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GfsA is a β1,5-galactofuranosyltransferase involved in the biosynthesis of the galactofuran side chain of fungal-type galactomannan in *Aspergillus fumigatus*

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Abstract

Previously, we reported that GfsA is a novel galactofuranosyltransferase involved in the biosynthesis of *O*-glycan, the proper maintenance of fungal morphology, the formation of conidia and anti-fungal resistance in *Aspergillus nidulans* and *A. fumigatus* (Komachi Y et al., 2013. GfsA encodes a novel galactofuranosyltransferase involved in biosynthesis of galactofuranose antigen of O-glycan in Aspergillus nidulans and Aspergillus fumigatus. *Mol. Microbiol.* 90:1054–1073). In the present paper, to gain an in depth-understanding of the enzymatic functions of GfsA in *A. fumigatus* (AfGfsA), we established an in vitro assay to measure galactofuranosyltransferase activity using purified AfGfsA, UDP-α-D-galactofuranose as a sugar donor, and *p*-nitrophenyl-β-D-galactofuranoside as an acceptor substrate. LC/MS, ^{1}H-NMR and methylation analyses of the enzymatic products of AfGfsA revealed that this protein has the ability to transfer galactofuranose to the C-5 position of the β-galactofuranose residue via a β-linkage. AfGfsA requires a divalent cation of manganese for maximal activity and consumes UDP-α-D-galactofuranose as a sugar donor. Its optimal pH range is 6.5–7.5 and its optimal temperature range is 20–30°C. ^{1}H-NMR, ^{13}C-NMR and methylation analyses of fungal-type galactomannan extracted from the Δ*AfgfsA* strain revealed that AfGfsA is responsible for the biosynthesis of β1,5-galactofuranose in the galactofuran side chain of fungal-type galactomannan. Based on these results, we conclude that AfGfsA acts as a UDP-α-D-galactofuranose: β-D-galactofuranoside β1,5-galactofuranosyltransferase in the biosynthetic pathway of galactomannans.

Key words: *Aspergillus*, cell wall, galactofuranose, galactomannan, glycosyltransferase

Introduction

Polysaccharides and glycoconjugates are present in the plasma membrane, cell wall, and extracellular environment of filamentous fungi. These polysaccharides form the three-dimensional structure of the cell wall and play an important role in many biological events (de Groot et al. 2009). Filamentous fungal cell walls contain several polysaccharides, including β1,3-, β1,3-/β1,4- and α1,3-linked glucans, chitin, galactosaminogalactan and galactomannan (GM), which is composed of D-mannose and D-galactofuranose (Gal_f) residues, as well as *O*- and *N*-glycans linked to proteins (de Groot et al. 2009). Of these polysaccharides, β1,3- and α1,3-linked glucans and chitin are common components of budding and/or fission yeast, and filamentous fungi. The glycosyltransferase genes involved in the synthesis of these components have been identified and characterized in filamentous fungi based on a previous study conducted on yeast (de Groot et al. 2009). Galactosaminogalactan and GM are specific to filamentous fungi. Research on the glycosyltransferase genes involved in these components has been slow to develop, although it seems that oligosaccharides are important for the physiological functioning of filamentous fungi. Recently, the genes involved in the synthesis of galactosaminogalactan, including putative glycosyltransferase genes, have been identified and characterized in *Aspergillus fumigatus* (Bamford et al. 2015; Sheppard and Howell 2016). We recently reported that *gfsA* encodes a novel galactofuranosyltransferase in *A. nidulans* and *A. fumigatus* (Komachi et al. 2013).

Gal_f functions as a component of glycans and glycolipids in a limited range of organisms, including eubacteria, some protists, *Caenorhabditis* species, Basidiomycota and some Ascomycota. Gal_f residues are immunogenic in mammals and are often associated with virulence in pathogenic fungi. They are therefore considered an important virulence factor in fungi and their biosynthesis is used as a target for anti-fungal agents without side effects (Bernard and Latgé 2001; Goto 2007; Gastebois et al. 2009; Latgé 2009; Jin 2012; Tefsen et al. 2012b; Oka and Goto 2016).

In *A. fumigatus*, Gal_f residues have been found to be linked to four different types of glycan structure: *N*-glycans, in which there is usually one Gal_f residue per chain (Morelle et al. 2005; Engel et al. 2009); *O*-glycans, which have one to eight Gal_f-chains, which we refer to as *O*-mannose-type galactomannan (OMGM) (Leitao et al. 2003; Kudoh et al. 2015); glycosylinositolphosphorylceramide (GIPC), with one or two Gal_f-compounds (Toledo et al. 2007; Simenel et al. 2008); and fungal-type GM (FTGM), which is a polysaccharide composed of a main α1,2/α1,6-mannan chain and a number of side chains of β1,5/β1,6-linked Gal_f residues (Latgé et al. 1994; Latgé 2009; Kudoh et al. 2015). FTGM is found in three forms: soluble and released in a medium; cross-linked to other cell-wall polysaccharides; and in membranes, anchored to a glycosylphosphatidylinositol (GPI) (Costachel et al. 2005; Latgé 2009; Kudoh et al. 2015). OMGM is attached to the hydroxyl groups of the serine or threonine residues of proteins. The initial reaction in the transfer of mannose to serine or threonine residues is catalyzed in the endoplasmic reticulum (ER) by protein-*O*-D-mannosyltransferase (Pmt), which requires dolichol phosphate-mannose as an immediate sugar donor in *Aspergillus* spp. (Shaw and Momany 2002; Oka et al. 2004; Oka et al. 2005; Goto et al. 2009; Mouyna et al. 2010).

Few reports are available on the enzymes involved in the initiation, extension, and termination of GMs. However, several enzymes involved in the biosynthesis of uridine diphosphate-α-D-galactofuranose (UDP-Gal_f), which is required for the transferring activity of galactofuranosyltransferase, have been studied in fungi (Tefsen et al. 2012b; Oka and Goto 2016). UDP-glucose 4-epimerase (Uge5/UgeA) is responsible for the conversion of UDP-glucose to UDP-galactopyranose (Gal_p) (El-Ganiny et al. 2010; Lee et al. 2014; Park et al. 2014), which is then converted to UDP-Gal_f by UDP-Gal_p mutase (GlfA/UgmA) 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 subsequently transported into the Golgi lumen by the Golgi-localized UDP-Gal_f transporter (GlfB/UgtA) (Engel et al. 2009; Afroz et al. 2011). There are two UDP-Gal_f-transporters, UgtA and UgtB, in *A. niger*. Deletion analysis of single and double mutants in this species indicates that UgtA and UgtB perform the same fuction with respect to UDP-Gal_f-transport, since only the double mutant displayes a Gal_f-negative phenotype. (Park et al. 2015). These genes are required for GM biosynthesis and are involved in hyphal growth, colony morphology, conidiation, and anti-fungal compound resistance, suggesting that Gal_f residues play an important role in cell-wall integrity and pathogenicity in *Aspergillus* spp. Several β-galactofuranosidases belonging to the GH2, GH51, and GH54 families have been identified, and these orthologs exist in the *A. fumigatus* genome (Tefsen et al. 2012a; Matsunaga et al. 2015; Matsunaga et al. 2017). It is possible that these β-galactofuranosidases affect the structural variation of GM.

Recently, we showed that GfsA in *A. nidulans* (AnGfsA) can transfer a Gal_f residue from UDP-Gal_f to galactomannoproteins or the WscA protein, one of the *O*-glycosylated proteins localized at the cell surface, extracted from the Δ*AngfsA* strain (Futagami et al. 2011; Komachi et al. 2013). At least three galactofuranosyltransferases appear to be involved in the synthesis of GMs; the first forms a (β1,3, β1,6 and/or β1,2)-Gal_f linkage between α-mannose residues, the second forms a β1,5-Gal_f linkage between β-Gal_f residues, and the third forms a β1,6-Gal_f linkage between β-Gal_f residues. The type of linkage catalyzed by GfsA in *A. fumigatus* (AfGfsA) and the substrate sugar it prefers are not clear. In addition, it is not clear whether AfGfsA is responsible for the biosynthesis of the galactofuran side chains of FTGM.

In this study, we constructed a vector for AfGfsA expression in *Escherichia coli* and established an in vitro assay to detect galactofuranosyltransferase activity using purified AfGfsA, UDP-Gal_f as a sugar donor, and *p*-nitrophenyl-β-D-galactofuranoside as an acceptor substrate. LC/MS, ^{1}H-NMR (nuclear magnetic resonance) and methylation analyses of the enzymatic products of AfGfsA revealed that it has the ability to transfer the Gal_f to the C-5 position of the β-Gal_f residue via the β-linkage. In addition, we used the assay to determine the enzymatic properties of AfGfsA. Furthermore, we demonstrated that AfGfsA is responsible for the biosynthesis of β1,5-Gal_f in the galactofuran side chains of FTGM.

Results

Features of GfsA in *Aspergillus fumigatus*

The *gfsA* gene in *A. fumigatus* (*AfgfsA*) encodes a 537-amino acid protein with a putative molecular mass of 61.3 kDa (Supplementary data, Fig 1A). A comparison of the cDNA and genome sequences revealed that no introns were present in the *AfgfsA* gene, similar to the *gfsA* gene in *A. nidulans* (*AngfsA*). Analysis of the secondary structure using TMHMM (Krogh et al. 2001) revealed that AfGfsA has a transmembrane domain (amino acids 20–42) at the *N*-terminus, suggesting that AfGfsA is a type-II membrane protein that is localized at the Golgi apparatus, similar to AnGfsA. In addition, AfGfsA has a metal-binding DXD motif (amino acids 260–262), which is conserved among Gfs proteins from other organisms, and three potential *N*-glycosylation sites (amino acids 97–99, 417–419 and 418–420).

