Repression of $N$-glycosylation triggers the unfolded protein response (UPR) and overexpression of cell wall protein and chitin in *Aspergillus fumigatus*

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*Aspergillus fumigatus* is the most common airborne fungal pathogen, causing fatal invasive aspergillosis in immunocompromised patients. The crude mortality is 60–90 % and remains around 29–42 % even with treatment. The main reason for patient death is the low efficiency of the drug therapies. As protein $N$-glycosylation is involved in cell wall biogenesis in *A. fumigatus*, a deeper understanding of its role in cell wall biogenesis will help to develop new drug targets. The *Af*stt3 gene encodes the essential catalytic subunit of oligosaccharyltransferase, an enzyme complex responsible for the transfer of the *N*-glycan to nascent polypeptides. To evaluate the role of $N$-glycosylation in cell wall biosynthesis, we constructed the conditional mutant strain CPR-stt3 by replacing the endogenous promoter of *Af*stt3 with the nitrogen-dependent *niiA* promoter. Repression of the *Af*stt3 gene in the CPR-stt3 strain led to a severe retardation of growth and a slight defect in cell wall integrity (CWI). One of the most interesting findings was that upregulation of the cell wall-related genes was not accompanied by an activation of the MpkA kinase, which has been shown to be a central element in the CWI signalling pathway in both *Saccharomyces cerevisiae* and *A. fumigatus*. Considering that the unfolded protein response (UPR) was found to be activated, which might upregulate the expression of cell wall protein and chitin, our data suggest that the UPR, instead of the MpkA-dependent CWI signalling pathway, is the major compensatory mechanism induced by repression but not abolition of $N$-glycosylation in *A. fumigatus*. Our finding is a key to understanding the complex compensatory mechanisms of cell wall biosynthesis and may provide a new strategy for drug development.

INTRODUCTION

*Aspergillus fumigatus* is one of the most important human opportunistic pathogens and causes life-threatening invasive aspergillosis in immune-suppressed patients (Latgé, 1999, 2001). Due to its essentiality and uniqueness, the cell wall has long been an important drug target. Although several cell wall-target drugs have been introduced as therapies, the mortality rate is still around 29–42 % (Zmeili & Soubani, 2007). One of the reasons for this high mortality rate is the low efficiency of the drug therapies due to the complicated mechanism of cell wall biogenesis in *A. fumigatus*. For example, echinocandin, an inhibitor of β-1,3-glucan synthesis, not only inhibits glucan synthesis but also triggers an increase of chitin via a protective mechanism (Bowman et al., 2002; Denning, 2003), which reduces the efficiency of treatment. Therefore, a more profound understanding of the mechanisms of cell wall biosynthesis in *A. fumigatus* would help to improve the efficiency of drug therapies, especially for drugs which target the cell wall.

Since many glycoproteins are directly or indirectly involved in the synthesis and organization of the fungal cell wall, it is of importance to assess the roles of $N$-glycosylation in cell wall synthesis in *A. fumigatus*. Generally, $N$-glycosylation occurs in the lumen of the endoplasmic reticulum (ER)
and can be separated into three steps (Silberstein & Gilmore, 1996; Helenius & Aebi, 2001). The first step is initiated with assembly of a lipid-linked oligosaccharide donor by a series of glycosyltransferases located on the cytoplasmic and luminal faces of the ER membrane. Subsequently, oligosaccharyltransferase (OST) transfers the lipid-linked oligosaccharide to an asparagine residue within an N-X-T/S consensus sequence of a nascent peptide. Then the N-glycan is trimmed sequentially by glucosidase I and glucosidase II to a mono-glycosylated state, which is one of the most important mechanisms to assure quality control of glycoprotein folding (Hebert et al., 2005).

Previously we have shown that the lack of AfCwh41, the gene encoding the x-glucosidase I removing the outmost glucose residue in N-glycans, leads to a temperature-sensitive deficiency of cell wall integrity (CWl), suggesting an important role for N-glycosylation in cell wall synthesis (Zhang et al., 2008). However, the role of N-glycosylation is still unclear in A. fumigatus. Obviously, investigation of the roles of N-glycosylation in cell wall biosynthesis would help to understand the mechanism of cell wall organization and thus facilitate a rational design of novel therapeutic strategies.

