N-Glycosylation of Gel1 or Gel2 is vital for cell wall β-glucan synthesis in *Aspergillus fumigatus*

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Fungal cell wall is a dynamic structure that communicates with and protects the cell from outside stress. In *Aspergillus fumigatus*, the cell wall β-glucans are mainly elongated by β-1,3-glucanosyltransferases Gels, which consist of seven family members (Gel1–7) utilizing β-1,3-glucan chains as substrates. Previously, we have shown that the mutant deficient of N-glycan processing displays a reduction in the cell wall β-glucans, suggesting that N-glycosylation is required for the proper function of β-1,3-glucanosyltransferase. To verify this hypothesis, in this study, the gene encoding β-1,3-glucanosyltransferase Gel1 or Gel2 was deleted in the Δcwh41 mutant to construct a double-mutant Δgel1Δcwh41 or Δgel2Δcwh41. The growth phenotypes of both double mutants were similar to the single-mutant Δcwh41, suggesting that Gel1 and Gel2 are proteins that are mainly affected by deficient N-glycan processing in Δcwh41. Furthermore, the mutant Δgel1Δgel1-NM or Δgel2Δgel2-NM, in which all potential N-glycosylation sites on Gel1 or Gel2 were removed by site-directed mutagenesis, showed phenotypes similar to the single-mutant Δgel1 or Δgel2. Biochemical analysis revealed that N-glycosylation was essential for the function of Gel1 or Gel2 and thus required for β-glucan synthesis in *A. fumigatus*.

**Keywords:** *Aspergillus fumigatus* / β-1,3-glucanosyltransferase / cell wall / cell wall integrity / N-glycosylation

**Introduction**

*Aspergillus fumigatus* is an opportunistic pathogenic fungus that causes diverse diseases, ranging from aspergillosis to invasive aspergillosis (IA), which has emerged as the primary mold pathogen among immunocompromised patients, especially solid organ transplant recipients, bone marrow transplant recipients and AIDS (Munro 2001; Kontoyiannis and Bodey 2002; Morgan et al. 2005; Maschmeyer et al. 2007). Due to the low efficacy of the methods to detect and treat, the lethality rate of IA is around 50–95 and 50% even when treated with antifungal drugs (Latgé 1999; Hagen et al. 2003).

As the main line of defense against adverse environmental conditions, the fungal cell wall is mainly coated with glycoproteins containing N- and O-glycans derived primarily from the process of glycosylation (Latgé et al. 2005; Balloy and Chignard 2009) and is responsible for the interaction with the host (Brown et al. 2002; Hohl et al. 2005; Aimanianda et al. 2009; Abad et al. 2010; Ben-Ami and Kontoyiannis 2010). The core skeleton of *A. fumigatus* cell wall is composed of β-1,3/1,6-branched glucans, on which other polysaccharides (α-1,3-glycans, linear β-1,3/1,4-glycans, chitin and galactomannans) and glycoproteins are cross-linked (Mouyna et al. 2005; Latgé 2007; Gasteloais et al. 2009).

In *A. fumigatus*, β-1,3-glucanosyltransferase is responsible for splitting a β-1,3-glucan chain with at least 10 glucose units and transfer the new generated reducing end (>5 glucose units) to the non-reducing end of another β-1,3-glucan chain, resulting in the elongation of the glucan chain (Fiedler and Simons 1995; Douglas 2001; Gasteloais, Fontaine, et al. 2010; Gasteloais, Mouyna, et al. 2010). Seven members of β-1,3-glucanosyltransferases have been annotated, namely Gel1 (AFUA_2G01170), Gel2 (AFUA_6G11390), Gel3 (AFUA_2G12850), Gel4 (AFUA_2G05340), Gel5 (AFUA_8G02130), Gel6 (AFUA_3G13200) and Gel7 (AFUA_6G12410). All of Gel family members are predicted as glycosylphosphatidylinositol (GPI)-anchored proteins that contain N-linked carbohydrates, but only gel1, gel2 and gel4 are expressed during mycelial growth (Fiedler and Simons 1995). Disruption of the gel1 does not result in any visible phenotype, whereas the Δgel2 and Δgel1Δgel2 exhibit reduced growth, abnormal conidiogenesis, attenuated virulence and a decrease in β-1,3-glucan content (Gasteloais, Mouyna, et al. 2010).

