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Characterization of the need for galactofuranose during the *Neurospora crassa* life cycle

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ABSTRACT

Galactofuranose is a constituent of the cell walls of filamentous fungi. The galactofuranose can be found as a component of N-linked oligosaccharides, in O-linked oligosaccharides, in GPI-anchored galactomannan, and in free galactomannan. The Neurospora genome contains a single UDP-galactose mutase gene (*ugm-1*/NCU01824) and two UDP-galactofuranose translocases used to import UDP-galactofuranose into the lumen of the Golgi apparatus (*ugt-1*/NCU01826 and *ugt-2*/NCU01456). Our results demonstrate that loss of galactofuranose synthesis or its translocation into the lumen of the secretory pathway affects the morphology and growth rate of the vegetative hyphae, the production of conidia (asexual spores), and dramatically affects the sexual stages of the life cycle. In mutants that are unable to make galactofuranose or transport it into the lumen of the Golgi apparatus, ascospore development is aborted soon after fertilization and perithecium maturation is aborted prior to the formation of the neck and ostiole. The Neurospora genome contains three genes encoding possible galactofuranosyltransferases from the GT31 family of glycosyltransferases (*gfs-1*/NCU05878, *gfs-2*/NCU07762, and *gfs-3*/NCU02213) which might be involved in generating galactofuranose-containing oligosaccharide structures. Analysis of triple KO mutants in GT31 glycosyltransferases shows that these mutants have normal morphology, suggesting that these genes do not encode vital galactofuranosyltransferases.

1. Introduction

Polysaccharides and glycoconjugates play key roles in many biological processes. In the fungi, polysaccharides are the major components of the cell wall, where they provide structural support and cell type-specificity to the cell surface. Galactofuranose, the five-membered ring form of galactose, is a component of fungal cell walls (Fig. 1A). Galactofuranose has been shown to be important for the growth and virulence of fungal pathogens (Tefsen et al. 2012; Senicar et al. 2020). Because galactofuranose is not found in mammalian cells, its biosynthetic pathway has been considered an excellent target for the development of antifungal agents (El-Ganiny, Sanders, and Kaminskyj 2008; Schmalhorst et al. 2008). Studies have shown that galactofuranose can be attached to N-linked glycans, O-linked glycans, GPI-anchored galactomannans, and to free galactomannans (Senicar et al. 2020; Tefsen et al. 2012).

The first step in the galactofuranose pathway is the conversion of UDG-galactopyranose to UDP-galactofuranose by the enzyme UDP-galactopyranose mutase (Fig. 1B). The gene encoding UDP-galactopyranose mutase has been identified and studied in *Aspergillus fumigatus* (Bakker et al. 2005; Oppenheimer et al. 2010; Schmalhorst et al. 2008), *A. niger* (Damveld et al. 2008), and *A. nidulans* (Alam et al. 2014; El-Ganiny, Sanders, and Kaminskyj 2008). Because of its relevance to human disease, the *A. fumigatus* UDP-galactopyranose mutase has been extensively studied. The crystal structure of the enzyme has been elucidated and key amino acids which play roles in the enzymatic reaction have been identified and characterized (Dhatwalia et al. 2012a; Dhatwalia et al. 2012b; Da Fonseca et al. 2014; Penman et al. 2012; van Straaten, Routier, and Sanders 2012a, 2012b; Tanner et al. 2014). Aspergillus mutants lacking UDP-galactopyranose mutase have a reduced growth rate, cell wall alterations, aberrant morphology, reduced virulence, sensitivity to antifungal agents, and abnormal conidiation (Alam et al. 2014; Oppenheimer et al. 2010; Damveld et al. 2008; El-Ganiny, Sanders, and Kaminskyj 2008).

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The second step in the galactofuranose pathway is the translocation of UDP-galactofuranose from the cytosol into the lumen of the secretory pathway, where it can be used for the synthesis of galactomannan and oligosaccharide structures (Fig. 1B). A UDP-galactofuranose translocator gene, located next to the UDP-galactopyranose mutase gene, was identified in *A. fumigatus* as being the responsible for transporting UDP-galactofuranose into the lumen of the Golgi apparatus (Engel et al. 2009; Engel, Schmalhorst, and Routier 2012). *A. nidulans* also contains a single UDP-galactofuranose translocator gene located next to the mutase gene (Afroz et al. 2011). There are two genes encoding UDP-galactofuranose translocators in *A. niger*, one of which is located next to the mutase gene (Park et al. 2015). Both of the *A. niger* UDP-galactofuranose translocator genes encode active translocators in *A. niger*. Loss of UDP-galactofuranose transport into the Golgi apparatus generates a phenotype very similar to that observed for the mutase mutant (Park et al. 2015; Afroz et al. 2011; Engel et al. 2009). This demonstrates the importance of UDP-galactofuranose in the formation of galactomannan and oligosaccharides within the Golgi apparatus.