Expression and purification of AfGfsA in *Escherichia coli*

In previous experiments conducted by our group, we constructed a strain expressing 3xFLAG-tagged AnGfsA (using chromosomal tagging) and a purified AnGfsA-3xFLAG protein from the solubilized membrane fraction. The purified AnGfsA-3xFLAG protein displayed galactofuranosyltransferase activity towards WscA-HA, an *O*-glycosylated sensor protein, purified from the Δ*AngfsA* strain (Komachi et al. 2013; Oka et al. 2014). However, we could not obtain a sufficient quantity of the AnGfsA-3xFLAG protein to investigate the function and properties of GfsAs using this approach. We therefore attempted to obtain a recombinant AfGfsA protein using the *E. coli* expression system. The gene encoding the soluble form of AfGfsA lacking an *N*-terminal region (amino acids 1–42), including both a putative signal sequence and a transmembrane domain, was introduced into an *E. coli* expression vector fused in frame with a 6xhistidine (6xHis) tag at the *N*-terminus. The AfGfsA was analyzed using SDS-PAGE (Supplementary data, Fig 1B). AfGfsA was visualized as a band of approximately 60 kDa, similar to the expected molecular weight of 58.0 kDa for AfGfsA lacking an *N*-terminal region. Purification of 1 L of the culture yielded approximately 1.5 mg of the AfGfsA protein.

Enzymatic function of AfGfsA

We previously described a galactofuranosyltransferase activity assay of the AfGfsA protein that involves using a monoclonal antibody (EB-A2) that binds Gal$_f$ residues with a high specificity (Komachi et al. 2013). Since GM contains β1,5/β1,6-galactofuranoses attached to the β-Gal$_f$ residue and β1,2/β1,3/β1,6-galactofuranoses attached to the α-mannose residue, we predicted that AfGfsA is either a β-galactofuranoside β1,5/β1,6-galactofuranosyltransferase, or a α-mannopyranoside β1,2/β1,3/β1,6-galactofuranosyltransferase. Because of the type of linkage catalyzed by AfGfsA, it is necessary to perform a galactofuranosyltransferase assay using a chemically defined compound and to directly determine the linkage of the enzymatic products. Consequently, we attempted to assay galactofuranosyltransferase activity using the chemically synthesized substrate *p*-nitrophenyl-β-D-galactofuranoside (*p*NP-β-D-Gal$_f$) and UDP-Gal$_f$ as a sugar donor. The galactofuranosyltransferase activity of the purified AfGfsA protein was assayed using UDP-Gal$_f$, 1 mM Mn^{2+} as a cofactor, and 1 mM *p*NP-β-D-Gal$_f$. The reacted mixture was analyzed using HPLC with an amino column. The products were detected using UV_{260} absorbance. The fraction containing heat-inactivated AfGfsA did not exhibit any new peaks. In contrast, the fractions containing AfGfsA displayed two new peaks (defined as *peak A* and *peak B*) at 18.0 and 21.5 min, suggesting that *peak A* and *peak B* are enzymatic reaction products (defined as *product A* and *product B*; Figure 1A). When other potential sugar nucleotides [uridine diphosphate 5′-α-D-galactopyranose (UDP-Gal$_p$) or guanosine 5′-α-D-mannopyranose (GDP-Man)] or potential acceptor substrates [*p*-nitrophenyl-β-D-galactopyranoside (*p*NP-β-D-Gal$_p$) or *p*-nitrophenyl-α-D-mannopyranoside (*p*NP-α-D-Man)] were used in the assay instead of UDP-Gal$_f$ or *p*NP-β-D-Gal$_f$, no new peaks were generated (Figure 1B and C). To determine the chemical structures of *product A* and *product B*, we collected the peaks for the substrate, *p*NP-β-D-Gal$_f$, and for *product A* and *product B*, and analyzed their molecular masses using LC/MS (Figure 2A). They were detected as compounds with respective *m/z* values of 346.1, 508.1 and 670.2 $[M+HCOO]^-$ (Figure 2A). The 346.1-*m/z* peak is identical to the molecular weight of verified *p*NP-β-D-Gal$_f$ (Figure 2A), and the 508.1-*m/z* peak is consistent with the theoretical molecular weight of Gal$_f$-Gal$_f$-D-β-*p*NP, suggesting that *product A* is Gal$_f$-Gal$_f$-D-β-*p*NP. In addition, the 670.2-*m/z* peak is consistent with the theoretical molecular weight of Gal$_f$-Gal$_f$-Gal$_f$-D-β-*p*NP, suggesting that *product B* is Gal$_f$-Gal$_f$-Gal$_f$-D-β-*p*NP.

To further determine the chemical structure, we collected approximately 500 μg of *product A* (Gal$_f$-Gal$_f$-D-β-*p*NP) using HPLC, and analyzed the sample using ^{1}H-NMR. The signals for *product A* agreed well with the reported chemical shift values for the terminal β1,5-linked Gal$_f$ residue with the β-Gal$_f$ structure (Figure 2B). The chemical shift values of the H-1 position of the underlined Gal$_f$ residue in the Gal$_f$β1→benzoic acid and Gal$_f$β1→5Gal$_f$→ structures are 5.805 and 5.226, respectively, according to previous reports (Varela et al. 1986; Kudoh et al. 2015). A chemical shift signal was detected at 5.805 ppm in the ^{1}H-NMR charts of both *p*NP-β-D-Gal$_f$ and *product A* (Figure 2B), but a signal at 5.226 ppm was only detected in the ^{1}H-NMR chart of *product A*. To obtain further evidence of the glycosidic linkage, we attempted a methylation analysis of *product A*. A 500-μg sample of *product A* was methylated, and then hydrolyzed using 0.15 M trifluoroacetic acid. The resultant sample was analyzed by GC/MS, and *p*NP-β-D-Gal$_f$ was used as the control (Figure 2C). The retention times for t-Gal$_f$→, 5-Gal$_f$1→ and 6-Gal$_f$1→ were 16.36, 18.40 and 19.56 min, respectively, under these analysis conditions (Shibata et al. 2009; Kudoh et al. 2015). Both *p*NP-β-D-Gal$_f$ and *product A* displayed a peak at 16.36 min (Figure 2C), indicating that they had terminal Gal$_f$ residues. Only *product A* had a peak at 18.40 min but not at 19.56 min (Figure 2C, *right panel*), indicating that the second Gal$_f$ residue was attached to the C-5 position of the first Gal$_f$ residue. The chemical structure of *product A* was therefore identified as Gal$_f$β1→5 Gal$_f$-D-β-*p*NP, indicating that the AfGfsA protein catalyzes a reaction that transfers the D-Gal$_f$ residue from UDP-Gal$_f$ to the C-5 position of the Gal$_f$ residue in *p*NP-β-D-Gal$_f$ via the β-linkage. In brief, AfGfsA protein acts as UDP-D-Gal$_f$: β-D-galactofuranoside β1,5-galactofuranosyltransferase.

Enzymatic properties of AfGfsA

The biochemical properties of AfGfsA were determined using established in vitro assays. In these assays, one enzyme unit corresponds to the generation of 1 nmol Gal$_f$β1→5 Gal$_f$-D-β-*p*NP/min/μg. We first determined the optimal temperature and pH ranges for AfGfsA activity. The optimal temperature was 20°C, although the enzyme appeared to be relatively active across a broad temperature range (20–30°C; Figure 3A). With respect to pH, the greatest enzyme activity was observed in 100 mM MOPS-NaOH buffer, pH 7.0, although activity was generally high for the range pH 6.5–7.5 (Figure 3B). Next, we studied the effect of several divalent cations (Mn^{2+}, Co^{2+}, Ca^{2+}, Mg^{2+} and Zn^{2+}) on this enzyme. Enzyme activity was enhanced by the addition of Mn^{2+}, Ca^{2+} or Mg^{2+}, with Mn^{2+} eliciting the greatest response, whereas it was almost zero in the presence of Zn^{2+} (Figure 3C).

Involvement of AfGfsA in GM biosynthesis in vivo

It was not clear whether AfGfsA is responsible for the biosynthesis of the galactofuran side chains of FTGM. Because the structure of these galactofuran side chains is similar to that of *O*-glycans, we predicted that AfGfsA also has the ability to biosynthesize them (Komachi et al. 2013). To test this hypothesis, we performed structural analyses of FTGM extracted from the Δ*AfgfsA* strain. First, we obtained a strain whose *AfgfsA* mutation was complemented, where a copy of the