N-Glycosylation is a fundamental and extensive post-translational modification that modulates a variety of the physicochemical and biological properties of proteins, such as intracellular trafficking, protein folding, secretion, stability, localization and function (Taniguchi et al. 2001; Helenius and Aebi 2004; Freeze and Aebi 2005; Wang et al. 2005; Yokoe et al. 2007; Hutzler et al. 2008). The *Saccharomyces cerevisiae* Mid2p is a glycoprotein and functions in response to cell wall stresses. Lack of the N-linked glycan affects neither the stability of Mid2p nor distribution at the plasma membrane during...
vegetative and sexual growth. However, non-N-glycosylated Mid2p fails to perceive cell wall challenging, suggesting that N-glycan is directly involved in maintaining cell wall integrity (Helenius and Aebi 2004). In A. fumigatus, although many membrane-bound proteins, such as Gels, are N-glycosylated, it is unclear how N-glycosylation affects the cell wall integrity. Previously, we have confirmed that A. fumigatus possesses a similar N-glycan-dependent quality control QC system found in mammalian cells (Weidner et al. 1998; Olivari et al. 2006; Zhang et al. 2008, 2009; Jin 2012). The A. fumigatus \(\alpha\)-glucosidase I, a key enzyme that cleaves the outmost glucose residue and initiates the N-glycan processing, is encoded by the \(cwh41\) gene. The deletion of the \(cwh41\) leads to a defective N-glycan processing of proteins and causes 38% decrease of the cell wall \(\beta\)-1,3-glucans, suggesting a correlation of the N-glycan processing to the \(\beta\)-1,3-glucan synthesis.

To explore how N-glycosylation affects the synthesis of cell wall \(\beta\)-glucans, in this study, we deleted the \(gel1\) and \(gel2\) genes in the \(\Delta cwh41\), respectively. Phenotypes of the double mutants were investigated when compared with their parental strain \(\Delta cwh41\). Additionally, multiple site mutations in the \(gel1\) or \(gel2\) gene were generated to remove potential N-glycosylation sites. Our results suggest that N-glycosylation is essential for the function of Gel1 and Gel2 in A. fumigatus.

**Results**

**Phenotypes of the \(\Delta gel1\Delta cwh41\) and \(\Delta gel2\Delta cwh41\)**

When the double mutants were cultured on complete medium (CM) with Congo red (CR) or Calcofluor white (CFW), a similar sensitivity was seen in both double mutants when compared with the \(\Delta cwh41\) (Figure 1A), which demonstrated that further deletion of the \(gel1\) or \(gel2\) in addition to the \(\Delta cwh41\) did not enhance the cell wall defect. We further analyzed the cell wall contents of the double mutants. When compared with the wild type (WT), the amount of \(\beta\)-glucan in the \(\Delta gel1\Delta cwh41\), \(\Delta gel2\Delta cwh41\) or \(\Delta cwh41\) was almost the same to each other, which reduced to 64, 66 and 65% at 37°C (Table I), which were consistent with their similar sensitivities to CR. And a similar result was observed in the content of chitin. These results clearly showed that no significant change in cell wall contents was observed in the double mutants when compared with the \(\Delta cwh41\). Interestingly, when the mutants were cultured at 50°C, the amount of \(\beta\)-glucan in the \(\Delta gel1\Delta cwh41\) (47%) and \(\Delta gel2\Delta cwh41\) (44%) was decreased when compared with the \(\Delta cwh41\) (50%) (Table I), suggesting an important role of Gel1 and Gel2 at elevated temperature, especially Gel2. Furthermore, real-time polymerase chain reaction (PCR) analysis revealed that the expression of the \(gel2\) or \(gel1\) was not changed in the \(\Delta gel1\Delta cwh41\) or \(\Delta gel2\Delta cwh41\), respectively, whereas the expression level of the \(gel4\) was increased by 12% in the \(\Delta gel1\Delta cwh41\) (P \(\leq\) 0.05) and decreased by 13% (P \(\leq\) 0.01) in the \(\Delta gel2\Delta cwh41\) when compared with the WT (Figure 2B), which demonstrated that, at least, the \(gel1\) or \(gel2\) did not compensate each in the double-mutant \(\Delta gel2\Delta cwh41\) or \(\Delta gel1\Delta cwh41\).

Both double mutants displayed a growth rate similar to the \(\Delta cwh41\) either at 37°C or at 50°C (Figure 1C). As shown in Figure 2, the WT conidia germinated in a typical isotropic pattern at an angle of 180°, and the second germ tube and the first septation generated after four rounds of mitosis (7-8 h) when incubated at 37°C in liquid CM (Figure 2A). In comparison with the WT, the earliest emergence of the second germ tube at a 120° angle occurred in the \(\Delta gel1\Delta cwh41\), \(\Delta gel2\Delta cwh41\) and \(\Delta cwh41\) after the second mitotic division (5 h), and the third germ tube or branching of the germing was found after the third or fourth nuclear division (6-7 h). About 60% conidia of the \(\Delta gel1\Delta cwh41\), \(\Delta gel2\Delta cwh41\) and \(\Delta cwh41\) mutants germinated in an apparently abnormal bipolar pattern. All three mutants did not form septum even after four rounds of mitosis (7-8 h). In addition, the septum occurred in these mutants were usually formed at the neck site of the newly emerged germ tube or germing, instead of the neck site of the first germling as that in the WT (Figure 2B). Frequency of random branching in the \(\Delta gel1\Delta cwh41\), \(\Delta gel2\Delta cwh41\) or \(\Delta cwh41\) was similar. More than 42% conidia of the \(\Delta gel1\Delta cwh41\), \(\Delta gel2\Delta cwh41\) and \(\Delta cwh41\) formed the second germ tube after four rounds of mitotic division (7 h; Table II).

