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gfsA encodes a novel galactofuranosyltransferase involved in biosynthesis of galactofuranose antigen of *O*-glycan in *Aspergillus nidulans* and *Aspergillus fumigatus*

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Summary

The cells walls of filamentous fungi in the genus *Aspergillus* have galactofuranose (Gal*f*)-containing polysaccharides and glycoconjugates, including *O*-glycans, *N*-glycans, fungal-type galactomannan and glycosylinositolphosphoceramide, which are important for cell wall integrity. Here, we attempted to identify galactofuranosyltransferases that couple Gal*f* monomers onto other wall components in *Aspergillus nidulans*. Using reverse-genetic and biochemical approaches, we identified that the *AN8677* gene encoded a galactofuranosyltransferase, which we called GfsA, involved in Gal*f* antigen biosynthesis. Disruption of *gfsA* reduced binding of β-Gal*f*-specific antibody EB-A2 to *O*-glycosylated WscA protein and galactomannoproteins. The results of an *in-vitro* Gal*f* antigen synthase assay revealed that GfsA has β1,5- or β1,6-galactofuranosyltransferase activity for *O*-glycans in glycoproteins, uses UDP-D-Gal*f* as a sugar donor, and requires a divalent manganese cation for activity. GfsA was found to be localized at the Golgi apparatus based on cellular fractionation experiments. Δ*gfsA* cells exhibited an abnormal morphology characterized by poor hyphal extension, hyphal curvature and limited formation of conidia. Several *gfsA* orthologues were identified in members of the Pezizomycotina subphylum of Ascomycota, including the human pathogen *Aspergillus fumigatus*. To our knowledge, this is the first characterization of a fungal β-galactofuranosyltransferase, which was shown to be involved in Gal*f* antigen biosynthesis of *O*-glycans in the Golgi.

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Introduction

Polysaccharides and glycoconjugates are present in the plasma membrane, cell wall and extracellular environment, and play important roles in many biological events. Filamentous fungal cell walls contain many polysaccharides, including β1,3-, β1,6- and α1,3-linked glucans, chitin, galactosaminogalactan and galactomannan, as well as *O*- and *N*-glycans linked to proteins (Bernard and Latgé, 2001; Gastebois *et al*., 2009; de Groot *et al*., 2009; Latgé, 2009; 2010; Fontaine *et al*., 2011; Jin, 2012). The components and polymeric organization of the *Aspergillus* cell wall have been determined, and several glycosyltransferase genes involved in the synthesis of these components have been identified and characterized (Kelly *et al*., 1996; Motoyama *et al*., 1997; Horiuchi *et al*., 1999; Shaw and Momany, 2002; de Groot *et al*., 2009; Yoshimi *et al*., 2013).

Galactofuranose (Gal*f*) is a component of several polysaccharides and glycoconjugates in a number of filamentous fungi, including various *Aspergillus* species, bacteria, trypanosomatids and nematodes, but is absent in yeast, mammals and plants (Latgé, 2009; Tefsen *et al*., 2012). Gal*f* residues are immunogenic in mammals (Reiss and Lehmann, 1979; Yuen *et al*., 2001; Morelle *et al*., 2005) and are speculated to be involved in pathogenicity in humans. Therefore, the inhibition and detection of the biosynthesis of Gal*f* might be exploited for chemotherapy and as a diagnostic tool for *Aspergillus* infection (Pedersen and Turco, 2003; Latgé, 2009; Tefsen *et al*., 2012).

Gal*f* residues are frequently found in *Aspergillus* glycoproteins, including *N*-glycans and *O*-mannose glycans, which modify many cell wall proteins and extracellular enzymes (Wallis *et al*., 1999; 2001; Goto, 2007; Tefsen *et al*., 2012). A single 1,2-linked Gal*f* residue is present at

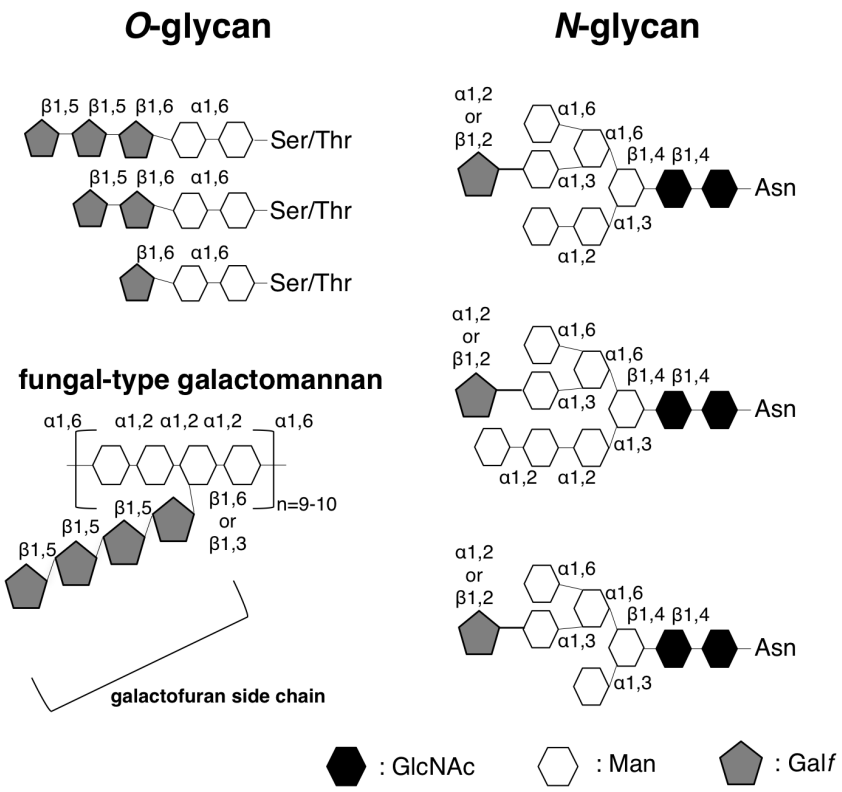

Fig. 1. Structures of galactofuranose-containing *O*-glycan, *N*-glycan and fungal-type galactomannan in *Aspergillus* spp. The *O*-glycan structure was described by Leitao *et al.* (2003). *O*-glycans exhibit structural diversity dependent on the species. *N*-glycan structures were described by Takayanagi *et al.* (1994) and Schmalhorst *et al.* (2008) and the structure of fungal-type galactomannan was described by Latgé *et al.* (1994). GlcNAc, *N*-acetylglucosamine; Man, mannose; Gal*f*, galactofuranose; Ser, serine; Thr, threonine; Asn, aspartic acid.

the non-reducing terminus of *N*-glycans (Takayanagi *et al.*, 1994; Wallis *et al.*, 2001; Morelle *et al.*, 2005; Schmalhorst *et al.*, 2008), and terminally β1,5-linked Gal*f* residues are present in *O*-mannose glycans (Leitao *et al.*, 2003) (Fig. 1). *O*-mannose glycan modification of proteins is widely distributed in bacteria and eukaryotic cells, from yeast to mammals, and is required for the formation of filamentous fungal cell walls (Shaw and Momany, 2002; Oka *et al.*, 2004; 2005; Zhou *et al.*, 2007; Goto *et al.*, 2009; Kriangkripipat and Momany, 2009; Lommel and Strahl, 2009; Mouyna *et al.*, 2010). The initial reaction in the transfer of mannose to serine or threonine residues is catalysed in the endoplasmic reticulum by protein-*O*-D-mannosyltransferase (Pmt), which requires dolichol phosphate-mannose as an immediate sugar donor (Strahl-Bolsinger *et al.*, 1993). Although *O*-glycans are elongated by glycosyltransferases in the Golgi apparatus, the responsible enzymes, such as galactofuranosyltransferases, remain to be identified in filamentous fungi.

Gal*f* is also found in fungal-type galactomannan of *Aspergillus* (Fig. 1). Fungal-type galactomannan of *Aspergillus* is composed of a linear mannan core with an α1,2-linked mannotetraose repeating unit attached via an α1,6-linkage and β1,5-Gal*f* oligomers (galactofuran side-chain) of up to five residues that are attached to the mannan backbone *via* β1,6- or β1,3-linkages (Latgé *et al.*, 1994; Fig. 1). Galactomannan is anchored to the plasma membrane by glycosylphosphatidylinositol and is covalently bound to the non-reducing end of a short β1,3-glucan chain in the cell wall (Fontaine *et al.*, 2000; Costachel *et al.*, 2005). However, galactomannan has not been identified in the glycosylphosphatidylinositol anchors of *Aspergillus* spp. (Tefsen *et al.*, 2012).

Although little is known about the enzymes involved in Gal*f* initiation, extension and termination, several enzymes involved in the synthesis of Gal*f*-containing sugar chains have been studied in fungi. For example, UDP-glucose 4-epimerase (UgeA) is responsible for the conversion of UDP-glucose to UDP-galactopyranose (Gal*p*) (El-Ganiny *et al.*, 2010), which is then converted to UDP-Gal*f* by UDP-Gal*p* mutase (UgmA/GlfA) in the cytosol (Bakker *et al.*, 2005; Damveld *et al.*, 2008; El-Ganiny *et al.*, 2008; Schmalhorst *et al.*, 2008). The synthesized UDP-Gal*f* is then transported into the Golgi lumen by the Golgi-localized UDP-Gal*f* transporter (UgtA/GlfB) (Engel *et al.*, 2009; Afroz *et al.*, 2011), which is unique to *Aspergillus* spp. and is required for Gal*f* deposition in the cell wall. UDP-Gal*f* plays an important role in the Gal*f* biosynthetic pathway, where it acts as a sugar donor for galactofuranosyltransferases (Engel *et al.*, 2009; Afroz *et al.*, 2011). An *Aspergillus fumigatus glfA* deletion mutant strain was shown to lack Gal*f* residues and displayed attenuated virulence in a mouse model of invasive aspergillosis (Schmalhorst *et al.*, 2008). In contrast, Lamarre reported that *glfA* deletion in a different *A. fumigatus* strain results in increased adhesion to host cells due to a lack of galacto-

furan (Lamarre *et al*., 2009), and no attenuation of virulence. Heesemann reported that the monoclonal antibody IgM L10-1, which specifically reacts with Gal*f*-containing glycostructures, fails to inhibit hyphal growth of *A. fumigatus* and is not able to protect infected mice (Heesemann *et al*., 2011). Furthermore, *Aspergillus nidulans* Gal*f*-deficient mutants exhibit compact colony growth, and abnormal conidiation and hyphal morphology (El-Ganiny *et al*., 2008). Taken together, these results suggest that Gal*f* residues play important roles in cell wall integrity and pathogenicity in *Aspergillus* spp. To elucidate the individual role of Gal*f*-containing polysaccharides and glycoconjugates in cell wall biosynthesis, it is necessary to identify and characterize the galactofuranosyltransferases that catalyse the addition of Gal*f* residues from UDP-Gal*f*.

Several bacterial genes reportedly encode galactofuranosyltransferases (Tam and Lowary, 2009). For example, the product of the *glfT2* gene is required for mycobacterial galactan polymerization and has both β1,5- and β1,6-Gal*f* transferase activities (Kremer *et al*., 2001; Rose *et al*., 2006). In addition, WbbI in *Escherichia coli* performs β1,6-coupling of Gal*f* to α-glucose (Wing *et al*., 2006) and WbbO in *Klebsiella pneumoniae* couples Gal*f* to Gal*p* by a β1,3-linkage (Guan *et al*., 2001). To date, LPG1 of *Leishmania* sp. is the only Gal*f* transferase described in eukaryotes and the encoding gene was isolated by functional complementation of the *Leishmania donovani* R2D2 mutant (Ryan *et al*., 1993). Evidence suggests that LPG1 adds β1,3-Gal*f* residue onto mannose (Huang and Turco, 1993). Although many laboratories have likely searched for galactofuranosyltransferases involved in the synthesis of Gal*f*-containing polysaccharides and glycoconjugates, to our knowledge, the identification of such an enzyme has not been reported in eukaryote other than *Leishmania*.

In the present report, we attempted to identify galactofuranosyltransferase genes involved in the synthesis of Gal*f* antigen in *A. nidulans* and *A. fumigatus* using reverse-genetic and biochemical approaches. We speculated that galactofuranosyltransferase candidate genes might be among the set of *A. nidulans* genes encoding unidentified and uncharacterized putative glycosyltransferases that are not orthologues of genes identified in other organisms. By constructing and screening a gene-disruptant library of candidate genes involved in the biosynthesis of Gal*f* antigen, we identified that the *AN8677* gene encoded a galactofuranosyltransferase, which we called GfsA, involved in Gal*f* antigen of *O*-glycan synthesis. Sucrose density gradient centrifugation analysis revealed that Gal*f* is localized to Golgi apparatus, similar to the UDP-galactofuranose transporter UgtA/GlfB (Engel *et al*., 2009; Afroz *et al*., 2011). Based on these results, we updated the biosynthetic pathway for Gal*f* antigen in *Aspergillus* to include the galactofuranosyltransferase GfsA.

