Functional analysis of three putative galactofuranosyltransferases with redundant functions in galactofuranosylation in Aspergillus niger

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Abstract
Galactofuranose (Galf)-containing glycostructures are important to secure the integrity of the fungal cell wall. Golgi-localized Galf-transferases (Gfs) have been identified in Aspergillus nidulans and Aspergillus fumigatus. BLASTp searches identified three putative Galf-transferases in Aspergillus niger. Phylogenetic analysis showed that they group in three distinct groups. Characterization of the three Galf-transferases in A. niger by constructing single, double, and triple mutants revealed that gfsA is most important for Galf biosynthesis. The growth phenotypes of the ΔgfsA mutant are less severe than that of the ΔgfsAC mutant, indicating that GfsA and GfsC have redundant functions. Deletion of gfsB did not result in any growth defect and combining ΔgfsB with other deletion mutants did not exacerbate the growth phenotype. RT-qPCR experiments showed that induction of the agsA gene was higher in the ΔgfsAC and ΔgfsABC compared to the single mutants, indicating a severe cell wall stress response after multiple gfs gene deletions.

Keywords Cell wall integrity · Galactofuranose · Galactomannan · Calcofluor white hypersensitive · Glycosylation · Golgi apparatus

Abbreviations
Galf Galactofuranose
CFW Calcofluor white
MM Minimal medium
CM Complete medium

Introduction
Galactofuranose (Galf) is an important constituent of the fungal cell wall (Tefsen et al. 2012; Oka and Goto 2016). Around 5% of the dry weight of the cell wall of A. fumigatus consists of Galf (Lamarre et al. 2009) and similar amounts of Galf are expected to be present in other Aspergilli. Galf is the five-membered ring form of galactose and is found in several cell surface fractions. It has been identified as a component of the cell wall galactomannan fraction in Aspergilli, as a part of N- and O-glycans of extracellular proteins, and within glycosphingolipids (Bardalaye and Nordin 1977; Baretto-Bergter et al. 1980; Wallis et al. 1999; Toledo et al. 2007). We previously reported on the identification of several genes involved in the biosynthesis of Galf-containing glycoconjugates in A. niger. The genes involved encode a UDP-glucose 4-epimerase (UgeA), a UDP-galactomutase (UgmA), and two UDP-Galf-transporters (UgtA and UgtB) (Damveld et al. 2008; Park et al. 2014, 2015). Several studies in A. fumigatus and A. nidulans have shown that similar gene sets of UDP-glucose 4-epimerases, UDP-galactomutases, and UDP-Galf-transporters are present in these fungi and are important for Galf biosynthesis (Lee et al. 2014; El-Ganiny et al. 2008, 2010; Schmalhorst et al. 2008; Engel et al.)
The genes encoding the final step in the synthesis of Galf-glycostructures, the galactofuranosyl (Galf)-transferases, have been identified in A. nidulans and A. fumigatus (Komachi et al. 2013; Katafuchi et al. 2017). Galf-transferases use UDP-Galf as a nucleotide sugar donor to transfer Galf to glycostructures such as galactomannans, N-chains, and O-chains. Galf-transferases are predicted to be present in the Golgi as Golgi-localized UDP-Galf transporters with a crucial function in Galf-biosynthesis (Engel et al. 2009; Afroz et al. 2011; Park et al. 2015). Since Golgi-localized transferases are mostly type II transmembrane proteins, Komachi et al. searched for type II transmembrane protein encoding genes in the genome of A. nidulans and systematically deleted these genes. Deletion mutants were analyzed for the presence of Galf on glycostructures, resulting in the identification of GfsA (AN8677) being required for galactofuranosylation of O-glycans (Komachi et al. 2013). Deletion of the A. fumigatus ortholog (GfsA, Afu6g02120) lead to similar reduction in the presence of Galf-antigens in O-glycans, indicating that also the A. fumigatus ortholog encodes a Galf-transferase (Komachi et al. 2013). GfsA of A. fumigatus and A. nidulans were shown to be localized in the Golgi via fractionation experiments or via GFP-tagging, respectively (Komachi et al. 2013; Oka 2018). The GfsA protein of A. fumigatus was further characterized biochemically and characterized as a β1,5-galactosyltransferase responsible for the biosynthesis of β1,5-galactosylfuranose in the galactofuran side chain of fungal-type galactomannans (Katafuchi et al. 2017).