Melanin, the outmost layer of conidial cell wall, is an important virulence factor to govern adherence, protecting the fungus against the host’s immune defense (Wheeler and Bell 1988; Langfelder et al. 1998; Younghim et al. 2004). Transmission electron microscope (TEM) analysis revealed that the conidia of the \(\Delta gel1\Delta cwh41\), \(\Delta gel2\Delta cwh41\) and \(\Delta cwh41\) produced a loosen and defective melanin layer which sometimes faded away at 37°C (Figure 3). Meanwhile, the expression level of the \(pksp\) or \(abr1\), which are involved in melanin synthesis, was decreased (Figure 4) in these mutants (P \(\leq\) 0.01). The double mutants produced similar amounts of conidia when compared with the \(\Delta cwh41\) (Figure 5). Average diameters of conidia produced by the \(\Delta gel1\Delta cwh41\), \(\Delta gel2\Delta cwh41\) and \(\Delta cwh41\) were 2.3 ± 0.2, 2.2 ± 0.2 and 2.3 ± 0.1 μm, respectively, whereas the average diameter of the WT was 1.7 ± 0.1 μm.

These observations clearly demonstrated that the phenotypes of the \(\Delta gel1\Delta cwh41\) and \(\Delta gel2\Delta cwh41\) were similar to those of the \(\Delta cwh41\), suggesting that Gel1 and Gel2 did not function in the \(\Delta cwh41\) mutant.

**N-Glycans are crucial for the function of Gel1 and Gel2**

To further explore the effect of N-glycosylation on Gel1 or Gel2, the mutant strains expressing the Gel1 and Gel2 lacking N-glycosylation sites were constructed, respectively, namely \(\Delta gel1^{gel1}_{gel1}NM\) and \(\Delta gel2^{gel2}NM\). The sensitivity of the \(\Delta gel1^{gel1}NM\) or \(\Delta gel2^{gel2}NM\) to CR or CFW was nearly the same as its corresponding parental strain \(gel1\) or \(gel2\) (Figure 6). When the mutants were cultured at 37°C, the cell wall contents of the \(\Delta gel1^{gel1}NM\) or \(\Delta gel2^{gel2}NM\) were almost the same as their parental strains (Table I). It should be pointed out that cell wall \(\beta\)-glucans in the \(\Delta gel1^{gel1}NM\) or \(\Delta gel2^{gel2}NM\) was similar to that in the \(\Delta cwh41\), indicating that the lack of N-glycan led to a loss of function of Gel1 or Gel2. When the mutants were cultured at 50°C, the cell wall contents of the \(\Delta gel1^{gel1}NM\) or \(\Delta gel2^{gel2}NM\) were the same as their parental strains (Table I). However, the content of \(\beta\)-glucan in the \(\Delta gel1^{gel1}NM\) or \(\Delta gel2^{gel2}NM\) was higher than that in the \(\Delta cwh41\). These results suggested that at an elevated temperature, other \(\beta\)-glucanosyltransferases, in addition to Gel1 and Gel2, were...
affected by a deficient N-glycan processing in the Δcwh41, which might be one of the causes for the severe cell wall defect in the Δcwh41 grown at higher temperatures. Additionally, the observation that Δgel2 or Δgel2 Gel2-NM produced less β-glucan than the Δgel1 or Δgel1 Gel1-NM also suggested an important role of Gel2 at an elevated temperature.