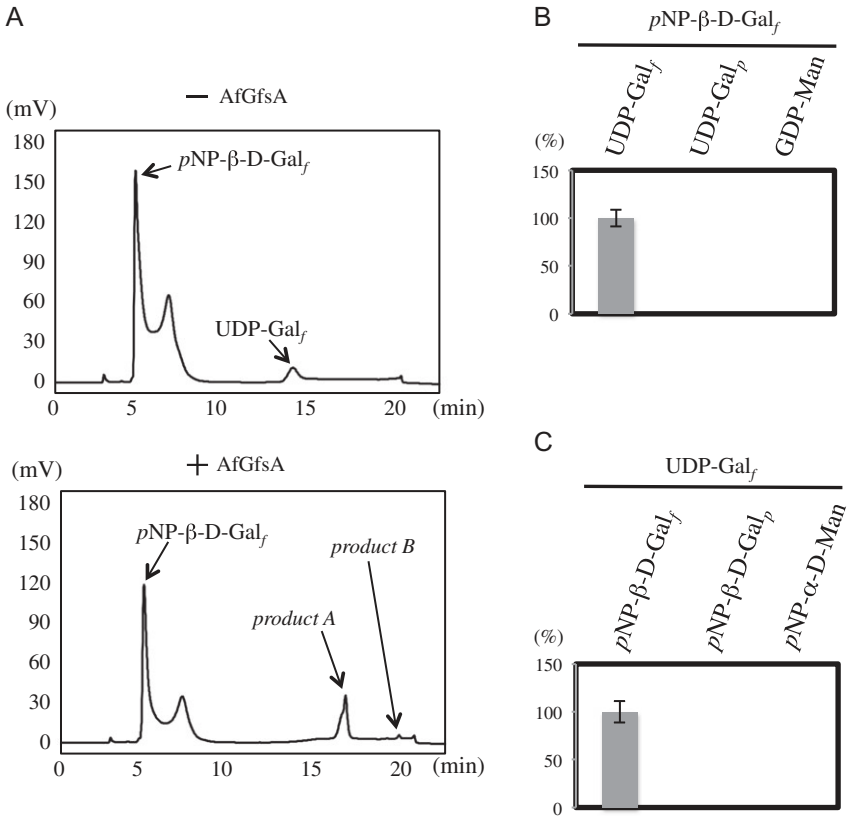

Fig. 1. Construction of in vitro assay for galactofuranosyltransferase activity of AfGfsA. (**A**) Chromatograms of in vitro assay of galactofuranosyltransferase activity. The galactofuranosyltransferase activity of 5 μg of purified AfGfsA protein was assayed using 0.5 mM UDP-Gal$_f$, 1 mM Mn^{2+} as a cofactor, and 1 mM *p*NP-β-D-Gal$_f$. The mixtures were incubated at 30°C for 6 h. Chromatograms indicate typical results of the assay without (upper panel) and with AfGfsA (lower panel). The assay without AfGfsA showed no new peaks, but fractions with AfGfsA contained two new products (termed *product A* and *product B*) at 18.0 and 21.5 min. (**B**) Determination of appropriate sugar nucleotides. Each assay used 0.1 mM of a sugar nucleotide, *p*NP-β-D-Gal$_f$ (1 mM) as the acceptor substrate, and 0.82 μg of purified AfGfsA protein. The mixtures were incubated at 30°C for 60 min. A value of 100% corresponds to the incorporation of 2.3×10^{-3} units (nmol/min/μg) of UDP-Gal$_f$. Data are presented as mean ± SD ($n = 3$ independent experiments). (**C**) Determination of appropriate acceptor substrates. Each assay used 1 mM of an acceptor substrate, 0.1 mM UDP-Gal$_f$ as the sugar donor, and 0.82 μg of purified AfGfsA protein. The mixtures were incubated at 30°C for 60 min. A value of 100% corresponds to the incorporation of 3.3×10^{-3} units (nmol/min/μg) of *p*NP-β-D-Gal$_f$. Data are presented as mean ± SD ($n = 3$ independent experiments).

whole *AfgfsA* gene was integrated into the Δ*AfgfsA* locus (Figure 4A). The correct integration of the expression cassette was confirmed using PCR (Figure 4B). Δ*AfgfsA* exhibits temperature sensitivity (Komachi et al. 2013), which was restored in the Δ*AfgfsA* strain expressing *AfgfsA* (Figure 4C). The colony growth rates of the WT, Δ*gfsA*, and Δ*AfgfsA*::*AfgfsA* strains were 0.35 ± 0.055, 0.24 ± 0.054, and 0.33 ± 0.018 mm/h, respectively. In addition, Gal$_f$ antigens in galactomannoproteins were detected by immunoblotting analysis with EB-A2, a monoclonal antibody that binds Gal$_f$ residues with high specificity (Figure 4D). In the Δ*AfgfsA* strain expressing *AfgfsA*, the EB-A2 immunodetection signal intensity was recovered to some extent, although it was weaker than WT levels (Figure 4D).

To identify the effect of gene disruption on the structure of the GMs, we analyzed GM structure using GM purified by cetyl trimethyl ammonium bromide (CTAB) precipitation with a boric acid buffer. The GMs designated as FTGM+OMGM contain both FTGM and OMGM. These purified FTGM+OMGMs were analyzed by ^{13}C- and ^{1}H-NMR spectroscopy (Figure 5A and C). The chemical shift signals at 5.195 ppm and 5.05 ppm of the ^{1}H-NMR spectra represent the H-1 signal of the underlined Gal$_f$ residue in the respective Gal$_f$β1→5Gal$_f$β1→5Gal$_f$1 (β1,5-Gal$_f$) and Gal$_f$β1→5Gal$_f$β1→6Gal$_f$1 (β1,6-Gal$_f$) structures, according to previous reports (Shibata et al. 2009; Kudoh et al. 2015). The H-1 signals of the chemical shift of the α1,2-mannan backbone appear from 5.0–5.1 ppm (Kudoh et al. 2015) in the ^{1}H-NMR charts. The intensity of the β1,5-Gal$_f$ signal was 4.1-fold higher than that of the mannan backbone in the ^{1}H-NMR chart for WT-FTGM+OMGM. In addition, the intensity of the β1,5-Gal$_f$ signal was 1.5-fold higher than that of the mannan backbone in the ^{1}H-NMR chart for Δ*gfsA*-FTGM+OMGM (Figure 5A). Furthermore, the intensity of the β1,5-Gal$_f$ signal in the ^{1}H-NMR chart for Δ*gfsA*-FTGM+OMGM was approximately one-third of the WT-FTGM+OMGM value, despite the comparable intensity of the mannan-related signals of WT-FTGM+OMGM and Δ*gfsA*-FTGM+OMGM, indicating that β1,5-Gal$_f$ was reduced in Δ*gfsA*-FTGM+OMGM. This defect was recovered in the complementary strain.

The signals at 107.87 ppm and 108.70 ppm represent the C-1 chemical shift of the underlined Gal$_f$ residues in the respective Gal$_f$β1→5 Gal$_f$β1→5 Gal$_f$1 (β1,5-Gal$_f$) and Gal$_f$β1→5 Gal$_f$β1→6 Gal$_f$1 (β1,6-Gal$_f$) structures, according to previous reports (Shibata and Okawa 2011; Kudoh et al. 2015). Both of these signals were detected in the ^{13}C-NMR chart for WT-FTGM+OMGM. The signal intensity of β1,5-Gal$_f$ was stronger than that of β1,6-Gal$_f$ in the ^{13}C-NMR chart for WT-FTGM+OMGM. In contrast, the signal intensity of β1,5-Gal$_f$