In Saccharomyces cerevisiae, protein N-glycosylation is catalysed by the OST complex, which consists of at least eight different subunits, including Ost1p, Ost2p, Wbp1, Stt3p, Swp1p, Ost4p, Ost5p and Ost3/Ost6p (Silberstein & Gilmore, 1996; Knauer & Lehle, 1999; Yan & Lennarz, 2005; Weerapana & Imperiali, 2006). Among these subunits, Stt3p is the catalytic subunit (Yan & Lennarz, 2003; Nilsson et al., 2003), and its homologues are found in almost all eukaryotes (Kelleher & Aebi, 2001). The first step is glucose residue in afN-glycans, leads to a temperature-

**METHODS**

**Strains and growth conditions.** A. fumigatus strain YJ-407 (CGMCC0386) was maintained on potato glucose (2%) agar slants (Xia et al., 2001). A. fumigatus strain CEA17, a gift from C. d’Enfert, Institut Pasteur, Paris, France, was propagated at 37°C on yeast extract-glucose agar (YGA) containing 5 mM uridine and uracil (Weidner et al., 1998). For protoplast transformation, a modified aspergillus minimal medium (AMM) was used, which contained 20 mM nitrate as the sole nitrogen source and 1% (w/v) glucose as the sole carbon source. For phenotypic analysis, complete medium (CM) (Cove, 1966) was supplemented with 5 mM (NH₄)₂SO₄ (RCM) for repressing conditions or supplemented with 10 mM NaNO₃ (ICM) for inducing conditions. For gene expression analysis, strains were grown in liquid RCM at 37°C for 20 h. Mycelia were harvested, washed with distilled water, and ground in liquid N₂ using a mortar and pestle. The powder was stored at −70°C for DNA and RNA isolation.

Conidia were prepared by growing A. fumigatus strains on solid ICM at 37°C for 48 h. The spores were collected, washed twice with 0.1% Tween 20 in physiological saline, and resuspended in 0.1% Tween 20 in saline. The concentration of spores was calculated by haemocytometer counting and viable counting. Vectors and plasmids were propagated in *Escherichia coli* DH5α (Bethesda Research Laboratories).

**Constrution of the conditional inactive mutant.** Briefly, *Aspergillus niger* pyrG and the A. fumigatus promoter P_nich were amplified by PCR using pCDAl4 (kindly provided by C. d’Enfert, Institut Pasteur, France) and A. fumigatus genomic DNA as template, respectively. Specific primers (pyrG5z and pyrG3 for pyrG, niiA/niiD5 and niiA/niiD3z for P_nich) containing multiple cloning site were used (Supplementary Table S1). PCR products of pyrG and P_nich were first digested with NotI/Ndel and Ndel/XhoI, respectively, and subcloned into pBluescript at the NotI/XhoI sites. The resulting plasmid pPyrG-
P_nich contained pyrG-P_nich flanked on either side with two cloning sites (Hu et al., 2007).

The inducible promoter replacement cassette was constructed in two steps. In the first step, approximately 1.5 kb of the 5'-flanking sequence (left-arm) that stops at 250 bp upstream of the start codon of AfStt3 was amplified by PCR from A. fumigatus genomic DNA with primers stt3L5 and stt3L3 (Supplementary Table S1) and then subcloned into pPyrG-P_nich using the NotI and MdiI sites. In the second step, approximately 1.5 kb of the downstream genomic flanking sequence (right-arm) that starts at the ATG codon was amplified from A. fumigatus genomic DNA with primers stt3R5 and stt3R3 (Supplementary Table S1) and then subcloned into pPyrG-
P_nich using the Ascl and PacI sites. The resulting pPyrG-PCR was digested with NotI and PacI, transformed into A. fumigatus strain CEA17 by protoplast transformation (Zhuo et al., 2007), and screened for transformants with uridine/uracil autotrophy.

The transformants were first confirmed by PCR and then by Southern blotting. For PCR analysis, two PCRs were performed to generate the promoter replacement junctions using two pairs of primers (L1 and L2 for the left-arm junction, R1 and R2 for the right-arm junction) (Supplementary Table S1), and the third PCR was performed to generate the endogenous promoter in the wild-type (WT) using primer pair L1/P (Supplementary Table S1). For Southern blotting, genomic DNA was digested with Xbal, separated by electrophoresis, and transferred to a nylon membrane (Zeta-probe⁺). The 5'-flanking sequence (left-arm) was used as a probe. Labelling and visualization were performed using the DIG DNA Labeling and Detection kit (Roche Applied Science) according to the manufacturer’s instructions.

