

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 *Afstt3* 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 *Afstt3* with the nitrogen-dependent *niiA* promoter. Repression of the *Afstt3* 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.

Received 20 December 2010
Revised 19 April 2011
Accepted 26 April 2011

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.

Abbreviations: ConA, concanavalin A; CWI, cell wall integrity; ER, endoplasmic reticulum; HRP, horseradish peroxidase; OST, oligosaccharyltransferase; UPR, unfolded protein response; WT, wild-type.

Two supplementary tables, showing primers for construction of the mutant strain and primers for real-time RT-PCR analysis, are available with the online version of this paper.

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-glucosylated 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 α -glucosidase I removing the outmost glucose residue in *N*-glycans, leads to a temperature-sensitive deficiency of cell wall integrity (CWI), 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, 2002; Kelleher *et al.*, 2003; Nilsson *et al.*, 2003), and its homologues are found in almost all eukaryotes (Kelleher & Gilmore, 2006). To evaluate the impact of *N*-glycosylation on the cell wall of *A. fumigatus*, a conditional inactive mutant of the *A. fumigatus stt3* gene was constructed and its phenotypes were analysed.

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 with shaking (250 r.p.m.). 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* DH5 α (Bethesda Research Laboratories).

Construction of the conditional inactive mutant. Briefly, *Aspergillus niger pyrG* and the *A. fumigatus* promoter *P_{niiA}* were amplified by PCR using pCDA14 (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_{niiA}*) containing multiple cloning site were used (Supplementary Table S1). PCR products of *pyrG* and *P_{niiA}* were first digested with *NotI/NdeI* and *NdeI/XhoI*, respectively, and subcloned into pBluescript at the *NotI/XhoI* sites. The resulting plasmid pPyrG-*P_{niiA}* contained *pyrG-P_{niiA}* 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_{niiA}* using the *NotI* and *MluI* 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_{niiA}* using the *AscI* and *PacI* sites. The resulting pNiiA-CPR 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 *XbaI*, 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 μ l drop containing 1×10^4 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 μ l of *A. fumigatus* conidia (10^5 , 10^4 , 10^3 and 10^2) was spotted on RCM or ICM agar plates supplemented with 50 μ g Calcofluor white ml⁻¹ or 100 μ g Congo red ml⁻¹ and incubated at 37 °C for 36 h.

RNA isolation and real-time RT-PCR analysis. *A. fumigatus* conidia (1×10^5 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 TRIzol reagent (Invitrogen). cDNA was synthesized with 1 μ g RNA using a RevertAid First Strand cDNA Synthesis kit (Fermentas). Twenty nanograms of cDNA template, primers (2.5 μ M 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 Δ C_T method (Livak & Schmittgen, 2001). Briefly, the amount of target gene was normalized to the

endogenous reference gene *tub1*. $\Delta C_T = C_T$ (target gene) $- C_T$ (*Tub1*), where C_T represents the cycle number required to reach a defined threshold target abundance. The relative mRNA levels were calculated as $x^{\Delta C_T}$ (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 (1×10^5) 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-phospho-p44/42 MAPK antibodies (Cell Signaling Technology). Primary antibodies were detected using Anti-Rabbit IgG (Fc) Alkaline Phosphatase Conjugate (Promega) and 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT) solution (Amresco).

Chemical analysis of the cell wall. Conidia (1×10^5) 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_4HCO_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), extracted 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 α -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; Schoffemeer *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 *Xba*I fragment in strain CPR-stt3 (Fig. 1b). These results confirmed that the native *Afstt3* promoter was replaced by P_{niiA} 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_{niiA} 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