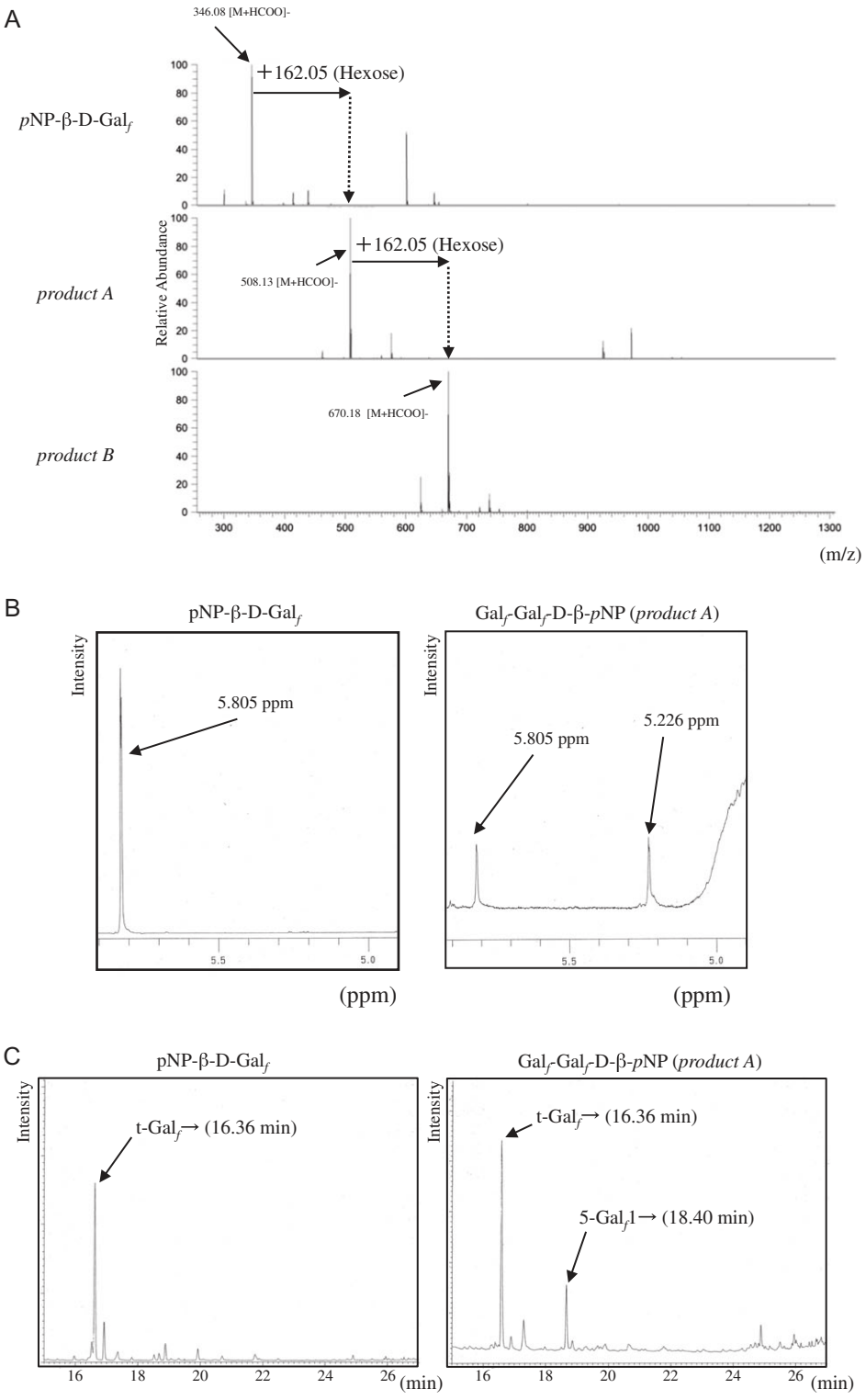

Fig. 2. Structural analyses of the enzymatic products of AfGfsA using *p*NP-β-D-Gal$_f$ as an acceptor. (**A**) LC/MS spectra of *product A* and *product B*. *Product A* and *product B* were collected from 17.5 to 18.5 min and from 21 to 22 min, respectively, as shown in Figure 3A (lower panel), and analyzed using LC/MS to determine their molecular masses. (**B**) ^{1}H-NMR analyses of *p*NP-β-D-Gal$_f$ and Gal$_f$-Gal$_f$-D-β-*p*NP (*product A*). ^{1}H-NMR charts for *p*NP-β-D-Gal$_f$ (left panel) and *product A* (right panel). The chemical shift values of the H-1 position of the underlined Gal$_f$ residue in the Gal$_f$β1→benzoic acid and Gal$_f$β1→5Gal$_f$→ structures are 5.805 and 5.226, respectively, according to previous reports (Varela et al. 1986; Kudoh et al. 2015). The 5.805 ppm signal was detected in the ^{1}H-NMR chart for *p*NP-β-D-Gal$_f$. Both signals were detected in the ^{1}H-NMR chart for Gal$_f$-Gal$_f$-D-β-*p*NP (*product A*). (**C**) Methylation analyses of *p*NP-β-D-Gal$_f$ and Gal$_f$-Gal$_f$-D-β-*p*NP (*product A*). A 500-μg sample of *product A* was analyzed. The retention times for t-Gal$_f$→, 5-Gal$_f$1→ and 6-Gal$_f$1→ were 16.36, 18.40, and 19.56 min, respectively, under these analysis conditions.

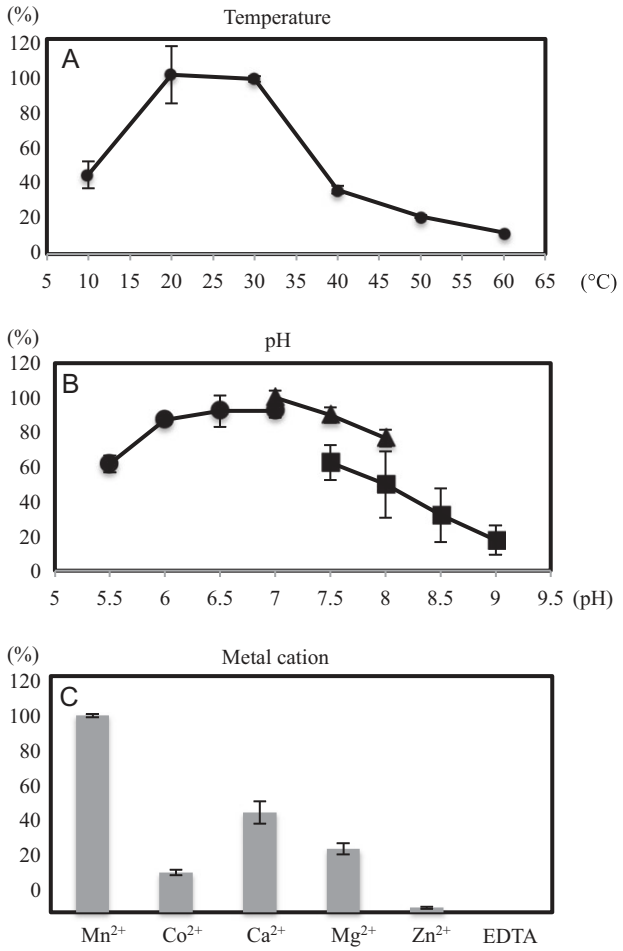

Fig. 3. Determination of optimal temperature and pH range and the metal cation requirements for AfGfsA activity. (**A**) Effects of temperature on AfGfsA enzymatic activity. The buffer used was 100 mM MOPS-NaOH (pH 7.0). A value of 100% corresponds to the incorporation of 6.0×10^{-3} units (nmol/min/μg) at 20°C. Each assay used 1 mM of an acceptor substrate, 0.1 mM UDP-Gal$_f$ as the sugar donor, and 0.82 μg of purified AfGfsA protein. The mixtures were incubated for 60 min. (**B**) Effects of pH on AfGfsA enzymatic activity. The buffers used were 100 mM MES-NaOH (circles), 100 mM MOPS-NaOH (triangles), or 100 mM Tris-HCl (squares). A value of 100% corresponds to the incorporation of 3.8×10^{-3} units (nmol/min/μg) of 100 mM MOPS-NaOH at pH 7.0. Each assay used 1 mM of an acceptor substrate, 0.1 mM UDP-Gal$_f$ as the sugar donor, and 0.82 μg of purified AfGfsA protein. The mixtures were incubated at 30°C for 60 min. (**C**) Metal cation requirements for AfGfsA activity. Reaction mixtures were incubated with 1 mM EDTA or 1 mM various divalent metals. A value of 100% corresponds to the incorporation of 6.3×10^{-3} units (nmol/min/μg) of manganese. Each assay used 1 mM of an acceptor substrate, 0.1 mM UDP-Gal$_f$ as the sugar donor, and 0.82 μg of purified AfGfsA protein. The mixtures were incubated at 30°C for 60 min. All data are presented as mean ± SD (n = 3 independent experiments).

was inverted in the ^{13}C-NMR chart for ΔAfgfsA-FTGM+OMGM, but was recovered in the ^{13}C-NMR chart for ΔAfgfsA::AfgfsA-FTGM+OMGM.

To verify whether GfsA is involved in the synthesis of FTGM, the extracted FTGM+OMGMs were subjected to β-elimination. The β-eliminated FTGM+OMGMs, designated as FTGMs, were also analyzed by ^{13}C- and ^{1}H-NMR spectroscopy (Figure 5B and D). The FTGMs charts were similar to those of the FTGM+OMGMs for both ^{1}H-NMR and ^{13}C-NMR. The signal intensity of β1,5-Gal$_f$ was 6.1-fold higher than that of the mannan backbone in the ^{1}H-NMR chart for WT-FTGM. The intensity of the ^{1}H-chemical shift of β1,5-Gal$_f$ was 1.9-fold higher than that of the mannan backbone in the ^{1}H-NMR chart for Δ*gfsA*-FTGM (Figure 5B), indicating that β1,5-Gal$_f$ was reduced in the ^{1}H-NMR chart for Δ*gfsA*-FTGM. This defect was recovered in the complementary strain. The signal intensity of β1,5-Gal$_f$ was stronger than that of β1,6-Gal$_f$ in the ^{13}C-NMR chart for WT-FTGM. In contrast, the intensity of the chemical shift of β1,5-Gal$_f$ was inverted in the ^{13}C-NMR chart of ΔAfgfsA-FTGM, but was recovered in the ^{13}C-NMR chart for ΔAfgfsA::AfgfsA-FTGM.

GC/MS analyses of O-methylalditol acetates derived from methylation analyses of FTGM+OMGMs and FTGMs were performed. Table I shows the methylation analysis results of the FTGM+OMGMs and FTGMs obtained from the WT, Δ*AfgfsA*, and Δ*AfgfsA::AfgfsA* strains. The ratio of the 5-O-substituted galactofuranose residue (5-Gal$_f$) of Δ*AfgfsA*-FTGM+OMGM (10.75 ± 4.32%) was lower than that of WT-FTGM+OMGM (18.16 ± 3.48%; $P < 0.05$; Table I). More specifically, the 5-Gal$_f$ ratio of Δ*AfgfsA*-FTGM (4.37 ± 0.39%) was lower than that of WT-FTGM (25.73 ± 1.50%; $P < 0.05$; Table I). These results clearly indicate that β1,5-Gal$_f$ was reduced in both Δ*AfgfsA*-FTGM+OMGM and Δ*AfgfsA*-FTGM. AfGfsA therefore acts as a UDP-Gal$_f$: β-galactofuranoside β1,5-galactofuranosyltransferase in the biosynthetic pathway of both OMGM and FTGM in vivo.

Discussion

Despite the discovery of FTGM approximately 50 years ago, it has not yet been clarified which enzyme is responsible for the biosynthesis of its galactofuran side chains. In this study, we showed that AfGfsA acts as a UDP-Gal$_f$: β-galactofuranoside β1,5-galactofuranosyltransferase in the biosynthetic pathway of both OMGM and FTGM in vivo. A few reports on the galactofuranosyltransferases in other organisms are available. The *LPG1* gene of the protist *Leishmania donovani* encodes a putative β1,3-galactofuranosyltransferase, which is required for synthesis of the lipophosphoglycan core (Huang and Turco 1993). In bacteria, *Rv3782* (*glfT1*) and *Rv3808* (*glfT2*) of the tuberculosis bacterium, *Mycobacterium tuberculosis*, have been identified as the genes encoding galactofuranosyltransferases (Belánová et al. 2008; Tam and Lowary 2009). WbbI of the *E. coli* strain K-12 is a UDP-Gal$_f$: β1,6-galactofuranosyltransferase capable of glycosylating octyl α-D-glucopyranoside (Wing et al. 2006). The WbbO protein of *Klebsiella pneumoniae* strain O1 is a bifunctional glycosyltransferase that sequentially adds one α1,3-Gal$_p$ and one β1,3-Gal$_f$ residue from UDP-Gal$_p$ and UDP-Gal$_f$, respectively, to form D-Galactan I, a polysaccharide with the disaccharide repeat unit structure of lipopolysaccharide O antigen (Kos and Whitfield 2010). AfGfsA has no significant amino acid sequence similarity to these previously reported galactofuranosyltransferases, indicating that it employs a different substrate recognition and/or enzymatic mechanism from the galactofuranosyltransferases.

