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Transcriptomic and molecular genetic analysis of the cell wall salvage response of *Aspergillus niger* to the absence of galactofuranose synthesis

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Summary

The biosynthesis of cell surface-located galactofuranose (Gal*f*)-containing glycostructures such as galactomannan, *N*-glycans and *O*-glycans in filamentous fungi is important to secure the integrity of the cell wall. UgmA encodes an UDP-galactopyranose mutase, which is essential for the formation of Gal*f*. Consequently, the Δ*ugmA* mutant lacks Gal*f*-containing molecules. Our previous work in *Aspergillus niger* work suggested that loss of function of *ugmA* results in activation of the cell wall integrity (CWI) pathway which is characterized by increased expression of the *agsA* gene, encoding an α-glucan synthase. In this study, the transcriptional response of the Δ*ugmA* mutant was further linked to the CWI pathway by showing the induced and constitutive phosphorylation of the CWI-MAP kinase in the Δ*ugmA* mutant. To identify genes involved in cell wall remodelling in response to the absence of galactofuranose biosynthesis, a genome-wide expression analysis was performed using RNAseq. Over 400 genes were higher expressed in the Δ*ugmA* mutant compared to the wild-type. These include genes that encode enzymes involved in chitin (*gfaB*, *gnsA*, *chsA*) and α-glucan synthesis (*agsA*), and in β-glucan remodelling (*bgxA*, *gelF* and *dfgC*), and also include several glycosylphosphatidylinositol (GPI)-anchored cell wall protein-encoding genes. *In silico* analysis of the 1-kb promoter regions of the up-regulated genes in the Δ*ugmA* mutant indicated overrepresentation of genes with RlmA, MsnA, PacC and SteA-binding sites. The importance of these transcription factors for survival of the Δ*ugmA* mutant was analysed by constructing the respective double mutants. The Δ*ugmA*/Δ*rlmA* and Δ*ugmA*/Δ*msnA* double mutants showed strong synthetic growth defects, indicating the importance of these transcription factors to maintain cell wall integrity in the absence of Gal*f* biosynthesis.

Introduction

The fungal cell wall is essential to prevent lysis during growth and development. The cell wall of filamentous fungi is composed of several different carbohydrate polymers (chitin, β-1,3-glucan, β-1,3/1,4-glucan, α-glucan and galactomannan) and glycoproteins (galactomannoproteins) (Guest and Momany, 2000; Gastebois *et al.*, 2009; Free, 2013). In *Aspergilli*, β-1,3-glucan together with chitin form the cell wall scaffold to which other components (galactomannan and α-glucans) bind and are retained (Fontaine *et al.,* 2000; Dichtl *et al.,* 2015). The β-1,3-glucan also acts as a scaffold to cell wall proteins as the *A. niger*, cell wall protein A (CwpA) and at least six other cell wall proteins have been shown to be covalently linked to the β-glucan part of the cell wall (Damveld *et al.,* 2005b).

Attacks by compounds that inhibit cell wall synthesis or by cell wall-degrading enzymes secreted by competing microorganisms or plants represent a serious threat for fungal survival. Fungi have developed several surviving strategies to cope with these threats. The best known strategy of cell wall reinforcement in response to cell wall stress is to

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activate the so-called cell wall integrity (CWI) pathway. This pathway is studied in *Saccharomyces cerevisiae* in most detail, but is conserved in several other species, including *Aspergilli* (Levin, 2011; Valiante *et al.,* 2015). The CWI signalling pathway is composed of cell wall stress sensors, which generate a signal that activates a Rho-Pkc1-MAP kinase module, resulting in the activation of the Rlm1 and Swi4/6 transcription factors. These transcription factors are required for modulating gene expression in response to cell wall stress (see for review: Levin, 2011). In *A. niger* the requirement of RlmA for the induction of the α-glucan synthase A gene (*agsA*) in response to Calcofluor white has been established (Damveld *et al.,* 2005). Deletion of RlmA renders *A. niger* sensitive to Calcofluor white, and to a lesser extent also to other cell wall disturbing agents such as caspofungin (CA), aureobasidinA (AbaA), FK506 or fenpropimorph (FP), indicating that other remodelling mechanisms might exist (Fiedler *et al.,* 2014). In the same study, transcription factor binding site enrichment analysis of differentially expressed genes in response to CA, AbaA, FK506 and FP was performed, which identified possible involvement of both RlmA and MsnA (homolog of Msn2/4 in *S. cerevisiae*) transcription factors in counteracting CA-induced cell wall stress. Indeed, deletion of the MsnA transcription factor, like deletion of the RlmA transcription factor, led to increased susceptibility towards CA (Fiedler *et al.,* 2014). MsnA has been shown to be required for coping with several stress responses, including osmotic and oxidative stress (Han and Prade, 2002; Bose *et al.,* 2005; Hong *et al.,* 2013).

The observation that *agsA* expression is induced in response to cell wall stress was previously used to set up a mutant screen for cell wall mutants (Damveld *et al.,* 2008). The rationale of this screen was that mutants with impaired cell wall strength, e.g. because of a mutation in a gene that encodes an enzyme involved in cell wall biosynthesis, would activate *agsA* expression. To isolate mutants showing constitutive activation of the *agsA* gene, the acetamidase reporter gene was cloned behind the *agsA* promoter, and mutants able to grow on acetamide were isolated. Using this screen, we identified the *ΔugmA* mutant, which lacks Gal*f* in its cell wall (Damveld *et al.,* 2008; Park *et al.,* 2014). The *ugmA* gene encodes a UDP-galactopyranose mutase, which converts UDP-galactopyranose to UDP-galactofuranose (UDP-Gal*f*). UDP-Gal*f* is subsequently used for the synthesis of Gal*f*-containing glycoconjugates such as cell wall galactomannan, *N*- and *O*-glycans, and glycolipids (Tefsen *et al.,* 2012).

In this study, we aimed at obtaining a broader view of understanding cell wall remodelling in the absence of Gal*f*-glycoconjugate biosynthesis. We therefore used the *ΔugmA* mutant to perform a genome-wide expression analysis using RNA-seq to identify differentially expressed genes. As expected, among the genes induced in the *ΔugmA* mutant, the *agsA* gene was included, as well as several other genes whose function could be directly linked to cell wall biosynthesis. A transcription factor binding site enrichment analysis of genes up-regulated in the *ΔugmA* mutant strain revealed several transcription factor binding sites to be overrepresented, including binding sites for the RlmA, MsnA, PacC and SteA transcription factors. The *ΔugmA/ΔrlmA* and *ΔugmA/ΔmsnA* double mutants showed severe synthetic growth defects, indicating that RlmA and MsnA play an important role in survival of the *ΔugmA* mutant. By aligning the new results obtained in this study to previous cell wall stress related transcriptomic studies in *A. niger*, a better understanding of the transcriptional response of the CWI signalling response is obtained which serves as a solid basis for comparing both differences and similarities in relation to CWI signalling in filamentous fungi.

Results

Constitutive phosphorylation of CWI-MAPK in the ΔugmA *mutant of* A. niger

The *ΔugmA* mutant of *A. niger* was isolated in a screen for cell wall mutants by selecting for mutants with induced expression of *agsA*. Targeted deletion of the *ugmA* gene confirmed its importance for CWI (Damveld *et al.,* 2008). In subsequent studies, we showed that the *ΔugmA* mutant does not contain Gal*f*-containing glycostructures in the cell wall (galactomannan) or the medium (galactomannoproteins) (Park *et al.,* 2014; Park *et al.,* 2015). Because the induction of *agsA* in response to Calcofluor White induced cell wall stress is RlmA dependent (Damveld *et al.,* 2005a) it was previously suggested that the transcriptomic responses in the *ugmA* mutant were mediated via the CWI pathway (Damveld *et al.,* 2008). To establish the role of the CWI pathway in cell wall remodelling in the *ΔugmA* mutant more firmly, the phosphorylation state of the CWI-related MAP kinase (CWI-MpkA) was evaluated. Therefore, proteins extracts were prepared from wild-type and *ΔugmA* strains and analysed by Western blot using anti-phospho p44/42. The total amount of MpkA was detected by using the anti p44/42 MAPK antibody. As shown in Fig. 1, the MAP kinase in the *ΔugmA* mutant displays at various time points during growth a consistent higher phosphorylation status compared the wild-type strain. Quantification of the signal indicates a threefold higher level of phosphorylation (Fig. 1). These results indicate that loss of galactofuranose biosynthesis signals via MpkA phosphorylation in the *A. niger* CWI pathway.