Fig. 1. Growth and sensitivity to antifungal reagents of the mutants. In (A), a series of 10-fold dilutions (10⁵–10² conidia) of the WT and mutant strains were spotted on a CM plate with or without the antifungal reagent and cultivated at 37 or 50°C for 24–36 h; in (B), quantitative real-time PCR was carried out using total RNAs extracted from strains grown at 37°C for 36 h as described under Materials and methods; in (C), the growth rate of the WT and mutant strains on CM were cultured at 37 or 50°C. Each point represents one growth diameter measurement (three experiments) and the tendency curves are indicated.
Table I. Cell wall components of the mutant strains

<table>
<thead>
<tr>
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<th>AS</th>
<th>AI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>α-Glucan (µg)</td>
<td>Mannoprotein (µg)</td>
</tr>
<tr>
<td>37°C</td>
<td>WT</td>
<td>507 ± 12</td>
<td>200 ± 8</td>
</tr>
<tr>
<td></td>
<td>Δcwh41</td>
<td>453 ± 24</td>
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<td></td>
<td>Δgel1Δcwh41</td>
<td>455 ± 11</td>
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<td>Δgel2Δcwh41</td>
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<td>Δgel1</td>
<td>473 ± 16</td>
<td>199 ± 11</td>
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<td></td>
<td>Δgel2</td>
<td>464 ± 32</td>
<td>180 ± 7</td>
</tr>
<tr>
<td></td>
<td>Δgel1Gel1-NM</td>
<td>472 ± 17</td>
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<tr>
<td></td>
<td>Δgel2Gel1-NM</td>
<td>456 ± 21</td>
<td>175 ± 23</td>
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<tr>
<td></td>
<td>Δgel2Gel2</td>
<td>498 ± 26</td>
<td>196 ± 27</td>
</tr>
<tr>
<td>50°C</td>
<td>WT</td>
<td>913 ± 27</td>
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<td>109 ± 13</td>
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<td>Δgel1</td>
<td>529 ± 11</td>
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<td>138 ± 17</td>
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<tr>
<td></td>
<td>Δgel2Gel1-NM</td>
<td>543 ± 17</td>
<td>110 ± 4</td>
</tr>
<tr>
<td></td>
<td>Δgel1Gel2</td>
<td>904 ± 42</td>
<td>143 ± 9</td>
</tr>
<tr>
<td></td>
<td>Δgel2Gel2</td>
<td>901 ± 38</td>
<td>147 ± 2</td>
</tr>
</tbody>
</table>

1 × 10^6 conidia were inoculated into 200 mL of liquid CM, incubated at 37 or 50°C with shaking at 200 rpm. The cell wall was extracted as described under Materials and methods. Three aliquots of 10 mg lyophilized cell walls were used as independent samples for cell wall analysis and the experiment was repeated three times. The values (mean ± SD) shown are cell wall component per 10 mg dry cell walls.

N-Glycosylation affects localization and degradation of Gel1 and Gel2

To determine how N-glycosylation affects Gel1 and Gel2, the antibodies against them were developed using synthesized peptides. Unfortunately, only the anti-Gel1 antibody was successfully developed. To develop the antibody against Gel2, we put a His-Tag behind the N-terminal signal peptide of the Gel2 and used the anti-His antibody to detect the localization of Gel2.

Using the anti-Gel1 or anti-His antibody as a primary antibody, we found that mature forms of the Gel1 and Gel2 from the WT were around 50 kDa (Figure 7A). After PNGaseF digestion, both of them shifted to ∼50 kDa, indicating that both Gel1 and Gel2 were N-glycosylated. Although the molecular weight of the mutated Gel1 or Gel2 in Δgel1Gel1-NM or Δgel2Gel2-NM was 50 kDa (Figure 7B).

As shown in Figure 8A, Gel1 or Gel2 was detected in the cytosol, membrane, cell wall and culture supernatant of the WT or Δcwh41 grown at 37°C. However, a significant increase in the cytosolic and membrane-bound Gel1 or Gel2 was detected in the Δcwh41. When compared with the WT, the cytosolic and membrane-bound Gel1 or Gel2 were remarkably degraded in the Δcwh41. When the strains were cultured at 50°C, both Gel1 and Gel2 in the WT began to degrade, but accumulation and degradation occurred in cytosol and membrane of the Δcwh41 were significantly increased in the Δcwh41. These results demonstrated that defect of N-glycan processing led to the accumulation and the degradation of cytosolic and membrane-bound Gel1 and Gel2.

To confirm this hypothesis, proteins from different subcellular fractions of the Δgel1Gel1-NM and Δgel2Gel2-NM were analyzed (Figure 8B). A large number of cytosolic and membrane-bound Gel1 and Gel2 were degraded in the Δgel1Gel1-NM and Δgel2Gel2-NM, respectively. The results indicate that N-glycosylation is required for the stability of Gel1 and Gel2 in A. fumigatus.