In *M. tuberculosis*, GlfT1 and GlfT2 are known to be involved in the biosynthesis of the arabinogalactan region of the mycolyl-arabinogalactan-peptideglycan complex, an essential component of the mycobacterial cell wall (Belánová et al. 2008; Tam and Lowary 2009). GlfT2 is a bifunctional galactofuranosyltransferase that transfers β1,5-Gal$_f$ and β1,6-Gal$_f$ alternately (Szczepina et al. 2009; Tam and Lowary 2009). In this study, we could not determine the second linkage of *product B*, of which the estimated structure was Gal$_f$-Gal$_f$-Gal$_f$-D-β-*p*NP. We therefore cannot exclude the possibility that the second linkage of *product B* is β1,6-Gal$_f$; in other words, that AfGfsA is also a bifunctional galactofuranosyltransferase that transfers

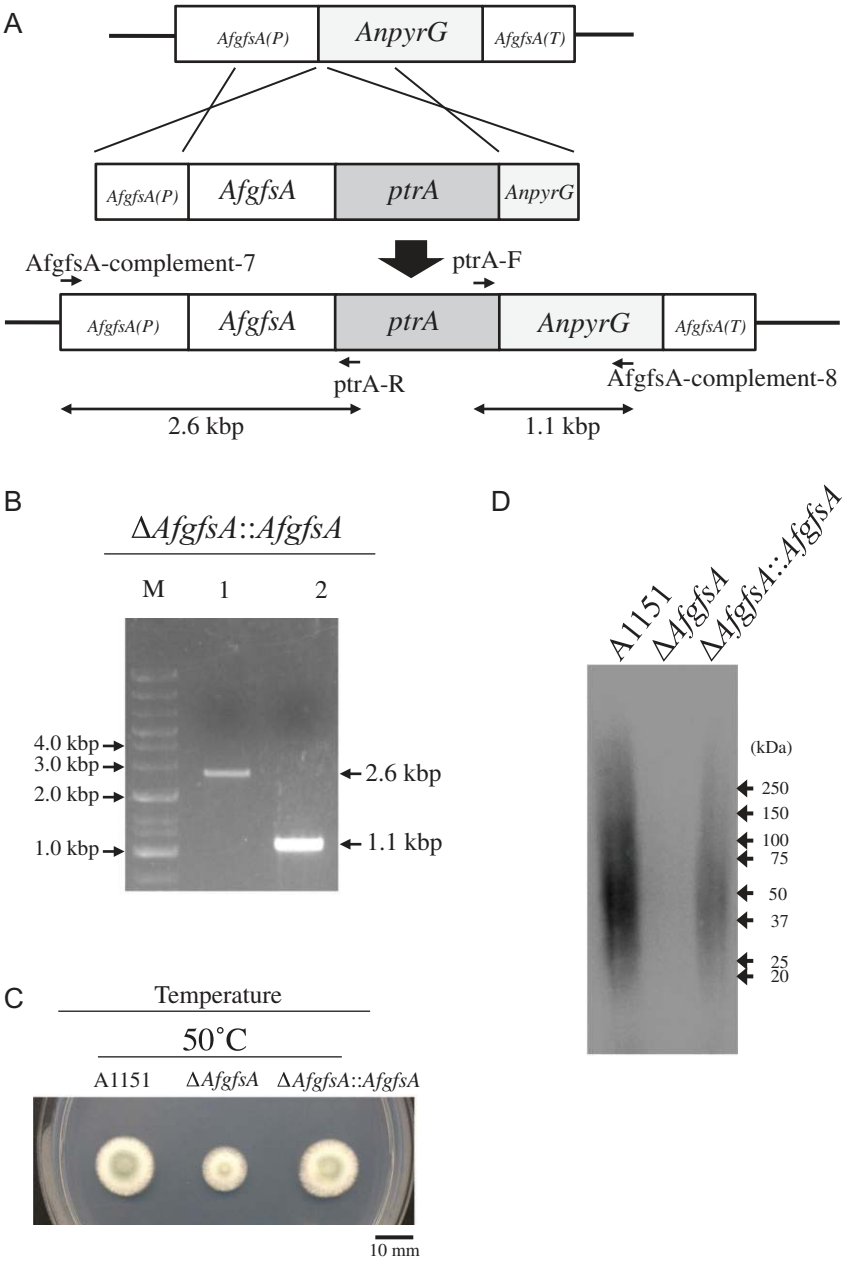

Fig. 4. Construction of the Δ*AfgfsA* complementary strain with *AfgfsA*. (**A**) Schematic representation of Δ*AfgfsA* complementation with *AfgfsA*. *AfgfsA (P)*, *AfgfsA* promoter; *AfgfsA (T)*, *AfgfsA* terminator; *AfgfsA*, open reading frame of *AfgfsA*; *ptrA*, pyrithiamine resistance gene, used as a selective marker. The positions of the primers are indicated by arrows. (**B**) Confirmation of correct recombination of the Af*gfsA* gene using PCR analysis. M, DNA size markers; lane 1, DNA fragment (2.6 kbp) amplified using PCR and the primers AfgfsA-complement-7 and ptrA-R; lane 2, DNA fragment (1.1 kb) amplified using PCR and the primers ptrA-F and AfgfsA-complement-8. (**C**) Colony phenotypic analysis of strains A1151, Δ*AfgfsA*, and Δ*AfgfsA::AfgfsA* at high temperature. Colonies of strains A1151 (left), Δ*AfgfsA* (middle), and Δ*AfgfsA::AfgfsA* (right) are shown. Conidia were incubated on minimal medium at 50°C for 3 days. (**D**) Functional rescue of the Δ*AfgfsA* strain with the *AfgfsA* gene. Immunoblot analysis for galactomannoproteins (1.7 μg per lane) from strain A1151 (left lane), Δ*AfgfsA* (middle lane), and Δ*AfgfsA::AfgfsA* (right lane) using antibody EB-A2. This figure is available in black and white in print and in color at *Glycobiology* online.

β1,5-Gal$_f$ and β1,6-Gal$_f$ alternately, similarly to GlfT2. However, AfGfsA has a very different protein sequence from GlfT2, so it is unlikely that AfGfsA is bifunctional in this way.

We established an in vitro quantitative assay for measuring galactofuranosyltransferase activity using a verified chemically synthesized compound, pNP-β-Gal$_f$, as the acceptor substrate, UDP-Gal$_f$, and recombinant AfGfsA (Figures 1 and 6A). Although the natural substrates of AfGfsA are expected to be [Gal$_f$β1]$_{0–2}$→5 Gal$_f$β1→(2, 3 or 6)]Man→ or [Gal$_f$β1]$_{0–3}$→5Gal$_f$β1→6 Gal$_f$→, based on the structures of FTGM and OMGM, it is difficult to obtain these substrates. We therefore used pNP-β-Gal$_f$ as a mimic acceptor substrate. If these substrates could be obtained, the detailed substrate recognition of AfGfsA could be investigated.

NMR and methylation analyses revealed that the β1,5-Gal$_f$ linkage with the Gal$_f$ residue remained in both Δ*AfgfsA*-FTGM+OMGM and Δ*AfgfsA*-FTGM, indicating that *A. fumigatus* contains other

