *CHIA1* 5′- and the 3′-flanking sequences used for this gene deletion (Table 2). Transformants that had lost the *PYRG* gene were selected on MM medium supplemented with 5-Fluoro-Orotic Acid (1 mg/ml).

2.3. Aspergillus transformation

Transformation of *A. fumigatus* strains were achieved either by polyethylene glycol-mediated transformation of protoplasts (deletion of *CHI1* in CEA17*akuB*^{KU80}*::PYRG PYRG*$^{-}$ and deletion of *CHI4* in the *chi1chi2chi3* triple mutant CEA17*akuB*^{KU80}*::PYRG PYRG*$^{-}$ *chi1::PYRG chi2::HPH, chi3::BLE,* respectively) or by electroporation of swollen conidia (all other mutants).

The protoplast transformation procedure was described by Paris and co-workers (Paris et al., 1993). Briefly, protoplasts were prepared from germlings obtained by growing conidia (5×10^6 cells ml^{-1}) in 500 ml of liquid minimal medium (MM) containing 5 mM ammonium tartrate; 1% (w/v) dextrose; 2% (v/v) salt solution (Cove et al., 1996) at 30 °C for O/N. For culturing *PYRG* deleted strains, MM medium was supplemented with uracil (5 mM) and uridine (10 mM). Protoplasts were obtained by enzymatic digestion of germ tubes using 3% (w/v) Glucanex (Novozymes, a kind gift of C Hjort, Copenhagen) in OM buffer (10 mM sodium phosphate, 1.2 M $MgSO_4$ pH 5.8). Transformation of protoplasts was achieved in 200 μl MSC buffer (10 mM MOPS (morpholinepropanesulfonic acid), pH 6.5, 1 M sorbitol, 10 mM $CaCl_2$) with DNA carrier, deletion cassette and PEG 60%. Transformed protoplasts were plated on MM agar medium containing 1 M Sorbitol (MMS). Transformants obtained with the pyrG marker were incubated at 37 °C until transformants arose (2–3 days). Transformants with the *PTRA* marker were obtained on the plates supplemented with pyrithiamine hydrobromide (500 ng/ml; Sigma) and incubated at RT for O/N. The day after, plates were overlaid with MMS top agar supplemented with pyrithiamine hydrobromide (500 ng ml^{-1}) and transferred at 37 °C until transformants arose. The electroporation procedure using KU80 strain was described previously (Lambou et al., 2010). Swollen conidia transformed by electroporation were spread on MM agar plates and selected with the appropriate drug added to melted MM top agar poured over the plates or in the absence of uridine/uracil for *PYRG*$^{-}$ parental strain.

2.4. Deletion verification using PCR and Southern analysis

Gene deletion was verified by PCR with the primers selected outside of the flanking regions used for each chitinase fusion vector construction, and the primers used for the resistance marker amplification. For confirmation of deletion mutants, genomic DNAs

Table 1
Aspergillus fumigatus strains used in this study.

Strain	Description	Reference
KU80Δ	CEA17*akuB*^{KU80}*::PYRG*	da Silva Ferreira et al. (2006)
KU80ΔpyrG	CEA17*akuB*^{KU80}*::PYRG PYRG*$^{-}$	da Silva Ferreira et al. (2006)
chi1	CEA17*akuB*^{KU80}*::PYRG PYRG*$^{-}$ *chi1::PYRG*	This study
chi1chi2	CEA17*akuB*^{KU80}*::PYRG PYRG*$^{-}$ *chi1::PYRG, chi2::HPH*	This study
chi1chi2chi3	CEA17*akuB*^{KU80}*::PYRG PYRG*$^{-}$ *chi1::PYRG, chi2::HPH, chi3::BLE*	This study
chi1chi2chi3chi4	CEA17*akuB*^{KU80}*::PYRG PYRG*$^{-}$ *chi1::PYRG, chi2::HPH, chi3::BLE, chi4::PTRA*	This study
chi1chi2chi3chi4 *pyrG*$^{-}$	CEA17*akuB*^{KU80}*::PYRG PYRG*$^{-}$ *chi1::PYRG, chi2::HPH, chi3::BLE, chi4::PTRA, PYRG*$^{-}$	This study
chi1chi2chi3chi4chi5	CEA17*akuB*^{KU80}*::PYRG PYRG*$^{-}$ *chi1::PYRG, chi2::HPH, chi3::BLE, chi4::PTRA, PYRG*$^{-}$*, chi5::PYRG*	This study

PYRG encodes an *A. niger* orotidine-5′-monophosphate decarboxylase conferring prototrophy to uracil and uridine; *HPH* encodes an *E. coli* hygromycin phosphotransferase conferring hygromycin B resistance; *BLE* encodes a bleomycin phosphotransferase conferring phleomycin resistance; *PTRA* confers pyrithiamine resistance; *PYRG*$^{-}$ corresponds to a mutation induced by 5-fluoroorotic acid conferring auxotrophy to uridine and uracil.

Table 2
Primers used in this study.