To examine the involvement of A. niger homologs of the A. nidulans and A. fumigatus Galf-transferases in galactofuranosylation, putative Galf-transferases in A. niger were identified by BlastP searches. Three putative Galf-transferases were identified and their possible redundant functions were examined by making single, double, and triple deletion mutants.

### Methods

#### Strains and culture conditions

The Aspergillus niger strains used in this study are listed in Table 1. Strains were grown on minimal medium (MM) (Bennett and Lasure 1991), containing 1% (w/v) glucose as carbon source or complete medium (CM) containing 0.5% (w/v) yeast extract and 0.1% (w/v) casamino acids in addition to MM. When required, plates were supplemented with 10 mM uridine. 5-fluoroorotic acid selection to obtain pyrG− strains was performed as described previously.

| Table 1 Strains used in this study | Description | References |
|-----------------------------------|-------------|------------|
| MA169.4 cspA1, pyrG378, kusA::DR-amdS-DR | ka70 disruption in AB4.1 | Carvalho et al. (2010) |
| MA234.1 cspA1, kusA::DR-amdS-DR | Restored pyrG in MA169.4 | Park et al. (2016) |
| MA87.6 cspA1, pyrG378, kusA::AMD, ugmA::AOpyrG | ΔugmA in MA70.15 | Damveld et al. (2008) |
| DL6.1 cspA1, pyrG378, kusA::DR-amdS-DR, An12g08720::AOpyrG | ΔgfsA in MA169.4 | This study |
| DL2.8 cspA1, pyrG378, kusA::DR-amdS-DR, An04g06900::AOpyrG | ΔgfsC in MA169.4 | This study |
| DL3.3 cspA1, pyrG378, kusA::DR-amdS-DR, An01g09510::AOpyrG | ΔgfsB in MA169.4 | This study |
| DL4.1 cspA1, pyrG378, kusA::DR-amdS-DR, An04g06900::AOpyrG, An12g08720::hph | ΔgfsAC | This study |
| DL5.1 cspA1, pyrG378, kusA::DR-amdS-DR, An12g08720::AOpyrG, An01g09510::hph | ΔgfsAB | This study |
| DL6.1 cspA1, pyrG378, kusA::DR-amdS-DR, An04g06900::AOpyrG, An01g09510::hph | ΔgfsBC | This study |
| MA314.1 cspA1, pyrG378, kusA::DR-amdS-DR, An04g06900::AOpyrG, An01g09510::hph, pyrG− | pyrG− mutant derived from DL6.1 | This study |
| MA316.3 cspA1, pyrG378, kusA::DR-amdS-DR, An04g06900::AOpyrG, An01g09510::hph, pyrG− | ΔgfsABC | This study |
| MA877.1 cspA1, pyrG378, An12g08720::AOpyrG | ΔgfsA, kusA restored in DL1.1 | This study |
| MA880.1 cspA1, pyrG378, An04g06900::AOpyrG, An12g08720::hph | ΔgfsAC kusA restored in DL4.1 | This study |
| MA881.1 cspA1, pyrG378, An12g08720::AOpyrG, An01g09510::hph | ΔgfsAB kusA restored in DL5.1 | This study |
| MA884.1 MA887.1+gfsA+pAN8.1 | ΔgfsA+gfsA | This study |
| MA887.1 MA881.1+gfsA+pAN8.1 | ΔgfsAB+gfsA | This study |
| MA884.1 MA881.1+gfsA+pAN8.1 | ΔgfsAC+gfsA | This study |
| MA885.1 MA880.1+gfsA+pAN8.1 | ΔgfsAC+gfsA | This study |
| MA886.1 MA880.1+gfsC+pAN8.1 | ΔgfsAC+gfsA | This study |
Calcofluor white (CFW) sensitivity was determined as described (Ram and Klis 2006). The presence of Galf reactive glycoproteins in the culture medium was performed by growing the strains in 25 ml CM in 50 ml tube Greiner tube for 24 h at 30 °C. Cultures were filtered over a Whatman glass microfiber filter and 2 µl medium was spotted on nitrocellulose blotting paper and labeled with the L10 monoclonal anti-Galf-antibody (Heesemann et al. 2011) as described (Park et al. 2014). Fungal transformations were performed according the protoplast method described by Arentshorst et al. (2012).