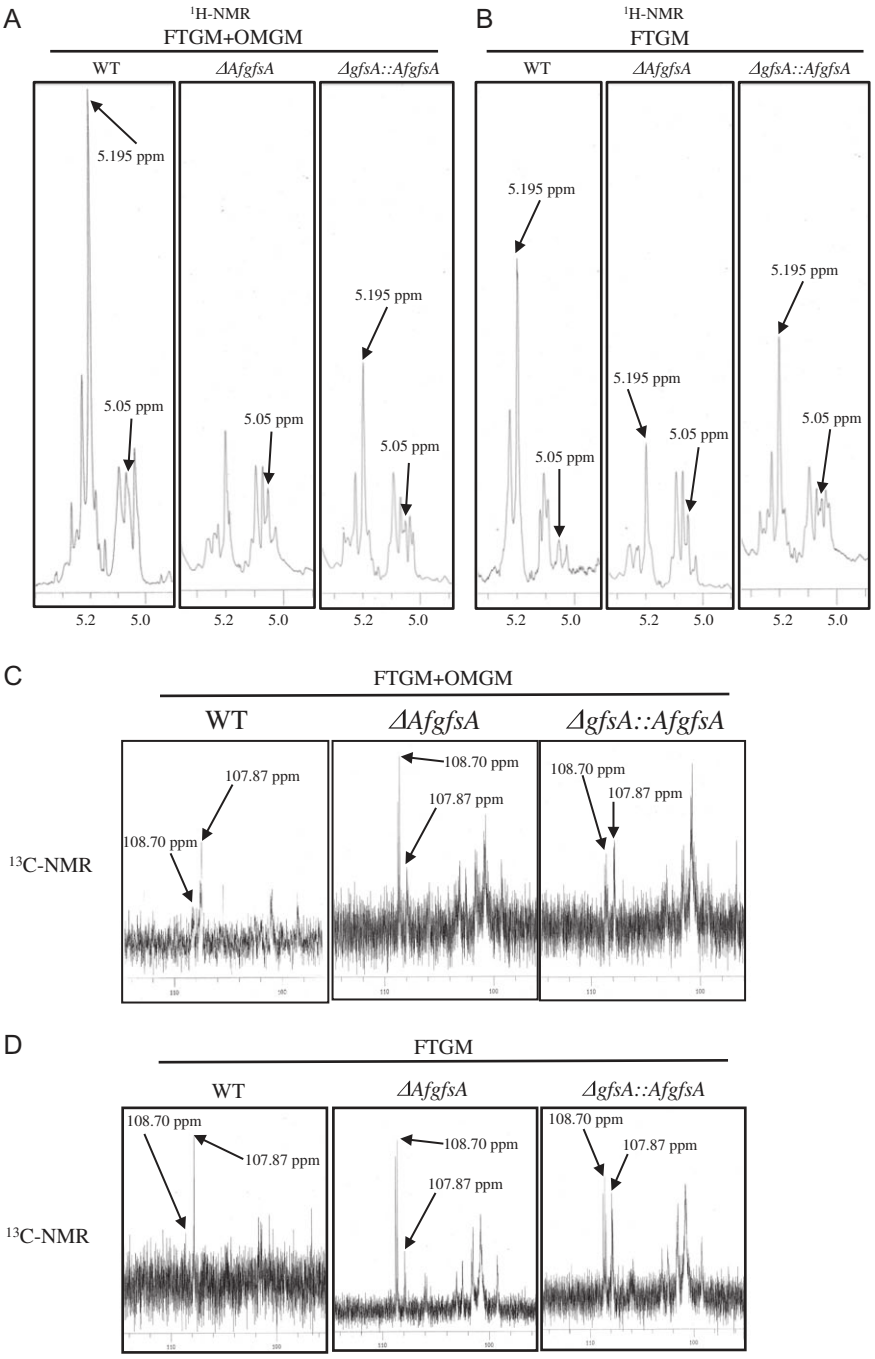

Fig. 5. NMR analyses of purified GMs from the WT, Δ*AfgfsA*, and Δ*AfgfsA::AfgfsA* strains. (**A**) ^{1}H-NMR analysis of FTGM+OMGM. (**B**) ^{1}H-NMR analysis of FTGM. (**C**) ^{13}C-NMR analysis of FTGM+OMGM. (**D**) ^{13}C-NMR analysis of FTGM. OMGM, O-mannose-type galactomannan; FTGM, fungal-type galactomannan; FTGM+OMGM, total GM (FTGM + OMGM). FTGM was prepared by β-elimination from total GM (FTGM+OMGM) treated with 0.5 M $NaBH_4$/0.1 M NaOH for 24 h. The signal at 5.195 ppm of the ^{1}H-NMR spectra is the H-1 signal of the C-1 position of the underlined Gal$_f$ residue in the Gal$_f$β1→5 Gal$_f$β1→5Gal$_f$1 (β1,5-Gal$_f$) structures (Kudoh et al. 2015). The signals at 107.87 ppm and 108.70 ppm represent a C-1 chemical shift of the underlined Gal$_f$ residue in the Gal$_f$β1→5 Gal$_f$β1→5 Gal$_f$1 (β1,5-Gal$_f$) and Gal$_f$β1→5Gal$_f$β1→6Gal$_f$1 (β1,6-Gal$_f$) structures, respectively (Shibata and Okawa 2011; Kudoh et al. 2015). The proton and carbon chemical shifts were referenced relative to internal acetone at δ 2.225 and 31.07 ppm, respectively.

β1,5-galactofuranosyltransferases in its cells (Figure 5, Table I). According to the genome information, *A. fumigatus* has seven putative *gfsA* paralogs with unidentified functions, and the proteins are termed AfGfsB (Afu4g13710/AFUB_070620), AfGfsC (Afu4g10170/AFUB_067290), AfGfsD (Afu6g00520/AFUB_096980), AfGfsE (Afu3g07220/AFUB_041840), AfGfsF (Afu3g00170/AFUB_048270), AfGfsG (Afu2g17320/AFUB_032980) and AfGfsH (Afu8g07260/AFUB_080490) (Oka and Goto 2016). Gfs proteins are phylogenetically classified into two groups: GT31-A (including GfsA) and GT31-B (Oka and Goto 2016). It is expected that these paralogs would be galactofuranosyltransferases that exhibit substrate recognition and form linkages in a manner different from AfGfsA (Oka and Goto 2016).

Table I. GC/MS analysis of *O*-methylalditol acetates derived from methylation analyses of galactomannans

		FTGM+OMGM			FTGM		
O-methylalditol acetate	Sugar linkage	WT	Δ*AfgfsA*	Δ*AfgfsA::AfgfsA*	WT	Δ*AfgfsA*	Δ*AfgfsA::AfgfsA*
2,3,4,6-Me4-Man	t-Man$_p$1→	34.02 ± 3.64	28.40 ± 9.78	19.12 ± 1.75	21.67 ± 1.60	24.62 ± 4.66	24.95 ± 0.762
3,4,6-Me3-Man	2-Man$_p$1→	17.64 ± 3.13	21.34 ± 3.78	15.99 ± 2.46	21.42 ± 1.59	23.28 ± 2.78	21.82 ± 3.920
2,3,4-Me3-Man	6-Man$_p$1→	8.08 ± 2.48	11.67 ± 0.53	14.96 ± 2.03	7.23 ± 0.96	*15.36 ± 1.70	15.78 ± 2.067
3,4-Me2-Man	2,6-Man$_p$1→	7.12 ± 3.57	8.65 ± 1.20	9.47 ± 1.47	12.02 ± 2.01	10.08 ± 0.80	10.05 ± 0.782
2,3,5,6-Me4-Gal	t-Gal$_f$1→	12.66 ± 1.02	14.94 ± 1.60	20.01 ± 0.94	9.27 ± 2.21	*15.46 ± 1.92	13.57 ± 3.001
2,3,6-Me3-Gal	5-Gal$_f$1→	18.16 ± 3.48	*10.75 ± 4.32	16.67 ± 1.27	25.73 ± 1.50	*4.37 ± 0.39	11.79 ± 1.438
2,3,5-Me3-Gal	6-Gal$_f$1→	2.32 ± 0.48	*4.21 ± 0.61	3.78 ± 1.37	2.66 ± 0.49	*6.82 ± 1.64	2.04 ± 0.378

Values presented are means ± SD. *$P < 0.05$.

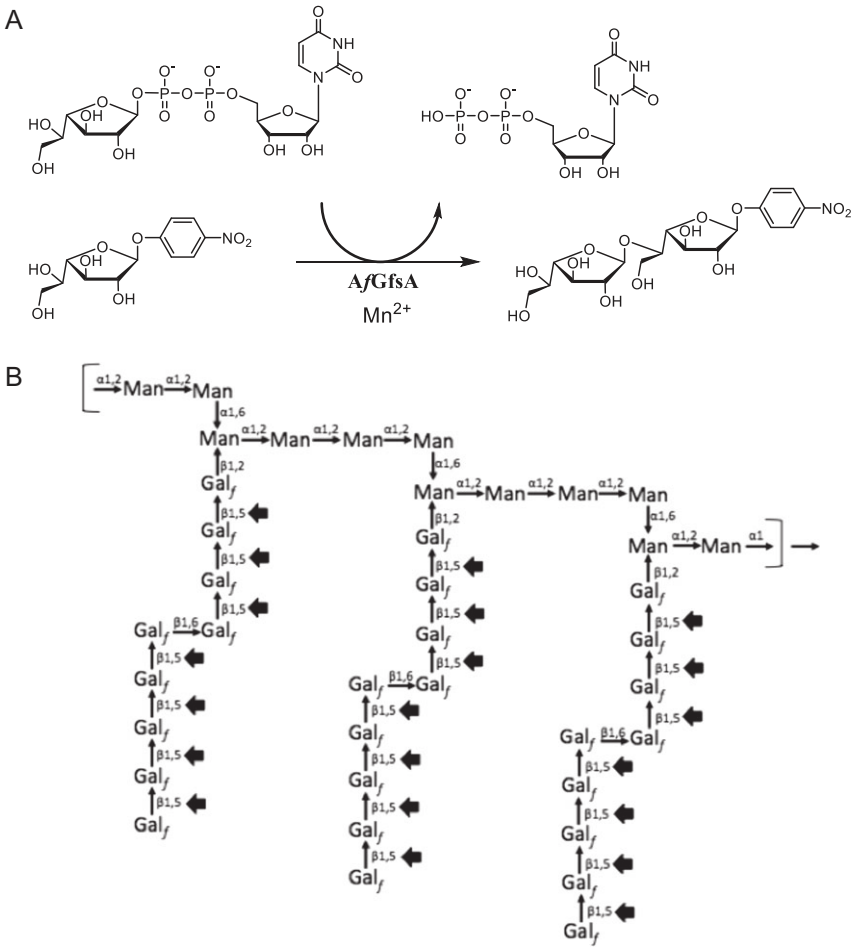

Fig. 6. Schematic of the AfGfsA reaction. (**A**) Schematic of the AfGfsA reaction in the in vitro assay. The AfGfsA protein transfers the D-Gal$_f$ residue from UDP-Gal$_f$ to the C-5 position of the Gal$_f$ residue in *p*NP-β-D-Gal$_f$ via the β-linkage. In brief, AfGfsA protein acts as UDP-D-Gal$_f$ β-D-galactofuranoside β1,5-galactofuranosyltransferase. At least two reactions occur consecutively. Mn^{2+}, divalent manganese cation. (**B**) Schematic of the biosynthesis of fungal-type galactomannan in *Aspergillus fumigatus*. Arrows indicate the potential reaction points of the β1,5-Gal$_f$ linkage catalyzed by AfGfsA in fungal-type galactomannan biosynthesis. Man, mannose; Gal$_f$, galactofuranose; Asn, asparagine.