Name	Sequence (5′ → 3′)	Purpose in this study
Afu5g03760		
5CHITA5	GACTTCCTCCATAGGCTAGTTCCAGCACAC	5′-flanking amplification
5CHITA3	GGAAAACCCTGGCGTTACCCAACTTAATCCAACTACCGCACAAGAAAGGAGTGTAGACG	5′-flanking amplification and fusion
3CHITA5	GCGTAATCATGGTCATAGCTGTTTCCTGTGCATTCCTCCCAGCTACACTCAAGAAGCTGT	3′-flanking amplification and fusion
3CHITA3	GGGTCCGTTCTCGATATTTATACGTTGCAT	3′-flanking amplification
5CHITAPYRG	CGTCTACACTCCTTTCTTGTGCGGTAGTTGGATTAAGTTGGGTAACGCCAGGGTTTTCC	pyrG amplification and fusion
3CHITAPYRG	ACAGCTTCTTGAGTGTAGCTGGGAGGAATGCACAGGAAACAGCTATGACCATGATTACGC	pyrG amplification and fusion
5VERIFCHITA	CGCTAAAATCTTCGCATTATGGTTGGTGTT	Transformants screening
3VERIFCHITA	GCCTTTTCAGCGAACTTTTCGATCATTTTA	Transformants screening
5CHITA5.PYRGPOP	GACTTCCTCCATAGGCTAGTTCCAGCACAC	pyrG marker recycling
5CHITA3.PYRGPOP	ACAGCTTCTTGAGTGTAGCTGGGAGGAATGCAACTACCGCACAAGAAAGGAGTGTAGACG	pyrG marker recycling
3CHITA5.PYRGPOP	CGTCTACACTCCTTTCTTGTGCGGTAGTTGCATTCCTCCCAGCTACACTCAAGAAGCTGT	pyrG marker recycling
3CHITA3.PYRGPOP	GGGTCCGTTCTCGATATTTATACGTTGCAT	pyrG marker recycling
Afu5g03530		
5CHITB5	CATGCCTCTCTTGTTACCGTCATAGGTCTG	5′-flanking amplification
5CHITB3.2	GGGTCTAATGAAGTTTCCATCAGCACATTG	5′-flanking amplification and fusion
3CHITB5.2	AAGCCGATCATCGAATACAGCAAGAAGTTT	3′-flanking amplification and fusion
3CHITB3	CAAGGACCGCTTAAATAGGTGATCTCAGCA	3′-flanking amplification
5CHITBPTR.2	CAATGTGCTGATGGAAACTTCATTAGACCCTACGGGATCCCATTGGTAACGAAATGTAAA	pyrG amplification and fusion
3CHITBPTR.2	AAACTTCTTGCTGTATTCGATGATCGGCTTACGGTATCGATAAGCTTGATGGCCTAGATG	pyrG amplification and fusion
5VERIFCHITB	GTGATTGGCATTCCACGGTGATCTCTTAG	Transformants screening
3VERIFCHITB	CCTCCACCGATTATCATCACCTCATAGTCA	Transformants screening
Afu3g07110		
5CHITC5	TACAAGCCCGAATATGCCGAGATCTTAGTC	5′-flanking amplification
5CHITC3	GGAAAACCCTGGCGTTACCCAACTTAATCATATCAGCCTCTACAACACCAAGCGAATGA	5′-flanking amplification and fusion
3CHITC5	GCGTAATCATGGTCATAGCTGTTTCCTGTGAGTTCATATGACGCAGGACAACCAATGACT	3′-flanking amplification and fusion
3CHITC3	ACCTTCCCTAGAGCTACAGGTCAGATCACG	3′-flanking amplification
5CHITCPYRG	TCATTCGCTTGGTGTTGTAGAGGCTGATATGATTAAGTTGGGTAACGCCAGGGTTTTCC	pyrG amplification and fusion
3CHITCPYRG	AGTCATTGGTTGTCCTGCGTCATATGAACTCACAGGAAACAGCTATGACCATGATTACGC	pyrG amplification and fusion
5VERIFCHITC	TGCATCGGCCCTTACTTTTTACTCAAACAT	Transformants screening
3VERIFCHITC	AACAATCAAGATCGTAGCAGAACGCCACTA	Transformants screening
Afu7g05140		
5CHITD5	TTCCTGACTGCCTGAGACTGAATGGAATAG	5′-flanking amplification
5CHITD3	TCGTGAATCTTTTACCAGATCGGAAGCAATAATACCTGATTGACGAGAAAACAGGGAGGA	5′-flanking amplification and fusion
3CHITD5	TGGTGCACTCTCAGTACAATCTGCTCTGATTATCTTACGCCATGGGCACATTATACCAGA	3′-flanking amplification and fusion
3CHITD3	AAACCCCGTATATTTCAAGCGACCACTGTA	3′-flanking amplification
5CHITDPHLEO	TCCTCCCTGTTTTCTCGTCAATCAGGTATTATTGCTTCCGATCTGGTAAAAGATTCACGA	pyrG amplification and fusion
3CHITDPHLEO	TCTGGTATAATGTGCCCATGGCGTAAGATAATCAGAGCAGATTGTACTGAGAGTGCACCA	pyrG amplification and fusion
5VERIFCHITD	CAGACCATGTTCGATTACATCTCGCAGTTT	Transformants screening
3VERIFCHITD	ATTGCTGATATTCATGCCGTTTGTTCTCAG	Transformants screening
Afu8g00700		
5CHITE5	GTTCCTCGGAATGGAACTGGTTCTGTACTC	5′-flanking amplification
5CHITE3	TCGTGAATCTTTTACCAGATCGGAAGCAATGGCTTTCCAACAGCACTATCACCAGACATA	5′-flanking amplification and fusion
3CHITE5	TGGTGCACTCTCAGTACAATCTGCTCTGATCTTGTTAGCCACAATTGCTGGATTTTCAGA	3′-flanking amplification and fusion
3CHITE3	ACTCGAACATGCATACTCCAACTTCTCTGC	3′-flanking amplifiation
5CHITEHYGRO	TATGTCTGGTGATAGTGCTGTTGGAAAGCCATTGCTTCCGATCTGGTAAAAGATTCACGA	pyrG amplification and fusion
3CHITEHYGRO	TCTGAAAATCCAGCAATTGTGGCTAACAAGATCAGAGCAGATTGTACTGAGAGTGCACCA	pyrG amplification and fusion
5VERIFCHITE	TTACGGGCGAAGATATACTCGACAAGAAGC	Transformants screening
3VERIFCHITE	GGGTAAATCGATTCATGACAGTTCATCCAA	Transformants screening
Class III gene family		
5CHITA.qPCR	CAACCACCGTGACGTTGACA	Afu5g03760 RT-PCR
3CHITA.qPCR	TTCAGAAAAGCTGCCGTTGC	Afu5g03760 RT-PCR
5CHITB.qPCR	TACCCTGACGCTGCCGATAA	Afu5g03530 RT-PCR
3CHITB.qPCR	AACCGTCTTGGCCCAGTTGT	Afu5g03530 RT-PCR
5CHITC.qPCR	TGCGATTTGGGTGCAGTTCT	Afu3g07110 RT-PCR
3CHITC.qPCR	TTTCCTGGCAGACCGATGAA	Afu3g07110 RT-PCR
5CHITD.qPCR	CAACCAGCTCCGCTCCTTGT	Afu7g05140 RT-PCR
3CHITD.qPCR	GCAGGCCGCAGTAGTTGTTG	Afu7g05140 RT-PCR
5CHITE.qPCR	CGGCTGGGACTTTGACATTG	Afu8g00700 RT-PCR
3CHITE.qPCR	AAACTGCGCCTTGGTGACAA	Afu8g00700 RT-PCR
Afu1g06390		
TEF1a	CCATGTGTGTCGAGTCCTTC	House keeping gene
TEF1b	GAACGTACAGCAACAGTCTGG	House keeping gene

from PCR positive transformants were digested with two different restriction enzymes, fractionated by electrophoresis through 0.7% agarose gels in TAE buffer and subjected to Southern blot analysis (Sambrook et al., 1989) probed with both [α-^{32}P]dCTP-labeled gene flanking sequences using the random Readyprime II labeling kit following the manufacturer's instructions (GE Healthcare, UK).

2.5. Quantitative real time polymerase chain reaction (qRT-PCR) analysis

Gene expression was quantified by RT-PCR analysis. Total RNAs were extracted from *A. fumigatus* resting and germinating conidia as described in Lamarre and coworkers (2007). RNA samples were

purified using RNeasy mini kit (Qiagen) following the RNA clean up protocol according to the manufacturer's instructions. 3 μg of total RNA were treated with RNasefree RQ1 DNase (Promega) in order to remove genomic DNA contamination. cDNAs were synthesized from 500 ng of DNaseI treated total RNA using the SuperScriptIII Reverse Transcriptase (Invitrogen) following manufacturer's instructions. A negative control without Reverse transcriptase enzyme was prepared as a control for each sample. cDNAs (2 μl) were used for qRT-PCR amplification in 20 μl reaction mixture containing specific primers (0.5 μM of each primer) (Table 2) and the SYBR Green JumpStart Taq Ready Mix (Sigma). The primer sequences were designed using the software Primer 3 available on-line at http://frodo.wi.mit.edu/. Primers were selected according to the following parameters: Tm between 64 °C and 66 °C, primer length between 20 and 22 base pairs, probe length between 150 and 155 nucleotides (Table 2).qRT-PCR was performed using a Rotor-Gene RG-3000 (Corbett Research, UK) with the following cycling parameters: initial incubation at 95 °C for 3 min. Next 40 cycles of 95 °C for 5 s, 58 °C for 5 s, 72C for 15 s, 78 °C for 4 s and 78 °C for one second. Amplification of one single specific target DNA was checked with a melting curve analysis (Melt (50–95 °C), hold 30 s on the first step, hold 5 s on the next steps) Absence of genomic DNA in the extracted RNA was confirmed using the negative controls in which reverse transcriptase were omitted as template for the qRT-PCR. Fold changes in expression were calculated using the $2^{-\Delta Ct}$ method for individual time points and normalized to the *TEF1* house keeping gene (Schmittgen and Livak, 2008). The experiment was repeated six times and all the gene expression values were used for graphical representation.