Results

Selection of candidate genes involved in the synthesis of galactofuranose antigens

A BLAST search (Altschul *et al*., 1997) of the *Aspergillus* genome did not identify any orthologues of *Leishmania* or bacterial galactofuranosyltransferase genes. Thus, we used a reverse-genetic approach that did not rely on primary sequence similarity and instead was based on the fact that galactofuranose residues are found in some filamentous fungi, protozoa and nematodes (Beverley *et al*., 2005), but not in yeasts, mammals or plants (Tefsen *et al*., 2012). A search of the CAZy database (carbohydrate-active enzymes database; http://www.cazy.org/) revealed that approximately 90 genes in the *A. nidulans* genome encode putative glycosyltransferases (Campbell *et al*., 1997). We selected candidate genes that might be involved in the synthesis of Gal*f* antigen using the following approach. First, we excluded glycosyltransferase family 1 (GT1) genes, which are thought to be involved in the synthesis of glycosides. Second, we excluded orthologues of genes identified in other organisms, including *Saccharomyces cerevisiae*. Finally, we selected genes common to filamentous fungi that contain Gal*f* antigen in their cell walls. Based on these criteria, 11 candidate genes encoding putative galactofuranosyltransferases in *A. nidulans* were selected (Table S1).

Screening of genes involved in the synthesis of galactofuranose antigen

To analyse the function of the 11 putative glycosyltransferase-encoding genes, each gene was disrupted in *A. nidulans* AKU89 by gene replacement with *argB* (Tables S1 and S2). Chromosomal targeting of the 11 genes was confirmed by PCR using the primers ANxxxx-1 and argB-R, and argB-F and ANxxxx-4 (Yu *et al*., 2004; Table S4, Fig. S1A and B), and at least two independent isogenic disruptant strains were isolated for each candidate gene. The colony phenotypes of all disruptant strains were observed after 3 days of growth at 30°C on minimal medium. Notably, strain Δ*AN8677* formed small white colonies, a phenotype similar to that of strain Δ*ugmA*, which lacks Gal*f* (Fig. 2A; El-Ganiny *et al*., 2008; Alam *et al*., 2012).

We next tested the affinity of EB-A2, a monoclonal antibody that binds Gal*f* residues with high specificity, towards galactomannoproteins extracted from the disruptant strains (Fig. 2B). Clinical screening with EB-A2 is considered to be a highly sensitive and specific assay for early detection of invasive aspergillosis (Klont *et al*., 2004), and the specificity of EB-A2 for Gal*f* antigen is well characterized (Stynen *et al*., 1992; Yuen *et al*., 2001; Leitao *et al*., 2003; Morelle *et al*., 2005). The main epitope

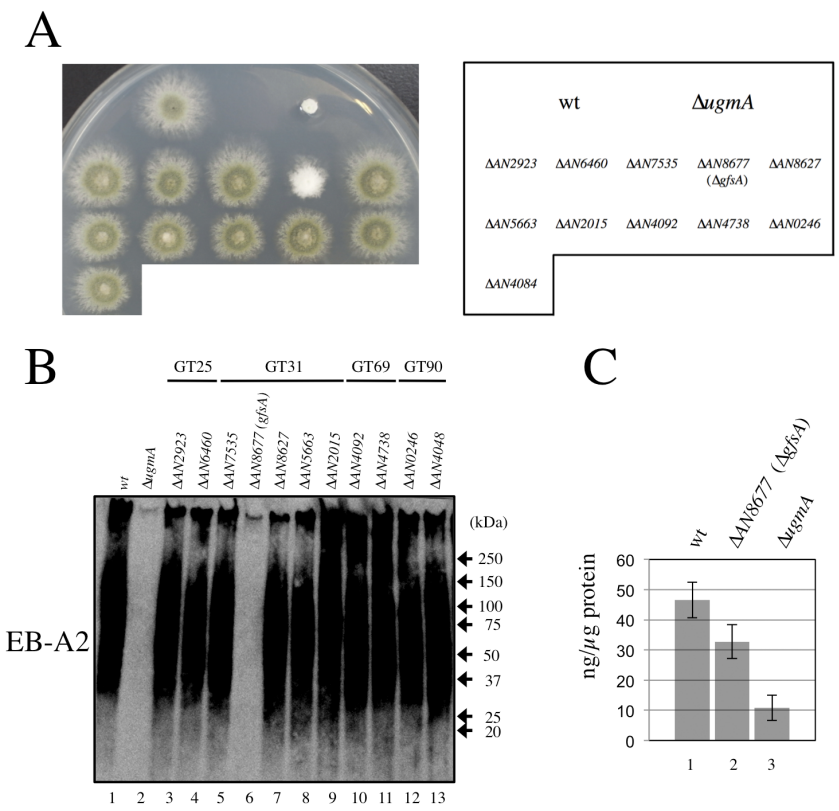

Fig. 2. Screening of genes involved in the synthesis of galactofuranose antigen.
A. Colony phenotypic analysis of disruptants of the indicated candidate genes. The image was taken after conidia were incubated on minimal medium for 2 days.
B. Immunoblotting analysis of galactomannoproteins from each disruptant. The presence of Gal*f* was detected with antibody EB-A2. Lanes 1–13: 5 μg of galactomannoproteins extracted from the wild-type (wt) strain (lane 1), strain Δ*ugmA* (lane 2) or individual disruptants (lanes 3–15) was loaded in each lane.
C. D-galactopyranose content of galactomannoproteins of wt (bar 1), strain Δ*AN8677* (Δ*gfsA*) (bar 2) and strain Δ*ugmA* (bar 3). The D-galactopyranose content was determined as described in the *Experimental procedures*. Error bars indicate standard error.

of EB-A2 is reportedly a tetrasaccharide of β1,5-linked Gal*f* (galactofuran side-chain) (Stynen *et al.*, 1992); however, EB-A2 also reacts with a single terminal non-reducing Gal*f* residue in *N*-glycans and terminal β1,5-linked Gal*f* residues in *O*-glycans (Yuen *et al.*, 2001; Leitao *et al.*, 2003; Morelle *et al.*, 2005). Based on these characterizations, EB-A2 is considered to represent a selective anti-β-Gal*f* antibody.

Previous work has shown that EB-A2 bound to cell wall proteins of *Aspergillus* spp. is detectable as a smear that migrates between 40 and > 200 kDa, whereas binding is absent in Gal*f*-defective mutants (Stynen *et al.*, 1992; Schmalhorst *et al.*, 2008; Engel *et al.*, 2009; Afroz *et al.*, 2011). Here, immunoblot analysis was used to examine the binding of EB-A2 to galactomannoproteins extracted from the 11 *A. nidulans* disruptants (Fig. 2B). The analysis revealed a marked reduction in the EB-A2 signal for strain Δ*AN8677* compared to wild type (wt), suggesting that *AN8677* is involved in the biosynthesis of Gal*f* antigen (Fig. 2B). Detection of EB-A2-positive signals in the wt and other disruptant strains demonstrated that Gal*f* antigen was present (Fig. 2B).

We next measured the levels of D-galactopyranose in galactomannoproteins extracted from the wt and Δ*AN8677* strains. It is generally known that furanoses, included in polymers, are detected as pyranoses after hydrolysis. The D-galactopyranose content of strain Δ*AN8677* was only 70% of that observed for wt (Fig. 2C), providing further evidence that the levels of Gal*f* antigen are reduced in strain Δ*AN8677*. Taken together, these results suggested that *AN8677* is involved in the biosynthesis of Gal*f* antigen and was therefore named *gfsA* (galactofuranose antigen synthase).

Southern blotting was used to examine the structure of the *gfsA* locus in strain Δ*AN8677* (Δ*gfsA*) (Fig. 3). The analysis revealed that site-specific recombination with the *argB* cassette occurred at the chromosomal *gfsA* locus (Fig. 3A and B). Next, we introduced the entire *gfsA* gene from *A. nidulans* into strain Δ*gfsA*, yielding strain Δ*gfsA*+pPTR-II-gfsA. As expected, introduction of *A. nidulans gfsA* into Δ*gfsA* rescued the abnormal colony phenotype and conidia formation at 30°C (Fig. 3C). In addition, Gal*f* antigen in galactomannoproteins from strain Δ*gfsA*+pPTR-II-gfsA was detected by immunoblotting with EB-A2 at signal intensities that were comparable to wt (Fig. 3D). These results are consistent with the speculation that *gfsA* is responsible for the defect in strain Δ*gfsA*.

To investigate the origin of the signal associated with the binding of EB-A2, we performed immunoblot analysis with proteinase K-treated galactomannoproteins extracted from wt (Fig. S2). We found that EB-A2 signal intensity decreased after proteinase K treatment, indicating that the signal originated from Gal*f* antigen attached to galactomannoproteins (Fig. S2).

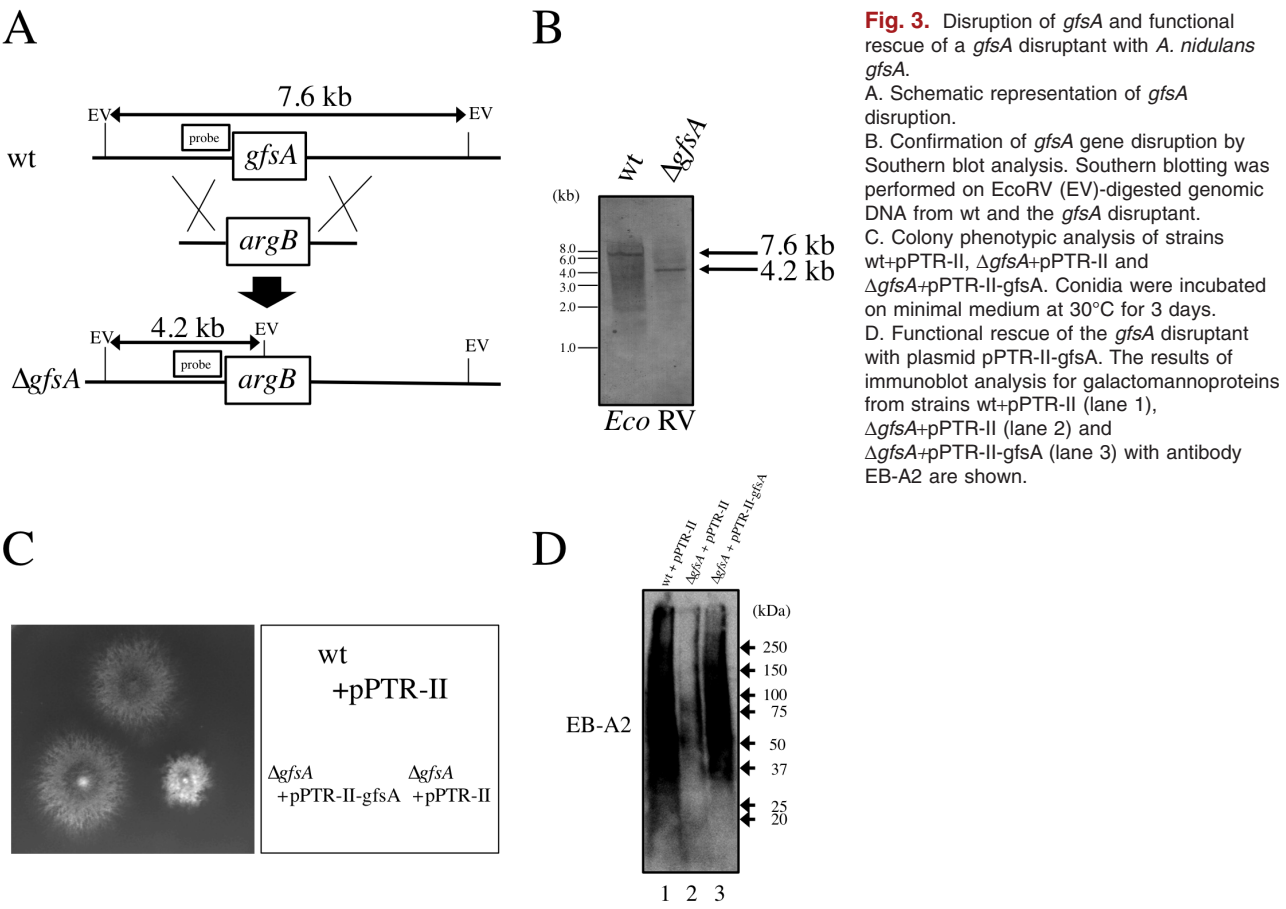

Fig. 3. Disruption of *gfsA* and functional rescue of a *gfsA* disruptant with *A. nidulans gfsA*.
A. Schematic representation of *gfsA* disruption.
B. Confirmation of *gfsA* gene disruption by Southern blot analysis. Southern blotting was performed on EcoRV (EV)-digested genomic DNA from wt and the *gfsA* disruptant.
C. Colony phenotypic analysis of strains wt+pPTR-II, Δ*gfsA*+pPTR-II and Δ*gfsA*+pPTR-II-gfsA. Conidia were incubated on minimal medium at 30°C for 3 days.
D. Functional rescue of the *gfsA* disruptant with plasmid pPTR-II-gfsA. The results of immunoblot analysis for galactomannoproteins from strains wt+pPTR-II (lane 1), Δ*gfsA*+pPTR-II (lane 2) and Δ*gfsA*+pPTR-II-gfsA (lane 3) with antibody EB-A2 are shown.