**Phenotypic analyses of the mutant.** For radial growth measurement, a 5 µl drop containing 1 x 10⁵ conidia was placed in the centre of an agar plate containing RCM or ICM. Plates were incubated at 37°C. For the antifungal reagent sensitivity test, 5 µl of A. fumigatus conidia (10⁴, 10³, 10² and 10⁰) was spotted on RCM or ICM agar plates supplemented with 50 µg Calcofluor white ml⁻¹ or 100 µg Congo red ml⁻¹ and incubated at 37°C for 36 h.

**RNA isolation and real-time RT-PCR analysis.** A. fumigatus conidia (1 x 10⁵ spores) were inoculated into flasks containing RCM and incubated in a shaker (250 r.p.m.) at 37°C for 20 h. Total RNA was isolated using TRZol reagent (Invitrogen). cDNA was synthesized with 1 µg RNA using a RevertAid First Strand cDNA Synthesis kit (Fermentas). Twenty nanograms of cDNA template, primers (2.5 µM each) and SYBR Premix Ex Taq (TaKaRa) were used to amplify gene-specific amplicons. Two steps of the real-time PCR were performed in an ABI 7000 PCR machine (Applied Biosystems). A dissociation curve of the PCR-amplified products was performed to confirm the absence of non-specific product and primer dimer. The amplified products were about 120 bp in length. Relative quantification of the mRNA levels was determined using the ΔCt method (Livak & Schmittgen, 2001). Briefly, the amount of target gene was normalized to the
endogenous reference gene tub1. ACG = C1 (target gene) − C1 (Tub1), where C1 represents the cycle number required to reach a defined threshold target abundance. The relative mRNA levels were calculated as $2^{ΔΔCt}$ (where $x$ is primer efficiency). All reactions were performed in duplicate. The primers used in this test are listed in Supplementary Table S2.

**Western blot analysis.** Conidia ($1 \times 10^5$) were inoculated into 200 ml liquid RCM and cultured at 37 °C for 20 h with shaking (250 r.p.m.). Mycelia were harvested, ground in liquid nitrogen to a fine powder using a mortar and pestle, and immediately suspended in pre-warmed SDS sample buffer [120 mM Tris/HCl, pH 8.8, 5 % SDS, 5 % mercaptoethanol, 10 % (v/v) glycerol, 1 mM sodium vanadate] without dye. The samples were quickly vortexed for 10 s and boiled at 100 °C for 10 min, and the cell debris was removed by centrifugation for 10 min at 15 000 g. Each sample (15 µg protein) was subjected to SDS-PAGE and transferred to a PVDF membrane (Millipore).

For glycoprotein and cell wall polysaccharide analysis, the blot was incubated with horseradish peroxidase (HRP)-coupled lectin concanavalin A (ConA) (Sigma-Aldrich) in a 1: 50 dilution. HRP activity was visualized by an enhanced chemiluminescence system (Pierce).

To analyse phosphorylation of the MpkA, the blot was detected using Anti-Rabbit IgG (Fc) anti-phospho-p44/42 MAPK antibodies (Cell Signaling Technology). To analyse phosphorylation of the MpkA, the blot was visualized by an enhanced chemiluminescence system (Pierce).