Identifying the galactofuranosyltransferases responsible for generating galactofuranose-containing oligosaccharide structures has proved to be difficult. The variety of known galactofuranose-containing structures include N-linked and O-linked structures in which galactofuranose is linked to mannoses in the N and O-glycans, and free and GPI-linked galactomannans. This suggests that several transferases may be functioning and that the phenotypes of mutants lacking these transferases could be quite variable. Progress has been made with the identification of the β-1,5-galactofuranosyltransfases GfsA, GfsB, and GfsC, which are responsible for generating the galactomannans found in *A. fumigatus* and *A. nidulans* (Komachi et al. 2013; Katafuchi et al. 2017; Chihara et al. 2020; Oka 2018). These enzymes belong to the GT31 family of glycosyltransferases.

We report on the characterization of the galactofuranose pathway in *Neurospora crassa*. The *N. crassa* genome encodes a single UDP-galactopyranose mutase and two UDP-galactofuranose transporters, one of which is adjacent to the mutase gene. Unlike the Aspergillus species previously characterized, *N. crassa* has not been reported to make GPI-attached galactomannans or free galactomannans. The known *N. crassa* galactofuranose-containing cell wall components include an N-linked galactomannan and O-linked oligosaccharides. The *N. crassa* N-linked galactomannan is quite different from that found in *A. fumigatus* and consists of a chain of α-1,6-mannoses with a galactofuranose attached at the 2 position of the mannoses (Nakajima et al. 1984a; Nakajima et al. 1984b; Kar et al. 2019; Leal et al. 1996). We show that mutants in the mutase or both transporters do not incorporate galactofuranose into their cell wall proteins. Mutants lacking the mutase or both transporters share a phenotype that includes all stages of the life cycle, but the phenotype of the mutants lacking both transporters is not as severe as that of the mutase mutant. A genetic analysis of the *N. crassa* GT31 glycosyltransferases, which we thought might encode important galactofuranosyltransferases, failed to uncover any mutant phenotypes.

2. Results

2.1. Isolation and characterization of mutants affected in galactofuranose synthesis

The UDP-galactomutase gene from *A. fumigatus* (ugmA/Afu3g12690) has been previously identified and characterized (El-Ganiny, Sanders, and Kaminskyj 2008; Bakker et al. 2005; Alam et al. 2014; Oppenheimer et al. 2010; Damveld et al. 2008). In *A. fumigatus* and *A. nidulans*, galactomutase mutants have reduced growth rates, cell wall alterations, aberrant morphologies, reduced virulence, sensitivity to antifungal agents, and abnormal conidiation (Damveld et al. 2008; Oppenheimer et al. 2010; El-Ganiny, Sanders, and Kaminskyj 2008; Alam et al. 2014). In the *N. Crassa* genome there is a single gene, *ugm-1*/NCU01824, with homology to the UDP-galactomutase gene of *A. fumigatus* (81% identity). The Neurospora deletion library contains a single *ugm-1* deletion isolate, which is mating type a. We noticed that perithecial development was aborted prior to the formation of the neck and ostiole when the *Δugm-1 mt*a isolate was used as a female in mating and that very few ascospores were formed. Therefore, a *Δugm-1 mt*A isolate was generated through mating, with a wildtype isolate being used as the female and the △*ugm-1 mt*a isolate as the male.

Upon isolating *Δugm-1* strains of both mating types, we began by characterizing how loss of galactofuranose affected the different stages of the *N. crassa* life cycle. The vegetative hyphae of △*ugm-1* mutants were compared with the vegetative hyphae of a wildtype isolate (Fig. 2). As can be seen, the mutant hyphae have a slightly altered morphology with an increased frequency of hyphal branching. The linear growth rate of the △*ugm-1* hyphae was found to be 0.056 +/- 0.009 cm/hr as compared to a growth rate of 0.303 +/- 0.017 cm/hr for the wildtype hyphae (Fig. 3). This represents an 83% reduction in linear growth rate. We conclude that the growth and morphology of the vegetative hyphae are affected by the loss of galactofuranose.

We also looked at how loss of galactofuranose affected the formation of conidia (asexual spores). Using the conidiation assay described in Materials and Methods, we found that the △*ugm-1* mutant produced 1.2 +/- 0.3 × 10^7 conidia per agar slant as compared to 1.77 +/- 0.26 × 10^8 conidia per slant being produced by a wildtype isolate (Fig. 4). This represents a 93% reduction in conidia formation in the absence of galactofuranose. The conidia produced by the mutants were normal in appearance.

We also looked at matings between two *ugm-1* isolates to assess how the development of perithecia and ascospores (sexual spores) was affected by the loss of galactofuranose. As mentioned above, when a △*ugm-1* mutant is the perithecial partner (female) in a mating, the development of the perithecia is aborted after a melanized perithecia is formed and the perithecia lack the typical neck and ostiole structures seen in normal matings. Perithecia squashes of such perithecia show that there are very few ascospores produced. We looked at matings in which both isolates were △*ugm-1* mutants to further characterize the mutant phenotype. We found that the perithecia formation was aborted after melanization (Fig. 5) and that the perithecia were completely devoid of developing ascospores (Fig. 6). Ascospores are generated from the diploid cells involved in the meiotic division, and the complete absence of ascospores indicates that galactofuranose is required for ascospore

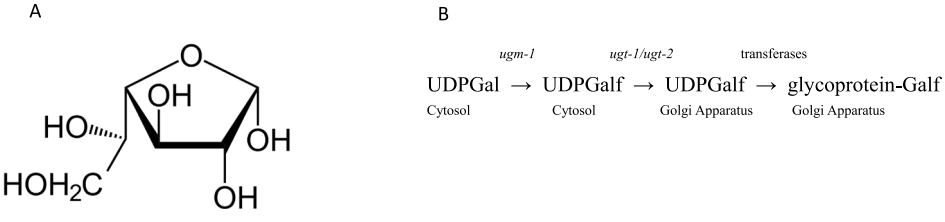

Fig. 1. A structural representation of galactofuranose with its five membered ring is shown.