Generation of A. niger deletion mutants

The A. niger gfsA, gfsB, and gfsC genes were deleted by replacing their respective open-reading frames (ORFs) with the A. oryzae pyrG resistance cassette using the split marker approach as was described in detail by Arentshorst et al. 2015. Approximately 800 bp flanking regions of each of the ORFs were PCR amplified from genomic DNA of the N402 strain using primer pairs as listed in Additional file 1: Table 1. The AopyrG gene was amplified from pAO4-13 (de Ruitter-Jacobs et al. 1989), using primers AOPyrGP12f and AOPyrGP13r (Additional file 1: Table 1). Subsequently, 5′ and 3′ split marker fragments were obtained in two separate fusion PCR amplifications using the respective flank and the AopyrG PCR products as a template and primer pairs according to Additional file 1: Table 1. The split marker fragments were transformed to A. niger strain MA169.4 (Carvalho et al. 2010) and homologous integration was confirmed by Southern blot analysis (data not shown). Double mutants (∆gfsAB, ∆gfsAC, and ∆gfsBC) were generated by transforming single mutants (∆gfsA for ∆gfsAB and ∆gfsC for ∆gfsAC and ∆gfsBC) with split marker fragments containing hygromycin as selection marker (Arentshorst et al. 2015). To create a triple deletion mutant, a pyrG− mutant of the ∆gfsBC strain was obtained from a 5-fluoroorotic acid plate. This strain (∆gfsBC, pyrG−) was subsequently transformed with ∆gfsA-AOPyrG split marker fragments, resulting in a triple deletion mutant (∆gfsABC). Proper deletion of the gfs genes in the respective deletion mutants was verified by Southern blot analysis (data not shown).

Complementation of the ∆gfs mutants was performed by transforming the PCR amplified gfs genes, including ~800 bp promoter and ~800 bp termination regions, to the gfs deletion strains by cotransformation with the phleomycin resistance marker on pAN8.1 (Punt and Hondel 1992). To allow ectopic integration of the gfs genes, strains DL1.1 (∆gfsA), DL4.1 (∆gfsAC), and DL5.1 (∆gfsAB) were cured for their disruption of ku70 by selection on 5′fluoro-acetamide to loop out the amdS marker used for disrupting ku70 (Carvalho et al. 2010). For the amplification of the genes, the gfs-specific P1 and P4 primers were used (Additional file 1: Table 1). Phleomycin-resistant transformants were purified and analysed by diagnostic PCR to confirm the expected deletion and the presence of an ectopically integrated gfs gene. PCR-positive transformants were further analysed for their sensitivity towards CFW using the CFW spot assay.

RT-qPCR experiments

Total RNA was extracted using TRIzol reagent (Invitrogen) from mycelium samples after growing the strains for 25 h in CM. RNA samples were further column purified using NucleoSpin RNA Clean-up kit (Macherey–Nagel) with rDNase treatment. The quantity and quality of the RNA samples were checked with a NanoDrop-1000 spectrophotometer (Thermo Fisher Scientific) and RNA gel electrophoresis, respectively. Primers for afsA and actA were designed using Primer-BLAST (Additional file 2: Table 2) (Ye et al. 2012). cDNA was synthesized using QuantiTect Reverse Transcription Kit (QIAGEN) according to the manufacturer’s instructions with 1 µg RNA per 20 µl total reaction volume and diluted afterwards 100 times. No reverse transcriptase samples, in which water was used instead of Reverse Transcriptase, were included to check for genomic DNA contamination. For each primer pair, efficiency of the reaction was calculated by generating a standard curve using cDNA obtained from 10 µg RNA per 200 µl total reaction volume and diluted to produce 10, 1, 0.1 and 0.01 ng RNA points. RNA obtained from ∆ugmA strain grown for 25 h was used for standard curve generation. qPCR was carried out in a C1000 CFX96 machine (BIO-RAD) with 20 µl total reaction volume containing 2 µl cDNA, 10 µl 2 × GoTaq qPCR Master Mix (Promega), 6 µl water, 1 µl 5 µM forward primer, and 1 µl 5 µM reverse primer. In no template control samples, water was used instead of cDNA. 96-well white-shell white-well PCR plate (Hard-Shell PCR Plates, BIO-RAD) and optically clear adhesive seals (Microseal ‘B’ seal, BIO-RAD) were used. Each reaction was performed in three technical replicates. The protocol of qPCR was as follows: 2 min 50 °C, 10 min 95 °C, 50 cycles of 15 s 95 °C, 30 s 60 °C, and 30 s 72 °C. Melting curves were generated by increasing the temperature from 65 °C to 95 °C gradually. Specificity of reactions and contamination was checked for each primer pair. Data was analyzed using the accompanying software Bio-Rad CFX Manager 3.1 Expression values (∆ΔCq) were normalized against that of the reference gene actA and represented relative to the wild-type strain.
Results and discussion