Growth analysis of the ΔugmA *mutant of* A. niger

To perform genome-wide expression analysis of the *ΔugmA* mutant, we initially cultivated it in a bioreactor under

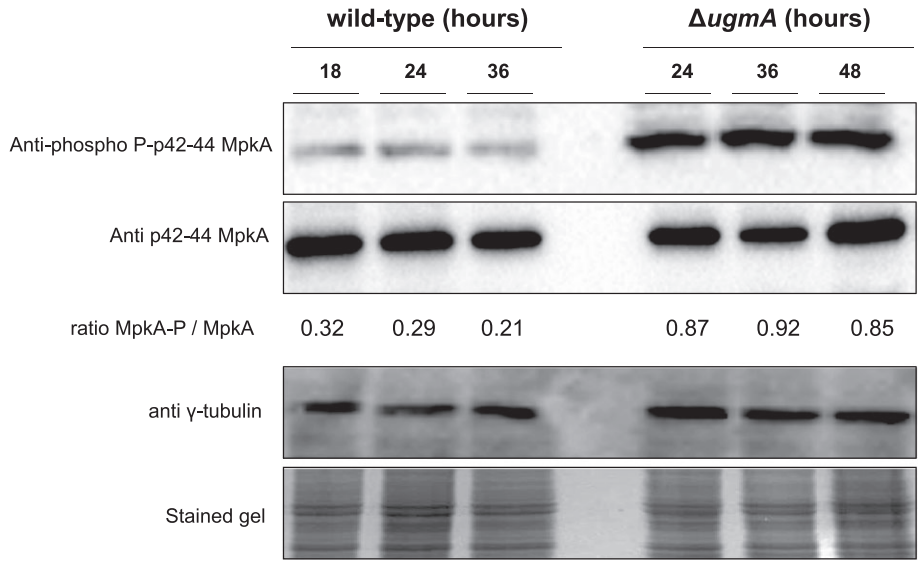


Fig. 1. The *ΔugmA* mutant strain has increased and constitutive CWI pathway activation. Conidia from the wild-type and mutant strain were grown in liquid CM medium. The samples were collected at the indicated time points for Western blot preparation. The phosphorylated fractions and the total MpkA amount were detected using anti-phospho p44/42 MAPK and anti-p44/42 MAPK antibodies respectively. The γ-tubulin antibody and the Coomassie Brilliant Blue stained gel were used as loading sample controls. Densitometry analysis of western blots showing the ratio of phosphorylated MpkA/MpkA expressed as the relative abundance (arbitrary units).

conditions normally used for wild-type *A. niger* batch cultivations (Jørgensen *et al.,* 2010; Nitsche *et al.,* 2012). According to this standard growth protocol, the pH of the cultivations is set at pH 3.0 during spore germination and kept constant during the exponential and post-exponential growth phases. Starting the cultivation at pH 3.0 prevents aggregation of the conidiospores and thereby prevents pellet formation. The pH is normally kept continuously at 3.0, which is close to the normal ambient pH created by *A. niger* through the secretion of acids. However, we noticed very poor growth of the *ΔugmA* at pH 3.0 (Table 1) compared to the wild-type strain (N402) and therefore also examined growth of the *ΔugmA* mutant at other pH values. In the bioreactor cultures run at pH 4.0, pH 5.0 and pH 6.0 were started at pH 3.0 to prevent spore aggregation and the pH was set to the desired pH after germination of the spores which takes about 5 h. The maximum growth rate of the *ΔugmA* was strongly improved at pH 4.0 and 5.0, but still slightly lower than in the wild-type (Table 1). At pH 6.0, the maximum growth rates of both the wild-type and the *ΔugmA* mutant were significantly lower (Table 1). The morphology of the *ΔugmA* mutant was still aberrant at pH 5.0 compared to the wild-type strain and characterized by short hyphal fragments and increased branching (Fig. 2A). At all pH conditions, the *ΔugmA* mutant did not produce any Gal*f*-containing glycostructures in the culture medium, whereas Gal*f* was present in the culture medium of the wild-type strain (Fig. 2B shows data for pH 5.0; data obtained at the other pHs not shown).

Table 1. Growth characteristics of the wild-type (N402) and the *ΔugmA* mutant in pH-controlled bioreactors.

	N402		*ΔugmA*	
pH	μ_{max} (h^{-1})*	Max. biomass (g/L)	μ_{max}(h^{-1})	Max. biomass (g/L)
3.0	0.22 ± 0.02	4.81 ± 0.53	0.04	2.43
4.0	0.23	3.79	0.20	4.24
5.0	0.25 ± 0.04	3.37 ± 0.02	0.20 ± 0	3.45 ± 0.01
6.0	0.14 ± 0.01	1.28 ± 0.18	0.11 ± 0.01	1.57 ± 0.05

*The averages and standard deviations of the growth rate are based on independent bioreactor cultivations.

Comparison of the transcriptome of the ΔugmA *mutant with the wild-type strain*

To identify genes in *A. niger* that are differentially expressed in the *ΔugmA* mutant compared to the wild-type strain, RNA was extracted from mycelium of the wild-type (N402) and the *ΔugmA* mutant after growth at pH 5. The maximum growth rate of the *ΔugmA* strain (0.20 h^{-1}) was slightly lower than the growth rate of the wild-type strain (0.25 h^{-1}). Biomass accumulation profiles of the wild-type (N402) and *ΔugmA* mutant at pH 5 were reproducible (Table 1) and mycelium for RNA extraction was harvested when 60% of the maximum biomass was reached. The RNA samples were analysed by Northern blot to examine whether marker genes known or expected to be induced in the *ΔugmA* mutant were up-regulated. As the *ΔugmA* mutant was identified in a screen for mutants with induced expression of an *agsA*-driven reporter gene (Damveld *et al.,* 2008), we expected the *agsA* gene to be higher expressed in the *ΔugmA*. As shown in Fig. 3, the *agsA* gene was indeed strongly induced in the *ΔugmA* mutant compared to the

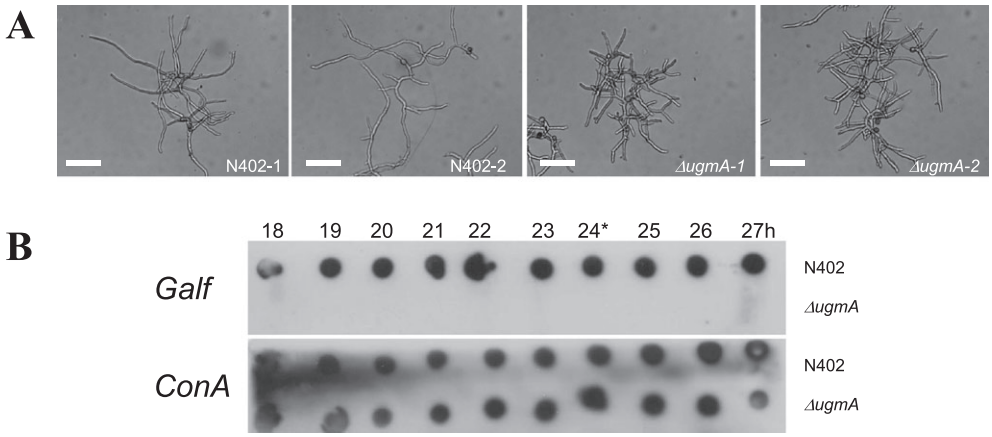
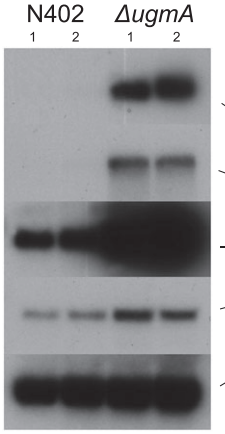


Fig. 2. Cultivation of the wild-type (N402) and the *ΔugmA* mutant in bioreactors.
A. Hyphal morphology of N402 and *ΔugmA* mutant. Note the compact growth phenotype of the *ΔugmA* mutant.
B. Dot blot assay to detect the presence of Gal*f* residues on secreted glycoconjugates from *A. niger* during batch growth. *A. niger* wild-type strain (N402) and *ΔugmA* mutant were grown for the times indicated from inoculation of the spores, and cell-free medium was spotted on nitrocellulose filter paper. The blots were incubated with the anti-Gal*f* antibody (L10) (Heesemann *et al.,* 2011) to detect the presence of Gal*f* or incubated with ConA-PO to detect mannoproteins.

wild-type strain. We also tested the expression of three other genes (*gfaA*: glutamine:fructose-6-phosphate amidotransferase, which is responsible for the first step in chitin synthesis) and two genes encoding putative cell wall proteins (PhiA and PirA), which were previously shown to be induced by cell wall stress (Ram *et al.,* 2004; Meyer *et al.,* 2007). These genes were also clearly induced in the *ΔugmA* mutant indicating that a typical cell wall stress response was activated in the *ΔugmA* mutant (Fig. 3).

Genome-wide expression analysis using RNA-seq revealed in total 741 genes that were differentially expressed between the wild-type and the *ΔugmA* mutant. Differential expression was defined as a statistically significant, differential expression with a false discovery rate (FDR) <0.05. In total, 432 genes were relatively higher expressed in the *ΔugmA* mutant, and we will refer to these genes as UgmA^{up} and 309 genes were relatively lower expressed in the *ΔugmA* mutant (UgmA^{down}). The normalized expression values for the genes monitored by Northern blot are shown in Fig. 3 and agree well with the Northern blot results. A comprehensive list of all differentially expressed genes including statistical significance and transcript ratios is presented in Table S1 (UgmA^{up}) and Table S2 (UgmA^{down}).

To get a better insight into the processes that are affected in the *ΔugmA* mutant, we performed GO-enrichment analysis of the genes that were significantly higher expressed in the *ΔugmA* mutant using FetGOat (Nitsche *et al.,* 2012) (Tables 2 and 3). Twenty-one GO terms (Biological Processes) were overrepresented in the *ΔugmA* mutant (Table S3). Among those GO terms, six terminal nodes were present (Table 2). GO enrichment analysis



Normalized expression data of genes selected for Northern analysis

Gene	Value N402	Value *ugmA*	Log²FC	Q-value
phiA	2,6	378,5	7,2	< 0.05
agsA	1,2	49,0	5,3	< 0.05
pirA	172,6	1756,6	3,3	< 0.05
gfaA	331,2	770,7	1,2	< 0.05
actA	1306,6	1303,7	-0,0	0,99

Fig. 3. Northern blot analysis of selected genes during batch growth in bioreactors. RNA was isolated from mycelium grown at pH 5.0 for 18 h, when about 60% of the glucose was consumed, and analysed by Northern blot. Hybridization with an actin probe was used to confirm similar loading.

Table 2. Enriched GO-terms in the up-regulated genes in the *ΔugmA* strain.