**Chemical analysis of the cell wall.** Conidia ($1 \times 10^5$) were inoculated into 200 ml liquid RCM and cultured at 37 °C for 20 h with shaking (250 r.p.m.). The mycelia were harvested, washed with deionized water and lyophilized. To isolate cell walls, 10 mg dry mycelial pad was added to a tube containing 50 mM NH$_4$HCO$_3$ at pH 8.0 and 0.2 g glass beads (1 mm diameter). The mycelia were then disrupted using a Disruptor Genie (Scientific Industries) five times (5 min each time). The cell homogenates were then centrifuged (12 000 g for 10 min) and washed several times. Three independent samples of lyophilized mycelial pad were used for cell wall analysis, and the experiment was repeated twice. The cell walls were boiled for 5 min in 1 ml 2 % SDS in 50 mM Tris/HCl buffer supplemented with 100 mM EDTA, 40 mM β-mercaptoethanol and 1 mM PMSF to remove noncovalently bound proteins and membrane fragments. Cell walls were collected by centrifugation (12 000 g for 10 min), washed for a second time and washed three times with deionized water. After washing, cell walls were treated with 1 M KOH and incubated at 70 °C for 30 min under N$_2$ to release glycoproteins and β-glucan. The alkali-soluble materials were acidified with acetic acid to pH 5.0 and the precipitated α-glucan was collected by centrifugation (12 000 g for 10 min) and washed with water. The glycoprotein in the supernatant was precipitated with two volumes of ethanol, washed twice with 64 % ethanol and dissolved in distilled water. The glycoprotein concentration was determined using the Bradford assay (Bradford, 1976). The alkali-insoluble materials were washed with water several times and hydrolysed in 6 M HCl at 100 °C for 2 h to release monosaccharides from β-glucan and chitin. Subsequently, HCl was evaporated and the residues were dissolved in 0.2 ml distilled water (Elorza et al., 1985; Hearn & Sietsma, 1994; Schoffelmeer et al., 1999). The amounts of α-glucan and β-glucan present were estimated by measuring released glucose using the phenol/sulfuric acid method (Dubois et al., 1956). Chitin content was determined by measuring the N-acetylglucosamine released after digestion using the method described by Lee et al. (2005).

**Microscopic analysis.** Mycelia were fixed with 2.5 % glutaraldehyde in 0.1 M phosphate buffer, pH 7.0, at room temperature for 4 h or at 4 °C overnight. After fixation, cells were washed three times in 0.1 M phosphate, post-fixed in 1 % osmium tetroxide and 0.1 M phosphate for 2–4 h, placed in increasing concentrations of methanol (30, 50, 70, 85, 95 and 100 %), and post-fixed in 2 % uranyl acetate and 30 % methanol. Cells were rinsed, dehydrated, and embedded in Epon 812 for the floating sheet method. Sections were examined with an H-600 electron microscope (Hitachi).

**RESULTS**

**Generation of the conditional inactive mutant**

The *A. fumigatus* Afstt3 gene contains five introns and its 2603 bp ORF encodes a protein consisting of 743 aa. The predicted Afstt3 shows an identity of 65 % with the *S. cerevisiae* Stt3p. Considering that *S. cerevisiae* STT3 is an essential gene (Yoshida et al., 1995; Zufferey et al., 1995), it is likely that *Afstt3* is also essential for *A. fumigatus*. Indeed, we initially tried to delete the *Afstt3* gene by replacement of *Afstt3* with *pyrG*; however, we failed to obtain any null mutant strain after several rounds of screening. Therefore, a promoter replacement strategy was used to generate a *Afstt3* conditional inactive mutant. As a result, a mutant, namely CPR-stt3, was obtained. PCR analysis showed that a 1.6 kb fragment of the left-arm junction and a 1.6 kb fragment of the right-arm junction were amplified from the genomic DNA of strain CPR-stt3, while neither of these two fragments was amplified from the WT. In contrast, the WT *Afstt3* promoter could be amplified from the WT, but not from strain CPR-stt3 (Fig. 1a). Southern blotting of the *Xba*I-digested genomic DNA confirmed that a 6.1 kb *Xba*I fragment in the WT was converted into a 5.4 kb fragment in strain CPR-stt3 (Fig. 1b). These results confirmed that the native *Afstt3* promoter was replaced by *P*nia in strain CPR-stt3.

**Phenotypes of strain CPR-stt3 under repressing condition**

The WT strain displayed normal growth on CM, RCM and ICM (Fig. 2a), while strain CPR-stt3 exhibited retarded growth on RCM and normal growth on ICM (Fig. 2b, c). When strain CPR-stt3 was grown in liquid RCM at 37 °C for 20 h, the expression level of *Afstt3* was only 9.4 % of that of the WT (Table 1). These results indicate that repression of the *P*nia promoter leads to a reduced expression of the *Afstt3* gene. As ConA is a lectin known to bind high mannose-type sugar chains, we used it to evaluate the status of *N*-glycosylation in the mutant grown under the repressing condition. When 15 µg protein from the WT or mutant (Fig. 3, left panel) was detected with ConA, binding of ConA to some of the proteins from strain CPR-stt3 was slightly reduced as compared with that in the WT (Fig. 3, right panel), which suggests that repression of *Afstt3* leads to a reduction of high mannose-type sugar chains on some proteins.