To determine if Gal*f* residues are attached to *N*-glycans and *O*-glycans in galactomannoproteins extracted from the wt strain, we first released *N*-glycans and *O*-glycans from galactomannoproteins by PNGase F treatment and β-elimination respectively (Fig. 4A). Treatment of galactomannoproteins with PNGase F did not affect EB-A2 signal intensity, suggesting that the signal is not related to the Gal*f* residues attached to *N*-glycans (Fig. 4A, lanes 1 and 2). By contrast, β-elimination treatment of galactomannoproteins resulted in a decrease in signal intensity (Fig. 4A, lanes 1, 3 and 4). Next, we tested the reactivity of EB-A2 to proteins extracted from *A. nidulans* strains disrupted for *pmtA* and *pmtC*, which encode enzymes that initiate the synthesis of *O*-mannose-type glycans (Shaw and Momany, 2002; Oka *et al.*, 2004; Goto *et al.*, 2009; Kriangkripipat and Momany, 2009). These disruptants have reduced levels of protein *O*-mannosylation (Oka *et al.*, 2004; Goto *et al.*, 2009; Kriangkripipat and Momany, 2009). We found that the signal intensity of both Δ*pmtA* and Δ*pmtC* strains was lower as compared to wt, indicating that the signal originated from Gal*f* residues in *O*-glycans (Fig. 4B). In addition, we confirmed that Gal*f* residues in *O*-mannosylated proteins were reduced in the Δ*gfsA* strain as compared with wt (Fig. 4B).

We previously reported that *A. nidulans* WscA, a putative stress sensor protein, is *N*- and *O*-glycosylated (Goto *et al.*, 2009; Futagami *et al.*, 2011). We therefore constructed wt, Δ*gfsA* and Δ*ugmA* strains expressing a WscA-HA fusion protein, which was then purified from each strain subjected to immunoblotting analysis with anti-HA antibody and EB-A2 after PNGase F treatment (Fig. 4C). The EB-A2 signal intensity of WscA-HA from Δ*gfsA* was less than that from wt (Fig. 4C, lanes 4 and 5). Densitometric quantification of the immunoblot bands was performed using Quantity One Ver. 4.4.1 (Bio-Rad). The signal intensity ratios for EB-A2 and anti-HA were then calculated and the ratio for wt was normalized to 1.0. The ratio of intensities of EB-A2 to anti-HA from Δ*gfsA* was less than half of that from wt (Fig. 4D). These data indicate that the *gfsA* gene is involved in the synthesis of the Gal*f* antigen in *O*-glycans.

Features of GfsA

The *gfsA* gene encodes a 532-amino-acid protein with a putative molecular mass of 60.9 kDa (Fig. S3). *gfsA* cDNA was amplified by PCR using a 24 h developmental cDNA library (obtained from the FGSC) as a template. Compari-

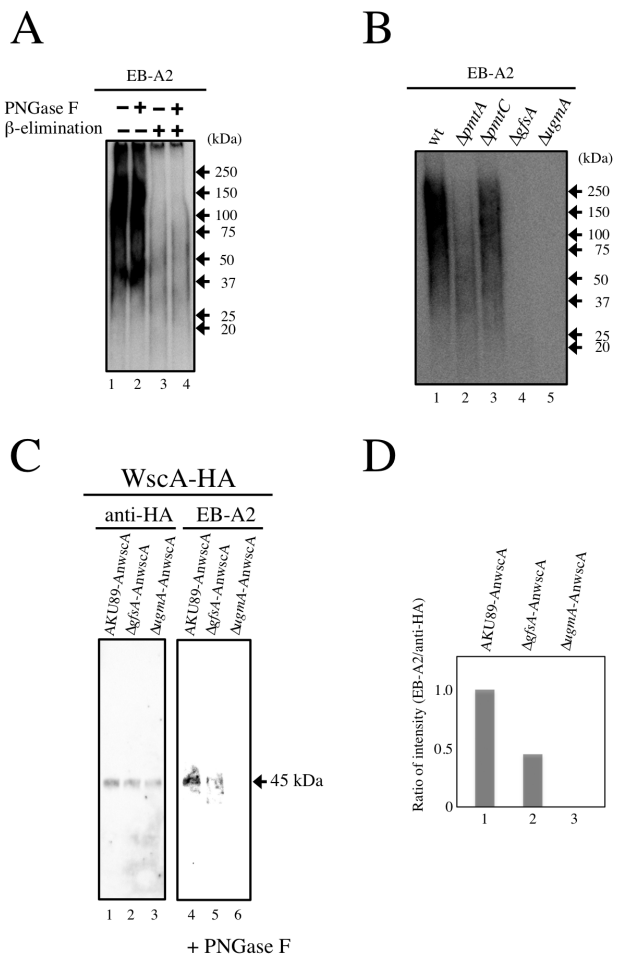

Fig. 4. Estimation of the origin of the EB-A2 signal.
A. Detection of Gal*f* antigen in galactomannoproteins with EB-A2 after treatment with PNGase F and β-elimination. Five micrograms of galactomannoproteins extracted from wt (lanes 1–4) were separated by SDS-PAGE prior to immunoblotting with EB-A2. Lanes 2 and 4, PNGase F-treated proteins; lanes 3 and 4; β-elimination treated proteins.
B. Detection of Gal*f* antigen with EB-A2 in galactomannoproteins from *O*-glycan-deficient mutants. Five micrograms of galactomannoproteins extracted from wt (lanes 1), Δ*pmtA* (lane 2), Δ*pmtC* (lane 3), Δ*gfsA* (lane 4) or Δ*ugmA* (lane 5) were separated by SDS-PAGE prior to immunoblotting with EB-A2.
C. *In-vivo* galactofuranose synthase activity of GfsA towards *O*-glycosylated WscA. *A. nidulans* strain AKU89-AnwscA expressing *wscA-ha* was used as the parental strain (Goto *et al*., 2009). The *gfsA* and *ugmA* genes were disrupted in strain AKU89-AnwscA to construct the strains Δ*gfsA*-AnwscA and Δ*ugmA*-AnwscA. WscA-HA was purified by rabbit polyclonal anti-HA-Tag-agarose and treated with PNGase F to remove *N*-glycans. A total of 2.5 μg WscA-HA from AKU89-AnwscA (lanes 1 and 4), Δ*gfsA*-AnwscA (lanes 2 and 5) and Δ*ugmA*-AnwscA (lanes 3 and 6) were separated by 5–20% SDS-PAGE and detected by immunoblotting with mouse monoclonal anti-HA antibody (left panel) and EB-A2 (right panel).
D. Comparison of the intensity ratios of EB-A2 to anti-HA signals for WscA-HA from wt (bar 1), strain Δ*gfsA* (bar 2) and strain Δ*ugmA* (bar 3). Densitometric quantification of immunoblotting bands was performed using Quantity One Ver. 4.4.1 (Bio-Rad). The intensity ratios of the EB-A2 to anti-HA signals for WscA-HA in Fig. 4C were calculated, and the ratio for wt was normalized to 1.0.

son of the cDNA and genome sequences revealed that no introns were present in the *gfsA* gene. Analysis of secondary structure using SOSUI (Hirokawa *et al*., 1998) revealed that GfsA has a putative signal sequence (amino acids 1–17) and transmembrane domain (amino acids 18–40) at the *N*-terminus, suggesting that GfsA is a type II membrane protein. GfsA also has a metal-binding DXD motif (amino acids 256–258), which is conserved among GfsAs from other organisms, and two potential *N*-glycosylation sites (amino acids 93–95 and 414–416) (Fig. S3). Two putative paralogues, *AN5663* and *AN2015*, were identified that have 27.5% and 26.0% sequence identity, respectively, with GfsA. However, disruption of these genes in *A. nidulans* did not result in any alteration of the colony phenotype under our experimental conditions (Fig. 2A).

BLAST analysis indicated that GfsA proteins are widely distributed in the subphylum Pezizomycotina (Table S3). However, no orthologues of GfsA were found in the genomes of *Caenorhabditis elegans*, *Trypanosoma cruzi*, or bacteria, including *E. coli*, *Klebsiella pneumoniae* and *Mycobacterium tuberculosis*. GfsA has 87% sequence identity with *Aspergillus flavus* NRRL 3357 (AFL2G_12474), 84% with *Aspergillus niger* CBS 513.88 (An12g08720), 84% with *A. fumigatus* A1163 (AFUB_096220), 84% with Af293 (Afu6g02120), 83% with *Aspergillus oryzae* RIB40 (AO090120000096), 76% with *Penicillium chrysogenum* Wisconsin 54-1255 (Pc22g24320), 62% with *Ajellomyces capsulatus* NAm1 (HCAG_03193), 57% with *Coccidioides immitis* RS (CIMG_05833), 56% with *Paracoccidioides brasiliensis* Pb03 (PABG_06492), 55% with *Trichophyton rubrum* CBS118892 (TERG_00129), 32% with *Neurospora crassa* OR74A (NCU02213) and 32% with *Fusarium graminearum* PH-1 (FGSC_05770).

Structural and functional analyses of GfsA

To enable a better understanding of GfsA, we constructed a strain expressing 3xFLAG-tagged GfsA by chromosomal tagging (Fig. 5A). The phenotype of the tagged strain was identical to wt, indicating that 3xFLAG-tagged GfsA has similar functional to that of wt GfsA. To confirm expression of 3xFLAG-tagged GfsA, a solubilized protein was prepared from the tagged strain and analysed with anti-FLAG antibody (Fig. 5B). The strain expressing 3xFLAG-tagged GfsA produced a protein of approximately 67 kDa (Fig. 5B, lane 1). After endoglycosidase H (Endo H*f*) treatment, 3xFLAG-tagged GfsA showed faster mobility during SDS-PAGE, with an apparent molecular weight of approximately 61 kDa (Fig. 5B, lane 2). These results show that GfsA is *N*-glycosylated.

To determine the enzymatic function of GfsA, we assayed Gal*f* antigen synthase activity using an *in-vitro* assay that was developed for measuring galactofurano-

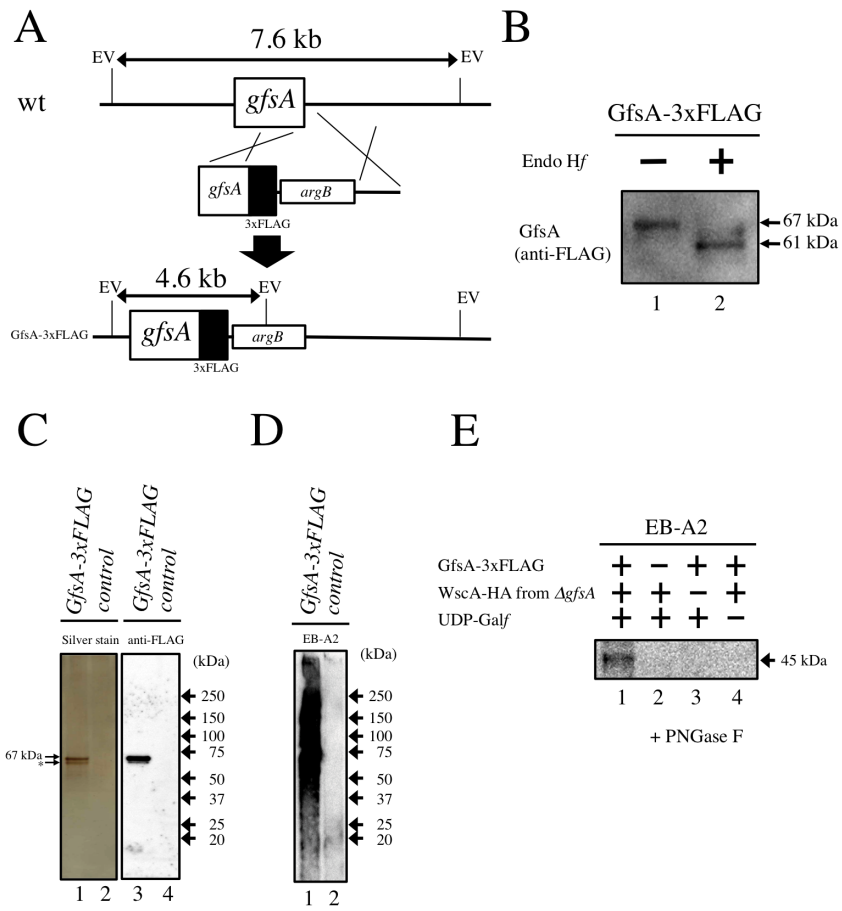

Fig. 5. Construction of GfsA-3xFLAG-expressing strain using PCR-based chromosomal tagging and purification of GfsA-3xFLAG protein.
A. Schematic representation of GfsA-3xFLAG strain construction.
B. Detection of GfsA protein in strain GfsA-3xFLAG. Microsomal fractions were mock treated (lane 1) or treated with endoglycosidase H (lane 2), separated by 5–20% SDS-PAGE, and analysed by immunoblotting with anti-FLAG antibody.
C. Purification of GfsA-3xFLAG protein. GfsA-3xFLAG was purified from strain GfsA-3xFLAG (Δ*ugmA*) (lanes 1 and 3) and the mock sample was similarly prepared from strain Δ*ugmA* (lanes 2 and 4). A total of 0.28 mg (silver staining) or 2.8 μg (immunoblotting) proteins were separated by 5%–20% SDS-PAGE, and were then assayed by silver staining (left panel) or immunoblot analysis with anti-FLAG antibody (right panel). GfsA-3xFLAG was detected as a 67 kDa protein. Asterisk indicates degraded GfsA-3xFLAG.
D. *In-vitro* analysis of Gal*f* antigen synthase activity of purified GfsA-3xFLAG by immunoblotting with EB-A2. Enzymatically synthesized UDP-Gal*f* was used as a sugar donor, and galactomannoproteins from strain Δ*gfsA* (GMPΔ*gfsA*) were used as acceptor substrates. Enzyme activities were assayed as described in *Experimental procedures*. Reaction products using purified GfsA-3xFLAG (14 μg) (lane 1) and the control sample (lane 2) are shown.
E. *In-vitro* analysis of Gal*f* antigen synthase activity for the purified GfsA protein fraction by immunoblotting with EB-A2. Enzymatically synthesized UDP-Gal*f* was used as a sugar donor and purified de-*N*-glycosylated WscA-HA protein from Δ*gfsA* was used as an acceptor substrate. Enzyme activities were assayed as described in *Experimental procedures*. Reaction products using purified GfsA-3xFLAG protein (14 μg) (lanes 1, 3 and 4), lacking the purified GfsA-3xFLAG protein (lane 2), purified WscA-HA protein from Δ*gfsA* (0.25 μg) after PNGase F treatment (lanes 1, 2 and 4), lacking the purified WscA-HA protein (lane 3), 0.72 mM UDP-Gal*f* (lanes 1, 2 and 3) and lacking UDP-Gal*f* (lane 4) are shown.