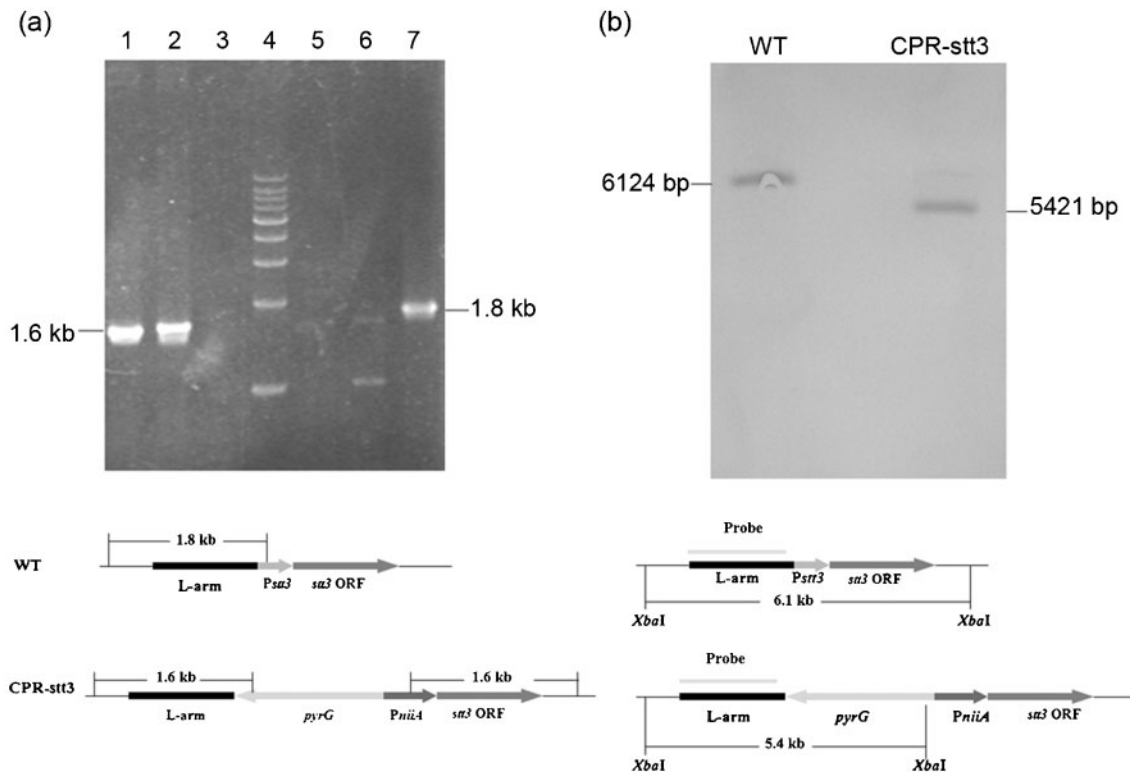


Fig. 1. Construction of the conditional mutant strain CPR-stt3. (a) PCR analysis was carried out as described in Methods. Lanes 1 and 5, primers L1 and L2 were used to amplify the 1657 bp L-arm-*pyrG* fragment; lanes 2 and 6, primers R1 and R2 were utilized to generate the 1681 bp *PniA-stt3* fragment; lanes 3 and 7, primers L1 and P were used to amplify the 1842 bp L-arm-*Pstt3* fragment. DNA from strain CPR-stt3 was used as template in lanes 1, 2 and 3, and DNA from the WT was used as template in lanes 5, 6 and 7. (b) Southern blot analysis. Genomic DNA was digested with *Xba*I, separated on agarose, then transferred to a nylon membrane and hybridized with L-arm.

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 glucanotransferases (*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 amido-transferase (*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