We predicted that AfGfsB and AfGfsC are β1,5-galactofuranosyltransferases similar to AfGfsA, but that their substrate recognition is different from that of AfGfsA. Methylation analyses of GMs revealed that O-5 substituted galactose is still present in the Δ*AfgfsA* strain, indicating that there is another Gal$_f$-transferase involved in this transfer (Figure 5; Table I). We therefore surmise that either AfGfsB or AfGfsC, or both, are involved in the biosynthesis of β1,5-galactofuranosyl residues. The potential positions catalyzed by AfGfsA are indicated by arrows in Figure 6B; however, it seems unlikely that all of these positions are catalyzed by AfGfsA. It is possible that *A. fumigatus* uses different β1,5-galactofuranosyltransferases at different catalyzing positions and/or under different growth conditions. Further studies of the paralogs allow us to understand the entire biosynthesis process of GMs in *A. fumigatus*.

We previously reported that AnGfsA is localized in the Golgi apparatus, which strongly suggests that AfGfsA is also localized there

(Komachi et al. 2013). The UDP-Gal_f transporter GlfB localizes in the Golgi apparatus membrane of *A. fumigatus* and transports cytosolic UDP-Gal_f into the lumen of the Golgi apparatus, where it is used for GM synthesis (Engel et al. 2009). In addition, FTGM is attached to the nonreducing terminal of *N*-glycans (Kudoh et al. 2015) and bound to a GPI anchor (Costachel et al. 2005). These facts indicate that FTGM is synthesized via a protein transport pathway from the ER to the Golgi apparatus. The biosynthetic pathways of *N*-glycans and the GPI anchor, which occur in the ER, are conserved in eukaryotic cells (Aebi 2013; Kinoshita and Fujita 2016). After the precursor of an *N*-glycan is transferred to the asparagine residues of a protein by an oligosaccharyltransferase, the *N*-glycosylated protein is transported to the Golgi apparatus. The remodeling of the *N*-glycans that occurs in the Golgi apparatus varies among proteins, cells, and species, and the *N*-glycans that are generated are structurally diverse (Aebi 2013). We therefore surmise that FTGM biosynthesis takes place during this remodeling process. The glycan remodeling of a GPI anchor also probably occurs in the Golgi apparatus, and we surmise that FTGM biosynthesis takes place during this remodeling process. However, how this works remains unclear, and further analysis is required. *O*-mannose-type glycan, in combination with a protein, is also transported to the Golgi apparatus, where elongation reactions occur via mannosyltransferases (Lussier et al. 1999). Previously, we have shown how galactofuranosylation happens during the biosynthesis of OMGM (Komachi et al. 2013). We suggest that in *A. fumigatus*, AfGfsA functions in the biosynthesis of the galactofuran side chains of FTGM and of the terminal region of the galactofuranoside in the OMGM of the Golgi apparatus.

GMs are present in many plant and animal pathogens. Analysis of AfGfsA facilitates elucidation of the role of GMs in pathogenicity, as well as of the infection mechanism. It is also expected that the biosynthesis pathway of GMs represents a new target for anti-fungal therapeutics. In this study, we have established an assay for GfsA activity (Figure 1A). In addition, an assay for measuring AfGfsA activity will be available for high-throughput screening of AfGfsA inhibitors that can be used as anti-fungal drugs. However, three-dimensional structural information would be useful for selecting the drug candidates, but crystallization and crystallographic studies will be required to elucidate the 3D structure of AfGfsA. Fortunately, in this study we found that AfGfsA is strongly expressed in *E. coli* and can be purified as a soluble recombinant enzyme, with an average yield of 1.5 mg/mL (Supplementary data, Fig 1B). We estimate that this yield and purity are sufficient for the necessary crystallization and structural studies (Supplementary data, Fig 1B).

Materials and methods

Microorganisms and growth conditions

The *A. fumigatus* strains used in this study are listed in Table II. *A. fumigatus* A1160 and A1151 (da Silva Ferreira et al. 2006) were obtained from the Fungal Genetics Stock Center (FGSC). The 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) $MgSO_4{\cdot}7\,H_2O$, 0.152% (w/v) KH_2PO_4, and Hunter's trace elements, pH 6.5) (Barratt et al. 1965). Standard transformation procedures for *Aspergillus* strains were used (Yelton et al. 1984). Plasmids were amplified in *E. coli* DH5α. The *E. coli* strain Rosetta-gami B (DE3) (Merck Millipore, Darmstadt, Germany) was used for protein expression.

Construction of expression vector for AfGfsA

All PCR reactions were performed using Phusion High-Fidelity DNA Polymerase (Daiichi Pure chemicals, Tokyo, Japan). The pET15b-KAI plasmid for protein expression in *E. coli* was constructed as follows. The PCR reaction was performed using the primers pET15-KAI-F and pET15-KAI-R with pET15b (Merck Millipore) as a template. After *Dpn* I digestion, the amplified fragment was purified and digested by *Bam* HI and self-ligated to yield pET15b-KAI.

Plasmids useful for the expression of *AfgfsA* (Afu6g02120/AFUB_096220) were constructed as follows. Total RNA was extracted from the mycelia of *A. fumigatus* strain A1160, grown in minimal medium for 18 h using TRIzol Reagent (Thermo Fisher Scientific, Waltham, MA) according to the supplier's manual. Single-strand DNA was synthesized by M-MLV Reverse Transcriptase (NIPPON GENE, Tokyo, Japan) using the oligo-dT-18 primer. The *AfgfsA* gene was amplified by PCR using single-strand DNA as a template for the primers pET15b-AfGfsA-NdeI-F and pET15b-AfGfsA-NotI-R. The amplified fragment was inserted into the *Nde* I and *Not* I sites of pET15b-KAI to yield pET15b-AfGfsA. The pET15b-AfGfsA plasmid carrying the *AfgfsA* gene was transformed into the Rosetta-gami B (DE3) cells.

Protein purification, quantification, and electrophoresis

Buffer A (50 mM HEPES-NAOH (pH 7.0), 30 mM KCl, 100 mM NaCl, 1 mM $MnCl_2$, 5% (w/v) glycerol, and the complete protease inhibitor cocktail EDTA-free; Roche Diagnostics, Basel, Switzerland) was used as a running buffer. Rosetta-gami B (DE3) cells harboring the plasmid pET15b-AfGfsA were grown in 1 L of Lysogeny broth (LB) medium containing ampicillin (40 μg/mL) and chloramphenicol (20 μg/mL) until they reached an optical density of 0.2 at 600 nm. The culture was induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG), and growth was continued for 65 h at 18°C in a shaker incubator to overexpress the soluble AfGfsA protein. Induced cells were collected by centrifugation. The collected cells were lysed using Bug Buster Protein Extraction Reagent (EMD Millipore, Billerica, MA). The supernatant was collected after centrifugation at 10,000 rpm for 10 min. All steps were performed at 4°C unless otherwise stated. His60 Ni Superflow Resin (50 μL; TAKARA Bio, Otsu, Japan) was added to the supernatant. The supernatant-agarose mix was gently shaken for 1 h. The His60 Ni Superflow Resin was collected by centrifugation at 3000 × *g* for 5 min and washed five times

Table II. *Aspergillus* strains used in this study

Strains	Genotype	Reference; Source
A. fumigatus A1151	*KU80::pyrG*	da Silva Ferreira et al. (2006); FGSC
A. fumigatus A1160	*KU80::pyrG, pyrG−*	da Silva Ferreira et al. (2006); FGSC
A. fumigatus Δ*AfgfsA*	*KU80::pyrG, pyrG⁻, AfgfsA::AnpyrG*	Komachi et al. (2013)
A. fumigatus Δ*AfgfsA::AfgfsA*	*KU80::pyrG, AfgfsA::AnpyrG,* Δ*AfgfsA::AfgfsA-ptrA*	This study

FGSC; Fungal Genetics Stock Center.

with 15 mL of buffer A. The enzyme was then eluted twice with 200 µL of buffer A containing 300 mM imidazole (WAKO Pure Chemical Industries, Osaka, Japan). The eluted enzyme was dialyzed in buffer A overnight, applied to an open Q Sepharose Fast Flow column (GE Healthcare, Chicago, IL), and eluted with buffer A containing 300 mM NaCl. The eluted enzyme was dialyzed in buffer A overnight. Protein concentrations were determined using the Qubit Protein Assay Kit (Thermo Fisher Scientific), and the purified AfGfsA was analyzed by SDS-PAGE to assess its purity and molecular weight.

Preparation of uridine diphosphate-α-D-galactofuranose

To prepare UDP-Gal$_f$, *E. coli* AG1 overexpressing UDP-Gal$_p$ mutase (*glf*) was obtained from the ASKA collection (a complete set of the *E. coli* K-12 ORF archive; National BioResource Project, Japan) (Kitagawa et al. 2005). The cells were grown in LB medium containing chloramphenicol (20 µg/mL) until they reached an optical density of 0.2 at 600 nm. The culture was induced with 1 mM IPTG, and growth was continued for 72 h at 18°C in a shaker incubator to overexpress the soluble Glf protein. The recombinant 6x-His tagged enzyme was purified by Ni^+ affinity chromatography.