Terminal GO term	Description GO term	Systematic name	Gene name	Description
GO:0045490	Pectin catabolic processes	An14g04200	*rhgB*	Rhamnogalacturonan hydrolase
		An01g11520	*pgaI*	Polygalacturonase
		An04g09690		Putative pectin methylesterase
		An16g02730		Putative arabinan endo-1,5-alpha-L-arabinosidase
		An02g10550	*abnC*	Putative endo-alpha-1,5-arabinanase
GO:0006031	Chitin biosynthetic processes	An09g04010	*chsB*	Putative chitin synthase, class III; induced by caspofungin
		An03g05940	*gfaB*	Putative glutamine:fructose-6-phosphate amidotransferase
		An07g05570	*chsA*	Chitin synthase class I
		An12g07840	*gnaA*	Putative glucosamine-6-phosphate N-acetyltransferase
		An18g06820	*gfaA*	Putative glutamine:fructose-6-phosphate amidotransferase
GO:0005996	Monosaccharide metabolic processes	An01g11520	*pgaI*	Polygalacturonase
		An02g10550	*abnC*	Putative endo-alpha-1,5-arabinanase
		An14g04200	*rhgB*	Rhamnogalacturonan hydrolase
		An03g00960	*axhA*	1,4-Beta-D-arabinoxylan arabinofuranohydrolase
		An14g01800		Putative alpha-galactosidase
		An04g02670		Predicted oxidoreductase activity
		An09g02240	*nag1*	N-Acetyl-beta-glucosaminidase
		An02g11150	*aglB*	Putative alpha-galactosidase variant B
		An01g12550	*msdS*	Mannosyl-oligosaccharide 1,2-alpha-mannosidase
		An04g03200		Mannose-6-phosphate isomerase
		An01g00780	*xynB*	Endo-1,4-xylanase
		An12g07840	*gnaA*	Putative glucosamine-6-phosphate N-acetyltransferase
GO:0010383	Cell wall polysaccharide metabolic processes	An14g02760	*eglA*	Putative secreted endoglucanase A; xylose-induced
		An02g11150	*aglB*	Putative alpha-galactosidase variant B; expression is induced on xylan
		An18g06820	*gfaA*	Putative glutamine:fructose-6-phosphate amidotransferase
		An03g05940	*gfaB*	Putative glutamine:fructose-6-phosphate amidotransferase
		An01g00780	*xynB*	Endo-1,4-xylanase
		An14g01800		Putative alpha-galactosidase
GO:0009251	Glucan metabolic processes	An02g00850		Glucoamylase (exo-1,4-glucosidase/amyloglucosidase); beta-glucanase
		An16g06800	*eglB*	Putative endoglucanase
		An15g04900		Putative endo-glucanase
		An03g05290	*bgtB*	Glucan 1,3-beta-glucosidase; putative glucanotransferase (caspofungin induced)
		An07g08950	*eglC*	Endoglucanase; specific for substrates with beta-1,3 and beta (XlnR controlled)
GO:0070882	Cellular cell wall organization or biogenesis	An05g00340		Unknown function, Has domain(s) with predicted hydrolase
		An14g02760	*eglA*	Putative secreted endoglucanase A; abundantly expressed on D-xylose; irreversibly inhibited by palladium ion; XlnR regulated
		An10g00430		Strong similarity to agglutinin core protein Aga1
		An08g03510		Serine-rich protein
		An06g01900		Similarity to polyphosphoinositide binding protein Ssh2p
		An07g05570	*chsA*	Chitin synthase class I
		An14g01800		putative Alpha-galactosidase
		An02g07670		Serine-rich protein
		An02g11150	*aglB*	Putative alpha-galactosidase variant B; expression is induced on xylan; XlnR regulated
		An18g06820	*gfaA*	Putative glutamine:fructose-6-phosphate amidotransferase (CA-induced)
		An03g05940	*gfaB*	Putative glutamine:fructose-6-phosphate amidotransferase (CA-induced)
		An01g09050		Ortholog(s) have FAD transmembrane transporter activity

ofthe genes down-regulated in the *ΔugmA* mutant identified three GO terms that were related to zinc metabolism (Table S4). The three genes in the terminal node (GO:0071577; zinc ion transmembrane transport) (Table 3) all encode putative zinc transporters, suggesting a lower need for zinc in the *ΔugmA* mutant. Closer examination of the genes and GO terms upregulated and enriched in the *ΔugmA* mutant revealed two groups of overrepresented genes. The first group of genes encodes proteins related to chitin biosynthesis and included genes

Table 3. Enriched GO-term in genes downregulated in the *ΔugmA* strain.

Terminal GO term	Description GO term	Systematic name	Gene name	Description
GO:0071577	Zinc ion transmembrane transport	An01g01620		Putative high-affinity zinc uptake transmembrane transporter
		An12g10320	*zrtA*	Putative high-affinity zinc transport protein
		An01g06690		Putative low-affinity zinc ion transmembrane transporter

Table 4. Differentially expressed cell wall modifying genes (FC > 1.3; FDR < 0.05).

Upregulated genes			Normalized expression value							
beta-1,3-glucan	Description	Gene name	N402	*ΔugmA*	$\log_2$ FC	Q-value		RlmA-box	MsnA-box	CrzA-box
An10g00400	Putative 1,3-beta-glucanosyltransferase GH72;	*gelA*	335.5	637.5	0.93	0.028	up	n	y	y
An16g06120	Putative 1,3-beta-glucanosyltransferase GH72	*gelF*	1.6	47.2	4.91	0.001	up	n	n	n
An02g09050	Putative 1,3-beta-glucanosyltransferase GH72	*gelG*	2.2	5.4	1.30	0.035	up	n	y	y
An03g05290	Putative beta-1,3-glucanosyltransferase GH17	*bgtB*	668.5	1601.5	1.26	0.013	up	y	y	y
An13g02510	Putative transglycosidase of GH16-family ScCrh1	*crhE*	1.5	11.4	2.91	0.001	up	n	y	n
An01g12450	Putative exo-beta-1,3-glucanase GH55-family	*bxgA*	73.2	193.5	1.40	0.001	up	n	y	n
Alpha-glucan										
An04g09890	Putative alpha1,3-glucan synthase	*agsA*	1.2	49.0	5.34	0.001	up	y	y	n
An08g09610	Putative alpha-1,3-glucanase GH71	*agnD*	51.8	91.8	0.82	0.035	up	n	y	y
Chitin										
An09g04010	Putative chitin synthase class-III	*chsB*	144.3	282.8	0.97	0.016	up	n	y	n
An12g10380	Putative chitin synthase class-III	*chsE*	80.8	160.3	0.99	0.007	up	y	y	n
An07g05570	Putative chitin synthase class-II	*chsA*	20.5	42.5	1.05	0.001	up	n	y	y
An04g04670	Putative class-V chitinase (GH18)	*cfcC*	36.8	72.4	0.98	0.006	up	y	y	y
Galactomannan										
An02g08670	UDP-galactofuranose transporter	*ugtA*	245.9	456.4	0.89	0.035	up	y	n	n
An12g08720	UDP-galactofuranose transferase	*gfsA*	98.0	203.7	1.06	0.002	up	y	y	n
CWP-linking										
An14g03520	Putative endo-mannanase (GH76-family) with a possible role in GPI-CWP incorporation	*dfgC*	42.0	107.8	1.36	0.001	up	y	y	n
Downregulated genes beta-1,3-glucan										
An19g00090	Putative exo-beta-1,3-glucanase GH55-family	*bgxC*	2.9	1.0	−1.55	0.011	down	n	n	n
Chitin										
An09g06400	Putative class-III chitinase (GH18)	*ctcA*	255.7	2.2	−6.86	0.001	down	n	n	n
Galactomannan										
An02g11320	UDP-glucose 4-epimerase	*ugeC*	72.6	33.0	−1.14	0.002	down	n	n	n
An02g08660	UDP-galactose mutase	*ugmA*	167.8	5.5	−4.93	0.001	down	n	n	n
CWP-linking										
An11g01240	Putative endo-mannanase (GH76-family) with a possible role in GPI-CWP incorporation	*dfgH*	10.2	4.1	−1.31	0.022	down	n	n	n

*Consensus bindings sites for RlmA (TAWWWWTAG), MsnA (AGGGG) and CrzA (GWGGCS) were used.

encoding proteins involved in UDP-N-acetyl-glucosamine biosynthesis (*gnaA, gfaA* and *gfaB*) as well as chitin polymerization (*chsA* and *chsB*) (Table 4). The second group encodes glycosyl hydrolases (GH) and includes genes encoding proteins that act on the plant cell wall polysaccharides cellulose, xylan and galactan. In total, 18 plant cell wall degradation genes were highly expressed in the *ΔugmA* mutant. (See below)

Cell wall remodelling in the ΔugmA *mutant*

Table 4 lists the differential expression of the genes directly involved in the biosynthesis of the cell wall polysaccharides β-glucan, α-glucan, chitin and galactomannan (Pel *et al.,* 2007). In addition to *agsA* and genes involved in UDP-N-acetyl-glucosamine biosynthesis (*gnaA*, *gfaA* and *gfaB*), differentially expressed genes include genes encoding proteins involved in chitin polymerization (*chsA* and *chsB*), a putative 1,3-β-glucanosyltransferase *gelF* and a gene encoding a putative glucan-chitin cross-linking enzyme (*crhE*). Although several chitin synthase-encoding genes showed a statistically significant induction, their fold-changes were limited, varying between 1.5 and 2. The strongest down-regulated gene in the *ΔugmA* mutants compared to wild-type is a chitinase (*ctcA*)-encoding gene showing a >100-fold decrease in expression (Table 4).