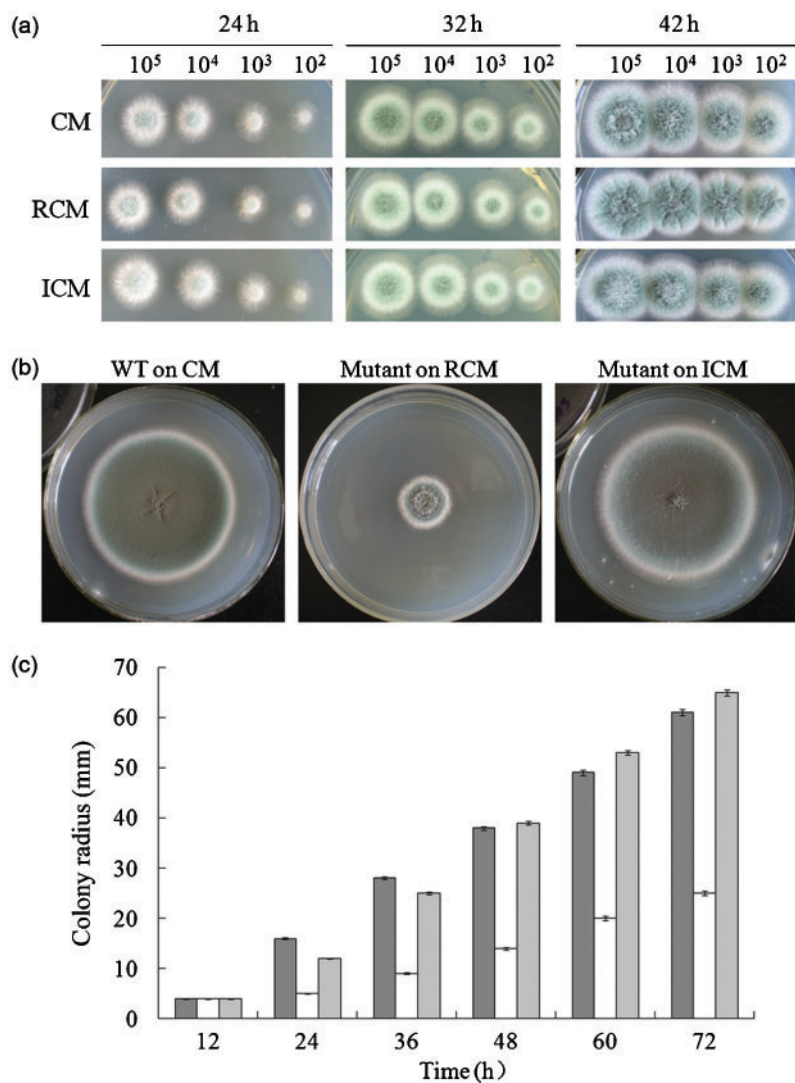


Fig. 2. Growth of *A. fumigatus* strains on various media. (a) Serially diluted *A. fumigatus* WT conidia (10^5 – 10^2 cells) were dotted and incubated on CM, RCM and ICM at 37 °C for 24–42 h; (b) freshly harvested conidia (10^4) were dotted and incubated on RCM and ICM at 37 °C for 2 days; (c) freshly harvested conidia (10^4) 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.

expression of which was only 60% of the WT. Although the function of *A. fumigatus* Ags2p is associated with hyphal morphology and conidiation, the Δ ags2 mutant of *A. fumigatus* does not show any alteration in cell wall composition (Beauvais *et al.*, 2005). In addition, AnagsA,

an orthologue of *A. fumigatus* ags2, does not show any induced expression under conditions of cell wall stress (Fujioka *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.

Table 1. Expression of *Afstt3* in strain CPR-stt3 under repression conditions

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.

Strain	ΔC_T (stt3-TUB)	$\Delta\Delta C_T$ (ΔC_T sample – ΔC_T WT)	$2^{-\Delta\Delta C_T}$
WT	9.55	0	1
CPR-stt3	12.96	3.41	0.094

Activation of *A. fumigatus* MpkA, a key protein in the CWI signalling cascade in yeast, is shown by its increased levels of transcription and phosphorylation (Valiante *et al.*, 2009). In this study, the transcription level of *A. fumigatus* mpkA increased by only 10% as compared with the WT (Table 3); furthermore, immunoblotting with anti-phospho-p44/42 MAP kinase antibody showed that phosphorylation of MpkA in the mutant was not increased (Fig. 6a). In addition, to exclude the possibility that repression of *N*-glycosylation disrupted the CWI signalling pathway in strain CPR-stt3, we treated the WT and CPR-stt3 with Calcofluor white and Congo red, compounds that interfere with chitin and glucan synthesis, respectively, for 2 h to