2.6. Chitinase activity assay and the determination of the cell wall chitin content

Fungal cells were broken using 0.5 mm glass beads in a FastPrep (MP Biomedicals) by 4–8 cycles of vortex depending on the morphotypes, for 30 s. Disrupted cells were centrifuged at 3000 g for 10 min and the intracellular chitinase activity was measured in the supernatant (intracellular fraction). The cell wall pellet was washed (×3) with water, suspended in a 50 mM sodium acetate buffer, pH 5.6 containing 5 mM sodium azide. After O/N incubation in a water bath (200 rpm agitation, 37 °C), the contents were centrifuged and the supernatant (cell wall autolyzate) was used as the source of cell wall bound chitinases. Chitinase activity was determined using carboxymethyl Remazol Brilliant Violet chitin (CM-Chitin-RBV, 4 mg/ml) as the substrate following the manufacturer's instructions (Loewe Biochemica, Nordring, Germany). Briefly, 50 μl of the intracellular fraction or cell wall autolyzate were treated with 50 μl of acetate buffer (pH 5.5, 50 mM) and 100 μl of CM-Chitin-RBV at 37 °C. The reaction was arrested by adding 0.5 M HCl and incubates on ice for 10 min. The reaction mixture was centrifuged at 10,000 g for 10 min and the absorbance of the supernatant was measured at 550 nm. The protein content in the intracellular fraction/cell wall autolyzate was determined by bicinchoninic acid (BCA) method using BCA protein assay reagent according to the manufacturer's instruction (Pierce Protein Research Products, Thermo Fisher Scientific Inc., Rockford, IL, USA). Chitinase activity was expressed as $\Delta A_{550}/10^8$ cells/h or ΔA_{550}/μg protein/h. Total activity was the sum of the soluble intracellular and cell wall associated activities.

Activity was also determined using 4-methylumbelliferyl-β-D-N-N′-N″-triacetylchitotrioside [4-MU-$(GlcNAc)_3$ as the substrate (Brurberg et al., 1996). The reaction mixture consisted of intracellular/cell wall autolyzate, 50 mM citrate–phosphate buffer (pH 6.2) and 20 μM substrate in a total volume of 50 μl. After incubating at 37 °C for 1 h, the reaction was arrested by the addition of 1.95 ml of 0.2 M Na_2CO_3. The amount of 4-MU released was measured using a TK0 100 Fluorometer (Hoefer Scientific Instruments, San Francisco) and the activity was expressed as 4-MU released/min/μg protein.

Cell wall chitin content was measured as the percentage of GlcNAc per cell wall polysaccharide by gas–liquid-chromatography analysis (GLC) as described earlier (Fontaine et al., 2000).

2.7. Fungal growth and germination

Flasks containing 50 ml Sabouraud (SAB: 2% glucose + 1% mycopeptone, Biokar Diagnostics, Pantin, France) and minimal medium (MM) (Mouyna et al., 2010) were inoculated with 1×10^8 conidia and incubated in a rotary shaker (150 rpm) at 37 °C. After 3- and 5-weeks, the mycelial mass obtained was extensively washed with water and freeze dried to measure the mycelial dry weights. The percentage of germination was quantified as described previously (Mouyna et al., 2010). Media containing N-acetyl-glucosamine (GlcNAc), carboxymethyl-chitin (CM-chitin), colloidal chitin or chitin (from Sigma) were also used to check the fungal growth: Brian medium (1% asparagine, 1% KH_2PO_4, 0.2% $MgSO_4{\cdot}7H_2O$, 0.24% NH_4NO_3, 2.6×10^{-3}% $ZnSO_4{\cdot}7H_2O$, 2.6×10^{-4}% $CuSO_4{\cdot}5H_2O$, 1.3×10^{-4}% $Co(NO_3)_2{\cdot}6H_2O$ and 6.5×10^{-3}% $CaCl_2$, pH 5.4) lacking glucose was supplemented with GlcNAc (1%) or CM-chitin (1%) or colloidal chitin (Gomez Ramirez et al., 2004) (1%) or chitin (5%) (Sigma). 1×10^5 conidia were inoculated per ml of medium, incubated at 37 °C with or without agitation. Growth was also monitored on the agar plates containing the same media with 2% agar.

2.8. Antifungal susceptibility testing (AST)

For the E-test, strips with concentration gradients from 0.004 to 32 mg/l of caspofungin, anidulafungin, voriconazole, posaconazole, and itraconazole were used (AB Biodisk, Tec-Laim, Madrid, Spain) in MM or potato dextrose Agar medium. The MICs and MECs were read after 24 and 48 h of incubation at 35 °C as per the manufacturer's instructions. Micro-dilution testing was performed in liquid RPMI-2% glucose following the EUCAST Definitive Document E.DEF 9.1 (Rodriquez-Tudela et al., 2008). The antifungal agents used in the study were amphotericin B (range 16–0.03 μg/ml) (Sigma Aldrich Química), itraconazole (range 8–0.015 μg/ml) (Janssen S.A., Madrid, Spain), voriconazole (range 8–0.015 μg/ml) (Pfizer, S.A.), posaconazole (range 8–0.015 μg/ml) (Schering-Plough Research Institute, Kenilworth, NJ, USA), terbinafine (range 16–0.03 μg/ml) (Novartis, Basel, Switzerland), caspofungin (range 16–0.03 μg/ml) (Merck & Co., Inc., Rahway NJ, USA) and micafungin (range 16–0.03 μg/ml) (Astellas Pharma Inc, Tokyo, Japan). The chitin synthase inhibitor Nikkomycin Z (range 64–0.125 μg/ml) and chitinase inhibitors Acetazolamide (Hurtado-Guerrero and van Aalten, 2007) (range 384–0.75 μg/ml), Caffein (range 384–0.75 μg/ml) and Theophylline (range 384–0.75 μg/ml) (Rao et al., 2005) (all from Sigma) were tested in a similar way. To test the effect of Calcofluor white and Congo red, series of 10-fold dilutions derived from starting suspensions of 10^7 conidia/ml, aliquots of 5 μl were spotted onto a MM agar plates alone or onto those supplemented with 200, 100, or 10 μg/ml of calcofluor white and with 50, 25, or 10 μg/ml of Congo red, and incubated at 37 °C for 48 h.

2.9. Statistical analysis

At least three biological replicates were performed per experiment and the statistical significance of the results was evaluated by a one way variance analysis using the JMP1 software (SAS Institute, Cary, NC, USA).

2.10. Phylogenetic analysis

Multiple alignments were created with ClustalX 2.0 (Larkin et al., 2007) using the default parameters, visually inspected in GeneDoc (Nicholas et al., 1997) and phylogenetic analysis was carried out with MEGA 4 (Tamura et al., 2007) using the Neighbour Joining algorithm and 500 bootstrap rearrangements.

3. Results

3.1. Chitinase Activity

In the parental strain (CEA17*akuB*^{KU80}*::PYRG*), an increase in the chitinase activity was associated to conidial germination in absence of any exogenous chitin addition (Fig. 1). Moreover, during germination, there was an increase in the chitinolytic activity associated with the cell wall (CW). The CW-associated/intracellular activity ratio during the course of germination increased from 0.06 in the resting conidia to 0.26 in the germinating conidia, indicating that during germination the chitinases were redirected to fungal cell wall, suggesting, chitinases were important for conidial swelling and germ tube formation.