The genome of *A. niger* is predicted to contain 117 glycosylphosphatidylinositol (GPI)-anchored proteins (Pel *et al.,* 2007). GPI-anchored proteins can either remain attached to the plasma membrane (mostly cell wall biosynthetic enzymes) or can become covalently linked to the β-glucan part of the cell wall (Frieman and Cormack,

Table 5. Differentially expressed genes encoding putative (GPI)-anchored cell wall proteins*.

			Normalized expression value						
Upregulated	Description	Gene name	N402	*ΔugmA*	log_2 FC	Q-value	RlmA-box	MsnA-box	CrzA-box
An01g12240	Unknown function		4.2	385.3	6.54	0.001	y	n	y
An02g00850	Putative glucanase related to MLG1 - *Neurospora crassa*		4.7	57.0	3.61	0.001	y	n	y
An03g02510	Unknown function		0.92	2.9	1.66	0.046	n	y	n
An03g05530	Putative endo-beta-1.4-glucanase		16.9	54.0	1.68	0.001	y	y	n
An03g05620	Putative hydrolases or acyltransferases		1.0	9.1	3.19	0.002	n	n	n
An04g07160	Unknown function		52.7	162.9	1.63	0.001	n	y	n
An09g03260	Endo-polygalacturonase D	*pgaD*	1.5	4.3	1.57	0.032	n	y	y
An10g00390	Putative cellobiose dehydrogenase CDH		2.9	11.8	2.05	0.001	n	y	n
An12g07430	Putative dioxygenase C		4.2	32.2	2.95	0.001	n	y	n
An13g02670	Unknown function		0.6	183.2	8.27	0.001	n	y	n
An14g01068	Unknown function		112.1	275.2	1.30	0.001	y	y	y
An14g01820	Cell wall protein binB		2.6	378.6	7.17	0.001	y	y	y
An14g01840	Temperature-shock induced protein Tir3		107.8	719.7	2.74	0.002	n	n	y
An14g02170	Putative cutinase		0.26	2.0	2.97	0.123	n	n	n
An15g07790	Unknown function		n.d.	1.3	inf	0.001	y	y	y
An18g03730	Unknown function		507.6	3073.2	2.60	0.001	n	y	y
Downregulated									
An02g09010	Unknown function		7.6	2.7	−1.47	0.007	n	n	n
An02g13220	Putative lysophospholipase phospholipase		397.4	156.5	−1.34	0.001	n	n	n
An07g04620	*N. crassa* Ham7 required for cell fusion.	*ham-7*	136.8	13.4	−3.35	0.001	n	n	n
An07g06210	Unknown function		45.9	19.1	−1.27	0.003	n	n	n
An08g07090	Similarity to protein Sim1		105.0	43.7	−1.27	0.001	n	n	n
An08g09850	Putative phosphatase precursor		3.5	0.9	−2.01	0.013	n	n	n
An09g02340	Similarity to self-pruning protein SP - *Lycopersicon esculentum*		76.4	26.1	−1.55	0.001	n	n	n
An12g07750	Unknown function		78.9	23.1	−1.77	0.001	n	n	n
An13g02130	Putative aspartyl protease;		12.0	2.6	−2.22	0.001	n	n	n
An14g02100	Cell wall protein A (CwpA) *Aspergillus niger*	*cwpA*	65.9	1.2	−5.77	0.001	n	n	n
An16g01780	Unknown function		94.8	45.6	−1.06	0.002	n	n	n
An16g07920	Unknown function		165.4	46.8	−1.82	0.001	n	n	n
An16g07950	Unknown function		872.5	359.7	−1.28	0.001	n	n	n
An18g00630	Unknown function		23.5	7.9	−1.58	0.004	n	n	n
An18g01320	Putative aspartyl protease		80.5	45.0	−0.84	0.037	n	n	n
An18g04070	Unknown function		1322.2	230.5	−2.52	0.001	n	n	n
An18g06360	Similarity to surface antigen Csa1 -		14.7	5.6	−1.38	0.035	n	n	n

*GPI-anchored proteins known to be directly involved in cell wall biosynthesis (Table 4) are not included in this table.

2003, 2004). Table 5 summarizes the differentially expressed, putative GPI-anchored protein-encoding genes. Expression of 17 GPI-anchored protein-encoding genes was up-regulated in the *ΔugmA* and 16 was down-regulated, suggesting important changes in the composition of membrane- and cell wall-localized GPI-anchored proteins. The set of down-regulated genes also includes the gene encoding CwpA, which is currently the only cell wall protein from *A. niger* for which a covalent linkage to the cell wall has been experimentally confirmed (Damveld *et al.,* 2005).

The major pathway for cell wall remodelling is the CWI pathway, which has been studied most extensively in *S. cerevisiae.* This pathway comprises among other proteins, the cell wall stress sensors (Wsc- and Mid-proteins), the Rho1 GTPase module, Pkc1 and the Bck1-Mkk1/Mkk2-Mpk1 MAP kinase signalling cascade. Activation of the CWI pathway results in activation of the Rlm1 transcription factor, which can bind to a conserved motif in promoter regions of cell wall stress-induced genes and activate their transcription. Homologs of these proteins have been identified in *Aspergilli* and have been shown to be also involved in the cell wall stress response (Damveld *et al.,* 2005; Dichtl *et al.,* 2012). From the list of genes related to the CWI pathway of *A. niger* (Pel *et al.,* 2007), three genes encoding a putative cell wall stress sensor protein WscC (An07g04070), a small GTPase RhoB (An16g04200) and MkkA (An18g03740) were transcriptionally induced (Table 6). No CWI-related genes were down-regulated in the *ΔugmA* strain.

Identification of transcription factors important to sustain growth of the ΔugmA *strain*

The 432 significantly up-regulated genes in the *ΔugmA* strain were analysed using the TFBSF tool (Meyer *et al.,* 2009). The available 1 kb promoter regions of 419 of the 432 up-regulated genes were analysed for the presence of binding sites established for 118 fungal transcription factors. The number of genes with these binding sites in the group of 419 induced genes was determined and it was evaluated whether the genes with these binding sites are statistically significant over- or under-represented compared to groups of the same size comprising randomly selected genes of *A. niger* (500 000 bootstrap samples, FDR ≤0.0005) (Table S5).

Using this *in silico* approach, four transcription factor binding sites were found to be enriched (Table 7). These are SteA (ScSte12-homolog), PacC (ScRim101-homolog), MsnA (ScMsn2/4-homolog) and RlmA (ScRlm1-homolog). The identification of the RlmA transcription factor binding site is in agreement with the function of the RlmA transcription factor in the CWI pathway and its requirement for the induction of *agsA* (Damveld *et al.,* 2005). A recent study addressing the role of transcription factors in response to cell wall perturbing drugs identified RlmA, MsnA and CrzA transcription factors as being involved in cell wall remodelling in *A. niger* (Fiedler *et al.,* 2014). Although CrzA binding sites were not identified as being overrepresented in the *ΔugmA* induced gene set, we decided to include CrzA in our subsequent studies.

Table 6. Upregulated cell wall integrity pathway-related genes.

			Normalized expression value						
Gene identifier	Description	Gene name	N402	*ΔugmA*	$\log_2$ FC	Q-value	RlmA-box	MsnA-box	CrzA-box
An07g04070	Putative plasma membrane cell wall sensor	*wscC*	105.5	310.7	1.56	0.001	n	y	y
An16g04200	Putative Rho-related GTPase; ScRho2- and SpRho2-like	*rhoB*	118.8	255.8	1.11	0.003	y	n	y
An18g03740	MAPKK with putative function in CWI-signalling	*mkkA*	44.8	122.8	1.46	0.001	n	n	n

Table 7. Transcription factor binding site enrichment analysis of *ΔugmA*-induced genes.

S. cerevisiae transcription factor	*A. niger* ortholog	ReqGN	ReqPG	RanGN	RanPG	*p*-Value	FDR
Rim101p	PacC	316.00	75.42	270.08	64.46	2.00E – 06	1.18E – 04
Ste12p	SteA	229.00	54.65	161.53	38.55	2.00E – 06	1.18E – 04
Msn2p/Msn4p	MsnA	291.00	69.45	246.47	58.82	4.00E – 06	1.57E – 04
Rlm1p	RlmA	81.00	19.33	51.10	12.20	1.00E – 05	2.95E – 04

ReqGN: number of genes with at least one putative TF binding site in the gene set up-regulated in the *ΔugmA* mutant.
ReqPG: percent of genes with at least one putative TF binding site in the gene set up-regulated in the *ΔugmA* mutant.
RanGN: average number of genes with at least one putative TF binding site in random gene sets.
RanPG: average percent of genes with at least one putative TF binding site in random gene sets.
p-Value: probability to get equal/more extreme number of genes with at least one putative TF binding site in random gene sets compared to the gene set up-regulated in the *ΔugmA* mutant.
FDR: FDR corrected *p*-value.