syltransferase activity using EB-A2 antibody. To exclude the influence of intracellular Gal*f* antigen, the assay was conducted in the Δ*ugmA* background (Δ*ugmA* and GfsA-3xFLAG (Δ*ugmA*) strains). GfsA proteins were first immunoprecipitated from strains GfsA-3xFLAG (Δ*ugmA*) and Δ*ugmA* (negative control) using anti-FLAG beads and then subjected to SDS-PAGE and silver staining (Fig. 5C, left panel). Purified GfsA was detected as two distinct protein bands of approximately 67 kDa (Fig. 5C, lane 1). The upper 67 kDa band is intact GfsA-3xFLAG and the lower band is a degradation product or an insufficiently *N*-glycosylated product that retained reactivity to FLAG antibody (Fig. 5C, lanes 1 and 3). Although a few weakly stained bands of lower molecular weight can be observed in the gel, these bands were also present in the control sample. These results indicate that GfsA-3xFLAG was highly purified.

Galactofuranosyltransferase activity of the purified protein was assayed using 0.72 mM UDP-Gal*f* as a sugar donor, 0.5 mM Mn^{2+} as a cofactor and 2.5 μg of galacto-

mannoproteins from strain Δ*gfsA* (GMPΔ*gfsA*). A strong signal of EB-A2 binding was detected in immunoblots when purified GfsA-3xFLAG was used (Fig. 5D, lane 1), whereas no detectable galactofuranosyltransferase activity was observed in the control fraction (Fig. 5D, lane 2). These results indicated that the purified GfsA fraction was not contaminated with any other galactofuranosyltransferase and that GfsA has galactofuranosyltransferase activity. To confirm these results, we also assayed the galactofuranosyltransferase activity of solubilized proteins extracted from Δ*ugmA* and Δ*ugmA*Δ*gfsA* (Fig. S4). A signal intensity of EB-A2 staining on immunoblots was only detected for solubilized proteins extracted from Δ*ugmA* (Fig. S4, lane 1). In contrast, the solubilized protein fraction obtained from Δ*ugmA*Δ*gfsA* did not have any galactofuranosyltransferase activity (Fig. S4, lane 3). This relatively simple experiment clearly demonstrates that proteins other than GfsA have no, or only very minimal, galactofuranosyltransferase activity.

We next attempted to determine if WscA-HA protein from strain Δ*gfsA* can serve as an acceptor substrate for GfsA-3xFLAG protein. The galactofuranosyltransferase activity of purified GfsA protein was assayed in the presence of 0.25 μg WscA-HA protein obtained from strain Δ*gfsA* (Fig. 5E). Prior to its use an acceptor substrate, WscA-HA was treated with PNGase F to remove *N*-glycans. Analysis of the reaction products of the assay by immunoblotting with EB-A2 antibody showed that purified GfsA-3xFLAG protein had galactofuranosyltransferase activity in the presence of both WscA-HA and UDP-Gal*f* (Fig. 5E, lane 1). In contrast, reaction mixtures without GfsA-3xFLAG, WscA-HA or UDP-Gal*f* did not have detectable enzymatic activity, as indicated by the absence of an EB-A2 staining signal (Fig. 5E, lanes 2, 3 and 4).

Biochemical analyses of GfsA from *A. nidulans* were also performed using the established *in-vitro* assay. EB-A2 signal intensity clearly increased in a time-dependent manner (Fig. 6A, lanes 1–5). GfsA was inactive towards galactomannoproteins from Δ*ugmA* (GMPΔ*ugmA*) (Fig. 6B, lane 4), and was also relatively inactive in the presence of UDP-Gal*p*, GDP-mannose and UDP-glucose compared to UDP-Gal*f*, which was associated with a strong EB-A2 signal (Fig. 6C, lanes 3–5). GfsA was also inactive in the presence of 10 mM EDTA, which can chelate Mn^{2+} (Fig. 6D, lane 2). Taken together, these results show that UDP-Gal*f* and Mn^{2+} are required for GfsA activity.

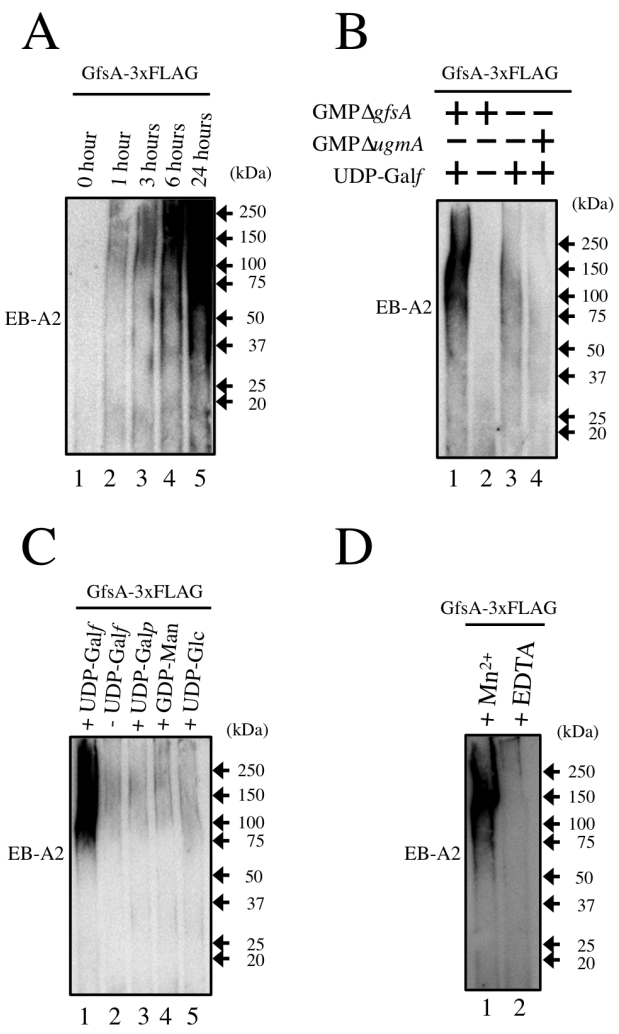

Fig. 6. Biochemical analysis of GfsA protein.
A. GfsA activity increased in a time-dependent manner in an *in-vitro* assay. Enzymatically synthesized UDP-Gal*f* was used as a sugar donor. Galactomannoproteins from Δ*gfsA* (GMPΔ*gfsA*) was used as acceptor substrates. Enzyme activities were assayed as described in *Experimental procedures*. Reaction products were stained with EB-A2 antibody after incubation with purified GfsA-3xFLAG for 0 h (lane 1), 1 h (lane 2), 3 h (lane 3), 6 h (lane 4) or 24 h (lane 5).
B. Substrate requirement for GfsA activity. Galactomannoproteins from GMPΔ*gfsA* or Δ*ugmA* (GMPΔ*ugmA*) were used as acceptor substrates. Reaction products using GMPΔ*gfsA* and UDP-Gal*f* (lane 1), lacking UDP-Gal*f* (lane 2), lacking GMPΔ*gfsA* (lane 3) and using GMPΔ*ugmA* in place of GMPΔ*gfsA* (lane 4) are shown.
C. Sugar nucleotide requirement for GfsA activity. Reaction products using 0.72 mM UDP-Gal*f* (lane 1), lacking UDP-Gal*f* (lane 2), 0.72 mM UDP-Gal*p* (lane 3), 0.72 mM GDP-Man (lane 4) and 0.72 mM UDP-Glc (lane 5) are shown.
D. Reaction products in the absence (lane 1) or presence (lane 2) of 10 mM EDTA to chelate Mn^{2+} are shown.

Subcellular localization of GfsA protein

To determine the subcellular localization of GfsA in *A. nidulans*, we performed sucrose density gradient centrifugation with total subcellular fractions extracted from the *A. nidulans* strain expressing GfsA-3xFLAG (Fig. 7). The presence of GfsA and distribution of marker proteins were analysed by immunoblotting with antibodies against UgtA/GlfB (a marker for the Golgi apparatus) and BipA (ER lumen). GfsA was predominantly detected in fractions 9 to 11. This pattern was similar to that observed for

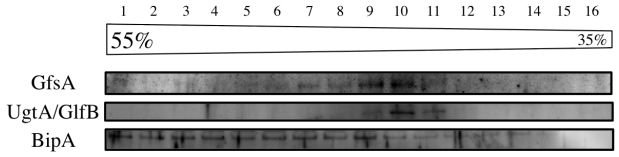

Fig. 7. Subcellular localization of GfsA. Sucrose density gradient centrifugation analysis. After centrifugation, each resulting gradient was separated into 16 fractions as described in *Experimental procedures*. Anti-UgtA/GlfB was used to detect markers for the Golgi apparatus, anti-BipA was used for endoplasmic reticulum markers, and anti-FLAG was used for GfsA.

UgtA/GlfB, suggesting that GfsA colocalizes with UgtA/GlfB (Fig. 7). In contrast, BipA was detected at similar levels in fractions 1 to 9 (Fig. 7). Thus, the distribution pattern observed for GfsA indicates that GfsA is principally localized to the Golgi apparatus.

Abnormal cell morphology of strain ΔgfsA

The disruption of *gfsA* inhibited hyphal extension and conidial formation. We observed that strain Δ*gfsA* forms smaller colonies than wt after cultivation on minimal medium at 30°C for 3 days (Fig. 8A). The colony growth rates of the wt and Δ*gfsA* strains were 0.40 ± 0.070 and 0.27 ± 0.060 mm h^{-1} respectively. The presence of an osmotic stabilizer (0.6 M KCl) slightly restored colony size in Δ*gfsA* (Fig. 8A). Moreover, under high-temperature conditions (42°C), the growth defects of strain Δ*gfsA* were restored (Fig. 8A).

We also examined the conidiation efficiency of the Δ*gfsA* and wt strains. Formation of normal green conidia was markedly repressed in Δ*gfsA* grown on minimal medium at 30°C for 3 days (Fig. 8A). The efficiency of conidiation in Δ*gfsA* was reduced to approximately 11% of that of the wt strain, but recovered to as much as 86% of wt on minimal medium plates in the presence of 0.6 M KCl at 42°C (Fig. 8B). Next, we tested the sensitivity of strain Δ*gfsA* to SDS, Congo red and calcofluor white treatments (Fig. 8C). We found that the growth of strain Δ*gfsA* was more sensitive than the wt strain to treatment with 0.002% (w/v) SDS (Fig. 8C), but was more resistant to 30 μg ml^{-1} calcofluor white (Fig. 8C). Hyphal growth of the Δ*gfsA* strain was affected by treatment with 30 μg ml^{-1} Congo red (Fig. 8C).

Conidiophores grown on minimal medium plates at 30°C for 3 days were next examined by scanning electron microscopy. Conidiophores of Δ*gfsA* were sparse and scattered compared with those of wt [Fig. 9A (a) and (b)]. In addition, the number of conidia per conidiophore was smaller in Δ*gfsA* than in wt [Fig. 9A (c) and (d)]. The structure of hyphae in liquid minimal medium was also investigated by fluorescence microscopy. The hyphae of wt *A. nidulans* grew linearly [Fig. 9B (a)], whereas strain Δ*gfsA* formed curved hyphae with abnormal branching. The distance between septa in hyphae of strain Δ*gfsA* was shorter than that in hyphae of wt [Fig. 9B (b)]. Together, these observations suggest that *gfsA* disruption causes abnormal hyphal formation, but does not influence septum formation.