To further evaluate the effect of reduced *N*-glycosylation on cell wall synthesis, we analysed the cell wall contents. As summarized in Table 2, when strains were grown in liquid
RCM at 37 °C, α-glucan and β-glucan levels in strain CPR-stt3 were similar to those in the WT, while the mannoprotein and chitin contents were increased by 130 and 78%, respectively. Electron microscopy revealed that the protein layer of strain CPR-stt3 was thickened as compared with that of the WT (Fig. 4). Based on these results, we conclude that repression of Afstt3 causes a reduction of N-glycosylation in A. fumigatus, which does not significantly affect glucan synthesis and leads only to overexpression of chitin and cell wall protein.

When A. fumigatus strains were grown on RCM agar plates containing Calcofluor white or Congo red at 37 °C for 36 h, strain CPR-stt3 showed a slight increase in sensitivity to these drugs as compared with strain CPR-stt3 grown on RCM plates without Calcofluor white or Congo red. When the strains were grown on ICM plates containing Calcofluor white or Congo red, the growth of strain CPR-stt3 was similar to that of the WT (Fig. 5). These results suggest that repression of Afstt3 causes only a minor defect of the CWI in A. fumigatus. Probably, the overexpression of cell wall protein and chitin partially compensates for the cell wall defect caused by reduced N-glycosylation.

Expression of cell wall-related genes in the mutant

Under conditions of cell stress, S. cerevisiae upregulates its chitin synthesis through activation of the PKC1-MPK1 CWI signalling pathway (Roncero, 2002; Valdivieso et al., 1991; Bulawa, 1992; Valdivia & Schekman, 2003; Levin, 2005). In this study, repression of the Afstt3 gene in strain CPR-stt3 also caused a minor cell wall defect and triggered overexpression of chitin. It was therefore reasonable to expect that a similar CWI signalling pathway would be activated. Thus, we analysed the transcription levels of the genes involved in cell wall biogenesis by quantitative real-time RT-PCR, including the genes encoding α-1,3-glucan synthases (ags1, ags2 and ags3) (Beauvais et al., 2005; Maubon et al., 2006), β-1,3-glucan glucanosyltransferases (gel1 and gel2) (Mouyna et al., 2000, 2005), β-1,3-glucan synthase (fskA) (Beauvais et al., 2000), chitin synthases (chsA, chsB, chsC, chsD, chsE, chsF and chsG) (Mellado et al., 2003) and glutamine fructose-6-phosphate amidotransferase (gfaA) (Ram et al., 2004). The transcription levels of these genes in the mutant were remarkably increased; the only exception was ags2 (Table 3), the
expression of which was only 60% of the WT. Although the function of \textit{A. fumigatus} Ags2p is associated with hyphal morphology and conidiation, the $\Delta$ags2 mutant of \textit{A. fumigatus} does not show any alteration in cell wall composition (Beauvais \textit{et al.}, 2005). In addition, AnagsA, an orthologue of \textit{A. fumigatus} ags2, does not show any induced expression under conditions of cell wall stress (Fujioka \textit{et al.}, 2007). Therefore, ags2 is thought to be dispensable for the compensatory mechanism. It appears that overexpression of ags1, ags3, gel1, gel2 and fskA in the repressed strain CPR-stt3 can restore glucan synthesis to a level similar to that of the WT.

\textbf{Fig. 2.} Growth of \textit{A. fumigatus} strains on various media. (a) Serially diluted \textit{A. fumigatus} WT conidia ($10^5$–$10^2$ cells) were dotted and incubated on CM, RCM and ICM at 37 °C for 24–42 h; (b) freshly harvested conidia ($10^4$) were dotted and incubated on RCM and ICM at 37 °C for 2 days; (c) freshly harvested conidia ($10^5$) were dotted and incubated on an agar plate and incubated at 37 °C. The colony radius was measured every 12 h for 3 days. Dark-grey bars, WT on RCM; white bars, CPR-stt3 on RCM; light-grey bars, CPR-stt3 on ICM.