Proteomic analysis of the Δcwh41 reveals that Hsp70, calnexin and Ubc, which contribute to proteasome and ubiquitin-mediated proteolysis, are overexpressed, suggesting that defective N-glycan processing leads to an increased accumulation of mis-folded proteins in the endoplasmic reticulum (ER) lumen (Zhang et al. 2009). Indeed, the Δcwh41, Δgel1Δcwh41 and Δgel2Δcwh41 showed a similar sensitivity to dithiothreitol (DTT) or tunicamycin, when compared with the WT (Figure 9), indicating an ER stress in the double mutants. Therefore, it is not surprising to observe an accumulation and degradation of Gel1 and Gel2 in these mutants. On the other hand, the Δgel1Gel1-NM and Δgel2Gel2-NM showed a similar sensitivity to DTT or tunicamycin when compared with the WT, Δgel1 or Δgel2. These observations suggested an accumulation of some proteins, in addition to Gel1 and Gel2, in the ER of the Δcwh41 and the disruption or mutation of the single gel1 or gel2 gene did not cause severe ER stress. Taken together, we conclude that Gel1 and Gel2 are proteins mainly affected by the N-glycan processing.

Discussion

Previously, we have shown that A. fumigatus possesses an N-glycan-dependent quality control QC system. α-Glucosidase I (Cwh41) initiates the trimming of the terminal α-1,2-glucose residue of the N-glycan in A. fumigatus. The deletion of this gene results in defective N-glycan processing, severe reduction in conidia formation and defective cell wall integrity (Zhang
et al. 2008). As the main contributor of the cell wall synthesis, gels are described as a family of the GPI-anchored \(\beta\)-1,3-glucanosyltransferase containing seven members. All of them are predicted as N-glycosylated proteins. As the reduction in cell wall \(\beta\)-glucans has been observed in the \(\Delta cwh41\) mutant, we hypothesized that Gel1 and Gel2 might be two of proteins affected by N-glycosylation. We therefore deleted the \(gel1\) and \(gel2\) in the \(\Delta cwh41\), respectively. The phenotypes of the double-mutant \(\Delta gel1\Delta cwh41\) or \(\Delta gel2\Delta cwh41\) were similar to the \(\Delta cwh41\), suggesting a loss of function of Gel1 or Gel2 in the \(\Delta cwh41\). We further removed potential N-glycosylation sites on Gel1 or Gel2 by site-directed mutagenesis. The phenotypes of the mutant \(\Delta gel1^{gel1-NM}\) or \(\Delta gel2^{gel2-NM}\) were identical to their parental strain \(\Delta gel1\) or \(\Delta gel2\), respectively, which confirmed that N-glycosylation is essential for the function of Gel1 and Gel2. When compared with the WT, Gel1 and Gel2 expressed in the \(\Delta cwh41\) were accumulated and degraded in the cytosol and membrane. Mutated Gel1 or Gel2 expressed in the

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**Fig. 2.** Germination and septation of the mutants. 10 mL of liquid CM was inoculated with \(10^6\) freshly harvested conidia in petri plates containing glass coverslips and incubated at 37°C. The germination conditions were observed (A). For each independent experiment, 100 conidia were counted and three independent experiments were carried out. The coverslips with adhering germlings were fixed and stained with CFW and 4,6-diamidino-2-phenylindole as described under Materials and methods (B).
Institut Pasteur, was maintained at 37°C on YGA medium

Table II. Germination and separation of different mutants

<table>
<thead>
<tr>
<th>Culture time</th>
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<th>Second germ tube</th>
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<td>46</td>
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<td></td>
<td>Age1</td>
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<td>34 ± 6</td>
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<tr>
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<td>Age1Δcwh41</td>
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</table>

10 mL of liquid CM was inoculated with $10^6$ freshly harvested conidia in petri plates containing glass coverslips and incubated at 37°C. The germination conditions were observed and counted per hour after cultured for 4 h. For each independent experiment, 100 conidia were counted and three independent experiments were carried out.

Age1<sup>Δcwh41</sup>-NM or Age2<sup>Δcwh41</sup>-NM was also accumulated and degraded in the cytosol and membrane. These results confirmed that N-glycosylation was essential for the function of Gel1 and Gel2. When the mutants were treated with ER stress inducing reagents, tunicamycin and DTT, an increased sensitivity was observed in the Δcwh41, Age1Δcwh41 or Age2Δcwh41. Therefore, it is reasonable to conclude that a reduction in the cell wall β-glucan in the Δcwh41, Age1Δcwh41 or Age2Δcwh41 was mainly due to the accumulation and degradation of mis-folded Gel1 and Gel2 proteins in the ER. On the other hand, the sensitivity of the Age1<sup>Δcwh41</sup>-NM or Age2<sup>Δcwh41</sup>-NM to tunicamycin or DTT was similar to that of the WT, Age1 or Age2. These observations suggested that the disruption or mutation of the single gel1 or gel2 gene did not cause ER stress.

Based on these observations, it can be concluded that N-glycosylation is required for folding and function of some other glycoproteins in addition to Gel1 and Gel2. Further identification of these proteins, e.g. other members of the Gel family, would help to understand how N-glycosylation affects and modulates cell wall synthesis in A. fumigatus.