Identification of three Galf-transferases in the A. niger genome

Galf-transferases in the genome of A. niger were identified by BlastP searches using the A. nidulans and A. fumigatus GfsA proteins as queries. We identified three putative homologs which were named GfsA, GfsB, and GfsC. A. nidulans and A. fumigatus also contain two additional candidates for Galf-transferases, as noticed previously (Komachi et al. 2013; Oka and Goto 2016). Phylogenetic analysis showed that the three orthologs cluster in distinct clades (Fig. 1) indicating an early triplication of this gene family. The three A. niger candidates (GfsA: An12g08720, GfsB: An01g09510 and GfsC: An04g06900) are all predicted to be type II transmembrane proteins [Center for Biological Sequence analysis (https://www.cbs.dtu.dk/services/TMHMM/)] and are of about 500 amino acids in length (Table 2). Whereas the ugmA/glfA and the ugtA/glfB genes are clustered in the genome (Engel et al. 2009), the location of any of the three candidate Galf-transferases in the genome was not clustered with other genes involved in Galf biosynthesis.

Functional analysis of the putative Galf-transferases

To analyze the function of the different putative Galf-transferases, ΔgfsA, ΔgfsB, and ΔgfsC single mutants, ΔgfsAB, ΔgfsAC, and ΔgfsBC double mutants, and a ΔgfsABC triple mutant were generated (Table 1), using the split marker method, with either the A. oryzae pyrG gene or the hygromycin resistance gene as a selection marker and MA169.4 (ku70−) as a host. The absence of galactofuranosylation, e.g., in the ΔugmA mutant, has been shown to result in a reduced growth phenotype, aberrant branching morphology, reduced conidiation, and increased sensitivity towards the cell wall assembly disturbing drug Calcofluor white (CFW) (Damveld et al. 2008; Park et al. 2016). Similar phenotypes were also observed in the A. niger ΔugeA mutant (Park et al. 2014) and the ΔugtAB double mutant (Park et al. 2015). When analyzing the growth phenotype of the different Δgfs mutants, we noticed, in the ΔgfsA single mutant, a reduced growth and an increased sensitivity towards CFW, although not as severe as in the ΔugmA mutant (Fig. 2). Deletion of gfsC alone did not result in an increased sensitivity to CFW; however, simultaneous deletion of gfsA and gfsC resulted in a severe phenotype, identical to the growth phenotypes of the ΔugmA strain, indicating that simultaneous deletion of gfsA and gfsC results in a complete galactofuranosylation defect. Deletion of gfsB does not seem to have an effect on the growth behaviour in the wild-type background as well as in combination with the deletion of gfsA and/or gfsC. To show that the deletions of gfsA and gfsC were responsible for the phenotypes of the single and double mutants, the mutants were complemented by transformation of the respective genes to the deletion mutants which restored the CFW sensitivity (Fig. 2).