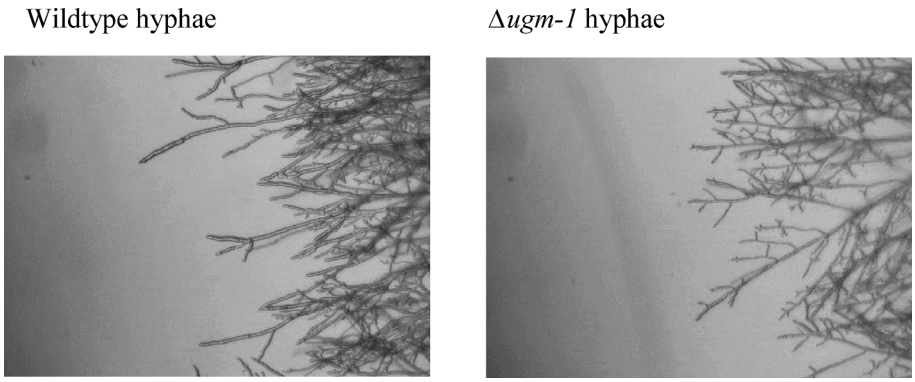

Fig. 2. Growth morphologies of wildtype and mutant vegetative hyphae. Wildtype and △*ugm-1* mutant cells were grown in a Petri dish with nutrient agar. An agar piece containing the edge of the colony was dissected from the agar, placed upside down on a microscope slide, and examined using an inverted microscope. The △*ugm-1* mutant has a reduced growth rate and a slightly increased branching pattern which is most easily observed in the hyphae at the growing edge of the colony.

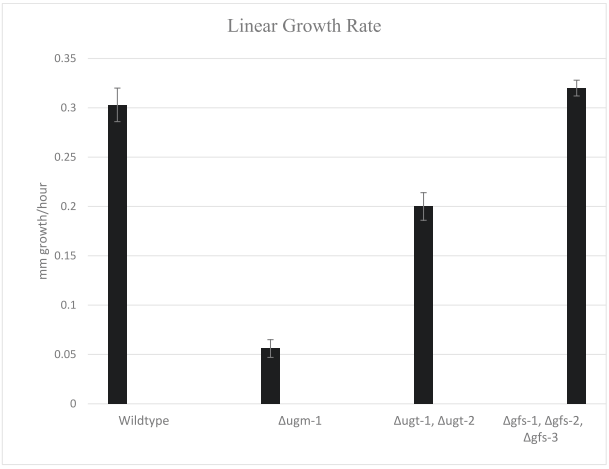

Fig. 3. Galactofuranose mutants are affected in their hyphae growth rates. Wildtype and mutant strains were inoculated near the edge of a Petri dish with agar medium and the rate of growth across the agar medium measured.

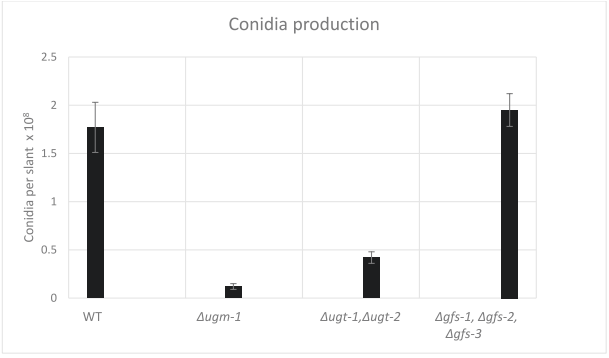

Fig. 4. Galactofuranose mutants are affected in conidia production. Conidiation efficiency was assessed by counting the number of conidia produced on 3 ml agar medium slant.

development. The fact that when the △*ugm-1* mutant serves as the female in a mating, perithecium development is aborted without the formation of the peridium-produced neck and ostiole structures suggests that galactofuranose may be needed for peridium development.

To demonstrate that these mutant phenotypes were due to the deletion of *ugm-1*, we generated a GFP-tagged version of the wildtype *ugm-1* gene and generated transformants of our *ugm-1* deletion strain in which the wildtype *ugm-1* gene was inserted it into the *his-3* location. We found that these transformants had a wildtype growth rate, produced conidia in wildtype numbers, and made fully fertile perithecia. We concluded that the wildtype *ugm-1* gene fully complemented the deletion mutation and that the mutant phenotypes were due to the loss of UDP-galactomutase.