Materials and methods

Strains and growth conditions

*Aspergillus fumigatus* strain YJ407 (China General Microbiological Culture Collection Center, CGMCC0386) was cultivated on potato glucose (2%) agar slants (Xia et al. 2001). *Aspergillus fumigatus* CEA17 strain (*pyrG*), a gift from Institut Pasteur, was maintained at 37°C on YGA medium (0.5% yeast extract, 2% glucose, 1.5% Bactoagar) with addition of 5 mM uridine and uracil (Pritchett and Baldwin 2004).

Strains were cultured in liquid CM with shaking at 200 rpm at 37 or 50°C, respectively. Mycelia cultured in different conditions were harvested and washed three times with distilled water, drained and frozen in liquid N$_2$ and then stored at −70°C for DNA genome, RNA and protein extraction.

Conidia were harvested from the solid CM with 0.1% Tween-20 in 1× phosphate-buffered saline (PBS) solution and were washed twice with double-distilled water, resuspended and stored at 4°C in sealed 1.5-mL centrifuge tubes. The conidial suspension concentration was confirmed by hemocytometer counting and viable counting. Vectors and plasmids were transferred and propagated in *Escherichia coli* DH5α (Bethesda Research Laboratories, Bethesda).

Construction of the Age1, Age2, Age1Δcwh41 and Age2Δcwh41 mutant

To delete the gel1 or gel2, two deletion cassettes containing the *pyrG* gene as the selectable marker were constructed, which was used to replace the entire coding region by inserting inside the gel1 or gel2 flanking sequence. Both the CEA17 strain and the Δcwh41 mutant strain were used as the initial strains for gene deletion. PCR primers were designed to amplify a 1.5-kb 5′-flanking sequence of the target gene before the ATG start codon (gel1, 5′-primer pair 5′-GCGGCGCCCATGAAACA TGCTGCCTGTAC3′ and 5′-GCTCTAGGATATC TATG ATGTA GTGTTGCTTGTC3′; gel2, 5′-primer pair 5′-GCGGC GCCATCTCCGATCAA AGTTGACTC3′ and 5′-GCTCTAGAGATATC TATGATGTTGTTGCTTGTC3′) and a 1.5-kb 3′-flanking sequence of the target gene after the terminator codon (gel1, 5′-GCTCTAGAGATACCCACTG TGAAT CAA TTGCA TCCGGTGTGC3′ and 5′-AATGCTGGAG CCGCCGCA TGAATTGATC3′; gel2, 5′-GCTCTAGAGATATC TATGATGTTGTTGCTTG3′ and 5′-AATGCTGG AGGCCCGCGCAACTTACGTTACCTTATG3′). The upstream and downstream non-coding regions were digested with NotI/XbaI and XbaI/PstI, respectively, and then separately cloned into pGEM-T Easy (Promega, USA) and confirmed by sequencing. As a fungal selectable marker, the *pyrG* gene cassette released from pCDA14 (Pritchett and Baldwin 2004) with *HpaI* was cloned into the junction between the 5′- and 3′-flanking regions of gel1 or gel2. The deletion vectors were linearized by digesting with NotI and then transformed into *A. fumigatus* CEA17 or Δcwh41 protoplasts and plated under uridine and uracil autotrophy selection. Plates were incubated in hypertonc medium at 30°C for ~3–5 days, and the deletion mutants were selected and then confirmed using PCR and Southern blotting.

For PCR analysis, primers *gel1*-up (5′-ATGAAAGGCCCT TGCTGTAC3′) and *gel1*-down (5′-CCGCTGATTAC GCAC AAGGAAGCGGCC3′) were used to amplify the encoding sequence of *gel1*. Primers *pyrG*-up (5′-CTAGCTGAGGA CAGTGC3′) and *pyrG*-down (5′-GCTGTCTGGATCCCA GTGC3′) were used to amplify the *pyrG* gene. Another pair of primers *cwh41*-up (5′-ATGGATCTTCCGATC3′) and *cwh41*-down (5′-TTCAACTCATGCCC3′) were used to amplify the encoding region of the *cwh41* gene. For Southern blotting, genomic DNA was extracted and digested
with HindIII, XbaI and EcoRI, separated by electrophoresis and transferred to a zeta-probe blotting membrane (Bio-Rad, USA). The 800-bp fragment of the gel1 downstream, the 800-bp fragment of the gel2 upstream and the 800-bp fragment of the cwh41 encoding region were used as probes and labeled by the digoxin (DIG)-labeled hybridization kit (Roche Applied Science, Germany; Figure 10).