To evaluate the importance of the five transcription factors (SteA, PacC, MsnA, RlmA and CrzA) with respect to growth of the *ΔugmA* strain, *ΔugmA/ΔTF* double mutants were generated and compared to the growth phenotype of the single mutants. The rationale of this analysis is that if a transcription factor is important for maintaining CWI in the *ΔugmA* mutant, the *ΔugmA/ΔTF* double mutant would have a more severe phenotype or even lead to synthetic lethality. As shown in Fig. 4, deletion of *ugmA* results in a severe reduction in radial growth and reduced conidiation, as previously observed (Damveld *et al.,* 2008). Single deletion of the transcription factors RlmA and SteA did not affect growth on MM. Deletion of the *msnA* gene, resulted in limited growth reduction but the *ΔmsnA* colony still produced spores. Single deletions of *pacC* and *crzA* severely reduced growth, indicative of an important function for these transcription factors during vegetative growth. Somewhat surprisingly the *ΔcrzA* strain grew and sporulated much better at 37 °C compared to growing this mutant at lower temperatures. The strong growth phenotype of the *ΔpacC* and the *ΔcrzA* by itself made it difficult to assess whether their function is specifically required for the *ΔugmA* mutant as it is likely that deletion of *pacC* and *crzA* in the *ΔugmA* mutant will further reduce growth anyway. Analysis of the *ΔugmA/ΔTF* double mutants showed a strong reduction in growth when the *ugmA* deletion was combined with the deletion of the *msnA* or *rlmA* transcription factor. In addition, the growth of the *ΔugmA/Δ crzA* double mutant at 37 °C also was severely reduced. The synthetic growth defects of the *ΔugmA/ΔrlmA*, *ΔugmA/ ΔmsnA* and *ΔugmA/ΔcrzA* double mutant (at 37 °C) suggest that RlmA, MsnA and CrzA target genes are important for survival of the *ΔugmA* mutant. Combining *ΔugmA* with

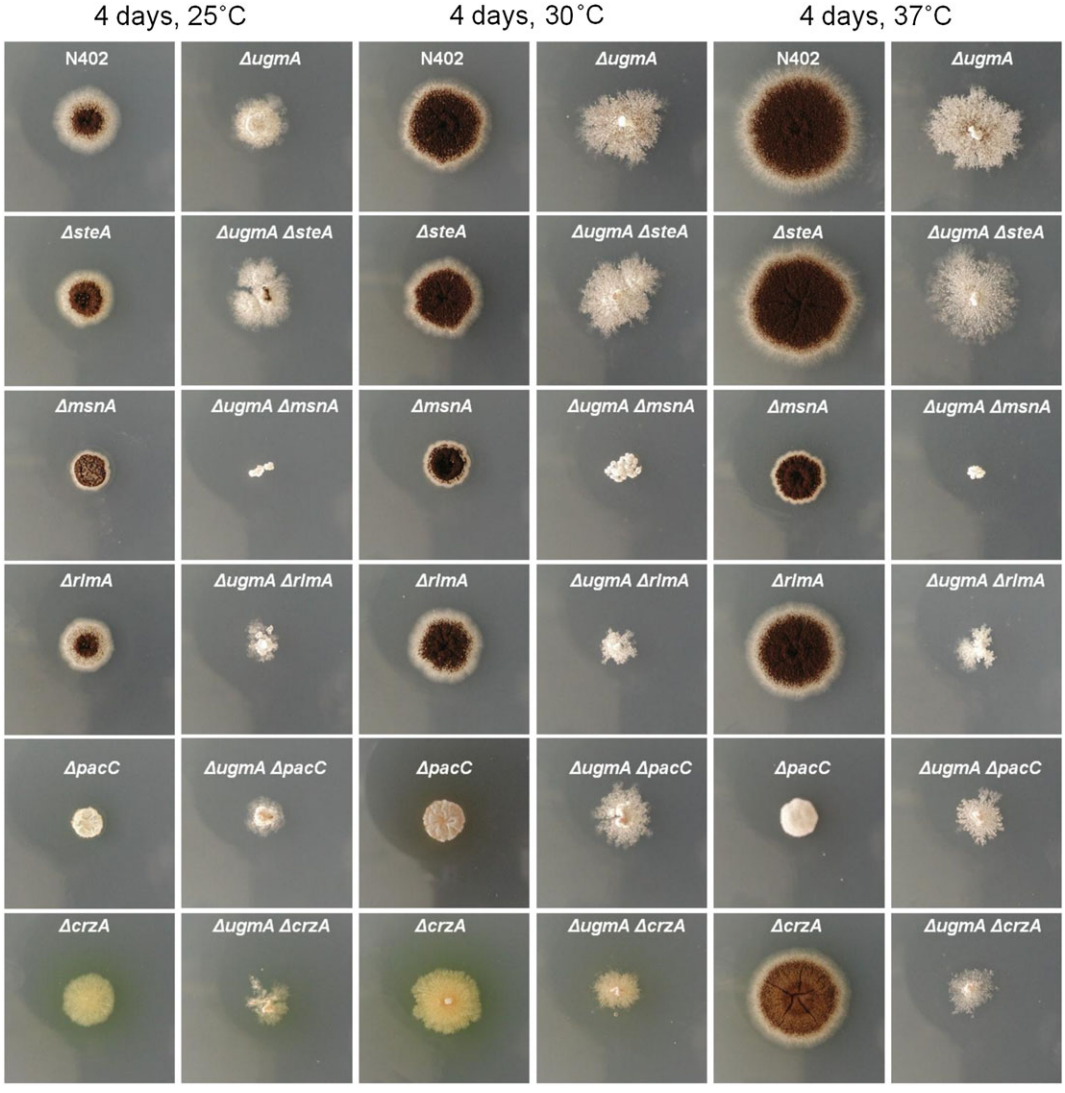


Fig. 4. Growth analysis of single and double mutants. Spores of single and double mutants were spotted in the centre of a MM plate and grown for four days at the indicated temperature. Synergistic growth reduction of the *ΔugmA/msnA* and *ΔugmA/rlmA* is indicative of an important role of the MsnA and RlmA transcription factors in survival of the *ugmA* mutant.

deletion of *ΔsteA* did not show a synthetic growth phenotype indicating that SteA and SteA target genes are not important for survival of the *ΔugmA* mutant. Combining the *ΔugmA* with *ΔpacC* resulted also in very poorly growing colonies, but because single deletion of *pacC* also resulted in poor growth, it is not possible to conclude whether PacC target genes are specifically required for *ΔugmA* survival.

Discussion

Biosynthesis of Gal*f*-containing glycoconjugates is important for securing the integrity of the fungal cell wall. Gal*f*-containing glycoconjugates are found in several ascomycetous clades, including the Eurotiales (*Aspergilli* and *Penicillium* ssp), Sordariales (*Neurospora* and *Magnaporthe* ssp), Hypocreales (*Trichoderma* and *Fusarium* spp), but are absent in the Saccharomycetales (*Saccharomyces* and *Candida* ssp). In the opportunistic pathogen *A. fumigatus*, Gal*f* is an important virulence factor and Gal*f*-mutants display reduced virulence (Schmalhorst *et al.,* 2008). In addition, Gal*f* is an important diagnostic tool to detect early-stage *A. fumigatus* infections in humans (Alexander and Pfaller, 2006). In the past few years the genes encoding the enzymes required for the synthesis of Gal*f*-containing glycostructures such as galactomannan, *N*- and *O*-glycans, and glycolipids have been identified. Analysis of mutants in genes involved in the biosynthesis of Gal*f* in several Aspergilli has shown that Gal*f*-mutants have an altered morphology, a different cell wall structure and are in general more susceptible to cell wall synthesis inhibitors as well as cell wall assembly-disturbing compounds (Damveld *et al.,* 2008; Engel *et al.,* 2009; Park *et al.,* 2014; Afroz *et al*., 2011; Alam *et al.,* 2012, 2014). The genes involved in Gal*f* biosynthesis include *ugeA* (encoding UDP-glucose-epimerase), *ugmA* (encoding UDP-galactose-mutase), *ugtA* and *ugtB* (encoding a UDP-Gal*f* transporters) and *gfsA* (encoding a galactofuranosyltransferase) (Komachi *et al*., 2013). Genes encoding these enzymes have been characterized in the three best studied *Aspergillus* species (*A. fumigatus*, *A. nidulans* and *A. niger*). In *A. niger*, the biosynthesis of Gal*f* has also been largely elucidated. By performing a genetic screen for cell wall mutants both the *ugeA* and *ugmA* genes have been identified (Damveld *et al.,* 2008; Park *et al.,* 2014). Because of genetic redundancy of the UDP-Gal*f* transporters and UDP-Gal*f* transferases in *A. niger* these genes were not identified in the genetic screen, but simultaneous disruption of the two redundant UDP-Gal*f* transporters (UgtA and UgtB) and simultaneous disruption of the three putative UDP-Gal*f* transferases lead to similar phenotypes as the *ΔugeA* and *ΔugmA* mutants (Park *et al.,* 2015; Arentshorst *et al*., 2015). In addition to increased α-glucan synthesis and, presumably, α-glucan levels, *ΔugmA* mutants have increased chitin levels (Afroz *et al*., 2011) and show increased expression of *gfaA and gfaB* (Fig. 3 and Table 4), which both encode glutamine:fructose-6-phosphate amidotransferases and are required for the biosynthesis of UDP-*N*-acetylglucosamine, the precursor of chitin. These observations indicated that the absence of Gal*f*-biosynthesis induces a cell wall remodelling programme to counteract the loss of Gal*f*-containing glycostructures.

In addition to cell wall remodelling enzymes, a group of 18 differentially expressed genes in the *ΔugmA* strain was identified which encode glycosyl hydrolases (GH) that act on the plant cell wall polysaccharides cellulose, xylan and galactan (Table 8). Several of these genes (*xynB*, *eglB*, *eglC*, *aglB*) are known to be controlled by the XlnR transcription factor (van Peij *et al.,* 1998b). XlnR encodes a $Zn(II)_2Cys_6$ transcription factor that positively regulates the expression of

Table 8. Differentially expressed genes encoding plant cell wall-degrading enzymes.