With the GFP-tagged version of UGM-1, we could examine the intracellular location of the enzyme. While the UGM-1 does not have a transmembrane domain, signal peptide, or any other features that would target it to an intracellular organelle, we considered the possibility that it might be associated with the Golgi apparatus to facilitate the transport of UDP-galactofuranose into the lumen of the Golgi apparatus, where the UDP-galactofuranose is used for post-translational modifications. Fig. 7 shows an image of the GFP-tagged UGM-1 in the tip of a growing hyphae. As can be seen, UGM-1 is located within the cytoplasm of the hyphae.

2.2. Isolation and characterization of UDG-galactofuranose translocation mutants

The *N. crassa* genome contains two genes, *ugt-1*/NCU01826 and *glt2*/NCU01456, with 65% and 63% identity to the UDG-galactofuranose translocase gene GlfB from *A. fumigatus*. One of these, *ugt-1*/NCU01826, is found adjacent to *ugm-1*/NCU01824. In *A. fumigatus*, the encoded translocator has been shown to function as a translocase to transport UDP-galactofuranose into the lumen of the Golgi apparatus (Engel et al. 2009). The Neurospora deletion library contains *Δugt-1 mta*, *Δugt-2 mta*, and *Δugt-2 mtA* isolates. These three strains have a wildtype phenotype. To get UDP-galactofuranose translocase double mutants of both mating types we mated the *Δugt-1 mta* isolate and the *Δugt-2 mtA* isolate and, using PCR analyses, identified progeny of both mating types with deletions of both *glt* genes.

We characterized UDP-galactofuranose translocase double mutants (△*ugt-1*, △*ugt-2* mutants) using the same approach described above for the △*ugm-1* mutants. We found that they generally phenocopied the △*ugm-1* mutants. Their morphology was indistinguishable from that of the △*ugm-1* mutant and their linear growth rates were reduced by 34% (0.200 +/- 0.014 cm/hr as compared with the wildtype growth rate of 0.303 +/- 0.017 cm/hr) (Fig. 3). This is somewhat less than the 83% reduction in growth rate seen in the △*ugm-1* mutant. They produced 4.20 +/- 0.6 × 10^7 conidia per agar slant (76% reduction in conidia production), which is a smaller reduction in conidiation than the 93% reduction in conidiation found in the △*ugm-1* mutant (Fig. 4). Mating experiments showed that perithecia development was aborted prior to the formation of the neck and ostiole structures mutants in △*ugt-1*, △*ugt-2* × △*ugt-1*, △*ugt-2* matings Fig. 5). Examination of perithecia squashes showed that no ascospores were formed within the perithecia

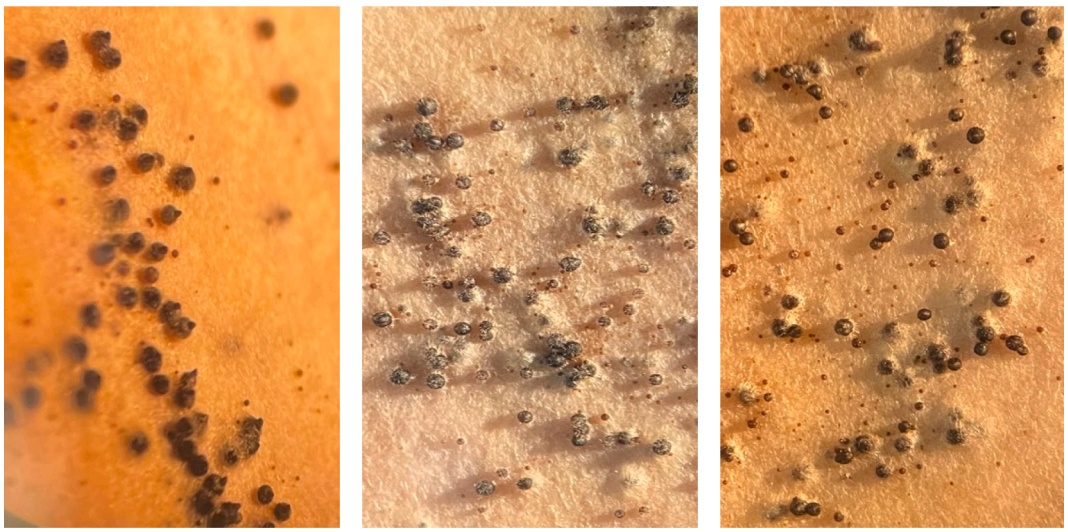

Fig. 5. Mutant isolates are defective in perithecium development. An angled view of the perithecia of *N. crassa* showing the beaks on the perithecia from the wildtype strain mating while the *Δugm-1* × *Δugm-1* mating and the *Δugt-1*, *Δugt-2* × *Δugt-1*, *Δugt-2* mating exhibit a mutant phenotype in which the beak is not generated during perithecium development. Representative wildtype perithecia with beaks can be seen in the wildtype × wildtype mating.

