GPI Anchored Proteins in *Aspergillus fumigatus* and Cell Wall Morphogenesis

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Contents

Abstract Glycosylphosphatidylinositol (GPI) anchored proteins are a class of proteins attached to the extracellular leaflet of the plasma membrane via a post-translational modification, the glycolipid anchor. GPI anchored proteins are expressed in all eukaryotes, from fungi to plants and animals. They display very diverse functions ranging from enzymatic activity, signaling, cell adhesion, cell wall metabolism, and immune response. In this review, we investigated for the first time

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an exhaustive list of all the GPI anchored proteins present in the *Aspergillus fumigatus* genome. An *A. fumigatus* mutant library of all the genes that encode in silico identified GPI anchored proteins has been constructed and the phenotypic analysis of all these mutants has been characterized including their growth, conidial viability or morphology, adhesion and the ability to form biofilms. We showed the presence of different fungal categories of GPI anchored proteins in the *A. fumigatus* genome associated to their role in cell wall remodeling, adhesion, and biofilm formation.

1 Introduction

The fungal cell wall is composed of polysaccharides and glycoproteins. The main central core of this cell wall is very similar in all fungal species but the nature of the carbohydrates and the degree and type of bridges between polysaccharides vary from one species to another. Synthases responsible for the biogeneration of linear polysaccharides are transmembrane proteins acting alone or in protein complexes (Latgé et al. 2017). The neosynthesized polysaccharides are extruded through the plasma membrane via as yet, undefined mechanisms. They are modified in the periplasmic space by remodeling enzymes. Many of the cell wall associated proteins responsible for the remodeling of these polysaccharides are anchored to the plasma membrane by a **g**lycosylphosphatidyl**I**nositol (GPI) anchor and designed as GPI anchored proteins.

The role of GPI anchored proteins has been previously investigated in *Saccharomyces cerevisiae* and *Candida albicans* (Caro et al. 1997; Plaine et al. 2008). In silico analysis suggested that *C. albicans* possesses 115 putative GPI anchored proteins, almost twice the number reported for *S. cerevisiae*. Moreover, it has been shown previously that some of the GPI anchored proteins play a major enzymatic role in cell wall morphogenesis like, for example, the elongation of β-(1–3)-glucans in yeasts and molds (Popolo and Vai 1999; Mouyna et al. 2000a; Gastebois et al. 2010a), whereas in yeast, it was also mentioned that these proteins are covalently bound to the cell wall polysaccharide (Caro et al. 1997; Kapteyn et al. 2000; Frieman et al. 2002). Herein, we describe our in silico analysis to provide comprehensive role of the cohort of genes that encode GPI anchored proteins in *A. fumigatus* genome. To aid our understanding of the role of these GPI proteins in the construction of the cell wall, we have generated and characterized null mutants for all of the genes we identified in this study.

2 Identification of putative GPI anchored proteins in the *A. fumigatus* genome

The identification of putative GPI anchored proteins in the *A. fumigatus* genome (AF293; http://fungi.ensembl.org/Aspergillusfumigatus/Info/Index) has been undertaken using the prediction programs PredGPI (http://gpcr.biocomp.unibo.it/

predgpi/proteome.htm) and big PI (http://mendel.imp.ac.at/sat/gpi/gpi_server.html) (Eisenhaber et al. 2004). In total, 86 proteins have been identified and predicted as being GPI anchored (see Table 1).

3 Comparative genomic analysis

By performing BLAST analysis (https://www.yeastgenome.org/blast-fungal and https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins) with these proteins, we were able to show that all had orthologues in a second *A. fumigatus* isolate A1163. Orthologues of only 28 proteins (32.5%) were commons to the yeasts *S. cerevisiae* and *C. albicans* and filamentous fungi and a further 38 proteins (44%) were restricted to filamentous fungal species. Interestingly, 20 GPI anchored proteins (23.5%) were found exclusively in the genomes of the *Aspergilli* (Table 1).

4 Functions of GPI anchored proteins

Of the GPI anchored proteins that we have identified, the role of 34 proteins has been previously characterized either in *A. fumigatus* or in other fungi. In the following section, we describe their known roles.

(a) GPI anchored common to yeast and filamentous fungi acting on cell wall morphogenesis

Among the GPI anchored proteins previously described, several enzymes, *GEL*, *BGT2*, *DFG*, *SUN,* and *CRH*, have been well studied and shown to have functions associated with remodeling cell wall polysaccharides. The GPI anchors on these proteins result in them being co-localized with other cell membrane proteins that have direct roles in cell wall biogenesis and hence allow them to modify neosynthesized polysaccharides. The most extensively studied of these enzymes belong to the *GEL* family (GH72 in the CaZy database http://www.cazy.org/ which describes families of structurally related catalytic and carbohydrate-binding modules). Seven members of this family are encoded in the *A. fumigatus* genome, whereas *S. cerevisiae* (GAS) and *C. albicans* (PHR) have five members each (Rolli et al. 2011; Popolo et al. 2017). *GEL/GAS/PHR* family enzymes are responsible for the elongation of β-(1,3)-glucans, which is an essential activity given that deletion of *GEL4* in *A. fumigatus* is lethal (Hartland et al. 1996; Mouyna et al. 2000a, b; Gastebois et al. 2010a). It was recently shown that some members of this family have a dual activity that allows them not only to elongate but also to branch the neo elongated β-(1,3)-glucan (Aimanianda et al. 2017). This branching activity is only seen in enzymes that have the carbohydrate-binding module, CBM43, and loss of this motif abolishes β-(1,3)-glucan branching (Aimanianda et al. 2017).