3.2. A. fumigatus chitinase gene family

BLAST analysis (http://www.cadre-genomes.org.uk/Aspergillus_fumigatus/) of the *A. fumigatus* genome using protein sequences of the published chitinase sequences identified 18-ORFs. All the *A. fumigatus* chitinases belong to the GH18 family of the CAZY database. A phylogenetic analysis of the *A. fumigatus* chitinases showed that they can be divided into three different subgroups previously identified in *Trichoderma* by Seidl (2008), Seidl et al. (2005) and Karlsson et al. (Ihrmark et al., 2010; Karlsson and Stenlid, 2008) (Fig. 2). Subgroup A corresponded to the Class V (fungal/bacterial) chitinases and contained six genes encoding for proteins with average molecular mass of 40–60 kDa. Most of these chitinases had an *N*-terminal signal peptide that targets them to the secretory pathway. *CHIB1* gene (AFU8G01410) previously analyzed by Jaques et al. (2003) belong to this family. Subgroup C was composed of six genes (AFU5G01400, AFU5G06840, AFU5G03960, AFU6G13720, AFU6G09310, AFU6G09780) and the proteins encoded by four of them were of higher molecular mass (120–140 kDa), contained one or two LysM motifs implicated in binding peptidoglycan and a CBM 18. One of them (AFU5G01400) had an immunoglobulin like motif. The two genes AFU1G00310 and AFU3G07160 were at the border of the subgroups A and C: phylogenetically these two proteins grouped with the subgroup A but they had a chitin binding module (CBM) that was only found in the subgroup C. Orthologues of the protein encoded by AFU5G03850 were included in the subgroup A in the analysis published by Karlsson and collaborators, but we found that for *A. fumigatus* AFU5G03850 the similarity was too low to include it with certainty within any subgroup. Subgroup B contained the fungal/plant (class III) chitinases (named *CHI1*, *CHI2*, *CHI3*, *CHI4* and *CHI5* respectively). They had relatively low molecular masses from 30 to 40 kDa and using the http://wolfpsort.org software they were predicted to be secreted proteins. Interestingly, the chitinase encoded by AFU5G03760, with a predicted M_r of ~90 kDa due to the presence of an additional CBM domain and several stretches of serine-rich motifs, contained a GPI-anchoring sequence (http://mendel.imp.ac.at/gpi/gpi_server.html). Gene expression analysis of the class III chitinases genes was performed for the *A. fumigatus* (CEA17*akuB*^{KU80}*::PYRG*) strain by real time PCR. Transcripts of the five genes were detected in the resting conidia and for all the genes except *CHI3* after 3 h and 12 h (swollen and germinating conidia, respectively) (Fig. 3). Because subgroup B was the only one shown to contain enzymes that play a role in yeast morphogenesis (Dunkler et al., 2005; Kuranda and Robbins, 1991; McCreath et al., 1995), it was chosen to delete the entire cluster of fungal/plant chitinases in *A. fumigatus* to analyze, for the first time, the putative function of this chitinase family in a filamentous fungus.

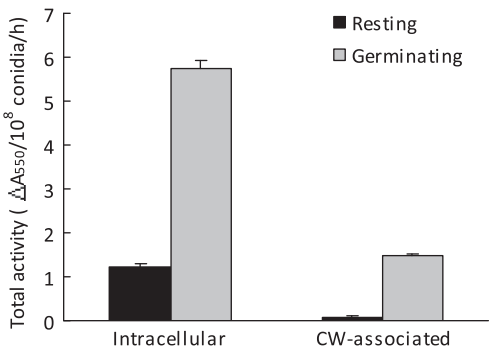

Fig. 1. Intracellular and cell wall (CW) associated chitinase activity of resting and germinating *A. fumigatus* conidia. Total activity estimated in unit = increase in the A_{550nm} by one OD/10^8 cells/h.

3.3. Generation of an A. fumigatus strain defective in Group B chitinase family

First, single mutants for each studied chitinase genes were generated in the *A fumigatus* parental strain (CEA17*akuB*^{KU80}*::PYRG*) in order to verify the functionality of each deletion cassettes. None of the single deletion mutants had a phenotype different from the wild type parental strain (data not shown). A quintuple mutant was then generated through sequential gene deletions as described in the Fig. 4A–E. First the four different selectable markers (the *PYRG* auxotrophic marker and three dominant markers; *HPH*, *BLE* and *PTRA* genes) routinely used for gene deletion in *A. fumigatus* were used to make a quadruple mutant. Attempts to use other resistance markers such as the *NAT*, *BAR* and *G418* genes to delete *CHI5* were unsuccessful because of the insensitivity of *A. fumigatus* to the corresponding drugs nourseothricin, glufosinate and geneticin, respectively. Thus, the fifth sequential deletion was obtained by recycling the *PYRG* marker using a pop-out cassette and selection with 5-FOA as described in materials and methods.

For each transformation, several transformants obtained on appropriate selective condition, were screened by PCR amplification using two pair of primers (Table 2): the first pair consisted of one primer designed outside the 5′-end of the deletion cassette coupled with the 3′ primer used for amplification of the resistance gene (for example for *chi1* deletant, 5VERIFCHITA-3CHITAPYRG). The second pair of primers consisted on one primer designed outside the 3′-end of the deletion cassette coupled with the 5′ primer used for amplification of the deletion cassette (3VERIFCHITA-5 CHITAPYRG). Amplicons obtained with both pair of primers indicated that the full-length deletion cassette was incorporated at the locus, and that transformant no longer contained the targeted chitinase gene. As transformations were performed in a parental strain (CEA17*akuB*^{KU80}*::PYRG*) that forms homologous recombination, the majority of transformants obtained for the five deleted genes had the deletion cassette incorporated at the targeted locus. To confirm the results obtained by PCR, genomic DNA was digested by appropriate enzymes (see Fig. 4A–E for choice of restriction enzymes) and analyzed by Southern blotting using both [α-^{32}P]dCTP-labeled flanking sequences for each gene targeted in this work. Each mutant generated in this work was analyzed for the five genes targeted (Fig. 4A–E).