	Description	Gene name	N402	*ΔugmA*	$\log_2$ FC	Q-value	XlnR site*
An02g00730	Similarity cutinase A gene		0	37.8	inf	0.001	Y (2x)
An03g00960	Arabinofuranosidase active on arabinoxylan	*axhA*	0.7	60.3	6.4	0.001	Y (3x)
An15g07760	Putative beta-mannanase		0.7	32.4	5.6	0.001	Y (2x)
An02g10550	Endo-alpha-1,5-arabinanase	*abnA*	2.1	78.5	5.2	0.001	Y (3x)
An15g04550	Xylanase A	*xynA*	18.2	323.5	4.2	0.001	Y (4x)
An15g04570	Putative endo-glucanase IV		8.5	112.7	3.7	0.001	Y (2x)
An14g04200	Rhamnogalacturonase	*rhgB*	6.3	69.6	3.5	0.001	Y (2x)
An15g04900	Putative endoglucanase IV		3.9	40.6	3.4	0.001	Y (6x)
An08g05230	Putative endoglucanase IV		8.4	86.7	3.4	0.001	Y (1x)
An06g02070	Rhamnogalacturonase rhgA -	*rhgA*	0.8	8.4	3.4	0.001	Y (1x)
An14g02670	Putative endoglucanase IV		3.6	31.1	3.1	0.001	Y (2x)
An04g09690	Pectin methylesterase	*pme1*	0.7	5.6	3.0	0.001	Y (6x)
An01g00780	Xylanase B	*xynB*	35.3	241.7	2.8	0.001	Y (6x)
An14g01800	Putative alpha-galactosidase		0.6	3.2	2.5	0.014	Y (1x)
An13g03140	Putative endo-beta-1,4-glucanase		2.8	13.4	2.2	0.001	N
An09g03260	Endo-polygalacturonase D	*pgaD*	1.5	4.3	1.6	0.03	Y (4x)
An02g11150	Alpha-galactosidase	*aglB*	60.3	141.8	1.2	0.04	Y (2x)
An16g02730	Endo-1,5-alpha-arabinase	*abnA*	42.3	91.6	1.1	0.003	Y (2x)

Consensus binding sites for XlnR (GGCTRR or GGNTAAA)

genes involved in xylose metabolism (van Peij *et al.,* 1998a, 1998b). Although the mechanism underlying the induced expression of plant cell wall-degrading enzymes in the *ΔugmA* mutant is unknown, it could be related to a possible accumulation of UDP-galactose as the pathway to UDP-Gal*f* is blocked by the absence of UgmA. Accumulation of UDP-galactose (in the pyranose form) could have an effect on the intracellular galactoglycome, which has been shown to be linked to the production of plant cell wall-degrading enzymes (Karaffa *et al.,* 2013).

We previously studied the genome-wide cell wall stress response in *A. niger* by challenging germlings with antifungals affecting cell wall synthesis or assembly. These treatments include the exposure to the β-1,3-glucan synthase inhibitor CA and the ergosterol biosynthesis inhibitor FP (Meyer *et al.,* 2007) as well as exposure to sphingolipid inhibitor aureobasidin A (AbaA), and calcineurin inhibitor FK506 (Fiedler *et al.,* 2014). Compound-induced genes in these studies were defined based on a FDR < 0.05 and resulted in 134 differentially expressed genes for CA, 178 for AbaA, 22 for FP and 49 for FK506 (Fiedler *et al.,* 2014; Table S6). We were interested whether the sets of genes up-regulated in the *ΔugmA* mutant showed overlap with the gene sets up-regulated by the various treatments. As shown in Fig. 5, the largest overlap of the 432 genes up-regulated in the *ΔugmA* mutant was observed with the CA-induced gene set (35 genes overlapping) and AbaA (33 genes overlapping) (Fig. 5). No overlapping genes were identified between the *ΔugmA*-induced genes and FP- or FK506-treated germlings (data not shown). The gene identities for the different groups of genes are given in Table S6. The numbers also show that there is only a limited overlap of genes that are commonly induced. In fact, when comparing CA-induced, AbaA-induced and *ΔugmA*-induced gene expression nine genes were commonly induced (Table 9). The majority of these nine genes encode proteins with

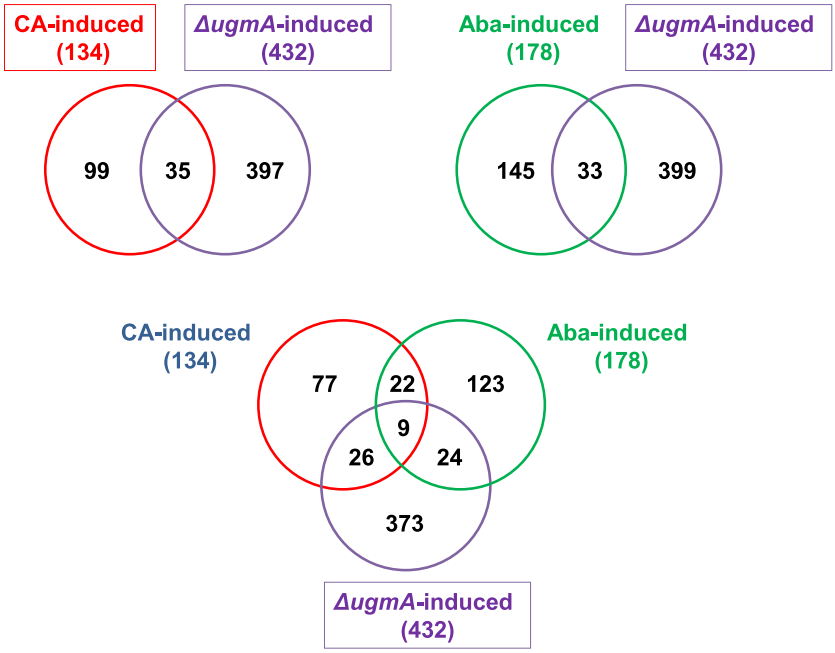


Fig. 5. Overview of the number of genes overlapping between different cell wall stress conditions. Caspofungin (CA)-induced genes (Meyer *et al.,* 2007) and Aureobasidin A (AbaA)-induced genes (Fiedler *et al.,* 2014) were compared to *ΔugmA*-induced genes (this study).

Table 9. Description of commonly induced genes after CA or AbaA treatments and in the *ΔugmA* mutant.

Gene#	Description*	*S. cerevisiae* homolog
An04g03870	Diacylglycerol diphosphate phosphatase activity	Ddp1
An05g00760	Protein with unknown function	
An06g01900	Sec14 family; member phosphatidylinositol transfer protein	Csr1
An07g05820	Protein with unknown function	
An12g06380	Protein of unknown function, highly repetitive; ortholog in *A. fumigatus* is farnesol-responsive	
An14g01070	Protein with unknown function	
An14g01840	GPI anchored protein; strongly induced in *ugmA*	
An15g07090	Protein with unknown function	
An17g01000	Putative anion transporter	

*Taken from AspGD (http://www.ncbi.nlm.nih.gov/pubmed/?term=aspGD).

unknown functions. Two proteins An04g03870 and An06g01900 encode homologs of the *S. cerevisiae* Dpp1p and Csr1p proteins. Dpp1p is a diacylglycerol diphosphate phosphatase, which synthesizes the second messenger diacylglycerol, a potential activator of protein kinase C of the CWI signalling cascade. Csr1p is a phosphatidylinositol transfer protein belonging to the Sec14-family, which has a potential role in regulating lipid and fatty acid metabolism and indicates turnover of phosphoinositides in response to different forms of cell wall stress, possibly, to relocate proteins involved in CWI signalling, such as Wsc1p, Rho1p, Rom2p and Pkc1p to the proper location at the membrane (Fernández-Acero *et al.,* 2015). The limited overlap of genes between the various treatments could have multiple reasons and might relate to the fact that treatments with antifungals provoke both short-term and transient responses, while the *Δugm*A mutant is fully adapted to the lack of Gal*f* biosynthesis and therefore represents a long-term adaptation. Alternatively, a noticeable difference between the *ΔugmA* transcriptome (this study) and transcriptome in response to chemical compounds (previous studies) is that the *ugmA* study was performed with mycelia while the chemical compound studies were performed with germlings. The limited overlap could also be well related to differences between the expression of cell wall biosynthetic genes during spore germination and vegetative growth.

It is therefore striking that, in contrast to the limited overlap of individual antifungal-responsive genes with *ΔugmA*-responsive genes, the transcription factor binding site enrichment analysis showed remarkable similarities and identified RlmA and MsnA transcription factor binding sites as overrepresented under all conditions (Fig. 6). Possibly, these transcription factors play an important role in combination with other factors that co-regulate and fine-tune the expression of different target genes.