\textbf{Table 1.} Expression of Afstt3 in strain CPR-stt3 under repression conditions

<table>
<thead>
<tr>
<th>Strain</th>
<th>$\Delta C_T$ (stt3-TUB)</th>
<th>$\Delta A C_T$</th>
<th>$2^{-\Delta A C_T}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>9.55</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>CPR-stt3</td>
<td>12.96</td>
<td>3.41</td>
<td>0.094</td>
</tr>
</tbody>
</table>

Total RNAs were prepared from mycelia cultured in liquid RCM at 37 °C for 20 h, and transcription levels of Afstt3 in strain CPR-stt3 and the WT were examined by real-time RT-PCR as described in Methods.
generate cell wall stress. It was found that an increased phosphorylation of MpkA was induced in the mutant by Calcofluor white, which was similar to that in the WT (Fig. 6a), while the transcription of mpkA in strain CPR-stt3 increased by over 100% upon induction by Congo red, which was as much as that in the WT strain treated with Congo red (Fig. 6b), suggesting that repression of Afstt3 did not disrupt the CWI signalling pathway in the mutant. Taken together, we conclude that overexpression of cell wall biogenesis genes is not activated by the MpkA-dependent CWI signalling pathway.

Expression of the UPR-related genes in the mutant

N-Glycosylation is known to be required for the proper folding of glycoproteins (Branza-Nichita et al., 2000). It has been shown that tunicamycin, a specific inhibitor of N-glycosylation, induces ER stress in A. fumigatus by impairing N-glycosylation (Richie et al., 2007). In addition, deficient N-glycan trimming also leads to ER stress and activation of the UPR in A. fumigatus (Zhang et al. 2009). Thus, it is reasonable to expect that repression of Afstt3 would result in an accumulation of misfolded unglycosylated proteins in the ER lumen and an activation of the UPR, which is accompanied by an overexpression of genes such as ER chaperone (Bip) and peptide disulfide isomerase (Pdi), which facilitate proper protein folding (Malhotra & Kaufman, 2007; Ron & Walter, 2007). Therefore, we measured the expression levels of four known UPR-related genes, including hacA, bipA, pdiA and tigA (Richie et al., 2009). As shown in Fig. 7, the expression levels of these genes were 5.3-, 23.4-, 10.5- and 4.7-fold that of the WT, respectively. These results clearly show that repression of Afstt3 triggers an activation of the UPR in strain CPR-stt3.

DISCUSSION

N-Glycosylation of proteins is catalysed by the OST enzyme complex, in which Stt3p plays a central role (Yan & Lennarz, 2002; Kelleher et al., 2003; Nilsson et al., 2003).
In mammalian cells, N-glycans promote maturation and quality control of glycoproteins (Mesaeli et al., 1999; Helenius & Aebi, 2004; Ruddock & Molinari, 2006). A similar quality control mechanism for glycoprotein folding in the ER is also found in Schizosaccharomyces pombe (Parodi, 1999). However, the mutant devoid of glucosidase II (encoded by the gls2 gene) shows no discernible phenotype. In addition, a further role for calnexin is suggested in Sch. Pombe, since the mammalian calnexin does not complement the lethal disruption of the Sch. pombe gene. In contrast, the quality control system of glycoprotein folding in S. cerevisiae is different from that in mammalian and Sch. pombe cells (Parodi, 1999). Our recent studies on Af cwh41 show that, unlike its counterpart in S. cerevisiae, N-glycosylation trimming in A. fumigatus is required for proper protein folding and trafficking, which suggests that the function of N-glycosylation in A. fumigatus is closer to that in mammalian cells (Zhang et al., 2008, 2009).

To evaluate the role of N-glycosylation in A. fumigatus, we constructed a conditional inactivation mutant strain, CPR-stt3. Two promoters have been successfully used to analyse the essential genes in A. fumigatus, including the A. nidulans alcA promoter and the A. fumigatus niiA promoter (Romero et al., 2003; Hu et al., 2007). The niiA promoter is induced by nitrate or other secondary nitrogen sources and repressed by ammonium or other primary nitrogen sources (Amaar & Moore, 1998; Murom-Pastor et al., 1999). In this study, we chose to place Afstt3 under the control of the promoter PniiA. Growth of the resulting CPR-stt3 strain was greatly inhibited by ammonium and restored by nitrate (Fig. 2). These results indicate that the expression level of Afstt3 is critical for normal growth of A. fumigatus; however, under repression, no fatal phenotype was observed. Therefore, due to potential leakage of the promoter PniiA (Hu et al., 2007), we are unable to exclude the possibility that Afstt3 is essential for viability. Indeed, when trying to delete the Afstt3 gene, we failed to obtain any null mutant. Therefore, N-glycosylation is likely to be essential for A. fumigatus.