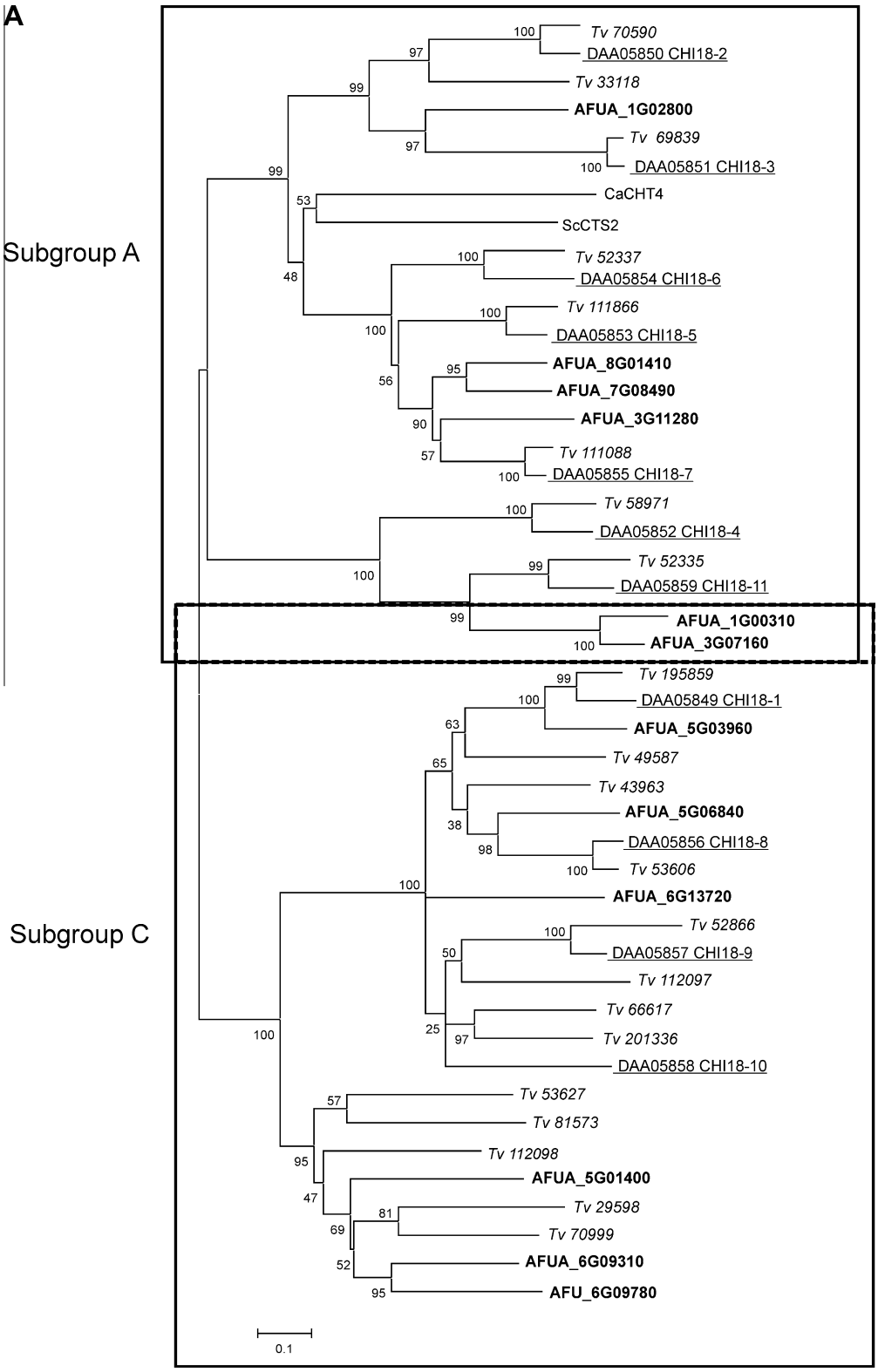

Fig. 2. (A and B): Phylogenetic trees showing the three subgroups of the *A. fumigatus* GH 18 chitinases. *A. fumigatus* chitinases are shown in bold letters (numbers corresponding to the protein models in the genome database), *T. reesei* chitinases are underlined (GenBank/EMBL/DDBJ accession numbers), *T. virens* chitinases are shown in italics (protein IDs in the JGI *T. virens* genome database v2.0, and *S. cerevisae* (Sc) and *C. albicans* (Ca) chitinases are indicated with normal black letters.

CHIA1 (AFU5G03760) was first replaced by the *PYRG* resistance marker using the CEA17*akuB*^{KU80}*::PYRG PYRG*$^{-}$ recipient strain. Correct integration of the *PYRG* at the *CHI1* locus was verified after DNA restriction with Bgl II and Hind III (Fig. 4A). The second round of transformation using *chi1* mutant (CEA17*akuB*^{KU80}*::PYRG PYRG*$^{-}$ *chi1::PYRG*) as the recipient strain and the fusion vector for *CHI2* (AFU8G00700) deletion containing hygromycin as selectable marker produced multiple transformants. Hyg-resistant transformants

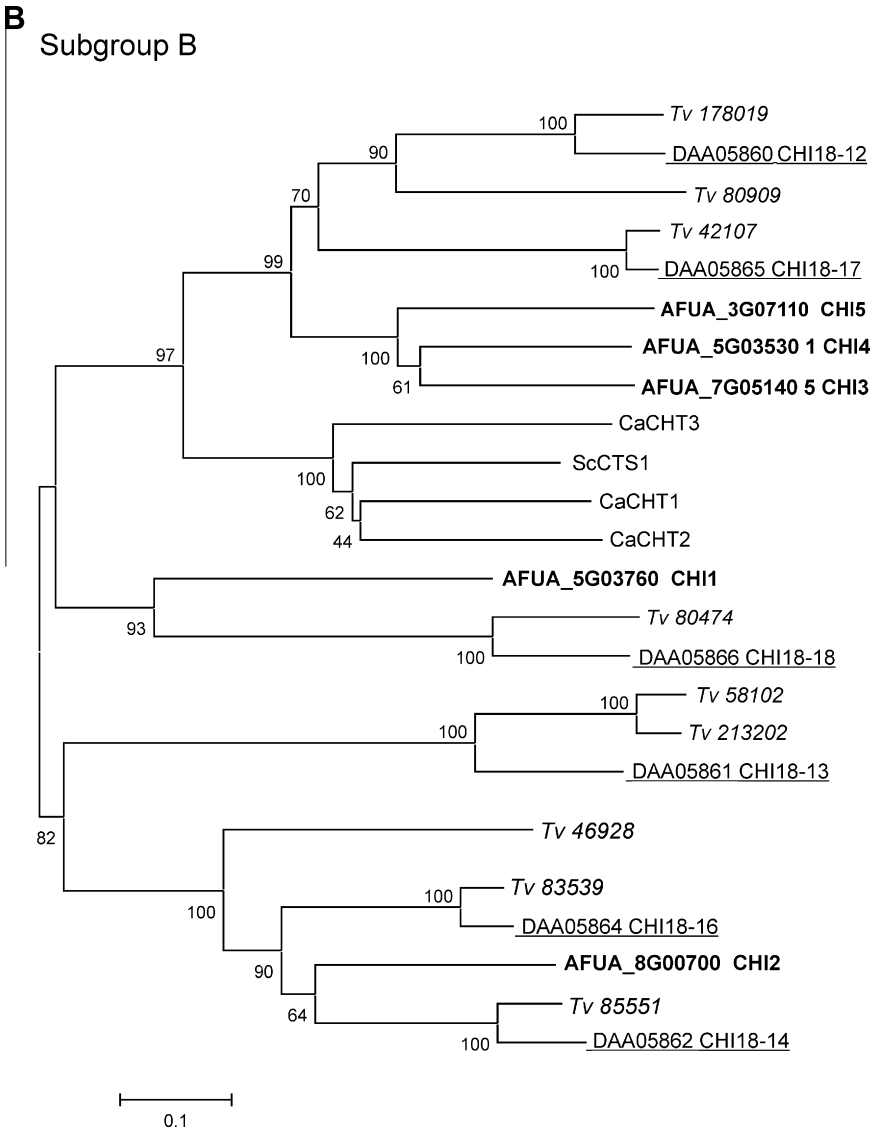

Fig. 2 (*continued*)

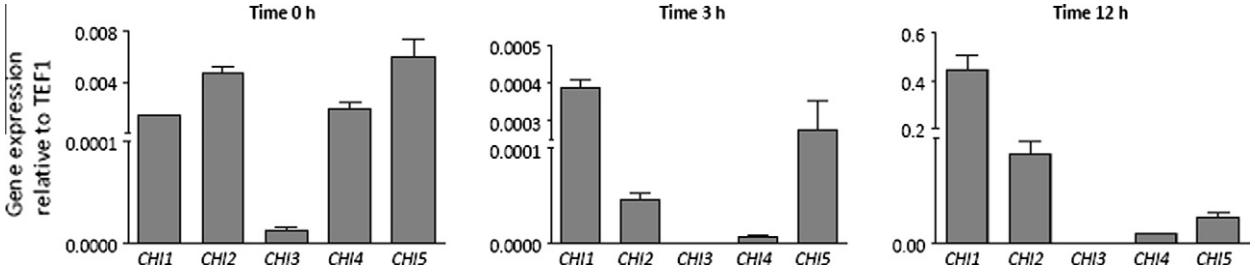

Fig. 3. Analysis of the transcripts of *A. fumigatus* fungal/plant-type chitinase genes by qRT-PCR. Zero, 3 and 12 h corresponded to resting, swollen and germinating conidia. *A. fumigatus* was incubated in YPD medium at 37 °C and 150 rpm. Ratios were normalized to TEF expression.

were verified by PCR and correct integration of the Hygromycin-resistance was verified by Southern blot after digestion with Eco RV and Pvu II on two transformants (Fig. 4B). Third round of transformation was using CEA17*akuB*^{KU80}*::PYRG PYRG*$^{-}$ *chi1::PYRG chi2::HPH* as the recipient strain and the fusion vector for *CHI3* deletion containing phleomycin resistant marker (*BLE*) produced multiple transformants. After PCR selection, correct integration of the resistance marker at the *CHI3* (AFU7G05140) locus was confirmed for transformants after Pvu II and Sac I digestion. The generation of the quadruple mutant was achieved on the fourth round of transformation, using CEA17*akuB*^{KU80}*::PYRG PYRG*$^{-}$ *chi1::PYRG, chi2::HPH, chi3::BLE* as the recipient strain. Integration of the *PTRA* cassette at the *CHI4* (AFU5G03530) locus was assessed by Southern blot on two pyrithiamine-resistant transformants. *CHI5* was replaced in the pyrG^{-} quadruple deletant obtained by transformation of the pyrG^{+} quadruple mutant with the pop-out cassette. Insertion of *PYRG* at the *CHI5* (AFU3G07110) locus was verified by Southern blots on two positive transformants after DNA restriction by Eco RV and Xba I digestion.