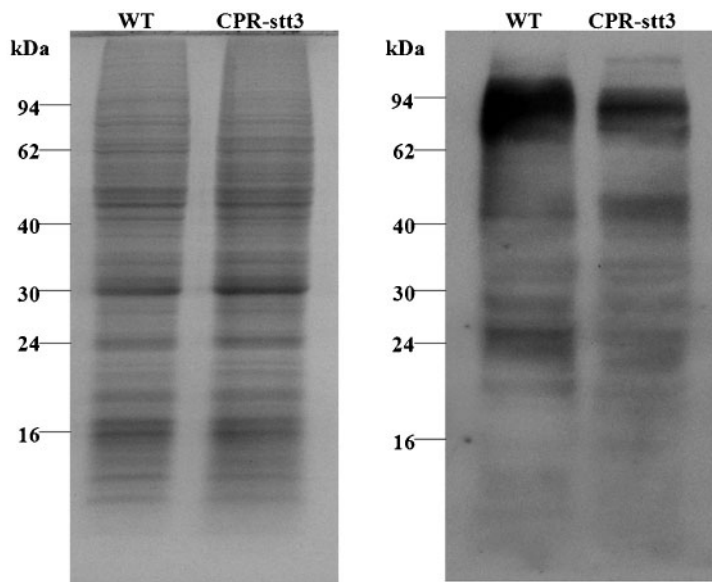


Fig. 3. SDS-PAGE analysis (left panel) and Western blot analysis of glycoproteins with an HRP conjugate of the lectin ConA (right panel). Conidia (1×10^5) were inoculated into 200 ml liquid RCM and cultured at 37 °C for 20 h with shaking (250 r.p.m.). Proteins were extracted from mycelia as described in Methods. Fifteen micrograms of protein were subjected to SDS-PAGE and transferred to a PVDF membrane, and then incubated with HRP-conjugated ConA at a 1:50 dilution. HRP activity was visualized by an enhanced chemiluminescence system.

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).

Table 2. Cell wall components of strain CPR-stt3 under inducing or repression conditions

Conidia (1×10^5) were inoculated and incubated in 200 ml RCM or ICM at 37 °C for 20 h with shaking (250 r.p.m.). The mycelia were harvested and lyophilized. Three aliquots of 10 mg dry mycelia were used as independent samples for the analysis of cell wall proteins and water-soluble sugars, as described in Methods. The experiments were repeated twice. Values are means \pm SD.

Strain	Cell wall component [μg (10 mg dry mycelium) $^{-1}$]			
	Mannoprotein	α -Glucan	Chitin	β -Glucan
WT	177 \pm 24	616 \pm 11	133 \pm 5	1490 \pm 78
CPR-stt3 (RCM)	409 \pm 28	554 \pm 40	237 \pm 6	1512 \pm 22
CPR-stt3 (ICM)	179 \pm 21	608 \pm 11	134 \pm 5	1463 \pm 53