Table 1 List of predictive GPI anchored proteins

AFUB number	AFUA.number	Gene name or function	Phenotype	Fungi	References
AFUB_018250	AFUA_2G01170	GEL1 §	no	Yeast and Filamentous	Hartland et al. (1996); Mouyna et al. (2000a, b)
AFUB_077400	AFUA_6G11390	GEL2 §	Conidia, Growth, S-D	Yeast and Filamentous	Mouyna et al. (2005)
AFUB_028470	AFUA_2G12850	GEL3	no phenotype	Yeast and Filamentous	Gastebois et al. (2010a
AFUB_022370	AFUA_2G05340	GEL4	Growth, S-D	Yeast and Filamentous	Gastebois et al. (2010a)
AFUB_084480	AFUA_8G02130	GEL5	no	Yeast and Filamentous	Gastebois et al. (2010a)
AFUB_036000	AFUA_3G13200	GEL6	no	Yeast and Filamentous	Gastebois et al. (2010a)
AFUB_078410	AFUA_6G12410	GEL7	no	Yeast and Filamentous	Gastebois et al. (2010a)
AFUB_048180	AFUA_3G00270	BGT2	no	Yeast and Filamentous	Gastebois et al. (2010b); Millet et al. (2018)
AFUB_002130	AFUA_1G01730	DFG1	no	Yeast and Filamentous	Muszkieta et al. (2019)
AFUB_017760	AFUA_2G00680	DFG2	no	Yeast and Filamentous	Muszkieta et al. (2019)
AFUB_048110	AFUA_3G00340	DFG3	Growth, S-D	Yeast and Filamentous	Muszkieta et al. (2019)
AFUB_047740	AFUA_3G00700	DFG4	no	Yeast and Filamentous	Muszkieta et al. (2019)
AFUB_101170	AFUA_4G00620	DFG5	no	Yeast and Filamentous	Muszkieta et al. (2019)
AFUB_100440	AFUA_4G02710	DFG7	no	Yeast and Filamentous	Muszkieta et al. (2019)
AFUB_013430	AFUA_1G13940	SUN2	no	Yeast and Filamentous	Gastebois et al. (2013)
AFUB_095070	AFUA_6G03230	CRH1 §	no	Yeast and Filamentous	Fang et al. (2019)
AFUB_020180	AFUA_2G03120	CRH2 §	no	Yeast and Filamentous	Fang et al. (2019)
AFUB_074470	AFUA_6G08510	CRH4	no	Yeast and Filamentous	Fang et al. (2019)
AFUB_015530	AFUA_1G16190	CRH5	no	Yeast and Filamentous	Fang et al. (2019)
AFUB_029980	AFUA_2G14360	ENG2	no	Yeast and Filamentous	Hartl et al. (2011)
AFUB_034540	AFUA_3G14680	PBL3	no	Yeast and Filamentous	Shen et al. (2004)
AFUB_052270	AFUA_5G03760	Chitinase A1	no	Yeast and Filamentous	Alcazar-Fuoli et al. (2011)
AFUB_063890	AFUA_4G06820	Ecm33 §	Conidia,virulence	Yeast and Filamentous	Chabane et al. (2006)

(continued)

Table 1 (continued)

AFUB number	AFUA.number	Gene name or function	Phenotype	Fungi	References
AFUB_076480	AFUA_6G10430	CDA6	no	Yeast and Filamentous	Mouyna et al (2020)
AFUB_092930	AFUA_6G05350	OPSB	no	Yeast and Filamentous	
AFUB_064130	AFUA_4G07040	CTSD	no	Yeast and Filamentous	Vickers et al. (2007)
AFUB_042000	AFUA_3G07050		no	Yeast and Filamentous	
AFUB_056560	AFUA_5G09020		no	Yeast and Filamentous	
AFUB_020300	AFUA_2G03230	AmylaseA	no	Filamentous fungi***	
AFUB_047500	AFUA_3G00900	Amylase	conidia, Growth,conidiation	Filamentous fungi***	
AFUB_000660	AFUA_6G14090	CFEMA	no	Filamentous Fungi	Vaknin et al. (2014)
AFUB_076620	AFUA_6G10580	CFEMB	no	Filamentous Fungi	Vaknin et al. (2020141 4)
AFUB_072620	AFUA_6G06690	CFEMC	no	Filamentous Fungi	Vaknin et al. (2014)
AFUB_057130	AFUA_5G09580	RODA #	Conidia,virulence	Filamentous Fungi	Aimanianda et al. (2009); Valsecchi et al. (2017a)
AFUB_016640	AFUA_1G17250	RODB #	no	Filamentous Fungi	Valsecchi et al. (2017a)
AFUB_042020	AFUA_3G07030	Glutaminase	no	Filamentous Fungi	
AFUB_081470	AFUA_8G06030	α(1-3) glucanase	no	Filamentous Fungi	
AFUB_097010	AFUA_6G00500	chitosanase	no	Filamentous Fungi	
AFUB_003980	AFUA_1G03570	PhoA §	no	Filamentous Fungi	Bernard et al. (2002)
AFUB_022180	AFUA_2G05150	AfMP2	Biofilm	Filamentous Fungi	Woo et al. (2018)
AFUB_099880	AFUA_4G03240	AFMP1	no	Filamentous Fungi	Woo et al. (2018)
AFUB_006180	AFUA_1G05790		Biofilm	Filamentous Fungi	
AFUB_087030	AFUA_7G00450		Biofilm	Filamentous Fungi	
AFUB_001030	AFUA_6G13710		no	Filamentous Fungi	
AFUB_004040	AFUA_1G03630		no	Filamentous Fungi	
AFUB_008960	AFUA_1G09510		no	Filamentous Fungi	