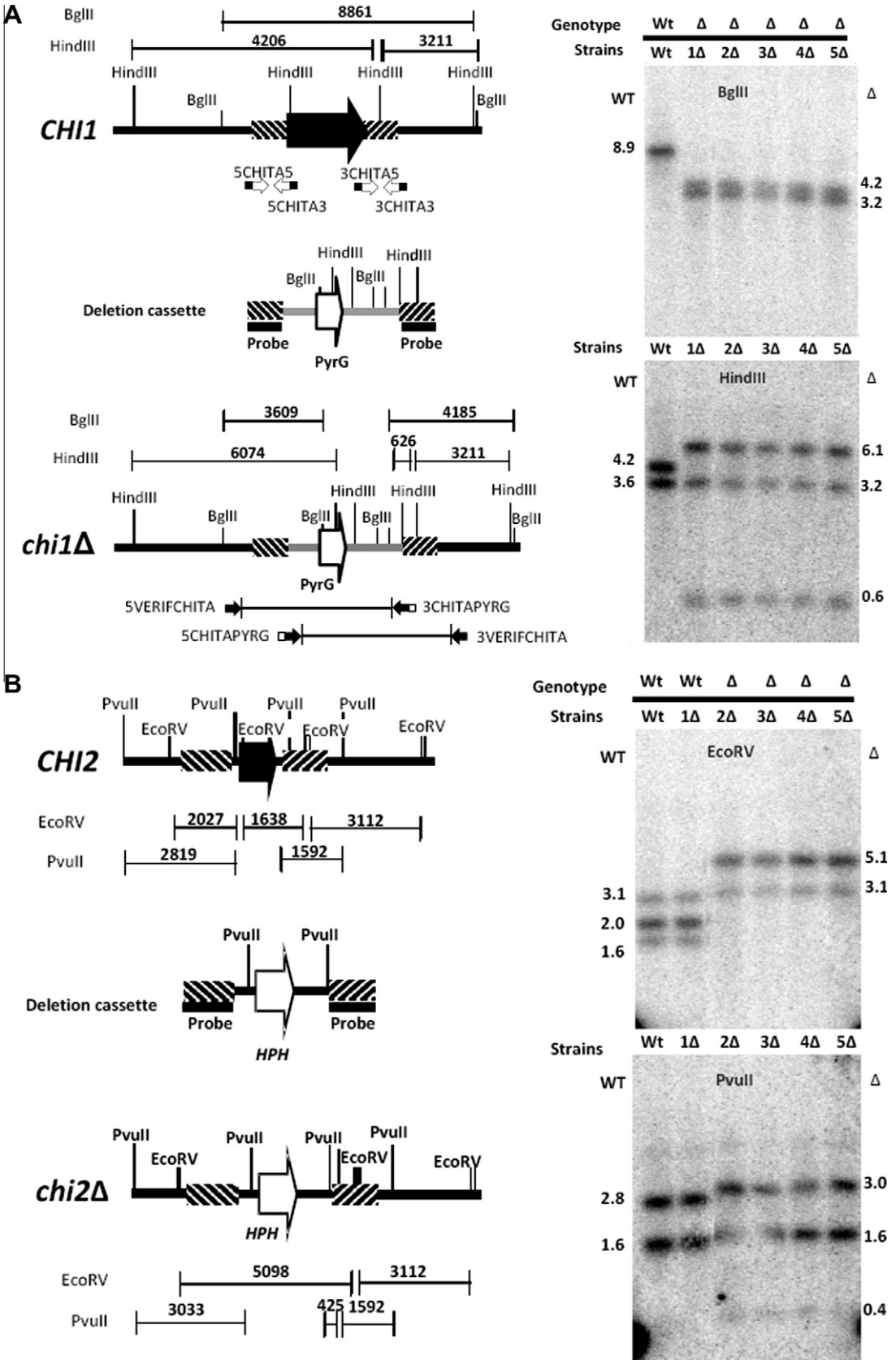

Fig. 4. Construction of a quintuple class III chitinase mutant in *A. fumigatus*. (A) Deletion of *CHI1* (Afu 5g 03760). In the left panel is the restriction map of the *CHI1* locus before (upper section) and after (lower section) gene replacement using deletion cassette encompassing the *PYRG* gene (middle section). Southern hybridization of genomic DNA with Bgl II and Hind III restriction enzyme digests of wild type (wt) and *CHI1* transformant (*chi1Δ*). The band size correlated with the predicted size from the left panel. (B) Deletion of *CHI2* using the hygromycin resistance gene (description, as in 4A). (C) Deletion of *CHI3* using the phleomycin resistance gene (description, as in 4A). (D) Deletion of *CHI4* using *PTRA* gene conferring resistance to pyrithiamine (description, as in 4A). (E) Deletion of *CHI5* using the *PYRG* gene (description, as in 4A). For all double, triple, quadruple and quintuple mutants, it was verified that the successive deletion did not result in any perturbation of the successive mutations (right panel in A–E).