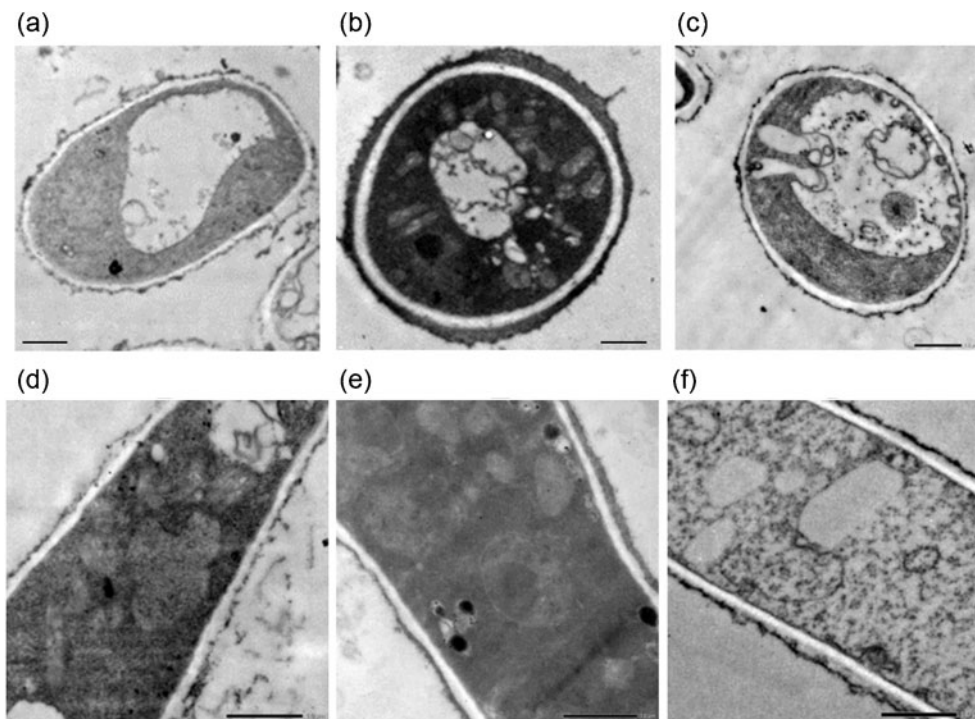


Fig. 4. Electron microscopy of the mycelial cell wall of the WT (a, d) and the mutant strain (b, e, in RCM; c, f, in ICM). Conidia (1×10^5) were added to 200 ml RCM and ICM, and incubated at 37 °C with shaking (250 r.p.m.) for 20 h. Mycelia were harvested and fixed in 2.5% glutaraldehyde in 0.1 M phosphate 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 μ m.

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 *Afcwh41* 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; Muro-Pastor *et al.*, 1999). In this study, we chose to place *Afstt3* under the control of the promoter P_{niiA} . 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 P_{niiA} (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

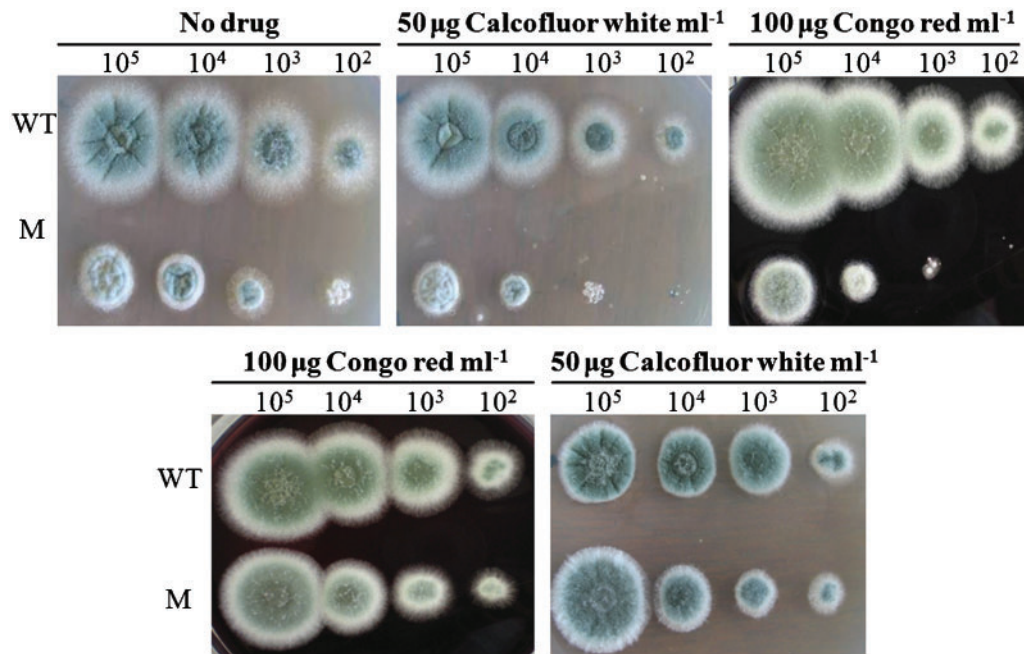


Fig. 5. Sensitivity of strain CPR-stt3 to cell wall-disturbing compounds. Serially diluted conidia (10^5 – 10^2 cells) were dotted and incubated on RCM (upper panel) and ICM (lower panel) containing Calcofluor white or Congo red. After incubation at 37 °C for 36 h, the plates were photographed.