(continued)

Table 1 (continued)

AFUB number	AFUA.number	Gene name or function	Phenotype	Fungi	References
AFUB_009040	AFUA_1G09590		no	Filamentous Fungi	
AFUB_009100	AFUA_1G09650		no	Filamentous Fungi	
AFUB_018780	AFUA_2G01710		no	Filamentous Fungi	
AFUB_035550	AFUA_3G13640		no	Filamentous Fungi	
AFUB_036090	AFUA_3G13110		no	Filamentous Fungi	
AFUB_044890	AFUA_3G03370		no	Filamentous Fungi	
AFUB_047260	AFUA_3G01150		no	Filamentous Fungi	
AFUB_047510	AFUA_3G00880		no	Filamentous Fungi	
AFUB_050450	AFUA_5G01920		no	Filamentous Fungi	
AFUB_056330	AFUA_5G08800		no	Filamentous Fungi	
AFUB_057570	AFUA_5G09960		no	Filamentous Fungi	
AFUB_057610	AFUA_5G10010		no	Filamentous Fungi	
AFUB_069330	AFUA_4G12370		no	Filamentous Fungi	
AFUB_082130	AFUA_8G05410		no	Filamentous Fungi	
AFUB_083170	AFUA_8G04370		no	Filamentous Fungi	
AFUB_084140	AFUA_8G02450		no	Filamentous Fungi	
AFUB_085740	AFUA_8G00830		no	Filamentous Fungi	
AFUB_088990	AFUA_7G02440		no	Filamentous Fungi	
AFUB_089500	AFUA_7G03970		no	Filamentous Fungi	
AFUB_095500	AFUA_6G02800		no	Filamentous Fungi	
AFUB_010650	AFUA_1G11220		Conidia, S-D	Aspergillus	
AFUB_066710	AFUA_4G09600		conidia, conidiation	Aspergillus	
AFUB_096850	AFUA_6G00620		Conidia	Aspergillus	
AFUB_099690	AFUA_4G03360		Conidia	Aspergillus	
AFUB_018220	AFUA_2G01140		Conidia	Aspergillus	

(continued)

Table 1 (continued)

AFUB number	AFUA.number	Gene name or function	Phenotype	Fungi	References
AFUB_040120	AFUA_3G08990	CSPA	Conidia, adhesion	Aspergillus	Levdansly et al. (2010); Valsecchi et al. (2017b)
AFUB_019530	AFUA_2G02440		no	Aspergillus	
AFUB_031860	AFUA_2G16180		no	Aspergillus	
AFUB_044000	AFUA_3G03960		no	Aspergillus	
AFUB_084580	AFUA_8G02030		no	Aspergillus	
AFUB_087170	AFUA_7G00580		no	Aspergillus	
AFUB_087560	AFUA_7G00970		no	Aspergillus	
AFUB_000740	AFUA_6G14010		no	Aspergillus	
AFUB_082630	AFUA_8G04860		no	Aspergillus	
AFUB_030420	AFUA_2G14780		no	Aspergillus*	
AFUB_037960	AFUA_3G11190		no	Aspergillus*	
AFUB_089000	AFUA_7G02460		no	Aspergillus*	
AFUB_016760	AFUA_1G17390		no	Aspergillus*	
AFUB_066570	AFUA_4G09450		no	Aspergillus*	
AFUB_084830	AFUA_8G01770		Conidia, Growth, S-D	Aspergillus*	Mouyna et al. (2020) in preparation

List of the putative GPI-anchored proteins identified by the two softwares in the A. fumigatus genome including the corresponding AFUB and AFUA number (http://fungi.ensembl.org/Aspergillus_fumigatus/Info/Index), the gene name when identified, the phenotype of the mutant and their presence in the other genomes. Yeast and Filamentous: Proteins which are present in *C. albicans*, *S. cerevisiae*, *A. fumigatus* and others filamentous fungi; Filamentous Fungi: proteins present in filamentous fungi and not in the yeast genome; Filamentous Fungi***: these proteins are not present in the *S. cerevisiae* and C. albicans genome but they are present in the S. pombe and C. neoformans genome. Aspergillus: proteins only present in *Aspergillus* species; *Aspergillus**: proteins only present in few species of Aspergillus like *A. clavatus*, *A. lentulus*, *A. thermomutatus*, and the *A. turcosus* species; S-D: sensitivity to drugs. The GPI mutant library was screened for the growth on different media (Malt or Minimal medium), or Minimal medium (MM) including calcofluor white (40mg/ml), or congo red (50mg/ml) after 48h at 37°C, conidial morphology, conidial viability as described by (Millet et al. 2018), adhesion (104 conidia were incubated at 37°C on MM medium + 0.01% tween 20 on plates TPP for 24h) as described by Fontaine et al., (2010) and the ability to form biofilm on agar plates on MM medium after 22h of growth at 37° as described by (Beauvais et al., 2007). NB: no=no phenotype; S-D: higher sensitivity to drugs; Conidia: mutants which are affected in their conidia (shape, linear chains); Conidiation: mutants which are affected in conidiation. # RODA and RODB predicted to be GPI in silico but proved biochemically to be non GPI. § Proteins proved to be GPI biochemically