Growth analysis of the *ΔugmA/ΔrlmA* and *ΔugmA/ΔmsnA* double mutants indicated an important role of the RlmA and MsnA transcription factors in securing CWI of the *ΔugmA* mutant. It will be of interest in future studies to determine the cell wall composition of the double mutants in comparison with single mutants and to identify RlmA or MsnA target genes via transcriptome analysis of CHIPseq analysis. In contrast to RlmA and MsnA, the role of SteA seems to be dispensable for the *ΔugmA* mutant, although its binding site was overrepresented in the *ΔugmA* up-regulated gene set. In. *S. cerevisiae,* the ScSte12 transcription factor is activated by the ScFus3/ScKss1 MAPK signalling cascade and is responsible for the activation of genes involved in mating or

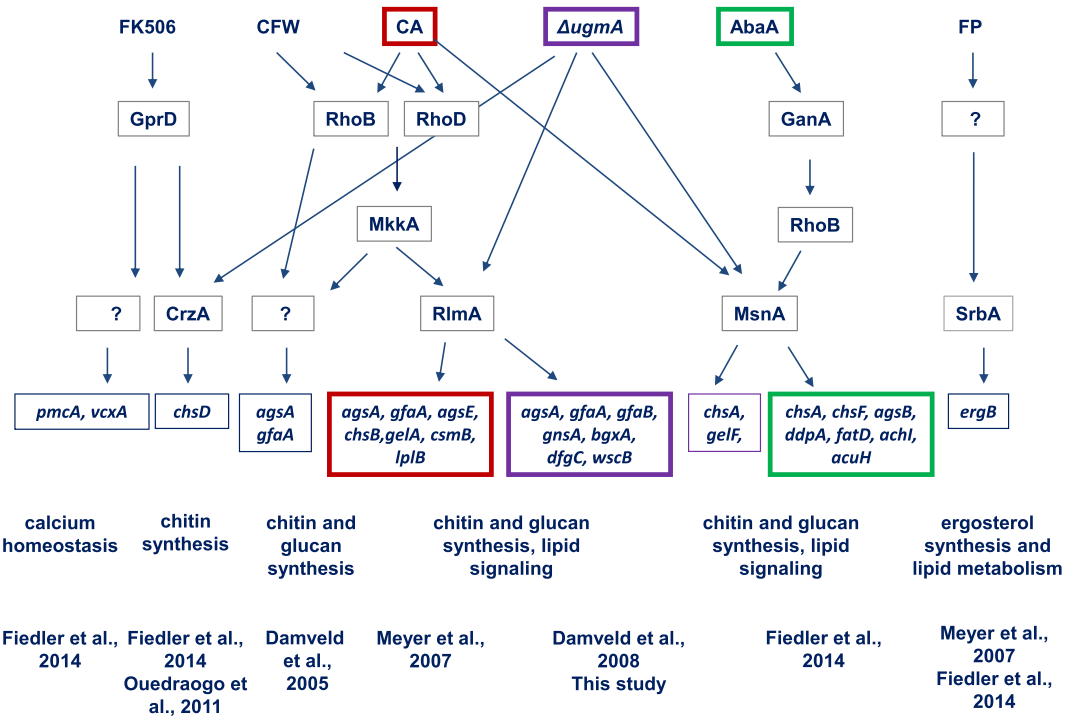


Fig. 6. Overview of key transcription factors and some of their putative target genes involved in the cell wall salvage gene network of *A. niger* deduced from transcriptomic and phenotypic analysis. On the upper row, several cell wall stress conditions including FK506 (Calcineurin inhibitor), CFW (Calcofluor white), caspofungin (CA), *ΔugmA* (lack of galactofuranose biosynthesis), Aureobasidin A (AbaA) and fenpropimorph (FP). Arrows indicate (putative) signalling events between and the proteins involved in signalling based on transcriptomic or functional genomics studies. Boxed genes are the possible target genes of various transcription factors and the relation with the inducing condition. Major biological processes affected by the different treatments are depicted under the boxed genes. Despite limited overlap of the target genes induced by chemical compounds and in the *ΔugmA* mutant, the RlmA, MsnA and CrzA transcription factors are likely to play a key role in orchestrating the response.

pseudohyphal growth (Roberts and Fink, 1994). In agreement with this, ScSte12 is involved in the regulation of fungal development and pathogenicity in various filamentous fungi (see for review Wong Sak Hoi and Dumas, 2010). Single deletion of the *ScSTE12* homolog in *A. niger* did not result in a detectable growth phenotype under the selected culture conditions (Fig. 4, and data not shown). The role of the PacC and CrzA transcription factors in rescuing the *ΔugmA* strain is more difficult to examine as single deletion of the corresponding genes in itself results in a severe growth phenotype (Fig. 4). However, both transcription factors have been shown to be involved in fungal cell wall remodelling. In *S. cerevisiae,* the ScRim101/PacC pathway is essential for cell wall assembly in the absence of CWI signalling created by disrupting the Sc*SLT2/ScMPK1* gene (Castrejon *et al.,* 2006), indicating that the ScPkc1–Slt2 CWI pathway and the ScRim101/PacC pathway are partially redundant. In *Cryptococcus neoformans*, deletion of Rim101 results in down-regulation of several cell wall-related genes, indicating that Rim101 is important for their expression (O'Meara *et al.,* 2014). Several studies have shown that the Crz1/CrzA transcription factor is also important for cell wall remodelling. An important role of the calcium/calcineurin-responsive transcription factor for cell wall maintenance was first discovered in *S. cerevisiae* (Yoshimoto *et al.,* 2002), but its role has been shown to be conserved in filamentous fungi, including *Aspergillus* species (Steinbach *et al*., 2007; Fortwendel *et al*., 2010; Fiedler *et al.,* 2014). A role for CrzA-mediated cell wall remodelling in the *ΔugmA* mutant was not inferred from the TF enrichment analysis as the CrzA transcription factor binding site was not enriched in the *ΔugmA*-upregulated gene set. However, the phenotype of the *ΔugmA/ΔcrzA* double mutant at 37 °C does suggest an important role for CrzA in cell wall remodelling in the *ΔugmA* mutant at least at higher temperatures. As in *Candida albicans* the calcineurin/Crz1p pathway is activated in response to membrane stress (Zakrzewska *et al.,* 2005), this might imply that deletion of *ugmA* does result in significant membrane stress.

Several studies have shown that cross-talk between several stress response pathways or pathways acting in parallel is important to cope with cell wall stress (Burgwun Fuchs and Mylonakis, 2009; Ouedraogo *et al.,* 2011; Verwer *et al.,* 2012; García *et al.,* 2015). Efficient inhibition of a fungal pathogen can be accomplished by the combination of two drugs, one targeting cell wall synthesis and the other by interfering with the adaptive mechanism. An elegant and promising example for such an approach is the recent study by Valiante *et al*., who showed that the efficacy of caspofungin treatment was strongly increased by simultaneous inhibition of the High Osmolarity Glycerol response pathway by the newly identified drug humidimycin (Valiante *et al.,* 2015). Our study has shown that inhibition of Gal*f* biosynthesis is by itself not fungicidal, but that Gal*f*-deficient cells become highly dependent on the RlmA and MsnA transcription factors. The strong synthetic growth defect of the double mutants shows the importance of activation of the CWI pathway to maintain CWI in the absence of Gal*f* biosynthesis and underscores the potential of using combinations of antifungals that act synergistically to combat fungal infections.

Experimental procedures

Strains and culture conditions

Aspergillus niger strains used in this study are listed in Table 10. Strains were cultivated in minimal medium (MM) (Bennett and Lasure, 1991) containing 1% (w/v) glucose (or other carbon sources as indicated), 7 mM KCl, 11 mM KH_2PO_4, 70 mM $NaNO_3$, 2 mM $MgSO_4$, 76 nM $ZnSO_4$, 178 nM H_3BO_3, 25 nM $MnCl_2$, 18 nM $FeSO_4$, 7.1 nM $CoCl_2$, 6.4 nM $CuSO_4$, 6.2 nM Na_2MoO_4, 174 nM EDTA; or in complete medium (CM) containing, in addition to MM, 0.1% (w/v) casamino acids and 0.5% (w/v) yeast extract. When required, 10 mM uridine, 2.5 μg/ml nicotinamide or 100 μg/ml hygromycin was added. Fermentation medium (FM) initially adjusted to pH 3 is composed of 0.75% glucose, 0.45% NH_4Cl, 0.15% KH_2PO_4, 0.05%KCl, 0.05% $MgSO_4$, 0.1% trace element solution and 0.003% yeast extract as described (Jørgensen *et al.,* 2010).

Protein extraction and immunoblotting analysis

1×10^7 conidia of *A. niger* N402 and *ΔugmA* strain were grown at 30 °C (200 rpm) in liquid CM during 18, 24 and 36 h or 24, 36 and 48 h for the wild-type and Δ*ugmA* strains respectively. For protein extraction, 0.5 ml of lysis buffer added to the ground mycelia as described previously (Rocha *et al.,* 2015). The supernatants were collected and the protein concentrations were determined according to the Lowry method modified by Hartree (Hartree, 1972). A 50 μg quantity of protein from each sample was resolved in a 12% (w/v) SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes (GE Health Care). The phosphorylation of MpkA was examined using anti-phospho p44/42 and the total amount of MpkA was detected by using the anti p44/42 MAPK antibody (9101 and 4370, respectively; Cell Signaling Technologies) according to the manufacturer's instructions. The primary antibody was detected with HRP-conjugated secondary antibody raised in rabbits (A0545; Sigma). Anti γ-tubulin (yN-20; Santa Cruz Biotechnology) was used as the loading control in these experiments. Anti γ-tubulin antibodies were detected with peroxidase HRP-conjugated secondary antibody (sc-2020; Santa Cruz Biotechnology). All the primary antibody detections here were performed at room temperature with the specified secondary antibody in TBST buffer (137 mM NaCl, 20 mM Tris and 0.1% Tween-20). Chemoluminescent detection was performed by using an ECL Prime Western Blot detection kit (GE Health Care). Images were generated by exposing the membranes to the ChemiDoc XRS gel imaging system (BioRad). The images were subjected to densitometric analysis in ImageJ software to achieve the ratio of phosphorylated/total MpkA for each time point.

Table 10. Strains used in this study.