Transcriptional and protein expression analyses of gfsA

To elucidate the growth stage at which *gfsA* is expressed, we analysed its transcription using real-time reverse transcription-PCR after 18, 24, 36 and 48 h of culture (Fig. 10A). The transcriptional levels of *gfsA* were relatively similar between 18 and 24 h, but markedly decreased between 24 and 48 h. We also assayed GfsA

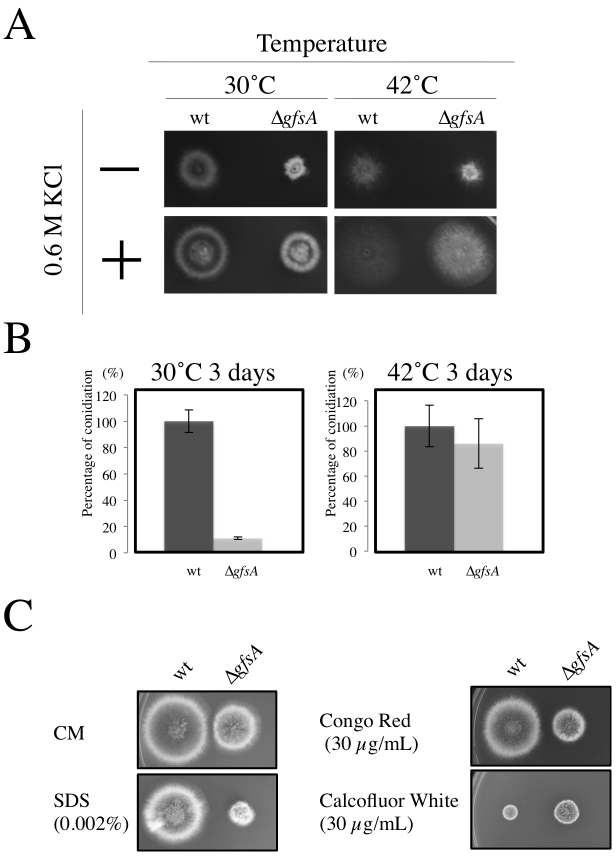

Fig. 8. Phenotypic analyses of strain Δ*gfsA*.
A. Colony phenotypic analysis of strain Δ*gfsA*. Strains were grown on minimal medium in the presence (+) or absence (–) of 0.6 M KCl at 30 or 42°C for 3 days.
B. Conidia formation by strain Δ*gfsA*. Efficiency of conidiation was analysed as described in *Experimental procedures*. Values in parentheses are percentages of growth relative to 7.65×10^5 conidia mm^{-2} (30°C) and 3.51×10^6 conidia mm^{-2} (42°C) for the wt strain.
C. Sensitivity of Δ*gfsA* strain to various drugs. Strains were grown in the presence of SDS (0.002%), Congo red (30 μg ml^{-1}) and calcofluor white (30 μg ml^{-1}) on CM medium for 3 days at 30°C.

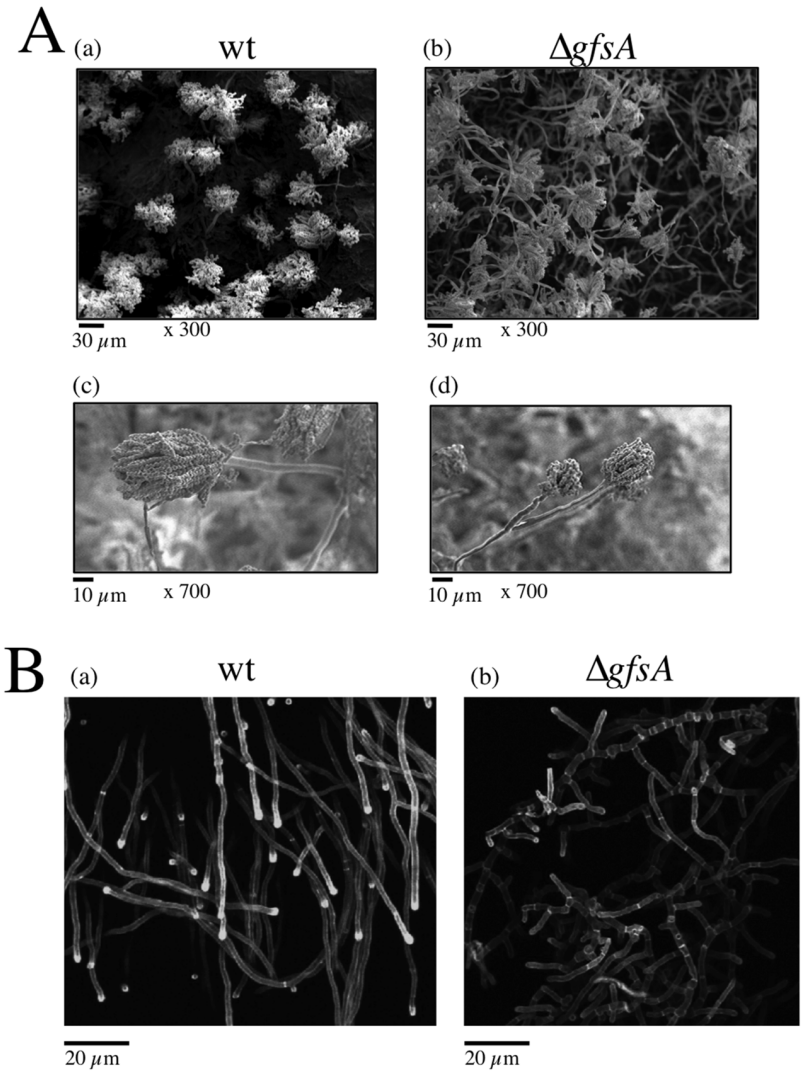

Fig. 9. Microscopic analyses of hyphae and conidiophores of strain Δ*gfsA* strain.
A. Density of conidiophores of the wt (a) and Δ*gfsA* (b) strains. Conidiophores and mycelia were cultured on minimal medium for 3 days prior to scanning electron microscope imaging. Conidiophore morphology of wt (c) and Δ*gfsA* strains (d). Conidiophores were cultured on minimal medium for 3 days prior to imaging.
B. Mycelial morphology of wt (a) and Δ*gfsA* (b) observed under a confocal laser-scanning microscope. The mycelia were grown for 24 h and harvested. Staining of chitin by calcofluor white was used as a marker for mycelia.

protein levels at 18, 24, 36 and 48 h (Fig. 10B). GfsA was expressed at high levels at 18 and 24 h, but the levels decreased after 24 h, whereas γ-actin was expressed almost constitutively expressed (Fig. 10B). This result was comparable with the result of *gfsA* transcription analysis. In addition, to determine if *gfsA* is transcribed in conidia, we compared transcription of *gfsA* in conidia with that in mycelia. We first tested the quality of the conidia RNA sample by assaying levels of the conidia-specific *spoC1-C1C* transcript. The results indicated that the sample RNA was successfully extracted from conidia (Fig. 10C, right panel). Moreover, the level of *gfsA* RNA in the conidia was higher than that in the mycelia after cultivation in minimal liquid medium for 18 h (Fig. 10C, left panel). These results are consistent with the speculation that GfsA has a significant role not only during hyphal development, but also during conidiation and in the conidia.

Functional analyses of a gfsA orthologue in A. fumigatus

It has been suggested that Gal*f*-containing molecules are related to virulence and infection of *A. fumigatus*, which is an opportunistic human pathogen (Schmalhorst *et al.*, 2008; Lamarre *et al.*, 2009). We identified a putative orthologue of *gfsA* (AFUB_096220) in *A. fumigatus* strain A1163. To analyse the function of this gene, which we termed *AfgfsA*, we generated an *AfgfsA* disruptant using *A. fumigatus* strains A1160 and A1151 as parental strains. Colonies formed by strain Δ*AfgfsA* in the A1160 background were smaller than those formed by strain A1160 at 47°C (Fig. 11A) and conidia formation was reduced. These phenotypes were restored in the presence of 0.6 M KCl at 47°C. To confirm that the mutation could be complemented, we introduced the plasmid pPTR-II-gfsA into strain Δ*AfgfsA*. The abnormal colony phenotype was

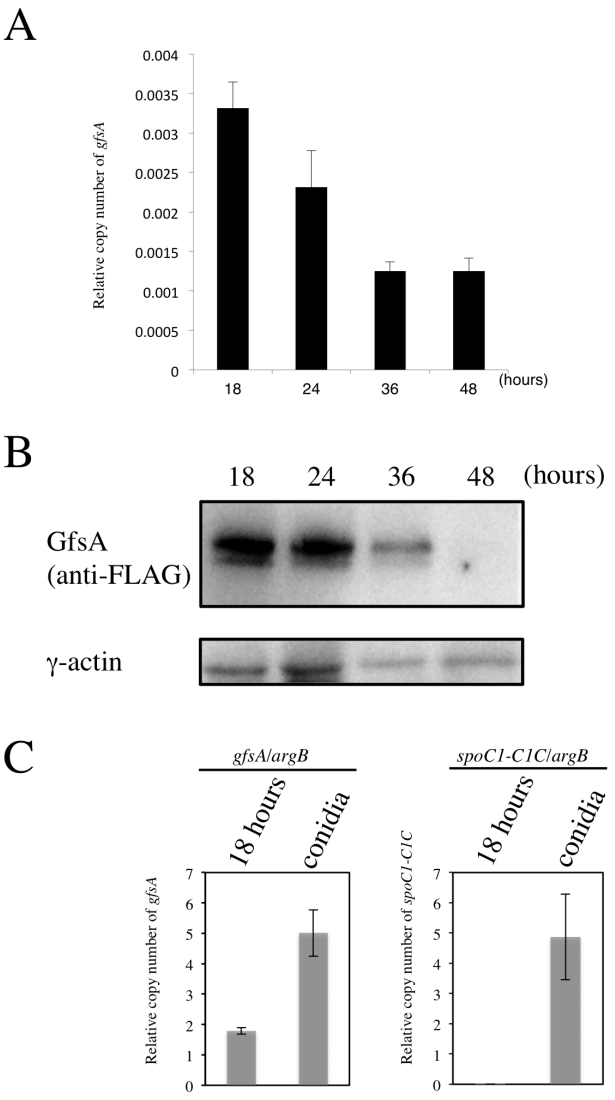

Fig. 10. Transcriptional and protein expression analyses of *gfsA*.
A. Real-time reverse transcription (RT)-PCR analysis of *gfsA* expression in the wt strain. Conidia (2×10^7) were inoculated into 100 ml liquid minimal medium and grown for 18, 24, 36 or 48 h at 30°C. *gfsA* expression was quantified by real-time RT-PCR analysis. The *y*-axis shows the level of mRNA relative to the histone 2B gene.
B. GfsA protein expression analysis in the GfsA-3xFLAG strain. Conidia (2×10^7) were inoculated into 100 ml liquid minimal medium and grown for 18, 24, 36 or 48 h at 30°C. The expression levels of GfsA (upper panel) and γ-actin (lower panel) were determined by immunoblot analysis. γ-Actin was used as a protein loading control.
C. Transcription analysis of *gfsA* in conidia. RNA was extracted from mycelia collected after 18 h of cultivation and conidia were harvested on a minimal medium plate after 3 days. Expression of *gfsA*, *spoC1-C1C* and *argB* were quantified by real-time RT-PCR analysis. The *argB* gene was used previously as a control for transcriptional analysis in conidia (Stephens *et al.*, 1999; Momany *et al.*, 2001), and *spoC1-C1C* is an established marker for conidia (Orr and Timberlake, 1982; Stephens *et al.*, 1999). The *y*-axis shows the level of mRNA relative to the *argB* gene.

restored in the Δ*AfgfsA* strain expressing *A. nidulans gfsA* (Fig. 11B). In addition, Gal*f* antigens in galactomannoproteins were detected by immunoblotting analysis with EB-A2. In the Δ*AfgfsA* strain expressing *gfsA* from *A. nidulans*, the signal intensity of EB-A2 immunodetection was also recovered to wt levels, indicating that *A. nidulans gfsA* can synthesize the Gal*f* antigen in both *A. nidulans* and *A. fumigatus* (Fig. 11C). This result also indicates that *AfgfsA* encodes a galactofuranosyltransferase.