The GH17 family in *A. fumigatus* contains five members (*BGT1–3, SCW4 and SCW11)*; however, *BGT2* is the only member of this family that is GPI anchored. Bgt1 transfers the donor β-(1,3)-glucan on the non-reducing end of the chain (Mouyna et al. 1998), whereas Bgt2 preferentially transfers within the β-(1,3)-glucan chain (Gastebois et al. 2010b). No phenotype has been associated to the deletion of *BGT2* alone in *A. fumigatus* or its ortholog *BGL2* in the yeast *S. cerevisiae* (Cappellaro et al. 1998). However, Millet et al. (2018) and Sestak et al. (2004) showed that in *A. fumigatus* and *S. cerevisiae,* the non-GPI-members of the

GH17 family, especially Scw4, Scw11, and Bgt3 and Scw4, Scw10, and Scw11, are important for cell wall integrity. The enzymatic activity of Scw4, Scw11, and Bgt3 is still unknown but the analysis of the quintuple null mutant showed that Scw4, Scw11, and Bgt3 have antagonistic and distinct functions to Bgt2 and Bgt1.

Recently, it has been shown in *A. fumigatus* that the *DFG* family (GH76 CaZy family) is involved in the covalent binding of Galactomannan (GM) to the β-(1,3)-glucan–chitin core of the cell wall. This family contains seven members in *A. fumigatus*, all of which are GPI anchored proteins, except *DFG6* (Muszkieta et al. 2019). The single mutant Dfg3 is playing the major role in the association of the GM to the glucan core. However, the phenotype defect was enhanced in the septuple *DFG* deleted mutant, such as highly reduced growth with hyper-branched hyphae and higher sensitivity to drugs, showing that Dfgs have additional activities on structural properties of the cell wall (Muszkieta et al. 2019). In both, *S. cerevisiae* and *C. albicans*, although single knockouts of *DFG5* and *DCW1* are viable, a double knockout is synthetically lethal (Kitagaki et al. 2002; Spreghini et al. 2003). Interestingly as yeasts do not have galactomannan in their cell wall, the biochemical function of these remodeling enzymes remains to be discovered.

The *SUN* family *in A. fumigatus* (also known as the GH132 CaZy family) comprises two members, *SUN1* and *SUN2* which is the only one predicted to be GPI anchored in *A. fumigatus*. They are so called as they encode a SUN domain originally identified in the yeast proteins *SIM1*, *UTH1*, *NCA3,* and *SUN4*. The SUN domain is closely related, at the sequence level, to a β-glucosidase of *Candida wickerhamii;* however, the yeast proteins have no detectable β-glucosidase activity. The deletion of *SUN2*, which is most closely related to the uncharacterized protein YMR244W in *S. cerevisiae*, did not induce any morphological alterations. In contrast, the deletion of the *SUN1* genes in yeasts and molds has been shown to exhibit defects in septum closure (Hiller et al. 2007; Norice et al. 2007; Firon et al. 2007; Gastebois et al. 2013) However, the baker's yeast *SUN1* and their ortholog in *C. albicans SUN41/SUN42,* which encodes an exo β-(1,3)-glucanase but are not a GPI anchored protein, play a role in cell wall morphogenesis. Inactivation of *SUN1* genes and orthologs leads to a defect in the separation of daughter cells from mother cells, and simultaneous inactivation of *SUN41* and *SUN42* is lethal in the absence of osmotic protection. Like for *A. fumigatus*, cell wall defects seen in this double mutant are mainly localized in the region surrounding the septa in mother yeast cells and subapical hyphal compartments. The role taken by each SUN protein remains unknown as well as the role of the GPI anchor in the function of *A. fumigatus SUN2* in the cell.

The *CRH* (for Congo Red Hypersensitivity) GH16 CaZy family has been associated to glucan/chitin linkage in yeast *S. cerevisiae* (Rodríguez-Peña et al. 2000; Cabib et al. 2008; Blanco et al. 2012; Arroyo et al. 2016). In *A. fumigatus*, five members are present in the genome (four proteins being GPI anchored proteins). The phenotype of the quintuple mutant is very weak and not associated to congo red resistance. Congo red toxicity is pleiopropic with this molecule acting not only on cell wall biosynthesis but also in oxido-reduction pathways. Moreover, the biochemical function of the Crh proteins has not been demonstrated and there is

not a definite proof that these genes could be essential for the establishment of chitin–glucan linkages (Fang et al. 2019).

Members of the *SPS2* family (which are not assigned to a CaZy family) play an essential role in the formation of the ascospore cell wall in *S. cerevisiae* (Coluccio et al. 2004), whereas the ortholog in *A. fumigatus*, *ECM33*, is important for conidial morphogenesis and virulence (Chabane et al. 2006). However, its enzymatic function remains unknown.

Three GPI anchored proteins, CFEM (A-C), containing fungal-specific CFEM domains (Common in Fungal Extracellular Membrane) are characterized by spaced cysteine residues (Kulkarni et al. 2003). Most CFEM-containing cell wall proteins studied to date have been shown to be involved in host-pathogen interactions and virulence. In *C. albicans*, deletion of the three GPI anchored-CFEM-encoding genes in the genome (Rbt5/Rbt51/Csa1) results in an increased sensitivity to cell wall damaging agents and a reduced ability to form a biofilm (Pérez et al. 2006, 2011). In contrast, in *A. fumigatus*, (Vaknin et al. 2014) showed that these proteins, even though their respective mutants display a higher sensitivity to congo red and calcofluor white than their parental strain, did not play any role in cell wall morphogenesis or virulence.