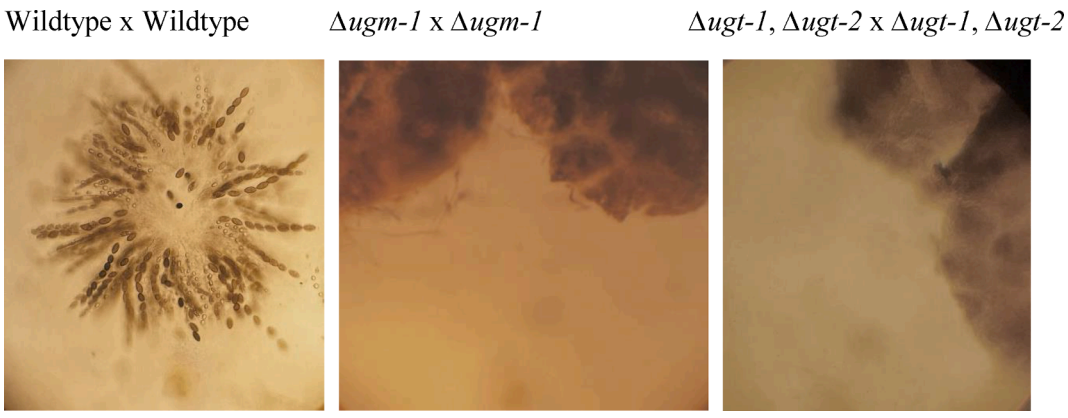

Fig. 6. UDP-galactofuranose is required for ascospore formation. Wildtype and mutant strains were mated and perithecia were collected and squashed between a microscope slide and cover glass to break the perithecia open and release "rosettes" of developing ascospores. The wildtype mating generated ascospore "rosettes" in which the individual developing ascospores could be clearly seen (left image). Squashes of perithecia produced by mutant matings were devoid of developing ascospore "rosettes". Images of squashed perithecia from the mutant △*ugm-1* × △*ugm-1* mating (middle image) and the △*ugt-1*, △*ugt-2* × △*ugt-1*, △*ugt-2* mating (right image) contain fragments of broken perithecia, which lack developing ascospores. The dark structures seen in the images from the mutant matings are fragments of the broken perithecia. Note the absence of any ascospores in the mutant matings.

(Fig. 6). Our results demonstrate that the translocation of UDP-galactofuranose from the cytosol into the Golgi apparatus is a major route of UDP-galactofuranose utilization in *N. crassa*. The results show that loss of UDP-galactofuranose from the lumen of the Golgi apparatus is responsible for the mutant morphology, growth rate, conidiation, perithecia, and ascospore development phenotypes we see in the △*ugm-1* mutant.

2.3. Cell wall analysis verifies that cell walls from the △ugm-1 and △ugt-1, △ugt-2 mutants lack galactofuranose

To demonstrate that the Neurospora *ugm-1* gene encodes a UDP-galactomutase and that the *ugt-1* and *ugt-2* genes encode redundant UDP-galactofuranose translocators, we isolated cell walls from the mutant strains and compared their composition with the composition of a wildtype cell wall. We have previously shown that the major source of galactofuranose in the wildtype cell wall was from the N-linked and O-linked oligosaccharides present on cell wall glycoproteins (Patel et al. 2022; Kar et al. 2019). As shown in Table 1, galactofuranose is found in the wildtype cell wall, but we were unable to detect galactofuranose in the △*ugm-1* cell wall and the cell wall from the △*ugt-1*, △*ugt-2* double mutant. We conclude that the *ugm-1* UDG-galactose mutase is needed for the synthesis of UDP-galactofuranose. We also conclude that the △*ugt-1*, △*ugt-2* translocators are needed for the import of UDP-galactofuranose into the Golgi apparatus and for its incorporation into the N-linked and O-linked oligosaccharides present in the cell wall.

2.4. Phenotypic characterization of GT31 enzymes

We also characterized the role of the three *N. crassa* GT31 UDP-galactofuranosyltransferases by looking at the phenotypes of triple mutants lacking all three transferase enzymes. In *A. fumigatus*, these enzymes are responsible for generating an O-linked galactomannan structure with a chain of β-(1-5)-/β-(1,6)-linked galactofuranoses (Oka 2018; Komachi et al. 2013; Chihara et al. 2020; Katafuchi et al. 2017). No similar galactofuranose chain structures have been identified in *N. crassa*, but we considered the possibility that the GT31 enzymes might function in adding galactofuranose residues to the *N. crassa* N-linked and

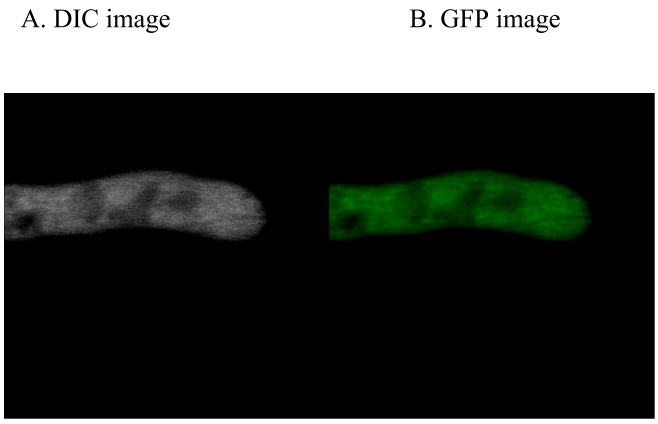

Fig. 7. UGM-1 is located within the cytosol. Cells expressing a chimeric UGM-1::GFP were examined with a confocal microscope. The images shown are from the growing tip of a germinating conidium. Panel A shows the DIC image and panel B the GFP fluorescence image.