**Complementation of gel1 and gel2 deletion strains**

Complementation of the Δgel1 or Δgel2 was constructed by the replacement of pyrG with a WT copy of the gel1 or gel2. PCR primers were designed to amplify a 3-kb sequence containing a 1.2-kb upstream sequence and the coding region of the target gene (gel1, 5'-GCGGCCGCGTTGTTTCCACGACCGGAC-3' and 5'-CGGGATCTCTTAGATCACAAGAGGACG

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**Fig. 3.** Morphology of the mutant conidia. Freshly washed conidia cultivated on solid CM at 37°C (A) or 50°C (B) were fixed as described under Materials and methods. Sections were examined with a Tecnai Spirit (120 kV) TEM (FEI).
AGGCCAG-3′; gel2, 5′-GCGGCCGCAAGACCGTC
CCTAAAG-3′ and 5′-CGGGATCTTGAATTACGCTGAA
GAAACGCCATG-3′). The product was cloned into pGEM-T
Easy Vector and sequenced. Transformants were screened by
PCR and then confirmed by Southern blotting using the non-
coding region as a probe (Figure 10). The probe was labeled by
the DIG-labeled hybridization kit (Roche Applied Science).

**Construction of the mutants expressing non-N-glycosylated
Gel1 or Gel2**

Two putative N-glycosylation sites (N\(^{249}-F-T\) and N\(^{337}-K-T\) in
Gel1 and four putative N-glycosylation sites (N\(^{236}-S-S, N^{311}-G-T, N^{339}-T-T\) and N\(^{357}-S-T\)) in Gel2 were predicted by
using the NetNGlyc program 1.0 (http://www.cbs.dtu.dk/services/

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**Fig. 4.** Quantitative real-time PCR analysis of gene expression in the mutants. The conidia cultured at 37°C for 36 h were used for total RNAs extraction and cDNA
synthesis as described under Materials and methods. Samples isolated from different strains were tested in triplicate. 18s rDNA protein was used as parameter. At
specific time, the gene expression level of the WT was taken as 1-fold.

**Fig. 5.** Conidia counting of the mutant stains. The conidial number was detected at 37°C (A) or 50°C (B) for selected time.
NetNGlyc) (Bause 1983; Clark et al. 2004). A QuikChange® Site-Directed Mutagenesis kit (Stratagene, USA) was used to generate the point-mutant constructs. To substitute Asn with Gln at the potential N-glycosylation sites in Gel1 (Asn249 and Asn337), mutations were generated by overlapping PCR amplification using the following primer sets: Asn249 5′-GCTGGGATCAAGAAGGTCAAGCAGTTCACTGGC-3′ and 5′-CTTGACCTTCTGATTCCCAGCCCGAGGTTTTGAAA-3′; Asn337 5′-TCCGGCGACGGCAACTACCAGAAGACTGGTG-3′ and 5′-GTAGTTGCCGTCGCCGGAGGGTTGGAGGTTC-3′. Then the mutated gene was transformed into the Δgel1 to construct the Δgel1Gel1-NM. The transformants were selected and confirmed by Southern blot (Figure 10) and sequencing.

All the mutated sequences were verified by DNA sequencing. 

The Δgel2Gel2-NM was selected and confirmed by Southern blot (Figure 10).

Fig. 6. Hyphal growth and sensitivity to antifungal reagents of the mutants. A series of 10-fold dilutions (10^5–10^2 conidia) of the WT and mutant strains were spotted on a CM plate with or without the antifungal reagent and cultivated at 37 or 50°C for 24–36 h.

Fig. 7. N-Glycosylation of Gel1 and Gel2 in the mutants. Membrane proteins were extracted from different strains, separated by SDS–PAGE and detected with western blotting using anti-Gel1 or anti-His antibody as the primary antibody (A). Proteins of the WT or mutants were treated with or without PNGaseF (B).
Phenotype analysis

Growth curve were detected by spotting $1 \times 10^6$ conidia onto the center of a solid CM plate at 37 or 50°C, respectively. The colony diameter was monitored intermittently until the stationary phase, and the mean diameter was used to plot the growth curves. The experiment was repeated three times.

For examining the process of conidial germination, 10 mL of liquid CM was inoculated with $10^6$ freshly harvested conidia in

Fig. 8. Detection of Gel1 and Gel2 in the mutants. To test the localization, 50 μg of proteins from the cytosol (1), membrane (2), cell wall (3) and culture supernatant (4) were extracted, separated and detected by western blotting using anti-Gel1 or anti-His as the primary antibody.
petri plates containing glass coverslips and incubated at 37 or 50°C. The coverslips with adhering germinated conidia were taken out, fixed in solution (3.7% paraformaldehyde, 50 mM phosphate buffer, pH 7.0, and 0.1% Triton X-100), observed and counted under a differential interference contrast microscope (Li et al. 2008; Zhang et al. 2008).