pathway (Carotti *et al.*, 2002). The CWI signalling pathway in *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 *A. fumigatus* MpkA, an orthologue of *S. cerevisiae* Mpk1, is also induced by cell wall damage (Valiante *et al.*, 2008). Furthermore, we have shown that deletion of *Afcwh41*, a gene encoding *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 *Afcdc42/CDC42*, *Afrho1/RHO1* and *Afrho3/RHO3*, suggesting a similar compensatory mechanism triggered by the MpkA-dependent CWI pathway in *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 signalling pathway in order to upregulate chitin synthesis. However, to our surprise, the expression level of *mpkA* was increased by only 10%. Further analysis confirmed that the phosphorylation level of MpkA was not increased. Therefore, it appears that repression of *Afstt3* does not activate the MpkA-dependent CWI pathway in *A. fumigatus*.

Among the cell surface sensors implicated in detecting and transmitting cell wall status to Rho1p in *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 *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 *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 *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 *mpkA* to a similar level. This result demonstrated that repression of *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 *A. fumigatus* (Richie *et al.*,

Table 3. Expression of cell wall-related genes in strain CPR-stt3 under repression conditions

A. fumigatus conidia (1×10^5 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 ΔC_T method (Livak & Schmittgen, 2001). The expression levels of all genes were normalized to the expression level of the endogenous control gene *tub1*.

Gene	Expression level relative to <i>tub1</i> ($\times 10^{-3}$)		
	WT	CPR-stt3	CPR-stt3/WT
<i>ags1</i>	3.6 ± 0.2	15.6 ± 3.0	4.3
<i>ags2</i>	0.5 ± 0.1	0.3 ± 0.1	0.6
<i>ags3</i>	1.8 ± 0.1	7.1 ± 1.0	3.9
<i>gell</i>	32.3 ± 5.0	289.0 ± 43.0	8.9
<i>gel2</i>	4.7 ± 3.0	124.1 ± 24.0	26.4
<i>fskA</i>	4.3 ± 1.0	230.0 ± 50.0	53.5
<i>gfaA</i>	22.1 ± 1.0	303.5 ± 60.0	13.7
<i>chsA</i>	1.9 ± 0.1	6.4 ± 1.0	3.3
<i>chsB</i>	4.4 ± 0.5	7.2 ± 1.0	1.6
<i>chsC</i>	1.2 ± 0.1	4.2 ± 0.8	3.5
<i>chsD</i>	0.2 ± 0.01	0.9 ± 0.2	4.5
<i>chsE</i>	2.8 ± 0.4	29.9 ± 1.2	10.7
<i>chsF</i>	3.2 ± 0.1	12.9 ± 0.9	4.0
<i>chsG</i>	4.3 ± 0.1	39.5 ± 1.0	9.2
<i>mpkA</i>	110.3 ± 12.0	124.1 ± 11.0	1.1

2007; Meusser *et al.*, 2005; Römisch, 2005; Kim *et al.*, 2006). However, the role of the UPR is not limited to the restoration of ER homeostasis. Transcriptome analysis of the UPR induced by DTT or tunicamycin in *S. cerevisiae* shows that only a quarter of 381 UPR-related genes are associated with the secretory pathway, including translocation, protein glycosylation, vesicular transport, cell wall biosynthesis, vacuolar protein targeting and ER-associated protein degradation (ERAD) (Travers *et al.*, 2000). Recently, Richie *et al.* (2009) have demonstrated that the UPR is also involved in CWI signalling in *A. fumigatus*. As the *N*-glycosylated proteins not only are involved in cell wall biosynthesis but also are components of the cell wall, it is not surprising to observe an activation of the UPR and the CWI signalling pathway in the previously described $\Delta Afcwh41$ mutant (Zhang *et al.*, 2008, 2009). In the case of $\Delta Afcwh41$, although all proteins can be normally *N*-glycosylated, the *N*-glycan trimming is completely blocked, and glycoproteins required for cell wall synthesis, probably including the postulated cell wall stress sensor molecule, cannot be transported to their proper sites, which therefore leads to a severe cell wall defect and in turn activates both the UPR and the MpkA-dependent CWI signalling pathway. Thus, it is interesting to know how strain CPR-stt3 responds to under-*N*-glycosylation. Unlike our previous finding with *cwh41*, we only observed an activation of the UPR when *N*-glycosylation was generally repressed, but not abolished, in the CPR-stt3 strain. Therefore, part of the protein population still undergoes *N*-glycosylation, and these molecules can still play a role in cell wall synthesis. To deal with the ER stress induced by repression of *N*-glycosylation, strain CPR-stt3 activates its UPR and upregulates the genes involved in protein folding and cell