Finally, no phenotype has been associated to the endo β-(1,3)-glucanase *ENG2* (Hartl et al. 2011) or the chitinase A1 (Alcazar-Fuoli et al. 2011) and the chitin deacetylase *CDA6* (Mouyna et al. 2020), which are the only GPI members in their respective family. However, the sequential deletion of *ENG2–5* belonging to the GH16 family altogether with *ENG1* (GH81) showed conidiogenesis defects, with linear chains of conidia unable to separate while the germination rate was not affected (Mouyna et al. 2016).

(b) GPI anchored proteins only found in filamentous fungi which are associated to cell wall structures

In addition to the GPI anchored proteins common to yeast and filamentous fungi which have been shown to be biochemically associated to cell wall construction, other GPI anchored proteins identified in silico are present only in the cell wall of filamentous fungi and are involved in adhesion and biofilm formation (Table 1).

The outer layer of the conidium is composed of melanin covered by a rodlet layer that confers hydrophobic properties to *A. fumigatus* conidia. This rodlet layer is exclusively composed of hydrophobins, which are low molecular weight proteins rich in cysteins residues. This rodlet layer masks conidial recognition by the human innate immune system (Aimanianda et al. 2009). Recently, (Valsecchi et al. 2017a) showed that seven hydrophobins (RodA–RodG) are present in the genome of *A. fumigatus*. RodA and RodB were identified as putative GPI anchored protein based on our in silico analysis. However, two lines of evidence indicate that the proteins are probably not GPI anchored: the predicted ω cleavage site which is the amino acid immediately upstream of the putative site of GPI anchor addition (the omega site) is located between Cys-residues C7 and C8, which would disrupt a conserved disulfide bridge that is important to stabilize the structure of the proteins; moreover,

it has been shown that the C-terminus of RodA extracted from conidia corresponds to that of the full-length protein (Pille et al. 2015; Valsecchi et al. 2017a).

It has been shown by Levdansky et al. (2010) that deletion of *CSPA*, a repeat rich GPI anchored protein only found in *Aspergillus* sp., is involved in reduced adhesion and increase speed of conidial germination. Moreover, Valsecchi et al. (2017b) showed that conidia of the *CSPA* mutant tended to stay grouped together in long chains and adhered also between themselves. This gene has been shown to be regulated by the Myb1 transcription factor (Valsecchi et al. 2017b).

5 Investigating the role of newly identified GPI anchored proteins in cell wall morphogenesis

Most of the previously analyzed GPI proteins were associated somehow to cell wall construction and fungal morphogenesis. These results suggested that all GPI anchored proteins may have essential functions in fungal growth some of them being undefined and this was at the basis of the study of the GPI proteins in *A.fumigatus*. In order to investigate exhaustively the role of the GPI anchored proteins, an *A. fumigatus* mutant library of all the genes identified in silico were constructed following the procedures outlined in Zhao et al. (2019) and Furukawa et al. (2020) using the oligonucleotide primers described in Supplementary Table 1 and screened for growth, conidiation, and biofilm formation.

From the screening analysis, three categories of GPI anchored protein null mutants were identified: proteins found in yeast and filamentous fungi, proteins found exclusively in filamentous fungi, and proteins found exclusively in *Aspergillus* species. Ten of the 57 new mutants (the previously published mutants are not counted) showed a distinct phenotype from the parental strain including conidial morphology, growth, sensitivity to congo red and calcofluor white, adhesion or biofilm formation (Table 1).

(a) **Proteins found in Yeast and filamentous fungi**

28 proteins are present in yeast and filamentous fungi genome, 23 being already described previously (see above) and 38 proteins are present exclusively in filamentous fungi genome.

- **Proteins with putative enzymatic functions**

Secreted proteases have always attracted attention as potential mediators of fungal invasion, conidophore development, or adhesion (Monod et al. 2002). We did not observe any distinct growth phenotype after the deletion of the aspartic proteases *CTSD* (AFUA_4G07040) (Vickers et al. 2007) and *OPSB* (AFUA_6G05350). Phospholipases (Plbs) activity which can destabilize host membranes are also considered to be virulence factors for pathogenic fungi like *C. albicans* (Leidich et al. 1998). In *A. fumigatus*, the mutant resulting from the deletion of the

phospholipase *PLB3* (AFUA_3G14680) (Shen et al. 2004) is not affected. Similarly, phosphatase plays a major role in the fungal life. In *A. fumigatus*, the acid phosphatase PhoA (AFUA_1G03570) which is specific to filamentous fungi (Bernard et al. 2002) are not directly associated to growth (data not shown). Moreover, the two genes encoding a putative chitosanase and a putative α-(1–3)-glucanase (respectively AFUA_6G00500 and AFUA_8G06030) which were predicted as GPI anchored proteins specific to filamentous fungi, do not play a role in the cell wall remodeling in *A. fumigatus* since the corresponding deleted mutant behaved like the parental strain (data not shown). However, non-GPI anchored homologs of these proteins (three for chitosanases and eight for α-(1–3)-glucanases) are present in the *A. fumigatus* genome and could be involved in compensatory mechanisms after the deletion of the GPI gene of the family.