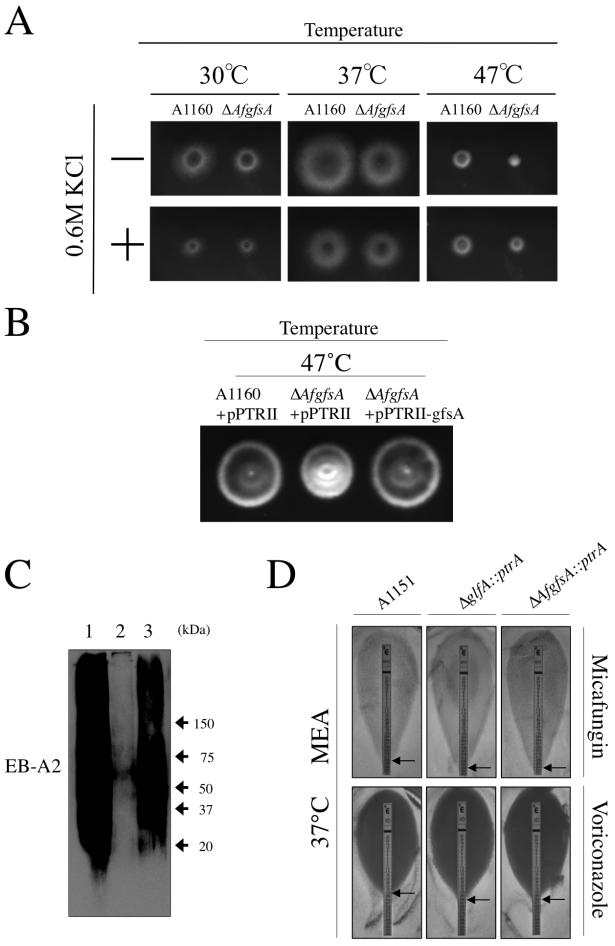

Fig. 11. Functional analyses of the *A. fumigatus AfgfsA* gene.
A. Colony phenotypic analysis of wt and Δ*AfgfsA* strains. Strains were grown on minimal medium in the presence (+) or absence (–) of 0.6 M KCl at 30°C, 37°C or 47°C for 3 days.
B. Functional rescue of a Δ*AfgfsA* strain with plasmid pPTR-II-gfsA. Strains were grown on minimal medium at 47°C for 3 days.
C. Immunoblot analysis of galactomannoproteins from strains A1160+pPTR-II, Δ*AfgfsA*+pPTR-II and Δ*AfgfsA*+pPTR-II-gfsA. Galactofuranose antigen was detected with EB-A2 antibody. Galactomannoproteins (5 μg) were extracted from strains A1160+pPTR-II (lane 1), Δ*AfgfsA*+pPTR-II (lane 2) and Δ*AfgfsA*+pPTR-II-gfsA (lane 3).
D. Sensitivity to micafungin and voriconazole assayed by the Etest. Conidia (6×10^5 per plate) were evenly spread on MEA plates and test strips were placed at the centre of the plate before incubation at 37°C for 24 h. Arrows indicate MIC values.

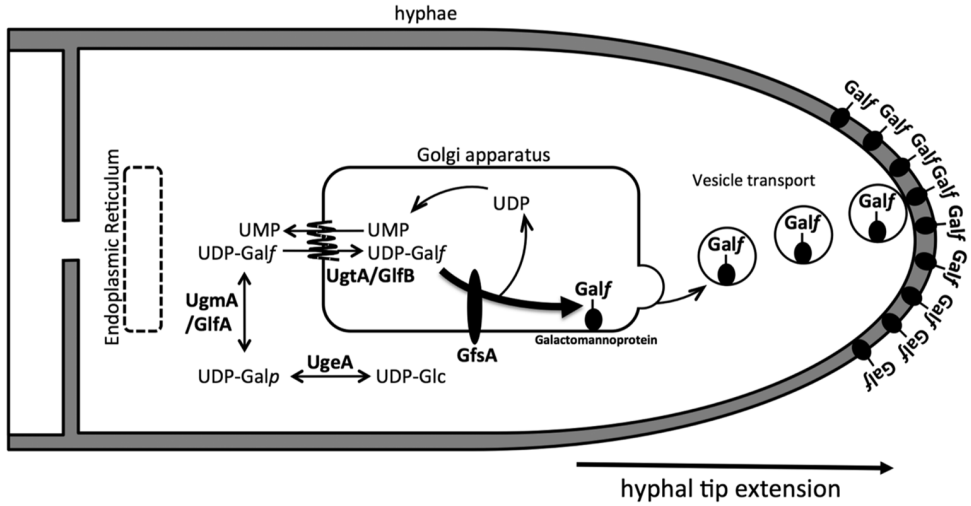

Fig. 12. Galactofuranose (Gal*f*) antigen in the *O*-glycan biosynthesis pathway. Updated model of biosynthesis of the Gal*f* antigen of *O*-glycan. UMP, uridine 5′-monophosphate; UDP, uridine 5′-diphosphate; Gal*f*, galactofuranose; Gal*p*, galactopyranose; Glc, glucose.

Finally, we tested the sensitivity of A1151, Δ*glfA* and Δ*AfgfsA* to micafungin and voriconazole on MEA medium. Plates with conidia covering the surface were incubated with test strips of the two antibiotics for 24 h at 37°C. Under these conditions, *A. fumigatus* strain A1151 formed a clear zone of growth inhibition around voriconazole [minimum inhibitory concentration (MIC), 0.125 μg ml^{-1}] and micafungin (MIC, 0.008 μg ml^{-1}). However, strain Δ*AfgfsA* showed a larger zone of growth inhibition around voriconazole (MIC, 0.064 μg ml^{-1}l) and micafungin (MIC, 0.004 μg ml^{-1}), similar to that formed by strain Δ*glfA* strain (Fig. 11D), indicating that Δ*AfgfsA* has increased sensitivity to antifungal agents.

Discussion

Strain Δ*ugmA* displays an abnormal phenotype that appears to result from the loss of all Gal*f*-containing polysaccharides and glycoconjugates, including *O*-glycans, *N*-glycans, galactofuran side-chains and glycosylinositolphosphoceramides. To help clarify the roles of individual Gal*f*-containing polysaccharides and glycoconjugates, it is necessary to identify and characterize genes that encode galactofuranosyltransferases involved in the synthesis of these molecules. Towards this goal, here, we attempted to identify a galactofuranosyltransferase gene involved in Gal*f* antigen synthesis in the filamentous fungus *A. nidulans*. Using reverse-genetic and biochemical approaches, we provide evidence that *gfsA* encodes a galactofuranosyltransferase with the ability to synthesize the Gal*f* antigen of *O*-glycans. To our knowledge, this is the first report describing a galactofuranosyltransferase-encoding gene that functions in the fungal Gal*f* synthetic pathway. Engel and colleagues proposed a novel schematic model of galactofuranosylation (Engel *et al*., 2009). Based on our present results, we have updated the model of galactofuranosylation in fungal cells to include GfsA protein (Fig. 12). In the updated model, UDP-Gal*f* is first synthesized from UDP-glucose *via* UDP-Gal*p* in the cytosol by UgeA and UgmA/GlfA (Damveld *et al*., 2008; El-Ganiny *et al*., 2008; 2010; Schmalhorst *et al*., 2008; Lamarre *et al*., 2009) and is then transported into the Golgi apparatus *via* the antiporter protein UgtA/GlfB (Engel *et al*., 2009; Afroz *et al*., 2011). GfsA then synthesizes Gal*f* antigen of *O*-glycans on galactomannoproteins in the Golgi apparatus during hyphal tip growth. Finally, the synthesized galactofuranosylated proteins are transported by vesicles and localized to the cell surface (Fig. 12).

The reactivity of monoclonal antibody EB-A2 with Gal*f* antigen present on galactomannoproteins was reduced in strain Δ*gfsA*, suggesting that *gfsA* is involved in the synthesis of Gal*f* antigen (Fig. 2B). EB-A2 reactivity towards purified WscA protein, an *O*-glycosylated protein, was also reduced following disruption of *gfsA* (Fig. 4D). In addition, the results of *in-vitro* assays provide evidence that purified GfsA protein has Gal*f* antigen synthase activity towards WscA protein and GMPΔ*gfsA*, using UDP-Gal*f* as a sugar donor (Fig. 5D and E). However, GfsA does not show activity towards GMPΔ*ugmA* (Fig. 6B, lane 4). Based on the structure of Gal*f* antigen in *O*-glycans (Fig. 1), at least two galactofuranosyltransferases appear to be involved in the synthesis of Gal*f* antigen; one forms a β1,6-linkage between Gal*f* and the α1,6-mannobiose backbone, and the other forms β1,5-Gal*f* linkages between Gal*f* residues at the non-reducing termini. As the latter reaction requires a Gal*f* residue as an acceptor substrate for catalysis, galactomannnoproteins extracted from Δ*ugmA* strain cannot serve as a substrate for this

enzyme. Therefore, it is highly likely that GfsA is a novel β1,5-galactofuranosyltransferase involved in *O*-glycan biosynthesis. However, details studies of the type of linkage for the Gal*f* residue and the substrate structure of GfsA are warranted.

Aspergillus nidulans strains Δ*ugmA* and Δ*ugtA* exhibit defects in colony growth, hyphal morphogenesis and conidiation (El-Ganiny *et al*., 2008; Afroz *et al*., 2011). Strain Δ*gfsA* exhibits defects similar to those of Δ*ugmA* (Fig. 1A), including abnormal cell morphology, poor hyphal extension, hyphal curvature and limited conidia formation (Figs 8A and B and 9A and B). Similar phenotypes were also observed in strains Δ*ugmA* and Δ*pmtA*, indicating that Gal*f* residues in *O*-mannose type glycans are required for normal cell morphology (Oka *et al*., 2004; El-Ganiny *et al*., 2008). If GfsA is also a galactosyltransferase involved in the synthesis of galactofuran side-chains, our findings also indicate that these side-chains are also required for normal cell morphology.

Genes involved in the Gal*f* biosynthetic pathway are thought to be functionally linked to cell wall formation and maintenance (El-Ganiny *et al*., 2008; 2010; Afroz *et al*., 2011). The growth defect of strain Δ*gfsA* was not observed under conditions of high temperature and hyperosmolarity, a finding that is also reported for mutants with defects in cell wall integrity (Horiuchi *et al*., 1999; Oka *et al*., 2004; Goto *et al*., 2009; Futagami *et al*., 2011). The molecular mechanisms that control the ability of *Aspergillus* spp. to maintain cell wall integrity under stress have not been fully elucidated. Nevertheless, it is reasonable to speculate that in strain Δ*gfsA*, the triggering of stress responses, such as those normally induced by heat and hyperosmotic stress, might help to compensate for the loss of Gal*f* antigen in cell wall *O*-glycans. Strain Δ*gfsA* shows sensitivity to Congo red (30 μg ml^{-1}), an inhibitor of glucan synthesis, but is resistant to calcofluor white (30 μg ml^{-1}), an inhibitor of chitin synthesis. These results imply that the balance of cell wall components is altered in strain Δ*gfsA*, perhaps due to the reduction in Gal*f* antigen and/or an increase in chitin levels. El-Ganiny reported that the abnormal hyphal phenotype that is observed in the *A. nidulans* Δ*ugmA* strain under normal growth conditions can be partially suppressed by growth on medium containing 10 μg ml^{-1}, but not 30 μg ml^{-1}, calcofluor white (El-Ganiny *et al*., 2008). This finding is different from that observed for strain Δ*gfsA*, suggesting that the function of the Gal*f* antigen of *O*-glycans differs from those of other antigens, including the galactofuran side-chain, *N*-glycan and glycosylinositolphosphoceramide, at least as they relate to resistance or sensitivity to calcofluor white.

Disruption of *ugmA* leads to a complete loss of galactofuranose residues in the cell, because UDP-Gal*f*, which is the sugar donor for all galactofuranosyltransferases, is not synthesized. In contrast, galactofuranose residues were detected in strain Δ*gfsA* (Fig. 2C), providing further evidence that GfsA protein is a galactofuranosyltransferase that can transfer galactofuranose from UDP-Gal*f* to *O*-glycans. Consistent with this speculation, the defects observed in strain Δ*gfsA* were less severe than those observed for Δ*ugmA* (Fig. 2A), demonstrating that other galactofuranose residues, in addition to *O*-glycans, are also important for normal cell growth.

We also found that galactomannnoproteins extracted from Δ*gfsA* are weakly recognized by EB-A2, even though no signal was detected with proteins from ΔugmA (Fig. 3D, lane 2; Fig. 4C, lane 5). A signal was seen for WscA purified from the Δ*gfsA* mutant because a larger number of WscA molecules were present on the gel compared to individual galactomannoproteins (Fig. 4C, lane 5). This observation indicates that galactofuranosyltransferase activity remained in the Δ*gfsA* cells. However, galactofuranosyltransferase activity was not detected in the solubilized protein fraction extracted from Δ*ugmA*Δ*gfsA* (Fig. S4, lane 3). Although these results are at first contradictory, they suggest that GfsA plays a major role in the transfer of galactofuranose residue to *O*-glycans in cells and that the conditions of our *in-vitro* assay were not suitable to detect the remaining galactofuranose activity in strain ΔugmA.

The *gfsA* gene was identified among candidate genes selected from the CAZy database and belongs to the GT31 family, which includes galactosyltransferases such as glycoprotein-*N*-acetylgalactosamine-β1,3-galactosyltransferase (beta3GalT1) (Bardoni *et al*., 1999), hydroxyproline-*O*-galactosyltransferase (Basu *et al*., 2013) and glycosaminoglycan-β1,3-galactosyltransferase (Zhou *et al*., 1999). In the *A. nidulans* genome, there are seven genes encoding GT31 family proteins (*AN2015*, *AN4824*, *AN5663*, *AN7535*, *AN8627*, *AN11144* and *AN11697*) in addition to *gfsA*. Of these genes, *AN2015* and *AN5663* appear to be the most closely related to *gfsA*. Thus, it is likely that these genes are also involved in the addition of Gal*f* residues to *O*-glycans. These genes will be examined in future experiments in an effort to identify additional galactofuranosyltransferases involved in the synthesis of Gal*f*-containing polysaccharides and glycoconjugates.