Table 1

Sugar linkage	WT	△*ugm-1*	△*ugt-1* △*ugt-2*
GLUCOSE			
3-linked glucopyranose	55.6%	74.1%	59.2%
4-linked glucopyranose	8.7%	15.8%	12.2%
Terminal glucopyranose	1.7%	2.4%	2.4%
3,4-linked glucopyranose	3.6%	1.2%	9.2%
2,3-lined glucopyranose	4.5%	0.0%	0.0%
3,6-linked glucopyranose	2.6%	2.1%	4.2%
2,3,4-linked glucopyranose	0.0%	0.0%	2.4%
3,4,6-linked glucopyranose	0.0%	0.0%	2.6%
MANNOSE			
2,3-linked mannopyranose	4.3%	2.0%	5.5%
6-linked mannopyranose	1.3%	1.6%	1.4%
Terminal mannopyranose	0.0%	1.0%	1.0%
GALACTOSE			
5-linked galactofuranose	6.9%	0.0%	0.0%
3-linked galactofuranose	6.7%	0.0%	0.0%
Terminal galactofuranose	4.0%	0.0%	0.0%

Glycosyl linkage analysis of the cell wall carbohydrates from wildtype cell wall, and the cell walls from the △*ugm-1* mutant and the △*ugt-1*, △*ugt-2* double mutant.

O-linked oligosaccharides. We found that the gt31 triple mutants did not phenocopy the △*ugm-1* mutants and △*ugt-1*, △*ugt-2* double mutants. The linear growth rate of a △*gfs-1*, △*gfs-2*, △*gfs-3* triple mutant was 0.320 +/- 0.008 cm/hr, as compared with the wildtype growth rate of 0.303 +/- 0.017 cm/hr. The morphology of the △*gfs-1*, △*gfs-2*, △*gfs-3* triple mutants was indistinguishable from that of the wildtype. Conidia produced was found to be 1.95 +/- 0.17 $\times$ 10^8 conidia per slant, as compared to a value of 1.77 +/- 0.26 $\times$ 10^8 conidia per slant for the wildtype (Fig. 4). Our results show that the △*gfs-1*, △*gfs-2*, △*gfs-3* triple mutants were indistinguishable from the wildtype, which suggests that the GT31 enzymes are not responsible for producing the galactofuranose-containing oligosaccharides, whose loss generates the mutant phenotypes we see in the △*ugm-1* and △*ugt-1*, △*ugt-2* mutants.

3. Discussion

Previous studies have shown the importance of the galactofuranose pathway for growth and virulence in the Aspergilli (Schmalhorst et al. 2008; Komachi et al. 2013; Park et al. 2015; Afroz et al. 2011; Engel, Schmalhorst, and Routier 2012; Engel et al. 2009; El-Ganiny, Sanders, and Kaminskyj 2008; Damveld et al. 2008; Alam et al. 2014). Our results show that the loss of galactofuranose in *N. crassa* not only affects vegetative growth and asexual development (conidiation) (Figs. 2, 3, and 4), but also dramatically affect female development and ascospore formation (Figs. 5 and 6). Our results lend credence to the idea that the galactofuranose pathway would be a good target for the development of antifungal agents.

In *N. crassa*, galactofuranose has been shown to be a component of O-linked oligosaccharides and in a galactomannan structure attached to N-linked oligosaccharides (Patel and Free 2019). The O-linked oligosaccharides are thought to consist of mannoses attached to a serine or threonine residue with terminal galactofuranose (Patel and Free 2019). For O-linked oligosaccharides, the initial mannose is attached by one of three PMT (protein mannose transferases), and loss of any of the PMT enzymes is a lethal event (Patel and Free 2019). Additional mannoses are thought to be added by MNT-1, and mutations to *mnt-1* result in a tight colonial morphology, an inability to generate conidia, and in female infertility (Bowman et al. 2005). Galactofuranose is the terminal sugar present on O-linked oligosaccharides.

The galactomannan structure attached to *N. crassa* N-linked oligosaccharides has some similarities to the outer-chain mannans seen in yeast. It contains a short chain of α-1,6-linked mannoses with a single galactofuranose attached as a side group to the mannoses at the C2 position (Nakajima et al. 1984b; Leal et al. 1996; Kar et al. 2019). Mutants lacking OCH-1, which attaches the initial mannose residue of the structure to the N-linked oligosaccharide and thus lack the galactomannan, have a tight colonial phenotype, have abnormal conidiation, and are female fertile but unable to release the ascospores from the perithecia (Maddi and Free 2010).