The GPI anchored protein encoded by AFUA_3G00900, is a putative amylase. The null mutant exhibits a twofold decrease in conidiation, a slight reduction in radial growth and increased resistance to congo red (data not shown). The protein encoded by this gene belongs to the GH13 family. This CAZYme family is a large family containing various hydrolyzing and transglycosylating enzymes, mostly acting on α-(1,4)- or α-(1,6)-glycosidic linkages, which can be involved in starch degradation or in the synthesis or modification of alpha-glucan in the fungal cell wall (Morita et al. 2006; Yuan et al. 2008). In addition to AFUA_300900, four other GH13 proteins are present in the *A. fumigatus* genome: AFUA_2G03230, another GPI anchored protein specific to filamentous fungi (Table 1), AFUA_2G00710, AFUA_4G10130, and AFUA_2G13460. In contrast to AFUA_3G00900, we saw no phenotype associated with the deletion of AFUA_2G03230. The phylogenetic tree of the GH13 family of *A. fumigatus* showed two distinct groups, the first group (with AFUA_2G00710 AFUA_4G10130) associated to proteins involved in starch degradation like AmyA and AmyB in *A. niger* (Korman et al. 1990) and the second group (AFUA_3G00900, AFUA_2G03230 and AFUA_2G13460) associated to proteins with transferase activities like AgtA and AgtB in *A. niger* and Aah3 in *S. pombe* (Morita et al. 2006; van der Kaaij et al. 2007b; Yuan et al. 2008) (Fig. 1). In *A. niger*, both enzymes showed transglycosylation activity on donor substrates with alpha-(1,4)-glycosidic bonds and at least five anhydroglucose units. The enzymes, designated AgtA and AgtB, produced new alpha-(1,4)-glycosidic bonds (van der Kaaij et al. 2007b). In *S. pombe*, disruption of *AAH3* encoding a GPI anchored protein resulted in hypersensitivity toward cell wall-degrading enzymes and an aberrant cell shape, indicating that normal cell wall biosynthesis was affected (Morita et al. 2006). Disruption of *AgtA* in *A. niger* also affected cell wall stability. The protein sequence of AFUA_3G00900 and AFUA_2G13460 is very closely related to AgtA and AgtB of *A. niger* (between 50 and 60% of identity) and notably the catalytic conserved domain characteristics of transferase activities of this GH13 families (van der Kaaij et al. 2007a) suggest they may be also transferases in *A. fumigatus.*

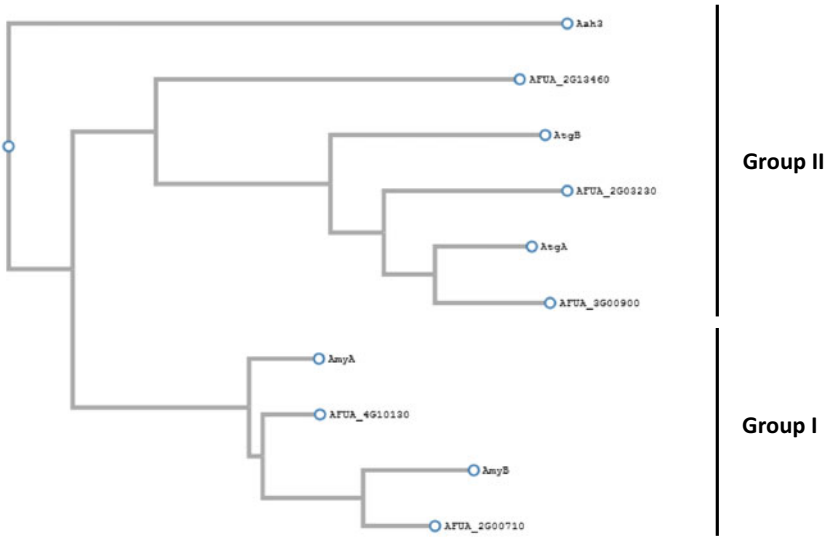

Fig. 1 Phylogeny of the GH13 family of *A. fumigatus*, AtgA-B and AmyA-B of *A. niger* and aah3 of *S. pombe*. Sequence alignment and phylogenetic reconstructions have been done using clustalW (https://www.genome.jp/tools-bin/clustalw). The tree was constructed using FastTree v2. 1.8 with default parameters

- **Proteins with unknown function**

Most of the proteins exclusively present in filamentous fungi genome display unknown functions (25 on the 38 identified).

Three null mutants corresponding to the genes (AFUA_2G05150, AFUA_7G00450, and AFUA_1G05790) showed a twofold reduced ability to form biofilm (Fig. 2a). AFUA_2G05150 is annotated as the cell wall galactomannoprotein Mp2. In contrast, the AFUA_4G03240 null mutant (also a GPI anchored protein) annotated as the galactomannoprotein Mp1 did not show any difference in biofilm formation in our study. Mp1 and Mp2 are homologous to *Penicillium marneffei* Mp1, a cell surface antigenic cell wall mannoprotein and a virulence factor (Cao et al. 1998; Woo et al. 2016). *A. fumigatus* Mp1 and Mp2 have been shown to be also immunogenic (Yuen et al. 2001; Woo et al. 2002; Chong et al. 2004). We constructed the double mutant Δ*mp*1/Δ*mp*2 but we did not observe additional decreases in biofilm formation or reduction in adhesion in comparison to the single mutant *Δmp*2 (data not shown). Recently, (Woo et al. 2018) identified two distantly others homologs in *A. fumigatus*, Mp3 and Mp4, containing also one lipid-binding domain and showed that Mp4 was involved in virulence.