Fungal-type galactofuran side-chains of galactomannan are β1,5-linked Gal*f* oligomers composed of up to five residues. Galactofuran side-chains are linked to α1,2-mannan by β1,6- or β1,3-linkages (Latgé *et al*., 1994). In yeast, the mannosyltransferase Mnn1 is responsible for the synthesis of both *O*-glycans and the outer chain of *N*-glycans (Lussier *et al*., 1999). Because the structure of galactofuran side-chains is similar to the structure of *O*-glycans (Fig. 1), we cannot exclude the possibility that GfsA, and/or its putative paralogues, have the ability to biosynthesize fungal-type galactofuran side-chains of galactomannan.

Genes related to *gfsA* are widely distributed in the subphylum Pezizomycotina of Ascomycota, but are not found in the subphyla Saccharomycotina or Taphrinomycotina (Table S3). In contrast, basidiomycete fungi, including *Cryptococcus neoformans* and *Ustilago maydis*, have both *ugmA/glfA* and *ugtA/glfB* orthologues, but no orthologue of *gfsA* (Table S3) (Beverley *et al*., 2005). Consistent with this finding, no binding of EB-A2 to exopolysaccharides from *C. neoformans*, *Candida albicans*, *Saccharomyces cerevisiae* or *Rhizopus stolonifer* was observed (Stynen *et al*., 1992; Kappe and Schulze-Berge, 1993). To our knowledge, Gal*f* antigen structures are not found in *C. neoformans*, despite the fact that a non-reducing terminal Gal*f* residue is observed in galactoxylomannans from *C. neoformans* (James and Cherniak, 1992). These observations are consistent with *C. neoformans* having both *ugmA* and *ugtA/glfB* orthologues, but no *gfsA* orthologue. In the phylum Zygomycota, which includes fungi such as *Rhizopus oryzae* RA 99-880, no genes involved in Gal*f* synthesis have been detected, including *gfsA* orthologues (Table S3). It seems likely that fungal species containing the *gfsA* gene would also have both *ugmA/glfA* and *ugtA/glfB*, as the products of these genes act upstream of GfaA in the Gal*f* synthesis pathway.

The Pezizomycotina subphylum contains fungi pathogenic to humans, animals and plants. It has been suggested that Gal*f*-containing polysaccharides and glycoconjugates from these fungi are related to virulence and infection (Pedersen and Turco, 2003; Latgé, 2009; Tefsen *et al*., 2012). Moreover, in the present study, we found that strain Δ*AfgfsA* of the opportunistic human pathogen *A. fumigatus* exhibits a small-colony phenotype and has increased sensitivity to antifungal treatments. These characteristics suggest that GfsA activity might be an appropriate new target for antifungal treatment (Fig. 11A and D). We anticipate that our findings will stimulate functional investigation into the medical and biotechnological implications of Gal*f*-containing polysaccharides and glycoconjugates.

Experimental procedures

Microorganisms and growth conditions

The *A. nidulans* and *A. fumigatus* strains used in this study are listed in Table S2. *A. nidulans* strain AKU89 was previously described (Goto *et al*., 2009). *A. fumigatus* A1160 and A1151 (da Silva Ferreira *et al*., 2006) were obtained from the Fungal Genetics Stock Center (FGSC). Strains were grown on minimal medium [1% (w/v) glucose, 0.6% (w/v) $NaNO_3$, 0.052% (w/v) KCl, 0.052% (w/v) $MgSO_4{\cdot}7H_2O$, 0.152% (w/v) KH_2PO_4, biotin (trace) and Hunter's trace elements, pH 6.5] (Barratt *et al*., 1965), complete medium (CM) with appropriate supplements for nutritional markers as described previously (Kaminskyj, 2001), YG medium (0.5% yeast extract and 1% glucose) or MEA medium (2% malt extract, 2% glucose, 0.1% peptone and 2% agar, pH 6.0). Growth experiments to allow hyphal development in submerged culture were begun by inoculation of 100 ml minimal medium with 2×10^7 conidia in 500 ml culture flasks. The flasks were shaken at 126 r.p.m. at 30°C for *A. nidulans* and 37°C for *A. fumigatus*. Standard transformation procedures for *Aspergillus* strains were used (Yelton *et al*., 1984). Plasmids were amplified in *E. coli* JM109 or XL1-Blue.

Construction of deletion cassettes and gene disruption strains

Aspergillus nidulans AKU89, *A. fumigatus* A1160 and *A. fumigatus* A1151 were used as wild-type (wt) control strains (Table S2). The *pyrG* gene was amplified by PCR using *A. nidulans* FGSC26 genomic DNA as template and primers AnpyrG–F and AnpyrG–R (Table S4). The amplified fragments were inserted into the EcoRV sites of pGEM-5Zf(+) using the pGEM-T Easy Vector System (Promega, Madison, WI) to yield pSH1. Genes were disrupted in *A. nidulans* by *argB* or *ptrA* insertion. *AfgfsA* was disrupted in strain A1160 by *pyrG* insertion, and *glfA* and *AfgfsA* were disrupted in strain A1151 by *ptrA* insertion. DNA fragments for gene disruption were constructed using a 'double-joint' PCR method, as described previously (Yu *et al*., 2004). All PCR was performed using Phusion High-Fidelity DNA Polymerase (Daiichi Pure Chemicals, Tokyo, Japan). Primers used in this study are listed in Table S4. Eight primers (from ANxxxx-1 to ANxxxx-8 for *A. nidulans* genes or from AFUB_xxxxxx-1 to AFUB_xxxxxx-8 for *A. fumigatus* genes; xxxx or xxxxxx indicates the systematic gene name) were used to construct deletion cassettes. The 5′- and 3′-flanking regions (approximately 0.9–1.2 kb each) of each gene were PCR amplified from genomic DNA with primer pairs ANxxxx-1/ANxxxx-2 or AFUB_xxxxxx-1/AFUB_xxxxxx-2 and ANxxxx-3/ANxxxx-4 or AFUB_xxxxxx-3/AFUB_xxxxxx-4 respectively (Table S4). The *argB*, *pyrG* and *ptrA* genes used as selective markers were amplified using plasmids pDC1 (Aramayo *et al*., 1989, obtained from the FGSC), pSH1 and pPTR-I (Takara Bio, Otsu, Japan), respectively, as template and the primer pairs ANxxxx-5/ANxxxx-6 or AFUB_xxxxxx-5/AFUB_xxxxxx-6. The three amplified fragments were purified and mixed and a second PCR was performed without specific primers, as the overhanging chimeric extensions act as primers, to assemble each fragment. A third PCR was performed with the nested primer pairs ANxxxx-7/ANxxxx-8 or AFUB_xxxxxx-7/AFUB_xxxxxx-8 and the products of the second PCR as template to generate the final deletion construct. The amplified final deletion constructs were purified by QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) and used directly for transformation. Transformants were grown on minimal medium plates containing 0.6 M KCl as an osmotic stabilizer under appropriate selection conditions and single colonies were isolated twice before further analysis.

Disruption of target genes was confirmed by PCR with the primer pairs ANxxxx-1/argB-R and argB-F/ANxxxx-4 or ANxxxx-1/ptrA-R and ptrA-F/ANxxxx-4 for *A. nidulans*, AFUB_xxxxxx-1/pyrG-R and pyrG-F/AFUB_xxxxxx-4 for *A. fumigatus* A1160, and AFUB_xxxxxx-1/ptrA-R and ptrA-F/AFUB_xxxxxx-4 for *A. fumigatus* A1151. Disruption of *gfsA* in transformants was confirmed by Southern blotting. The 5′-UTR of *gfsA* was amplified by PCR with the primer pair

gfsA-probe-F/gfsA-probe-R. The amplified fragment was labelled with digoxigenin and then used as a probe.

To construct an *argB*$^+$ derivative of the *A. nidulans* parent strain AKU89, the entire *argB* gene was amplified from genomic DNA of *A. nidulans* FGSC26 by PCR with the primer pair argB-RESCUE-F and argB-RESCUE-R (Table S4). The 2.5 kb amplified fragment was purified using a QIAquick Gel Extraction Kit (Qiagen) and used directly for transformation to generate strain AKUARG89.

Construction of strains ΔgfsA-AnwscA and ΔugmA-AnwscA

Aspergillus nidulans AKU89-AnwscA (Goto *et al*., 2009) was used as a parental strain for the construction of strains Δ*gfsA*-AnwscA and Δ*ugmA*-AnwscA (Table S2). The *gfsA* and *ugmA* genes were disrupted in AKU89-AnwscA by *ptrA* insertion. Eight primers [from AN8677-1 (*gfsA*::*ptrA*) to AN8677-8 (*gfsA*::*ptrA*) or AN3112-1 (*ugmA*::*ptrA*) to AN3112-8 (*ugmA*::*ptrA*)] were used to construct a deletion cassette. DNA fragments for gene disruption were constructed using a double-joint PCR method (described above). Disruption of *gfsA* and *ugmA* was confirmed by PCR with the primer pairs AN8677-1 (*gfsA*::*ptrA*)/ptrA-R and ptrA-F/AN8677-4 (*gfsA*::*ptrA*) or AN3112-1 (*ugmA*::*ptrA*)/ptrA-R and ptrA-F/AN3112-4 (*ugmA*::*ptrA*).

Purification of WscA-HA protein

HA-tagged WscA was purified from strains AKU89-AnwscA, Δ*gfsA*-AnwscA and Δ*ugmA*-AnwscA that were grown in 100 ml minimal medium at 30°C for 24 h and harvested by filtration. The cells (1 g wet cells) were ground in liquid nitrogen with a mortar and pestle, and the lysed cells were resuspended in buffer B (50 mM HEPES-NaOH (pH 6.8), 100 mM NaCl, 30 mM KCl and 1% Triton X-100). After cell debris was removed by centrifugation at 3000 *g* for 10 min, 20 μl of rabbit polyclonal anti-HA-Tag-agarose (Medical & Biological Laboratories, Nagoya, Japan) was added to the supernatant and the mixture was gently shaken for 3 h. The anti-HA-Tag-agarose was collected by centrifugation at 1400 *g* for 10 min, and was then washed five times with 1.5 ml buffer B.

Amplification, cloning and sequencing of gfsA cDNA

The *gfsA* cDNA was amplified by PCR using a 24 h developmental cDNA library from *A. nidulans* that was constructed by R. Aramayo (obtained from the FGSC) as a template and the primers AN8677-cDNA-F/AN8677-cDNA-R (Table S4). The amplified cDNA fragments were inserted into the EcoRV sites in pGEM-5Zf(+) using the pGEM-T Easy Vector Systems (Promega) to yield pTOGFSA. The sequence of *gfsA* was confirmed using an Applied Biosystems 3130*xl* Genetic Analyser (Life Technologies, Carlsbad, CA).

Extraction of galactomannoproteins

Galactomannoproteins were extracted according to the method described by Chiba and Jigami (2008). *Aspergillus* cells were grown in minimal medium at 30°C or 37°C for 24 h. The cells were harvested by filtration, washed twice with distilled water, resuspended in 100 mM citrate buffer (pH 7.0), and then autoclaved at 121°C for 120 min. After cell debris was removed by filtration, the extracted galactomannoproteins were precipitated by ethanol, dialysed with dH_2O and then lyophilized. The protein concentration of the purified galactomannoproteins was measured based on absorbance at 280 nm (A_{280}).

Determination of D*-galactopyranose content of galactomannoproteins*

The extracted galactomannoproteins (2.4 μg) were lyophilized, incubated in 4 M TFA at 100°C for 4 h, and then dried at room temperature. The hydrolysates were labelled with fluorescent *p*-aminobenzoic acid ethyl ester (ABEE) using an ABEE Labelling Kit (J-Oil Mills, Tokyo, Japan) according to the manufacturer's protocol. Protein levels were measured using a Qubit protein assay kit (Life Technologies). ABEE labelled D-galactopyranose was analysed using a high-performance liquid chromatography system equipped with a Honenpak C18 column (4.6 mm × 75 mm) (J-Oil Mills). D-galactose (Sigma Aldrich, St. Louis, MO, USA) was used as a standard for quantification.

Release of O*- and* N*-glycans from extracted galactomannoproteins*

To release *N*-glycan, galactomannoproteins (72 μg) were treated with PNGase F (New England Biolabs, Ipswich, MA) according to the manufacturer's protocol. To release *O*-glycan from cell wall proteins, β-elimination was performed using the GlycoProfile Beta-Elimination Kit (Sigma-Aldrich) according to the manufacturer's protocol. The β-elimination reaction was performed at 25°C for 24 h.