It is interesting and instructive to compare the phenotypes of the △*ugm-1* and △*ugt-1*, △*ugt-2* mutants with the phenotypes of the △*mnt-1* and △*och-1* mutants, which are affected in the initial steps of O-linked and N-linked oligosaccharides biosynthesis. The △*mnt-1* and △*och-1* mutants have very tight colonial growth phenotypes while the △*ugm-1* and △*ugt-1*, △*ugt-2* mutants have what would be considered a spreading or semi-colonial growth phenotype. This suggests that the loss of the galactofuranose is not as deleterious as losing the entire oligosaccharide structures. Since the △*och-1* and △*mnt-1* mutants are affected in conidiation and in sexual development, the mutant conidiation and ascospore development phenotypes seen in the △*ugm-1* and △*ugt-1*, △*ugt-2* mutants might be expected, since both O-linked and N-linked oligosaccharides are affected in the galactofuranose pathway.

What was a little surprising was that the phenotype of the △*ugt-1*, △*ugt-2* mutant was clearly less severe than that of the △*ugm-1* mutant. We hypothesize that, in addition to UGT-1 and UGT-2, the cell contains another sugar nucleotide transporter (a transporter dedicated to a sugar other than galactofuranose) which might be able to promiscuously translocate very small amounts of UDP-galactofuranose across the GA membrane. This would allow for a small amount of galactofuranose to be added to N-linked and O-linked oligosaccharides and provide for a less severe phenotype.

In summary, we have shown that the galactofuranose pathway is important for all stages of the Neurospora life cycle. UDP-galactopyranose mutase (*ugm-1*) mutants have a spreading colonial growth morphology, reduced conidiation, and are dramatically affected in ascospore development (Figs. 2, 3, 4, 5 and 6). Neurospora contains two functional UDP-galactofuranose translocators, *ugt-1* and *ugt-2*. The double mutant lacking both translocators generally phenocopies the △*ugm-1* mutant but is slightly less severe. Efforts to identify galactofuranosyltransferases among the GT31 family glycosyltransferases encoded in the Neurospora genome failed to identify a galactofuranosyltransferase. Further experiments will be needed to identify the enzymes responsible for adding the terminal galactofuranose present on O-linked oligosaccharides and the galactomannans found on N-linked oligosaccharides.

4. Materials and methods

4.1. Growth conditions and strains

Neurospora cells were grown on a Vogel's agar and liquid media containing 2% sucrose and supplemented with histidine and/or hygromycin as needed. Matings were performed as described by Davis and DeSerres (Davis and DeSerres 1970) using either agar synthetic crossing medium containing 0.5% sucrose or by using discs of 3MM filter paper as a carbon/energy source in a Petri dish with 5 ml of liquid synthetic crossing medium.

Deletion mutants for the steps in galactomannan synthesis were obtained from the Fungal Genetics Stock Center. These deletion mutants were generated as part of the Neurospora genome project and have their coding regions replaced by a hygromycin resistance cassette (Colot et al. 2006). The deletion strains used included *Δugm-1* (NCU01824) *mt*a, *Δugt-1* (NCU01826) *mt*a, *Δugt-2* (NCU01456) *mt*A, *Δgfs-1* (NCU05878) *mt*a, *Δgfs-2* (NCU7762) *mt*A, and *Δgfs-3* (NCU02213) *mt*a. The *Δtre-1* (NCU00943) *mt*a and *mt*A strains (trehalase deletion mutants) were chosen as representative deletion library strains and used as control/wildtype isolates in our analysis. Double mutants for UDP-galactofuranose translocases and possible galactofuranosyltransferases were generated by matings between strains containing single deletion mutations. Triple mutants lacking all three possible gt31 galactofuranosyltransferases were generated by mating two double mutants. The presence of the wildtype and deletion alleles for each of the genes being analyzed in these matings was determined by isolating genomic DNA from several isolate progeny and using PCR assays to examine their genomic DNA sequences. Two sets of primers were used in these PCR reactions that allowed for the specific amplification of the deletion allele and the wildtype allele. To determine if the wild type allele of a gene was present in a progeny genome, the PCR reaction used the forward primer positioned upstream (F primers) of the deletion crossover location and a reverse primer from within the gene's coding region (R primers). The primers used in these reactions are found in Table S1. To determine if a deletion allele of a gene was present, we carried out a PCR assay using the forward primer positioned upstream of the crossover site involved in generating the deletion and a reverse primer from within the hygromycin resistance cassette (Table S1).

The wildtype *ugm-1* gene was cloned by using primer 1824 CF (ACATGCGGCCGCTGTAGTGTTGCAGGCCATGCC) and primer 1824 CR (CATCTTAATTAAGTTAGGAAGCGCCGACCTCGC) to PCR amplify the *ugm-1* gene and insert it into the pMF272 vector (Bowman et al. 2009) using NotI and PacI restriction enzymes. The insertion of the *ugm-1* coding region into the pMF272 generates a chimeric *ugm-1*::GFP gene encoding a protein with the GFP coding region located at the carboxyl terminus of UGM-1. The gene was inserted into the *his-3* locus of a △*ugm-1*, *his-3 mt*a isolate by homologous recombination between *his-3* sequences in the pMF272 vector and the genomic *his-3* gene (Bowman et al. 2009). Transformants containing the wildtype *ugm-1* gene were isolated and tested for their ability to complement the △*ugm-1* mutant phenotypes. A transformant was mated with a △*ugm-1* mtA isolate to obtain a complemented isolate of mating type A (*mt*A), and matings between two complemented strains were used to assess the ability of the wildtype copy of *ugm-1* to complement the △*ugm-1* mating phenotype.

