**GPI anchored proteins in *Aspergillus fumigatus* and cell wall morphogenesis**

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**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 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 constructedand 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.

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**lycosyl **P**hosphatidyl**I**nositol (GPI) anchor and designed as GPI anchored proteins.

The role of GPI anchored proteins have 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 moulds(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 characterised null mutants for all of the genes we identified in this study.

**1) 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).

**2) 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).

**3) Functions of GPI anchored proteins**

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

1. 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-localised with other cell membrane proteins that have direct roles in cell wall biogenesis and hence allows 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](http://www.cazy.org/%20%20which) 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.*, 2000b; Mouyna *et al.*, 2000a; 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).

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 7 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 GH132CaZyfamily) comprises 2 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 uncharacterised protein YMR244W in *S. cerevisiae*, did not induce any morphological alterations. In contrast, the deletion of the *SUN1* genes in yeasts and moulds have 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 (4 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; Pérez *et al.*, 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).

1. 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.*, 2017) 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.*, 2017).

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.*, (2017) 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.*, 2017).

**4) Investigating the role of newly identified GPI anchored proteins in cell wall morphogenesis**

Most of the previously analysed 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).

1. **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 play 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 hydrolysing 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) (Figure 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 towards 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 are very closely related to AgtA and AgtB of *A. niger* (between 50 to 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.*

* **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 2 fold reduced ability to form biofilm (Figure 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.

**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 Figure 2b). In the case of AFUA\_1G11220, the deletion of this gene was also associated with a 2 fold 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 suggest 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 three fold reduction in conidiation compared to the parental strain (data not shown). We also observed linear chains of conidia in this mutant (Figure 2c). This phenotype is reminiscent of the *CSPA* null mutant phenotype (Valsecchi *et al.*, 2017).

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) (Figure 2d).

**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 Figure 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 moulds 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 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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**Table 1**: 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).

**Figure 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.

**Figure 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 48h at 37°C in comparison to the parental strain.

**Figure 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.

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