Construction of an expression vector for gfsA

pPTR-II (Takara Bio), which is an autonomously replicating plasmid containing the *AMA1* sequence (Kubodera *et al*., 2002), was used an expression vector for *gfsA*. The *gfsA* gene was amplified by PCR using *A. nidulans* genomic DNA as template and the primers gfsA-In-fusion-F and gfsA-In-fusion-R. The amplified fragments were inserted into the SmaI sites of pPTR-II using an In-Fusion™ Advantage PCR Cloning Kit (Clontech Laboratories, Mountain View, CA) to yield pPTR-II-gfsA. *A. nidulans* strain Δ*gfsA* was transformed with pPTR-II-gfsA or pPTR-II. Transformants were selected on minimal medium supplemented with 0.1 mg ml^{-1} pyrithiamine and 0.6 M KCl. For each construct, three different transformants were randomly selected for subsequent experiments.

Construction of GfsA-3xFLAG-expressing strain

All PCR was performed using Phusion High-Fidelity DNA Polymerase (Daiichi Pure Chemicals). A 3xFLAG-tagged DNA fragment was synthesized by PCR without template

using the primer pair 3xFLAG-F/3xFLAG-R. The amplified fragments were inserted into the SphI sites of pDC1 to yield pDC1-3xFLAG. The open reading frame and upstream promoter of *gfsA* were amplified from genomic DNA by PCR with the primer pair AN8677-3xFLAG-1/AN8677-3xFLAG-2. Similarly, the terminator regions of the *gfsA* gene were amplified with the primer pair AN8677-3xFLAG-3/AN8677-3xFLAG-4. The *argB* gene, including the 3xFLAG tag, was amplified with the primer pair AN8677-3xFLAG-5/AN8677-3xFLAG-6 using plasmid pDC1-3xFLAG as template. The three DNA fragments were mixed and a second PCR was performed with the nested primers AN8677-3xFLAG-7/AN8677-3xFLAG-8 to generate the final DNA construct for chromosomal tagging. The final amplified deletion constructs were purified using a QIAquick Gel Extraction Kit (Qiagen) and used directly for transformation.

Construction of strain GfsA-3xFLAG (ΔugmA)

The *ugmA* gene was disrupted in *A. nidulans* GfsA-3xFLAG (Table S2) by *ptrA* insertion. Eight primers [from AN3112-1 (*ugmA*::*ptrA*) to AN3112-8 (*ugmA*::*ptrA*)] were used to construct a deletion cassette. DNA fragments for gene disruption were constructed using the double-joint PCR method (described above). Disruption of *ugmA* was confirmed by PCR with the primer pairs AN3112-1 (*ugmA*::*ptrA*)/ptrA-R and ptrA-F/AN3112-4 (*ugmA*::*ptrA*).

Preparation of solubilized protein

Cells from GfsA-3xFLAG (Δ*ugmA*), Δ*ugmA* or Δ*ugmA*Δ*gfsA* were collected by centrifugation and were then ground in liquid nitrogen with a mortar and pestle. The lysed cells were resuspended in buffer A [50 mM HEPES-NaOH (pH 6.8), 100 mM NaCl, 30 mM KCl, 1 mM $MnCl_2$ and 5% glycerol (w/v)]. All procedures were performed on ice or at 4°C. Cell debris was removed by centrifugation at 10 000 *g* for 10 min, and the obtained supernatants were further centrifuged at 100 000 *g* for 30 min. The resultant pellet was resuspended in buffer A with 0.2% CHAPSO (Dojindo Laboratories, Kumamoto, Japan). To obtain solubilized membrane proteins, the sample was centrifuged at 100 000 *g* for 30 min after stirring for 1 h.

Purification of GfsA-3xFLAG protein

GfsA protein purified using anti-FLAG beads from strain GfsA-3xFLAG (Δ*ugmA*) was used as a source of enzyme. A protein sample purified from strain Δ*ugmA* using the same procedure was used as a negative control. The strains GfsA-3xFLAG (Δ*ugmA*) and Δ*ugmA* were grown in 2 l minimal medium at 30°C for 24 h and then harvested by filtration. The solubilized proteins were prepared from 25 g of wet cells as described above. Mouse-IgG-agarose (100 μl; Sigma-Aldrich) was added to the supernatant and the mixture was gently shaken for 1 h. Mouse-IgG-agarose was removed by centrifugation at 1400 *g* for 10 min. A total of 200 μl anti-FLAG M2 affinity gel (Sigma-Aldrich) was then added to the supernatant and the resultant mixture was gently shaken for 1 h. Anti-FLAG M2 affinity gel was collected by centrifugation at 1400 *g* for 10 min and then washed five times with 50 ml buffer A containing 0.2% CHAPSO. GfsA protein was then eluted with 200 μl buffer A with 0.2% CHAPSO containing 0.5 μg μl^{-1} 3xFLAG peptide (Sigma-Aldrich). The eluted proteins obtained from strains GfsA-3xFLAG (Δ*ugmA*) and Δ*ugmA* were designated GfsA-3xFLAG and control respectively. The protein concentration was determined using the bicinchoninic acid protein assay reagent (Thermo Fisher Scientific, Waltham, MA) with bovine serum albumin as a standard.

In-vitro *assay of galactofuranose antigen synthase activity*

UDP-Gal*f* was synthesized and then purified by HPLC, as previously described (Oppenheimer *et al*., 2010). UDP-Gal*f* was used as a sugar donor and purified WscA-HA, galactomannoproteins from Δ*gfsA* (GMPΔ*gfsA*), or galactomannoproteins from Δ*ugmA* (GMPΔ*ugmA*) were used as acceptor substrates. Gal*f* antigen synthase was assayed in a reaction mixture containing 25 mM HEPES-NaOH (pH 6.8), 50 mM NaCl, 15 mM KCl, 0.5 mM $MnCl_2$, 2.5% glycerol (w/v), 0.72 mM UDP-Gal*f* acceptor substrate, and 14 μg purified GfsA-3xFLAG protein in a total volume of 10 μl. For the assay, 2.5 μg GMPΔ*gfsA*, GMPΔ*ugmA* or 0.25 μg purified WscA-HA was used as an acceptor substrate. The mixture was incubated at 37°C and the reaction was stopped by incubation at 99°C for 5 min. The reaction products were analysed by immunoblotting (described below).

Antibodies

The following antibodies were used for immunoblotting. Anti-BipA antibodies were described previously (Goto *et al*., 2004). Anti-FLAG M2 and anti-UgtA/GlfB antibodies were purchased from Sigma-Aldrich. Anti-UgtA/GlfB antibody was raised by subcutaneous immunization of a rabbit with a synthetic peptide (CEPTLPTVNPAVDKPEPPK) conjugated to KLH, and the serum was tested using an enzyme-linked immunosorbent assay. Anti-FLAG M2 mouse monoclonal antibody (1:5000; Sigma-Aldrich), anti-HA F-7 mouse monoclonal antibody (1:5000; Santa Cruz Biotechnology, Santa Cruz, CA), anti-γ-actin monoclonal antibody clone C4 (1:200; MP Biomedicals, LLC., Santa Ana, CA), UgtA/GlfB (1:1000) and BipA (1:200) were used as primary antibodies. Anti-mouse IgG conjugate HRP (Santa Cruz Biotechnology) was used to detect anti-FLAG M2; anti-γ-actin and anti-UgtA/GlfB was used to detect UgtA/GlfB; and anti-rabbit IgG conjugate HRP (Santa Cruz Biotechnology) was to detect anti-BipA. EB-A2 antibody, which is one of the components of Platelia Aspergillus EIA (Bio-Rad Laboratories, Hercules, CA), was used at a dilution of 1:10 to detect β-Gal*f* antigen. All secondary antibodies were used at a dilution of 1:5000.

Immunoblotting analysis

Galactomannoproteins were separated by SDS-PAGE and then transferred to a polyvinylidene fluoride (PVDF) membrane using an AE-6677 electroblotter (Atto, Tokyo, Japan) at 100 mA for 1.5 h. After incubation of the membrane for 1 h in

a blocking buffer containing 10 mM phosphate (pH 7.4), 4% skim milk (Wako Pure Chemical Industries, Osaka, Japan), 0.1% Tween 20 and 0.9% (w/v) NaCl, the membrane was transferred to a 5 ml solution of primary antibody. The membrane was incubated for 12 h at 4°C, washed three times with a buffer containing 10 mM phosphate (pH 7.4), 0.1% Tween 20 and 0.9% (w/v) NaCl for a total of 30 min, and then incubated for 1 h with secondary antibody. An ECL Prime Western Blotting Detection System (GE Healthcare, Little Chalfont, UK) was used to visualize the immunoreactive proteins. Chemical fluorescent signals on the membrane were recorded using a MicroChemi imaging system (Berthold Technologies, Bad Wildbad, Germany). Imaging of the ECL-treated membranes was stopped before saturation of the signals.

Colony growth rate determination

Colony growth rates were measured as described previously (Kellner and Adams, 2002). Briefly, conidia from each strain were point-inoculated into the centre of agar medium plates. Colony diameters were measured after 24, 48, 72, 96 and 120 h of incubation at 30°C. The growth rates were determined for each colony in millimetres per hour during each of the incubation intervals, i.e. 24–48, 48–72, 72–96 and 96–120 h, and were then averaged across the entire time interval. Measurements of growth rates for all individual strains were performed 20 times.

Analysis of the efficiency of conidiation

The efficiency of conidiation was analysed as described previously (Oka *et al*., 2004). Briefly, approximately 10^5 conidia were spread onto a minimal medium plate (90 mm diameter). After 5 days of incubation at 30°C, the conidia were suspended in 5 ml of 0.01% (w/v) Tween 20 and counted using a haemocytometer.

Microscopy

Hyphae cultured in liquid minimal medium were observed using a Fluoview FV10i confocal laser-scanning microscope (Olympus, Tokyo, Japan). The mycelia were harvested and incubated with 10 ng ml^{-1} Fluorescent Brightener 28 (calcofluor white; Sigma-Aldrich) for 10 min. Conidiophores were observed using a VE-8800 scanning electron microscope (Keyence, Osaka, Japan).

Transcription analysis of gfsA

For RNA extraction from mycelia, conidia (2×10^7) of *A. nidulans* wt strain (AKUARG89) were inoculated into 100 ml liquid minimal medium and were then grown for 18, 24, 36 and 48 h at 30°C. For RNA extraction, approximately 2×10^5 conidia were spread onto an 84 mm agar plate of minimal medium. After a 3-day incubation at 30°C, conidia were collected and then ground for 90 s at 2000 r.p.m. with a Multi-Beads Shocker (Yasui Kikai, Osaka, Japan). RNA was extracted using RNAiso PLUS (Takara Bio) according to the manufacturer's protocol and was then quantified using a Quant-iT RNA Assay Kit (Life Technologies). cDNA was synthesized from the RNA using a PrimeScript Perfect Real-Time Reagent kit (Takara Bio) according to the manufacturer's protocol. Real-time reverse transcription (RT)-PCR analysis was performed using a LightCycler Quick 330 system (Roche Diagnostics) with SYBR Premix DimerEraser (Perfect Real Time; Takara Bio). The following primers were used: gfsA-RT-F and gfsA-RT-R for *gfsA*, histone-RT-F and histone-RT-R for the histone H2B, argB-RT-F and argB-RT-R for *argB*, and spoC1-C1C-RT-F and spoC1-C1C-RT-R for *spoC1-C1C* (Table S4). The histone H2B gene was used to standardize the mRNA levels of the target genes (Fujioka *et al*., 2007).

Organelle separation

Organelles were separated on sucrose gradients as described previously (Engel *et al*., 2009). Briefly, *A. nidulans* cells were grown in minimal medium at 30°C for 24 h. All manipulations were performed on ice or at 4°C. Cells were harvested and ground in liquid nitrogen with a mortar and pestle. The lysed cells were suspended at 0.5 ml g^{-1} in 50 mM MOPS-NaOH, pH 7.0. Cell debris was removed by centrifugation at 3000 *g*. A discontinuous gradient was formed by sequentially adding 2.4 ml each of 55% sucrose, 50% sucrose, 45% sucrose, 40% sucrose and 35% sucrose. A total of 1.7 ml of supernatant was then loaded onto the upper 35% sucrose solution, and the tube was centrifuged at 100 000 *g* for 18 h, and a total of 16 fractions were collected. Specific membrane fractions were identified by immunoblotting using antibodies against organelle-specific markers.

Sensitivity testing

To assay the sensitivity of *A. fumigatus* to antifungal treatment, Etests for micafungin and voriconazole was performed using the appropriate test strips (Sysmex bioMérieux, Lyon, France) on MEA plates. For the assay, conidia were harvested, washed twice with distilled water, and then counted using a haemocytometer. Conidia (6×10^5) were then evenly spread and test strips were placed in the centre of the plate before incubation at 37°C for 24 h.

Software and database searches

The structure of GfsA was analysed using the SOSUI tool (http://bp.nuap.nagoya-u.ac.jp/sosui/) (Hirokawa *et al*., 1998). For similarity searches, we used the Multi-fungi BLAST program (http://www.broadinstitute.org/annotation/genome/FGI_Blast/Blast.html) and the AspGD Multi-Genome Search using NCBI BLAST+ (http://www.aspgd.org/cgi-bin/compute/blast_clade.pl) (Altschul *et al*., 1997).

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