Increased chitin synthesis is known to be an important compensatory response to cell wall stress both in S. cerevisiae and in filamentous fungi (Terashima et al., 2000; Ram et al., 1994; Popolo et al., 1997; Lagorce et al., 2002; Ram & Klis, 2006). In S. cerevisiae, the compensatory mechanism is triggered through the CWI signalling

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**Fig. 4.** Electron microscopy of the mycelial cell wall of the WT (a, d) and the mutant strain (b, e, in RCM; c, f, in ICM). Conidia \((1 \times 10^5)\) were added to 200 ml RCM and ICM, and incubated at 37 °C with shaking (250 r.p.m.) for 20 h. Mycelia were harvested and fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.0, at room temperature for 4 h or at 4 °C overnight. After fixation, cells were washed three times in 0.1 M phosphate, post-fixed in 1% osmium tetroxide and 0.1 M phosphate for 2–4 h, placed in increasing concentrations of methanol (30, 50, 70, 85, 95 and 100%), and post-fixed in 2% uranyl acetate and 30% methanol. Cells were rinsed, dehydrated, and embedded in Epon 812 for the floating sheet method. Sections were examined with an H-600 electron microscope (Hitachi). Bars, 1 μm.
pathway (Carotti et al., 2002). The CWI signalling pathway in \textit{S. cerevisiae} comprises a family of cell surface sensors coupled to the small G-protein Rho1p, which activates the CWI MAPK cascade via protein kinase C (Pkc1p), and allows a specific activation of the genes encoding cell wall proteins that are required to stabilize the cell wall in response to low osmolarity, thermal stress, or mating pheromone and polarized growth (Levin, 2005). Similarly, an increased expression of \textit{A. fumigatus} MpkA, an orthologue of \textit{S. cerevisiae} Mpk1, is also induced by cell wall damage (Valiante et al., 2008). Furthermore, we have shown that deletion of \textit{Afchw41}, a gene encoding \textit{A. fumigatus} glucosidase I, leads to a cell wall defect and then triggers an increase of mannoprotein and chitin in the cell wall through activation of \textit{Afcdc42}/CDC42, \textit{Afrho1}/RHO1 and \textit{Afrho3}/RHO3, suggesting a similar compensatory mechanism triggered by the MpkA-dependent CWI pathway in \textit{A. fumigatus} (Zhang et al., 2008). In the present study, we showed that the content of chitin was increased by 78\% in strain CPR-stt3 grown under repressing conditions. Meanwhile, the transcriptional levels of chitin synthase genes were upregulated by 1.6- to 13.7-fold. Our first hypothesis was that cell wall stress occurred in strain CPR-stt3 and so activated the MpkA-dependent CWI pathway in \textit{A. fumigatus}. However, to our surprise, the expression level of \textit{mpkA} was increased by only 10\%. Further analysis confirmed that the phosphorylation level of MpkA was not increased. Therefore, it appears that repression of \textit{Afstt3} does not activate the MpkA-dependent CWI pathway in \textit{A. fumigatus}.

Among the cell surface sensors implicated in detecting and transmitting cell wall status to Rho1p in \textit{S. cerevisiae} (Levin, 2005), Wsc1 and Mid2 appear to be the most important and serve a partially overlapping role in CWI signalling. More recently, N-glycans have been shown to be directly involved in Mid2 sensing in \textit{S. cerevisiae} (Hutzler et al., 2008). This observation demonstrates that N-glycosylation is important for CWI sensing and thus important for activation of CWI signalling in yeast. Although analyses regarding the cell wall stress sensor molecule in \textit{A. fumigatus} have yet to be initiated, it is likely that N-glycosylation is also important for the function of this as yet to be identified molecule. Given the possibility that repression of \textit{Afstt3} might affect the function of this unknown cell wall stress sensor molecule, we incubated the WT and CPR-stt3 strains with Congo red to induce cell wall stress. Our results showed that both WT and CPR-stt3 exhibited an upregulation of \textit{mpkA} to a similar level. This result demonstrated that repression of \textit{Afstt3} did not disrupt the MpkA-dependent CWI signalling pathway in the CPR-stt3 strain. Taken together, although we have observed overexpression of the genes required for cell wall biogenesis, their expression levels may be regulated by some other signalling pathway(s) instead of the typical MpkA-dependent CWI signalling pathway.