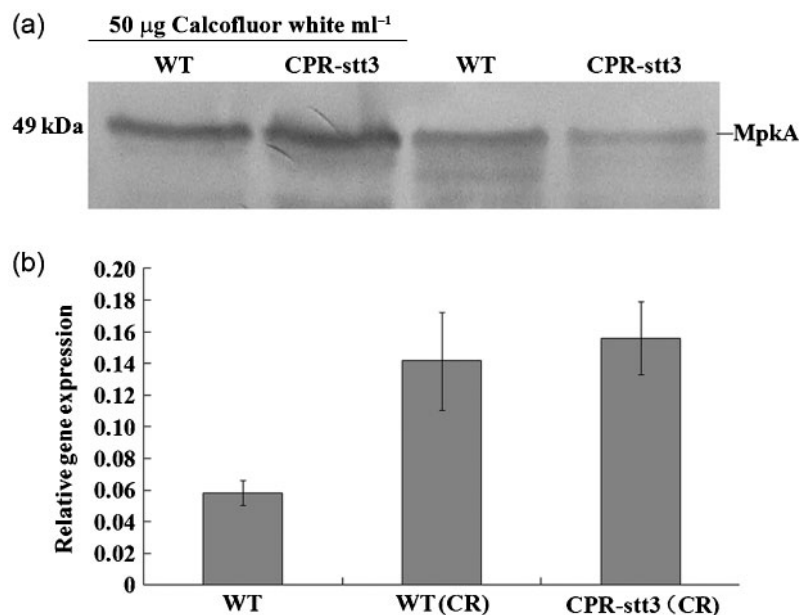


Fig. 6. Phosphorylation and expression of MpkA in strain CPR-stt3. (a) *A. fumigatus* strains were grown in RCM at 37 °C for 18 h. Cell wall damage was induced by the addition of 50 µg Calcofluor white ml⁻¹ for 2 h. Cell extracts were prepared and detected using anti-phospho-p44/42 antibodies as described 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.

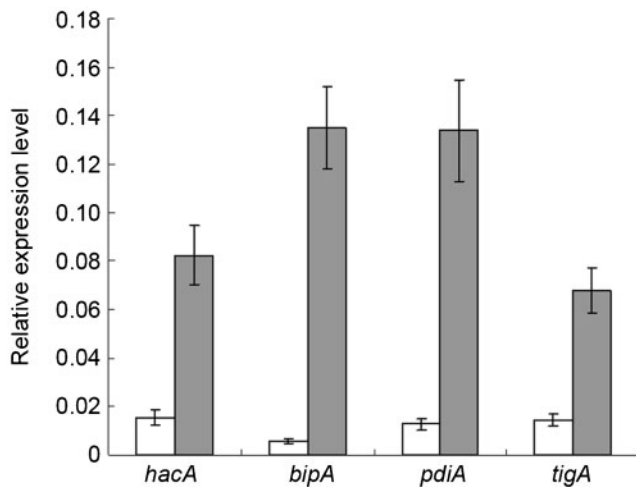


Fig. 7. Expression of the URP-associated genes in strain CPR-stt3. The white and grey bars indicate the relative transcript levels in the WT and CPR-stt3, respectively. The expression levels of all genes were normalized to the expression level of the endogenous control gene *tub1*. Total RNAs were extracted as described in 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.

ACKNOWLEDGEMENTS

This project was supported by the National Natural Science Foundation of China (31030025 and 31011130030) to C. J.

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Edited by: N. P. Keller