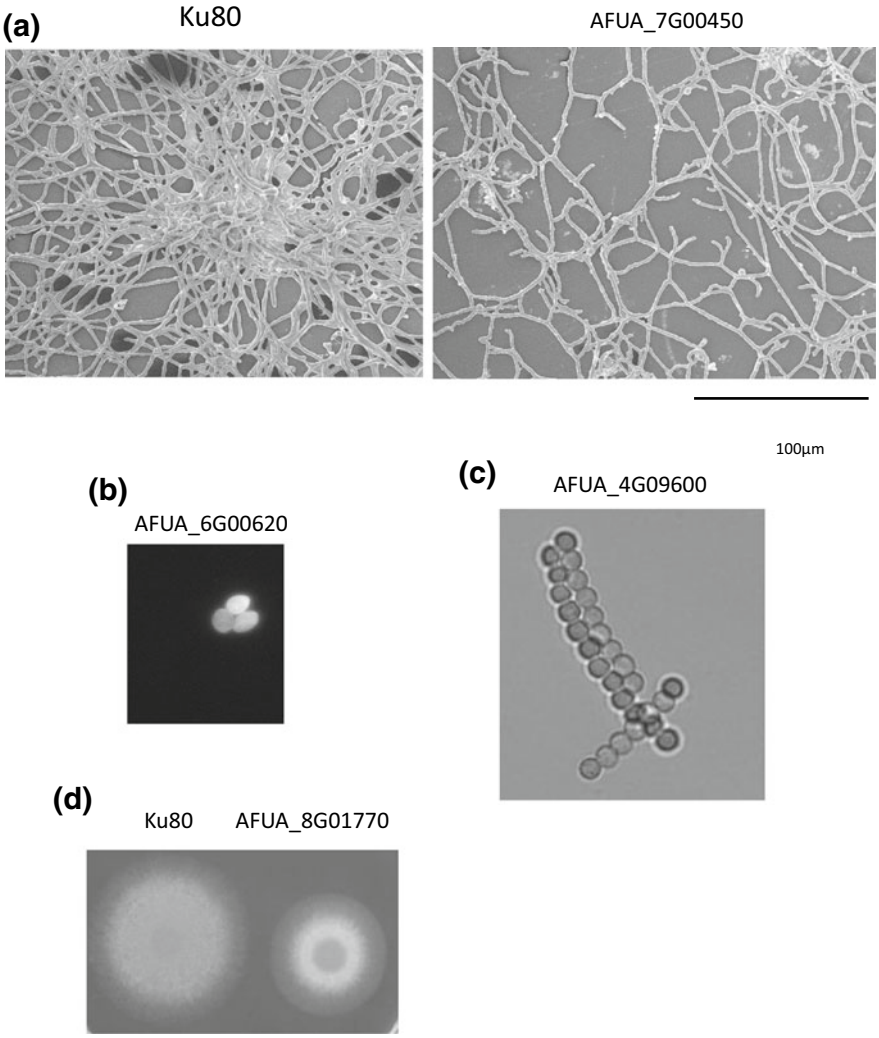

Fig. 2 Phenotype analysis of some GPI anchored protein mutants: **a** SEM of the AFUA_1G05790 deletion mutant involved in biofilm formation compared to the parental strain Ku80. **b** Light microscopy of the shape of conidia after deletion of AFUA_6G00620 gene (63x). **c** Light microscopy of the linear chains of conidia after the deletion of AFUA_4G09600 gene. **d** Growth on Malt medium of the AFUA_8G01770 deletion mutant after 48 h at 37 °C in comparison to the parental strain

(b) **Proteins found exclusively in *Aspergillus* species**

For the deletion of AFUA_2G01140, AFUA_4G03360, AFUA_6G00620, and AFUA_1G11220 which encode proteins of unknown function, we observed that the shape of 5% of the conidia were ovoids (an example is given in Fig. 2b). In the case

of AFUA_1G11220, the deletion of this gene was also associated with a twofold increase in congo red and calcofluor white sensitivity (data not shown). This modification of the morphology of the conidia and of the sensitivity to cell wall drugs suggests that the proteins encoded by these genes could be involved in the construction of the conidial cell wall.

Deletion of AFUA_4G09600, a protein containing several repetitions of amino acid motif GGPSGNDGGN and VKDAYTDDHSV also found only in *Aspergillus* sps, is correlated to a threefold reduction in conidiation compared to the parental strain (data not shown). We also observed linear chains of conidia in this mutant (Fig. 2c). This phenotype is reminiscent of the *CSPA* null mutant phenotype (Valsecchi et al. 2017b).

Six GPI proteins (AFUA_2G14780, AFUA_3G11190, AFUA_7G02460, AFUA_1G17390, AFUA_4G09450, AFUA_8G01770) are only present in the *Aspergillus* species close phylogenetically of *A. fumigatus* (*A. clavatus*, *A. lentulus*, *A. thermomutatus,* and *A. turcosus* (Table 1). No significant homology or domain has been found with any known proteins. Only the deletion of AFUA_8G01700 showed a distinct phenotype from the parental strain, reduced growth, higher sensitivity to drugs and reduced adhesion (Mouyna et al. 2020, manuscript in preparation) (Fig. 2d).