On the other hand, tunicamycin-induced ER stress is known to activate the UPR to slow down protein synthesis, upregulate the genes involved in protein folding and degrade misfolded proteins in \textit{A. fumigatus} (Richie et al., 2008).
Table 3. Expression of cell wall-related genes in strain CPR-stt3 under repression conditions

<table>
<thead>
<tr>
<th>Gene</th>
<th>Expression level relative to tub1 (×10⁻³)</th>
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<td></td>
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<td>ags1</td>
<td>3.6 ± 0.2</td>
</tr>
<tr>
<td>ags2</td>
<td>0.5 ± 0.1</td>
</tr>
<tr>
<td>ags3</td>
<td>1.8 ± 0.1</td>
</tr>
<tr>
<td>gel1</td>
<td>32.3 ± 5.0</td>
</tr>
<tr>
<td>gel2</td>
<td>4.7 ± 3.0</td>
</tr>
<tr>
<td>fskA</td>
<td>4.3 ± 1.0</td>
</tr>
<tr>
<td>gfaA</td>
<td>22.1 ± 1.0</td>
</tr>
<tr>
<td>chsA</td>
<td>1.9 ± 0.1</td>
</tr>
<tr>
<td>chsB</td>
<td>4.4 ± 0.5</td>
</tr>
<tr>
<td>chsC</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td>chsD</td>
<td>0.2 ± 0.01</td>
</tr>
<tr>
<td>chsE</td>
<td>2.8 ± 0.4</td>
</tr>
<tr>
<td>chsF</td>
<td>3.2 ± 0.1</td>
</tr>
<tr>
<td>chsG</td>
<td>4.3 ± 0.1</td>
</tr>
<tr>
<td>mpkA</td>
<td>110.3 ± 12.0</td>
</tr>
</tbody>
</table>

A. fumigatus conidia (1 × 10⁵ spores) were inoculated in flasks containing RCM and incubated in a shaker (250 r.p.m.) at 37 °C for 20 h. cDNAs were synthesized as described in Methods. Twenty nanograms of cDNA template, primers (2.5 μM each) and SYBR Premix Ex Taq (TaKaRa) was used to amplify gene-specific amplicons. PCR was performed in an ABI 7000 instrument (Applied Biosystems). The amplified products were about 120 bp in length. Relative quantification of the mRNA levels was determined using the ΔCₗ method (Livak & Schmittgen, 2001). The expression levels of all genes were normalized to the expression level of the endogenous control gene tub1.

Fig. 6. Phosphorylation and expression of MpkA in strain CPR-stt3. (a) A. fumigatus strains were grown in RCM at 37 °C for 18 h. Cell wall damage was induced by the addition of 50 μg Calcofluor white ml⁻¹ for 2 h. Cell extracts were prepared and detected using anti-phospho-p44/42 antibodies as described in Methods. (b) A. fumigatus strains were grown in RCM at 37 °C. After 18 h of incubation, 50 μg Congo red ml⁻¹ was added and expression levels of mpkA were measured after 2 h. The values shown are the mean and SD of three repeated experiments.
important to consider a wide range of molecular players thereby indicate that the ‘titration’ of glycosylation does not highly expressed Gel1, Gel2, FskA, ChsE and ChsG. Our data restore cell wall glucan and produce more chitin with the 3). Although the expression of Af
gel1 particularly

Methods. The values shown represent the mean and SD of three repeated experiments.

wall synthesis to ensure adequate synthesis of the functional glycoproteins. Indeed, we observed overexpression of the genes responsible for glucan and chitin synthesis, particularly gel1, gel2, fskA, chsE and chsG. Their expression levels varied from nine- to 50-fold of those in the WT (Table 3). Although the expression of Afstt3 was only one-tenth of that in the WT, the CPR-stt3 strain could still completely restore cell wall glucan and produce more chitin with the highly expressed Gel1, Gel2, FskA, ChsE and ChsG. Our data thereby indicate that the ‘titration’ of glycosylation does not consist simply of ‘all-or-nothing’ effects, and that it is important to consider a wide range of molecular players when examining the complete role of this important post-translational modification.

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