4.2. Cell wall isolation and compositional analysis

Cell walls were isolated from wildtype and mutant isolates that had been grown in liquid Vogel's sucrose medium for 48 h at 30 °C on an orbital shaker (120 rpms) as previously described (Maddi, Bowman, and Free 2009). Briefly, the hyphae were collected on a Buchner funnel, ground to a fine powder under liquid N2 in a mortar and pestle, suspended in PBS, and the cell walls isolated by centrifugation at 6,000 × G. The cell walls were suspended in PBS containing 1% SDS, and subject to 100 °C for 15 min to remove non-covalently attached proteins and lipids, and then washed 3 times with PBS and 3 times with distilled water. A glycosyl linkage analysis was performed by combined gas chromatography-mass spectrometry (GC–MS) of partially methylated alditol acetate (PMAA) derivatives from the cell wall samples (Anumula and Taylor 1992). Permethylation of the samples was achieved by two rounds of treatment with sodium hydroxide (15 min) and methyl iodide (30 min). The samples were then hydrolyzed using 2 M trifluoroacetic acid (2 hr at 120 °C), reduced with $NaBD_4$, and acetylated using acetic anhydride/trifluoroacetic acid. The resulting PMAAs were analyzed on an Agilent 7890A GC interfaced to a 5975 MSD (mass selective detector, electron impact ionization mode); separation was performed on a 30 m Supelco SP-2331 bonded phase fused silica capillary column for the neutral residues and an EC-1 column for the amino containing residues.

4.3. Phenotypic characterization

To determine the linear growth rate of the mutant strains, a 5 μl drop of conidia was inoculated at the edge of a Petri dish containing Vogel's 2% sucrose 2% agar medium and grown at 30ºC. These linear growth rate experiments were performed in triplicate. The extension of the hyphae across the agar medium was monitored by marking the location of the leading edge of the colony at 10 h and 20 h post-inoculation. The linear growth rate was calculated as the average hourly rate of extension of the colony leading edge in the time interval from 10 to 20 h post-inoculation. An average growth rate with a standard deviation was determined for the wildtype and for the mutant strains. To examine colony morphology, the growing edges of the colonies were viewed in a dissecting microscope with overhead illumination and photographed with a Canon Powershot A620 camera fitted with a microscope adaptor.

The production of conidia was assessed by inoculating test tubes with 3 ml slants of Vogel's sucrose medium with mutant and control isolates and allowing the cells to grow at 30 °C for 10 days. The conidia produced were harvested by adding 2 ml of water with 0.01% NP40 to each slant and vortexing the slant for 30 s. The number of conidia produced was determined by placing a drop of the harvested conidia on a hemocytometer and counting the conidia present. The average number of conidia per slant and a standard deviation was calculated for the wildtype and mutant strains.

How the lack of galactomannan affected the sexual stage of the life cycle (perithecium and ascospore development) was assessed by doing matings using Whatman 3MM paper as an energy source with synthetic crossing medium (Davis and DeSerres 1970). Two circles of Whatman 3MM paper (8 cm in diameter) were sterilized by autoclaving, placed in sterile Petri dishes with 5 ml of liquid synthetic crossing medium, and inoculated with conidia from both strains participating in the mating. The formation of protoperithecia and perithecia was followed by visual examination of the Petri dishes. To observe the formation of beaks in perithecial development, the Petri dishes were placed at a 30-degree angle and photographed with a Canon Powershot A620 digital camera with overhead illumination. The development of ascospores was followed by doing perithecia squashes as described by Davis and DeSerres (Davis and DeSerres 1970).

4.4. Confocal microscopy

To observe the intracellular location of UGM-1, Neurospora transformants expressing the chimeric UGM-1::GFP protein were for grown eight hr in Vogel's sucrose liquid medium. Germlings were placed on a microscope slide and examined with a Zeiss LSM710 confocal laser scanning microscope. A plan-aprochromat 40X oil DIC M27objective lens was used for GFP imaging. The images were collected at 493–598 nm with excitation at 488 nm.

CRediT authorship contribution statement

Hayden Schaff: Investigation, Methodology. **Protyusha Dey:**

Investigation, Methodology, Writing – review & editing. **Christian Heiss:** Formal analysis, Investigation, Methodology. **Griffin Keiser:** Investigation. **Tatiana Rojo Moro:** Investigation. **Parastoo Azadi:** Funding acquisition, Project administration, Resources. **Pavan Patel:** Investigation. **Stephen J. Free:** Conceptualization, Formal analysis, Funding acquisition, Project administration, Resources, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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We declare that the research was conducted in the absence of any commercial or financial relationships that could be considered as a potential conflict of interest.

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