To analyze the effect of the gfsA, gfsB, and gfsC deletion on the presence of Galf-containing glycoconjugates in the growth medium, medium samples were spotted in nitrocellulose membrane and labelled with a Galf-specific antibody (L10) as described (Park et al. 2014). As shown in Fig. 3, deletion of gfsA results in the absence of detectable amounts of Galf. Based on the growth phenotype of the ΔgfsA mutant, however, which is not as severe as the ugmA mutant, it seems that some

| An number | Protein name | length (aa) | TM domain | Probability TM prediction |
|-----------|--------------|-------------|-----------|--------------------------|
| An12g08720 | GfsA         | 532         | 20–37     | 0.99759                  |
| An01g09510 | GfsB         | 568         | 13–35     | 0.97105                  |
| An04g06900 | GfsC         | 461         | 7–24      | 0.91848                  |

*Center for Biological Sequence analysis (https://www.cbs.dtu.dk/services/TMHMM/)

Fig. 1 Phylogenetic tree of putative galactofuranosyltransferase from A. niger, A. nidulans, and A. fumigatus. Protein sequences were retrieved from AspGD (https://www.aspergillusgenome.org) and DNAman2.0 was used to make the homology tree. % of homology between the proteins is indicated. Saccharomyces cerevisiae galactotransferase Bed1p (Mnn10p) was used as an outgroup

Table 2 Characteristics of putative Galf-transferase A. niger

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galactofuranosylation still occurs in the absence of gfsA. In the dot-blot experiment, it is likely that Gal\textsubscript{f}-residues on N- and O-glycans are detected. Therefore, the absence of detectable Gal\textsubscript{f} in the ΔgfsA mutant suggests that GfsA is required for the galactofuranosylation of N- and O-glycans.

**Activation of the cell wall integrity pathway in gfs-deficient mutants**

The galactofuranose-deficient ugeA and ugmA mutants were identified in a screen for cell wall mutants with increased expression of the alpha-glucan synthase (agsA) (Damveld et al. 2008; Park et al. 2014). To identify additional mutants that are defective in Gal\textsubscript{f} biosynthesis, our collection of 240 mutants with induced expression of agsA was screened for lack of Gal\textsubscript{f} in the culture medium. However, screening of the collection failed to identify the gfsA mutant. Since the gfsA mutant is negative in the dot-blot assay (Fig. 3), we anticipated that a gfsA mutant could in principle be isolated in the mutant screen, if the agsA gene is strongly induced in the gfsA mutant. To analyze whether deletion of gfsA results in strong induction of the agsA gene, all single, double, and triple deletion strains as well as the ΔugmA strain were grown in liquid cultures for 25 h at 30 °C and RNA was isolated. The agsA expression in the mutants was determined by performing RT-qPCR experiments on these RNA samples, using actA expression as reference (Fig. 4). The RT-qPCR results show that the agsA expression in the ΔugmA strain is about fourfold higher than in the ΔgfsA strain, indicating that agsA induction in the ΔgfsA strain was probably not sufficient to be detected in the screen for cell wall mutants. Double deletion of both gfsA and gfsC as well as deletion of all three gfs genes causes a higher agsA induction, indicating again a redundant function of the gfs genes for the synthesis of Gal\textsubscript{f}-containing glycostructures in *A. niger* and activation of the cell wall stress response when multiple gfs genes are inactive.
Fig. 4 RT-qPCR analysis of agsA expression in single, double, and triple ∆gfs strains, the ∆ugmA mutant, and the wild-type (wt) strain. All strains were grown for 24 h at 30 °C, RNA was isolated and agsA expression was determined via RT-qPCR, using actA expression as reference. The agsA expression in all mutants is relative to the agsA expression in the wild-type strain, which was set to 1

Conclusions

The biosynthesis of cell surface-located galactofuranose (Gal)‐containing glycostructures such as galactomannan, N‐glycans, O‐glycans, and glycolipids in filamentous fungi is important to secure the integrity of the cell wall. A. niger as well as A. nidulans and A. fumigatus contain three galactofuranosyltransferases encoding genes in their genomes. By constructing single, double, or triple gfs mutants and comparing the phenotype to the ugmA mutant, we show that GfsA together with GfsC are most important for galactofuranosylation in A. niger. The next step in our understanding of the function of the different galactofuranosyltransferases will be to elucidate whether individual genes are involved in the galactofuranosylation of the different glycostructures (galactomannan, N-glycans, O-glycans, and glycolipids) which contain Galf.

Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests. Strains and plasmids are available upon request. We thank Frank Ebel for the L10 antibody.

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