An in vitro reaction for UDP-Gal$_f$ synthesis was performed as follows. The reaction mixture contained 250 mM UDP-Gal$_p$, 250 mM sodium dithionite, 1 mM $MgCl_2$, 100 mM Tris-HCl buffer (pH 8.0), 6% glycerol, and 27 µg of purified Glf enzyme in a total volume of 100 µL (Oppenheimer et al. 2010; Poulin and Lowary 2010). The mixtures were incubated at 37°C for 6 h, and the reaction was stopped by vortex mixing with 100 µL of ice-cold phenol/chloroform/isoamyl alcohol (25: 24: 1). The supernatants were analyzed using HPLC with a CarboPac PA1 anion-exchange column (250 mm × 4.0 mm; Dionex, Sunnyvale, CA), according to a previously described method, with slight modification (Oka et al. 2007). After sample injection, the column was equilibrated with 150 mM ammonium acetate (pH 4.4) at a flow rate of 0.7 mL/min for 30 min. The retention times for UDP-Gal$_p$ and UDP-Gal$_f$ were 14.5 min and 18.0 min, respectively. UDP-sugars were detected using UV_{260} absorbance. UDP-Gal$_f$ was collected using HPLC and was lyophilized, after which it was resuspended in distilled water and used as a sugar donor.

Enzyme assays

p-nitrophenyl-β-D-galactofuranose (*p*NP-β-D-Gal$_f$) was synthesized according to a previously described method (Varela et al. 1986; Marino et al. 1998; Matsunaga et al. 2015). Standard assays were performed with *p*NP-β-D-Gal$_f$ acceptor substrate, UDP-Gal$_f$, and purified AfGfsA protein, in a total reaction volume of 20 µL. The mixtures were incubated at 30°C for 60 min, and the reaction was stopped by heating (99°C) for 3 min. The reacted mixtures were analyzed using HPLC with a Shodex Asahipak NH2P-50 4E amino column (250 × 4.6 mm; Showa Denko, Tokyo, Japan), as previously described but with some modification (Hirayama et al. 2010). The elution was performed using two solvent gradients as follows: eluent A, 93% acetonitrile in 0.3% acetate (pH adjusted to 7.0 with ammonia); eluent B, 20% acetonitrile in 0.3% acetate (pH adjusted to 7.0 with ammonia). The gradient program was set at a flow rate of 0.8 mL/min (expressed as a percentage of solvent B): 0–5 min, isocratic 3%; 5–15 min, 3–25%; 15–30 min, 25–32%; 30–35 min, 32–3%; and 35–60 min, isocratic 3%. Para-nitrophenol derivatives were detected using UV_{260} absorbance. The molecular mass of the enzymatic products of AfGfsA was determined using an LTQ-OrbitrapXL liquid chromatography mass spectrometer (Thermo Fisher Scientific).

Construction of the complementary strain of ΔAfgfsA using the AfgfsA gene

A. fumigatus Δ*gfsA* was used as the parental strain (Table II), and DNA fragments for gene complementation were constructed using the "double-joint" PCR method (Yu et al. 2004). All PCR assays were performed using Phusion High-Fidelity DNA Polymerase (Daiichi Pure Chemicals, Tokyo, Japan). The primers used in this study are listed in Table III. Eight primers—AfgfsA-complement-1, -2, -3, -4, -7, and -8, ptrA-F, and ptrA-R—were used to construct a complementation cassette for Δ*AfgfsA*. The relevant region of the *AfgfsA* gene was PCR-amplified from genomic DNA using the primer pair AfgfsA-complement-1/AfgfsA-complement-2 (Table III). The relevant region of the *pyrG* gene from *A. nidulans* (*AnpyrG*) was PCR-amplified from pSH1 using the primer pair AfgfsA-complement-3/AfgfsA-complement-4 (Table III; Komachi et al. 2013). The *ptrA* genes that were used as selective markers were amplified using the pPTR-I plasmid (Takara Bio, Otsu, Japan) as a template and the primer pair ptrA-F/ptrA-R. The three amplified fragments were purified and mixed, and a second PCR assay was performed without specific primers, as the overhanging chimeric extensions acted as primers, to assemble each fragment. A third PCR assay was performed using the nested primer pair AfgfsA-complement-7/AfgfsA-complement-8 and the products of the second PCR as a template to generate the final DNA construct. The amplified final

Table III. Oligonucleotides used in this study

Oligonucleotide primers	Sequence
pET15-KAI-F	5′- GCGCGGATCCGCGGCCGCACTCGAGTGAGGCTGCTAACAAAGCC -3′
pET15-KAI-R	5′- GCGCGGATCCCATATGGCCGCTGCTGTG -3′
oligo-dT-18	5′- TTTTTTTTTTTTTTTTTT -3′
pET15b-AfGfsA-NdeI-F	5′- AAAAACATATGCATGCGGCCCTTCGGTTC -3′
pET15b-AfGfsA-NotI-R	5′- AAAAGCGGCCGCTTAATGCTGCTTTCTCACAGCA -3′
ptrA-F	5′- CATATGTAAATGGCTGTGTCCCG -3′
ptrA-R	5′- TTTAGCTTTGACCGGTGAGC -3′
AfgfsA-complement-1	5′- TACGCCGCTTGCTACTTGG -3′
AfgfsA-complement-2	5′- TCGTTACCAATGGGATCCCTTAATGCTGCTTTCTCACAGCATA -3′
AfgfsA-complement-3	5′- ATGCAAGAGCGGCTCATCGCCCTTCAACGCTATCACGCC -3′
AfgfsA-complement-4	5′- AGACATGATGGCGGTTCTCC -3′
AfgfsA-complement-7	5′- AAAAGAGGAATTCGCGGGAGG -3′
AfgfsA-complement-8	5′- AAGCTGGAAGTGGGATGGCT -3′

deletion constructs were purified using the Fast Gene Gel/PCR Extraction Kit (NIPPON GENE) 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. Correct replacement of the DNA fragments for gene complementation was confirmed by PCR using the primer pairs AfgfsA-complement-1/ptrA-R and ptrA-F/AfgfsA-complement-4 (Figure 4B).

Colony growth rate determination

Colony growth rates were measured as described previously (Kellner and Adams 2002; Komachi et al. 2013). Briefly, conidia from each strain were point-inoculated into the center of agar medium plates. Colony diameters were measured after 24, 48, and 72 h of incubation at 30°C. The growth rates were determined in millimeters per hour for each colony during each of the incubation intervals, i.e., 24–48 and 48–72 h, and were then averaged across the entire time interval. Growth rate measurements were performed six times for each strain.

Immunoblotting analysis

The immunoblotting analysis was performed as described previously (Komachi et al. 2013). The EB-A2 antibody, which is one of the components of the Platelia Aspergillus EIA assay (Bio-Rad Laboratories, Hercules, CA), was used at a dilution of 1:10 to detect the β-Gal*f* antigen.

Preparation of cell-wall galactomannoproteins

Aspergillus spores were grown in minimal medium at 37°C for 18 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 dialyzed with dH_2O and then lyophilized (Komachi et al. 2013). The cell wall extract was fractionated by CTAB using a previously described method (Lloyd 1970). A CTAB fraction precipitated at pH 9.0 with NaOH in the presence of borate was recovered as the galactomannoprotein fraction. A β-elimination reaction was performed by exposing the fractionated GM to reducing conditions (0.5 M $NaBH_4$/0.1 M NaOH, 10 mL, at 25°C for 24 h; Takahashi et al. 2012).

Methylation analysis

Glycosidic linkage analysis of the polysaccharides and oligosaccharides was performed by methylation analysis, using a previously described method (Ciucanu and Kerek 1984). The polysaccharide (2 mg) was separately dissolved in dimethylsulfoxide, and powdered NaOH was added. After stirring for 3 h, 500 μL of methyl iodide was added, and the suspension was stirred for 24 h. The methylated products were extracted in chloroform and back-washed three times using dH_2O. The methylated samples then were hydrolyzed using 2 M trifluoroacetic acid, reduced and acetylated. The partially methylated alditol acetates were analyzed by GC/MS using a capillary column (30 m × 0.25 mm; DB-5, Agilent), with helium as the carrier gas, and the following gradient temperature program: 210–260°C at 5°C/min. The GC/MS analyses were carried out on a JMS-K9 spectrometer (JEOL, Tokyo, Japan).

NMR spectroscopy

NMR samples were exchanged twice in D_2O, with intermediate lyophilization, and then dissolved in D_2O (99.97% atom ^2H). The NMR spectra were recorded using a JNM-LA600 spectrometer (JEOL) at 45°C. The proton and carbon chemical shifts were referenced relative to internal acetone at δ 2.225 and 31.07 ppm, respectively.

LC/MS analysis

Molecular mass was determined using reversed-phase liquid chromatography with a LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific) in the data-dependent acquisition mode at 5.0 μL/min. The instrument was operated in negative-ion mode, under the following conditions: source voltage 3.0 kV; capillary voltage −30 V; capillary temperature 275°C; and tube lens voltage −60 V. Samples were dissolved in 50% methanol containing 0.1% formic acid and injected into the instrument.

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Supplementary data

Supplementary data is available at *Glycobiology* online.

Conflict of interest statement

The authors declare no conflicts of interest.

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Abbreviations

CTAB, cetyl trimethyl ammonium bromide; ER, endoplasmic reticulum; FGSC, Fungal Genetics Stock Center; FTGM, fungal-type galactomannan; GDP-Man, guanosine 5′-α-D-mannopyranose; GIPC, glycosylinositolphosphorylceramide; GM, galactomannan; GPI, glycosylphosphatidylinositol; IPTG, isopropyl β-D-1-thiogalactopyranoside; LB, lysogeny broth; NMR, nuclear magnetic resonance; OMGM, *O*-mannose-type galactomannan; UDP-Gal$_f$, uridine diphosphate-α-D-galactofuranose.

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