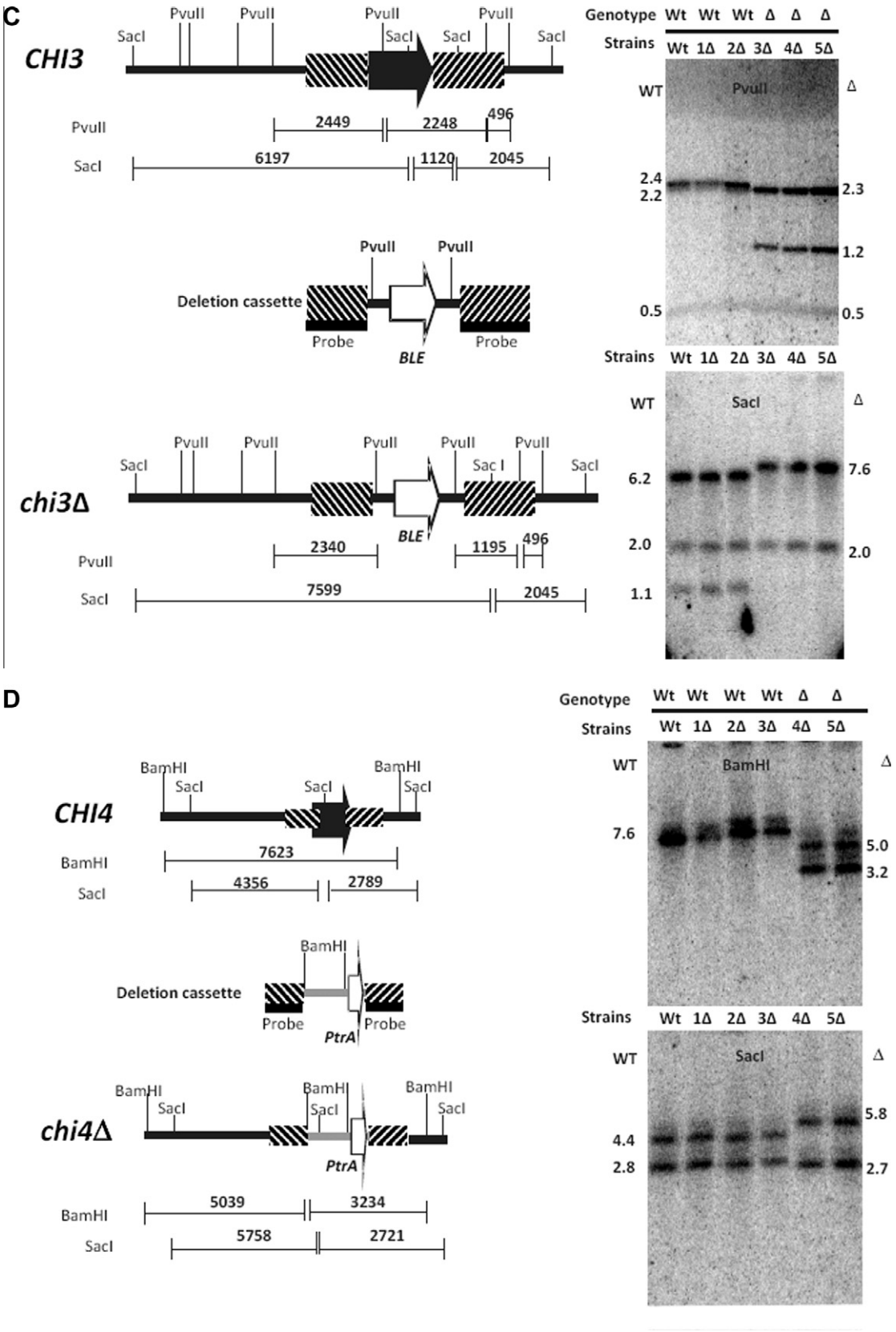

Fig. 4 (*continued*)

3.4. Chitinase activity assay and the cell wall chitin content

Successive deletion of class III chitinase genes resulted in a reduction of the both total and specific chitinase activities (Fig. 5) that were highest in the germinating and resting conidia, respectively. The higher was the number of genes deleted, the greater was the reduction in the activity. This reduction remained however limited and never exceeded ~20% of the total activity and ~30% of the

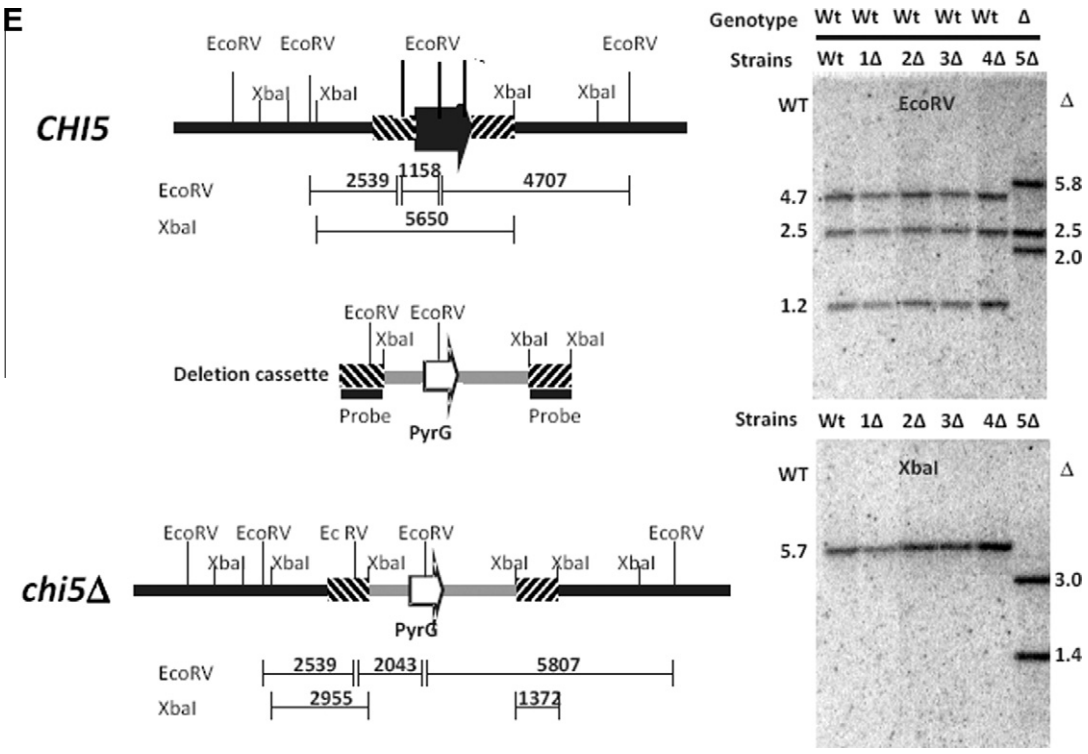

Fig. 4 (*continued*)

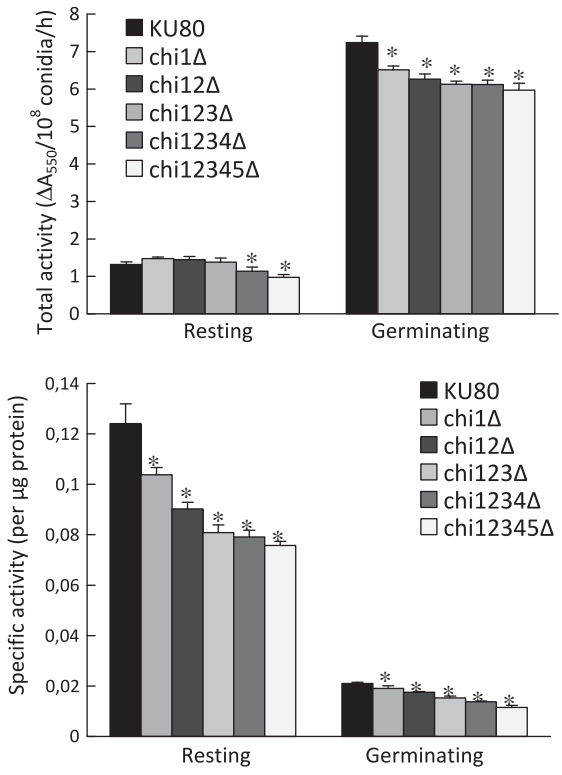

Fig. 5. Total (A) and specific (B) chitinolytic activities (increase in the A_{550nm} by one OD/h) of resting and germinating conidia of wild type, single, double, triple, quadruple and quintuple fungal/plant chitinase mutants of *A. fumigatus*. Note the decrease in the activity over the increasing number of gene deletion (∗indicates a significant difference with the parental strain).

specific activity. Similar results were seen when the chitinase activity was analyzed with CM-Chitin-RBV and 4-MU-GlcNAc$_3$ (data not shown). Also, there was no difference in the cell wall chitin content (measured as the % GlcNAc per cell wall polysaccharide) after growing the strains in the SAB medium at 37 °C for 20 h (19.1 ± 0.4% and 20.6 ± 1.4% for parental and the quintuple mutant strains, respectively).

3.5. Growth phenotype

The quintuple *chi*-mutant had a growth phenotype similar to the wild type parental strain at all temperatures tested in the SAB or the MM medium (data not shown). The radial growth of the mutants was almost indistinguishable from that of the parental strain (Supplementary Fig. 1A). There was no difference in growth of the parental, quintuple *chi*-mutant as well as the other sequential deletant strains in the Brian medium containing 1% GlcNAc, 1% soluble CM-Chitin, 1% colloidal chitin or 5% crude chitin as carbon source (both on the solid agar and in the liquid medium), even after 1 week of incubation at 37 °C. The frequency of hyphal branching was similar for parental and quintuple mutant strains in SAB/Brian media at different growth intervals (Supplementary Fig. 1B). Also, calcofluor white staining did not show any difference in the septum length, number and morphology (Supplementary Fig. 1C). The only growth phenotype noticed was a significant decrease of mycelia dry weight of the chitinase mutants compared to wild type strain during autolysis (Fig. 6). Accordingly, the mycelium of the quintuple mutant was more fragmented due to fungal death than the parental strain after 3- and 5-weeks of cultures (data not shown).