6 Discussion and Conclusion

Even if we try to dress an exhaustive list of all the GPI anchored proteins present in the *A. fumigatus* genome using different algorithms, some proteins could have been wrongly identified as GPI proteins (RodA and RodB) or missed. For example, the conidial surface protein CcpA has been shown to be GPI anchored (Voltersen et al. 2018) while it was not identified using the prediction softwares. Only few proteins have been demonstrated biochemically to be GPI anchored proteins after cleavage of the anchor by a phospholipase C releasing the protein in the Triton X-114 fraction and recognized by a cross-reacting determinant antibody. A proteomic analysis identified biochemically Gel1 and Gel2, Crh1, Crh2, Ecm33, PhoA as GPI anchored proteins (Bruneau et al. 2001). All of these proteins were identified in our bioinformatics predictions.

The localization of GPI anchored proteins has been also controversial. In the yeast *S. cerevisiae*, and *Candida* (Kapteyn et al. 2000; Frieman et al. 2002), it has been demonstrated that many GPI proteins (called GPI anchored cell wall proteins or GPI-CWPs) arrive at the plasma membrane but are then liberated. A remnant of the GPI anchor reacts with β1,6 glucan resulting in cross-linking of the GPI-CWP into the cell wall (Van der Vaart et al. 1997) suggesting that there are two terminal fates for GPI proteins—residence at the plasma membrane (GPI anchored plasma membrane proteins or GPI-PMPs) and residence at the cell wall (GPI-CWPs) (Lu et al. 1994). Moreover, based on in silico analysis of GPI anchored proteins in *S. cerevisiae*, Caro et al. (1997) proposed that a signal of two basic amino acids in the four amino acids upstream of the ω site acts to retain the protein at the plasma

membrane. In the absence of this retention signal, the proteins are mobilized to the cell wall. Using fusions of the GPI signal sequences from *S. cerevisiae* to alpha-galactosidase, (Hamada et al. 1998) found a good correlation between presence or absence of the dibasic motif and partitioning of the fusion protein to the plasma membrane or cell wall. Analysis of various point mutations in specific GPI anchor signal sequences also supported the importance of the dibasic motif in GPI anchored protein localization. In contrast, in *A. fumigatus*, the structural cell wall composition did not reveal the presence of β(1–6)glucan (Fontaine et al. 2000). Moreover, no proteins have been shown to be covalently attached to the cell wall after their release from the membrane (Bernard et al. 2002). In addition, none of the FLO, CWP or TIR family proteins identified in the *S. cerevisiae* genome (Caro et al. 1997) and predicted to be associated to the cell wall, have been found in the *A. fumigatus* genome.

The different categories of GPI anchored proteins found in *A. fumigatus* and their function are summarized in Fig. 3. The first category of proteins is highly conserved in all fungi (yeast as well as filamentous fungi) and is essential in cell wall morphogenesis. Indeed, the structural core of the cell wall between yeasts and molds is conserved. Most of them belong to multigenic families of proteins. Their analysis showed that most of the time, one or two genes in a family are responsible for the phenotype observed (Gastebois et al. 2010a; Millet et al. 2018; Muszkieta et al. 2019). Accordingly, all proteins in the same family are unlikely to have a shared function, which supports the redundancy of genes already observed in the *Aspergillus* genome. In the second category, we identified and characterized proteins present only in filamentous fungi, which are mostly involved in biofilm formation, adhesion, and virulence process. However, 60% of the proteins belonging to this category did not present any domain or identity with previously annotated

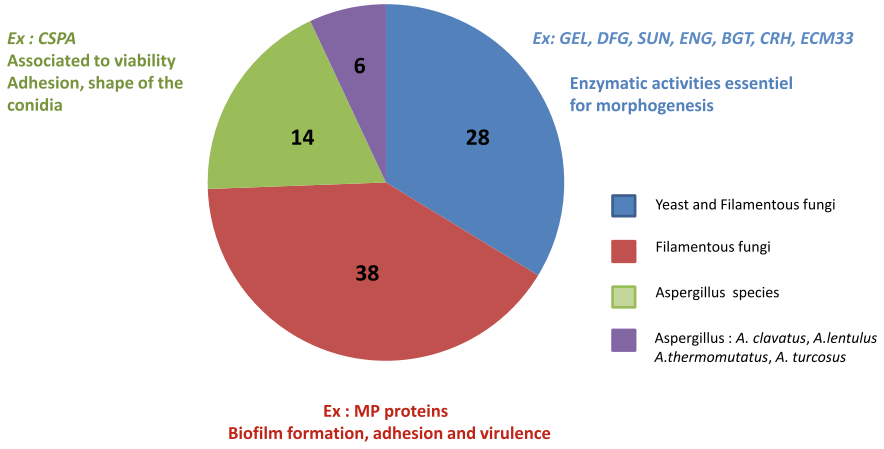

Fig. 3 Different fungal categories of GPI anchored proteins, which show an association between their putative role (cell wall remodeling, adhesion, biofilm or virulence) and their category

proteins or a distinct phenotype associated to their gene mutation. Finally, the third category of proteins is only present in *Aspergillus* species, or even in few related species of *Aspergillus*. These proteins seem to be mostly associated with the formation of the conidial stage but again their function is unknown. This review suggests that other non-GPI-bound transglycosidases are important for the remodeling of cell wall construction and remain to be discovered.

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