Strain	Genotype	Description	Reference
N402	*cspA1*	derivative of N400	Bos *et al.,* 1988
MA87.6	*cspA1, pyrG378, kusA::amdS, ugmA::AOpyrG*	*ΔugmA* in MA70.15	Damveld *et al.,* 2008
MA169.4	*cspA1, pyrG378, kusA::DR-amdS-DR*	*ku70* disruption in AB4.1	Carvalho *et al.,* 2010
MA234.1	*cspA1, kusA::DR-amdS-DR*	Restored *pyrG* in MA169.4	This study
MA417.1	*cspA1, kusA::DR-amdS-DR, rlmA::hygB*	*ΔrlmA* in MA234.1	This study
MA306.1	*cspA1, kusA::DR-amdS-DR, crzA::hygB*	*ΔcrzA* in MA234.1	This study
MA513.1	*cspA1, kusA::DR-amdS-DR, msnA::hygB*	*ΔmsnA* in MA234.1	This study
MA527.2	*cspA1, kusA::DR-amdS-DR, steA::hygB*	*ΔsteA* in MA234.1	This study
EA13.1	*cspA1, kusA::DR-amdS-DR, pacC::hygB*	*ΔpacC* in MA234.1	Alazi *et al.,* in prep
MA322.1	*cspA1, pyrG378, kusA::DR-amdS-DR, ΔnicB::AOpyrG*	*ΔnicB::AOpyrG* in MA169.4	Niu *et al.,* 2016
MA323.1	*cspA1, pyrG378, kusA::DR-amdS-DR, ΔnicB*	*ΔnicB::AOpyrG* in MA169.4, followed by *AOpyrG* loopout	Niu *et al.,* 2016
MB2.1	*cspA1, pyrG378, kusA::DR-amdS-DR, ΔnicB, ugmA::AnidnicB*	*ΔugmA* in MA323.1	This study
MB3.1	*cspA1, pyrG378, kusA::DR-amdS-DR, ΔnicB, ugmA::AnidnicB, rlmA::AOpyrG*	*ΔugmAΔrlmA* (*ΔrlmA* in MB2.1)	This study
MB4.1	*cspA1, pyrG378, kusA::DR-amdS-DR, ΔnicB, ugmA::AnidnicB, crzA::AOpyrG*	*ΔugmAΔcrzA* (*ΔcrzA* in MB2.1)	This study
MB5.1	*cspA1, pyrG378, kusA::DR-amdS-DR, ΔnicB, ugmA::AnidnicB, msnA::AOpyrG*	*ΔugmAΔmsnA* (*ΔmsnA* in MB2.1)	This study
MB6.1	*cspA1, pyrG378, kusA::DR-amdS-DR, ΔnicB, ugmA::AnidnicB, steA::AOpyrG*	*ΔugmAΔsteA* (*ΔsteA* in MB2.1)	This study
MB7.1	*cspA1, pyrG378, kusA::DR-amdS-DR, ΔnicB, ugmA::AnidnicB, pacC::AOpyrG*	*ΔugmAΔpacC* (*ΔpacC* in MB2.1)	This study

Construction of single and double mutant knock-out mutants

For the construction of single deletion mutants, flanks of the gene to be deleted were PCR-amplified and split marker fragments with selection markers *AOpyrG* or *hygB* were created using fusion PCR (Arentshorst *et al.,* 2015). The split marker fragments were either transformed to strain MA169.4 (*ku70*$^{-}$, *pyrG*$^{-}$) for *pyrG* selection (Carvalho *et al.,* 2010) or to strain MA234.1 (*ku70*$^{-}$) for *hygB* selection. Strain MA234.1 (*ku70*$^{-}$) was obtained by transformation of strain MA169.4 (*ku70*$^{-}$, *pyrG*$^{-}$) with a 3.8 kb *Xba*I fragment containing the *A. niger pyrG* gene, resulting in the full restoration of the *pyrG* locus in strain MA234.1. For the construction of *ΔugmA*/transcription factor double deletion mutants, parental strain MA323.1 (*ΔnicB*$^{-}$, *pyrG*$^{-}$) (Niu *et al*., 2016) was transformed with *ugmA-nicB* split marker fragments (Arentshorst *et al.,* 2015) to obtain *ΔugmA* mutant strain MB2.1 (*ΔugmA::nicB*, *pyrG*$^{-}$). *Aspergillus oryzae pyrG* (*AOpyrG)* split marker fragments of all transcription factors were transformed to this strain, resulting in double deletion mutants. All strains used in this study are listed in Table 10. All primers used in this study are listed in Table S7 Proper deletion of the genes in the various mutants was verified by Southern blot analysis or diagnostic PCR (data not shown).

Bioreactor cultivation conditions

Details on the medium composition and batch cultivation conditions with 6.6 L BioFlo3000 bioreactors (New Brunswick Scientific, NJ, USA) were performed as described by Jørgensen *et al.* (2010), except that glucose was used as a carbon source in this study. The cultivation program involves two consecutive phases: in the first phase, carried at out 30 °C and at pH 3, head space aeration was used and the agitation speed was kept at 250 rpm for the first 5 h. In the second phase, after spore germination, the pH was slowly increased by the addition of NaOH to the desired pH value (set at 3.0, 4.0, 5.0 or 6.0) and increased agitation speed (750 rpm) and sparger aeration. At the same time, 0.01% (v/v) polypropyleneglycol P2000 was added as antifoam agent. Biomass was determined by weighing lyophilized mycelium from a known mass of culture broth. Culture broth was filtered through GF/C glass microfiber filters (Whatman). The filtrate was collected and frozen for use in solute analyses. The mycelium was washed with demineralised water, rapidly frozen in liquid nitrogen and stored at −80 °C for RNA isolation and microscopic analyses. Mycelium samples harvested from bioreactor runs JH04 and JH08 (N402 at pH 5.0) and JH05 and JH09 (*ΔugmA* at pH 5.0) were used for RNA extraction. Dot blot analysis using the anti-galactofuranose antibody L10 (Heesemann *et al.,* 2011) was performed as described (Park *et al.,* 2014)

RNA isolation and quality control

Mycelium intended for gene-expression analyses was separated from culture medium and frozen in liquid nitrogen within 15–20 s from sampling and stored at −80 °C. RNA was extracted from mycelium in liquid nitrogen using TRIzol reagent (InVitrogen). Frozen ground mycelium (≈200 mg) was directly suspended in 800 μl Trizol reagent and vortexed vigorously for 1 min. After centrifugation for 5 min at 10 000 ×*g*, 450 μl of the supernatant was transferred to a new tube. Chloroform (150 μl) was added and after a 3 min incubation at room temperature, samples were centrifuged and the upper aqueous phase was transferred to a new tube to which 400 μl of isopropanol was added, followed by a 10 min incubation at room temperature and centrifugation for 10 min at 10,000 ×*g*. The pellet was washed with 75% (v/v) ethanol and finally dissolved in 100 μl H_2O. RNA samples for RNA sequencing were additionally purified

on NucleoSpin RNA II columns (Machery-Nagel) according to the manufacturer's instructions. RNA quantity and quality were determined using a Nanodrop ND1000 spectrophotometer.

RNA seq analysis

RNA sequencing was outsourced to ServiceXS (Leiden, The Netherlands). cDNA library constructions were performed using the Illumina mRNA-Seq Sample preparation kit according to the instructions of the supplier. In brief, mRNA was isolated from total RNA using oligo-dT magnetic beads. After fragmentation of the mRNA, cDNA synthesis was performed and the cDNA was ligated with the sequencing adapters before PCR amplification of the resulting product. The quality and yield after sample preparation were measured with a DNA 1000 Lab-on-a-Chip. Clustering and mRNA sequencing using the Illumina cBot and HiSeq 2000 were performed according to the manufacturer's protocol. For each RNA sample at least 3.2 Gb of sequence data were obtained that passed the quality control (% $\geq$ Q30). Image analysis, base calling and quality checks were performed with the Illumina data analysis pipeline RTA v1.13.48 and/or OLBv1.9 and CASAVA v1.8.2. Quality-filtered sequence tags are available upon request.

Transcriptomic data analysis

RNA-Seq analysis was performed essentially as described previously (Wang *et al.,* 2015) except for the transfer of genome annotations. Annotations were transferred from the CBS 513.88 genome to the N402 genome based on bidirectional alignments created with LAST (version 417) (Kiełbasa *et al*., 2011), with tandem repeat sections masked with TRF (Benson, 1999). Alignments were combined and chained using CLASP (Otto *et al*., 2011). Gaps in the global alignment were improved by performing realignment using either LAST (for large gaps, in sensitive mode) or Needleman–Wunsch (for smaller gaps). Chains with scores $>$ 150 were accepted for alignment transfer. In total, 13 412 (of 14070) genes were transferred. The normalized, RNAseq-based expression data are summarized in Table S8.

Gene ontology (GO) and transcription factor binding site enrichment analysis

Over-represented GO terms in sets of differentially expressed genes (FDR at $q < 0.05$) were determined by Fisher's exact test (Fisher, 1922). An improved GO annotation for the *A. niger* CBS 513.88 genome was based on orthology mappings from *A. nidulans* FGSC A4 (Nitsche *et al.,* 2012). Transcription factor binding site enrichment analysis of genes differentially higher expressed in the *ΔugmA* mutant was performed using a Perl script named the transcription factor binding site finder (TFBSF) as described previously (Meyer *et al.,* 2009).

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Supporting information

Additional supporting information may be found in the online version of this article at the publisher's web-site:

Table S1. Differentially expressed genes up-regulated in *ΔugmA* mutant.
Table S2. Differentially expressed genes down-regulated in *ΔugmA* mutant.
Table S3. GO-terms (Biological Processes) over-represented in the genes up-regulated in the *ΔugmA* mutant.
Table S4. GO-terms (Biological Processes) over-represented in the genes down-regulated in the *ΔugmA* mutant.
Table S5. Transcription factor binding sites enriched in the promoter of regions of genes up-regulated in the *ΔugmA* mutant.
Table S6. Gene identities of genes up-regulated after treatment with caspofungin, fenpropimorph, aureobasidin A, FK506 and in the *ΔugmA* mutant.
Table S7. Primers used in this study.
Table S8. RNAseq expression data N402 and *ΔugmA* mutant.