3.6. Antifungal susceptibility testing

The parental *A. fumigatus* (CEA17*akuB*^{KU80}*::PYRG*) used in the present study, the entire single *chi* deleted mutants and the quintuple mutant were tested against all class of antifungal in clinical use: Polyenes, amphotericin B (AmB); azole drugs, itraconazole

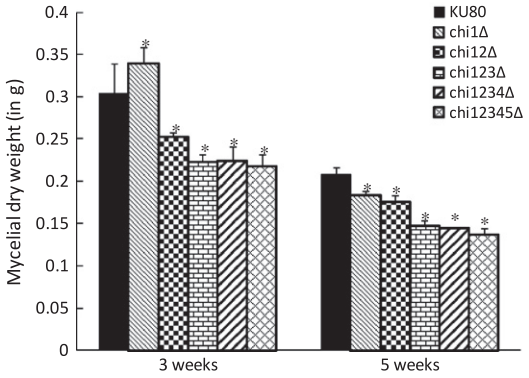

Fig. 6. Mycelial growth estimated by dry weight after 3- and 5-weeks of culture in shake conditions (150 rpm) in Sabouraud medium at 37 °C. Note that the lowest amount of the mycelial mass was obtained with the quintuple mutant.

(ITC), voriconazole VCZ, and posaconazole (POS); allylamines, terbinafine (TRB); and echinocandins, caspofungin (CAS), and anidulafungin (AND) in both liquid and solid media. There was no difference in the MICs for any of the single or multiple mutants to any class of antifungals. MICs ranges, in μg/ml, were AmB (0.25–0.5), ITC (0.12–0.25), VCZ (0.25–0.5), POS (0.03–0.06), TRB (4–8), CAS (0.25–0.5), AND (<0.03) for both parental strain and mutant strains. In addition, the quintuple mutant and the parental strain showed no difference in sensitivity to the cell wall-interfering agents such as calcofluor white or Congo red, even when high concentrations (200 μg/ml and 50 μg/ml respectively) were tested. Also, no susceptibility differences were found when the chitin synthase inhibitor Nikkomycin Z (64 μg/ml) and chitinase inhibitors (Caffein, 192 μg/ml; Acetazolamide, 384 μg/ml; Theophylline 384 μg/ml) were tested with any of the single or the quintuple mutants.

4. Discussion

This is the first report of a functional analysis of an entire fungal/plant chitinase (class III) family in a filamentous fungus. This chitinase family was selected because many arguments suggested its involvement in fungal morphogenesis. First, fungal/plant (class III) enzymes are endo- non-processing chitinases whereas fungal/bacterial (class V) enzymes show exo-acting processive activity (Hurtado-Guerrero and van Aalten, 2007). Second, chitinase with morphogenetic function in yeast belong to this class III family. Third, they are constitutively expressed and fourth, this family contained GPI-anchored proteins. The presence of a transmembrane spanning domain or GPI-anchoring domain would allow cell wall polysaccharide remodeling enzymes to face the carbohydrates of the cell wall matrix as in insect chitinases (Arakane and Muthukrishnan, 2010) or in fungal transglycosidases (Gastebois et al., 2010).

Our data showed however, that fungal/plant chitinases do not have a morphogenetic role in *A. fumigatus*. This study was in agreement with earlier single gene deletion studies showing that none of the single chitinase mutants constructed to date had an altered growth phenotype in filamentous fungi (Jaques et al., 2003; Yamazaki et al., 2008, 2007).

Several reasons could explain the lack of phenotype associated to the quintuple fungal/plant chitinase mutant. First, the lack of fungal/plant chitinase could be compensated by the other chitinases from class A and C. Indeed 12 of the 13 non-class III chitinases were expressed in resting and germinating conidia (data not shown). Second, the selection of genes was erroneous. Chitinases with chitin binding modules may have been more appropriate candidates since the presence of a CBM in this class of hydrolases could direct the chitinases to the proper cell wall locus. Such an important role of the CBM was reported for β-(1,3)-glucanases and β1,3)-glucanosyltransferases (de Medina-Redondo et al., 2008; Gastebois et al., 2010). To date not a single gene of the subgroup C coding for proteins with CBM has been disrupted leaving open the possibility that these chitinases may be the ones modulating the cell wall structures in filamentous fungi, at least in *A. fumigatus*. Against this hypothesis, is the fact that subgroup C genes have not been found yet in Basidiomycetes (Seidl, 2008). The essentiality of the CBM was however shown in bacterial and insect chitinases (Arakane et al., 2003; Huang et al., 2009; Uchiyama et al., 2001) and the addition of a CBM to a chitinase lacking CBM increases its activity towards insoluble chitin substrates (Limon et al., 2001).

Third, activation of glycosylhydrolases may not be necessary for conidial germination or hyphal branching that may only require an increase in the intracellular osmotic pressure as suggested previously (d'Enfert, 1997; Osherov and May, 2001). Accordingly, the specific activity was higher in the resting conidia than in the germinated conidia, suggesting that there was not a specific increase in the expression of chitinases during the early phases of germination.

Fourth, chitinases may have other functions than a putative role in degrading cell wall polymers. According to http://wolfpsort.org, some of the fungal/bacterial chitinases contain mitochondrial retention signal that would not be in agreement with a cell wall degrading activity. In addition, there are examples of chitinases that are not primarily associated with chitin degradation even though they are able to display a chitinolytic activity. Although mammals do not synthesize chitin, they do express enzymatically active chitinases (Boot et al., 2001). Moreover, many chitinase-like proteins have been associated to the activation of the immune and allergy response (Lee, 2009; Lee et al., 2008) and can act as a cellular survival function in response to environmental stress (Ohi et al., 2010). Chitinases of the subgroup C have a significant homology with the α-subunit of the *Kluyveromyes lactis*-type killer toxins of yeast. In this case, the binding property of this chitinase may be more important than the chitinolytic activity since it is suggested that it is the association of the α- and β-subunits of the toxin that allows the entrance of the γ-subunit into the cell (Seidl, 2008). These enzymes may belong to an ancient mechanism of defence against microbial aggressions.

Fifth, the primary function for fungal chitinases is to degrade chitin extracellularly and not intracellularly. This role has been especially seen among entomopathogenic or mycoparasitic fungi (Seidl, 2008). Similarly, both wild type parental and mutant *A. fumigatus* strains could grow when chitin (insoluble) or CM-chitin (soluble) as well as GlcNAc were used as the carbon sources. The only phenotype of the quintuple fungal/plant chitinase mutant is associated to mycelial autolysis. Several reports in the past have focused on this aspect and have indicated that starvation induced transcription of chitinases resulted in an exacerbation of autolysis (Emri et al., 2008, 2006; Jaques et al., 2003; Seidl et al., 2005; Yamazaki et al., 2007). The direct involvement of a specific chitinase family in this process is not clear since both members of the class III and V were associated to autolysis (Emri et al., 2008; Jaques et al., 2003; Yamazaki et al., 2007). Based on our data, class III chitinases may play a role in providing energy to the fungus following the degradation of its cell wall ghosts in the autolytic phase. The role of chitinases protecting against cell death due to nutrient starvation (Shin et al., 2009) is currently investigated.

5. Transparency declarations

E.M. and L.A-F. do not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.fgb.2010.12.007.

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