Fig. 9. Sensitivity of the mutants to tunicamycin or dithiothreitol (DTT). A series of 10-fold dilution (10^7–10^4) of conidia were inoculated into 1 mL of liquid CM containing 1% dimethyl sulfoxide (DMSO), 4 mM DTT or 5 μg mL^-1 tunicamycin (Sigma) and incubated at 37°C for 36 h. The experiment was repeated three times.

Fig. 10. Confirmation of the mutant strains. Genomic DNA of the WT, Δgel1, Δgel1Δcwh41, Δgel1^Gel1 and Δgel1^Gel1-NM digested with HindIII was probed with a downstream non-coding region of the gel1 or gel2. WT, wild type; Δgel1^Gel1, the Δgel1 complemented with the gel1; Δgel1^Gel2, the Δgel2 complemented with the gel2; Δgel1^Gel1-NM, the Δgel1 complemented with non-N-glycosylated Gel1; Δgel2^Gel2-NM the Δgel2 complemented with non-N-glycosylated Gel2.
To detect the nuclei, septa and cell wall, the coverslips were taken out and fixed in a fixative solution (4% formaldehyde, 50 mM phosphate buffer, pH 7.0, and 0.2% Triton X-100) for 30 min. The coverslips were then washed with PBS, incubated for 20 min with 1 μg 4,6-diamidino-2-phenylindole mL⁻¹ (Sigma, Germany), washed with PBS, then dyed for 10 min with 10 μg CFW mL⁻¹, washed again, observed and photographed under a fluorescence microscope.

For the sensitivity detection of the mutants to different antifungal reagents, fresh washed conidia were collected from the WT, the mutants and the different complemented strain. For each strain, the same numbers of conidia were spotted on the CM medium containing 100 μg mL⁻¹ CFW or 200 μg mL⁻¹ CR and incubated at 37°C or 50°C for 36 h.

Chemical analysis of cell wall
1 × 10⁸ conidia were inoculated into 100 mL of liquid CM with shaking at 250 rpm under 37 or 50°C. The mycelium were harvested, washed twice with distilled water and lyophilized. As independent sample for cell wall analysis, 10 mg dry mycelium was added into a 1.5-mL tube containing 50 mM NH₄HCO₃ at pH 8.0 and 0.2 g of glass beads (1 mm diameter), and disrupted by disruptor genie (Scientific Industries) for five rounds (5 min each round). Then, the cell homogenates were centrifuged and washed with distilled water. Three independent lyophilized mycelia were used in each test. The experiment was repeated three times.

Cell wall was treated with 1 M KOH and incubated at 70°C for 30 min and separated into two groups including the alkali-soluble (AS) and the alkali-insoluble (AI) part. The AS parts were acidulated to pH 5.0 using acetic acid, centrifuged. Then, soluble (AS) and the alkali-insoluble (AI) part. The AS parts for 30 min and separated into two groups including the alkali-soluble and the alkali-insoluble part. The AS parts were acidulated to pH 5.0 using acetic acid, centrifuged. Then, soluble (AS) and the alkali-insoluble (AI) part.

For protein extraction and western blotting
1 × 10⁸ conidia were inoculated in 100 mL of liquid CM at 37°C for 36 h. Total RNA was extracted using the TRIZOL method according to the manufacturer’s instruction (Invitrogen, Carlsbad, CA). cDNA synthesis was performed using the RevertAid TM First Strand cDNA Synthesis Kit (Fermentas, Canada). Primers 18sr-up (5’-CGAGTCTTTTGACGCACATT-3’) and 18sr-down (5’-GTGCTTGGAGGGCA-GCAAT-3’) were used to amplify an 80-bp fragment of 18s rRNA as control. The target genes were amplified with the length of 80–100 bp. To exclude the contamination of cDNA preparations with genomic DNA, primers were designed to amplify regions containing one intron in these gene (Bustin 2000, 2002). The expression was measured by quantitative real-time PCR using the fluorescent reporter SYBR Green (Fermentas) and ABI 7300 thermocycler (Applied Biosystems, USA). And the quantification of mRNA levels of different genes were performed using the 2⁻ΔΔCt method (Livak and Schmittgen 2001; Nolan et al. 2006).

Electron microscopy
Freshly washed conidia which cultivated on solid CM at 37 or 50°C were 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, conidia were washed three times in 0.1 M phosphate, post-fixed in 1% osmium tetroxide and 0.1 M phosphate for 2–4 h, then 15–20 min in methanol 30, 50, 70, 85, 95 and 100%, respectively, and post-fixed in 2% of 30% uranyl acetate–methanol. Conidia were rinsed, dehydrated and embedded in Epon 812 for the floating sheet method. Sections were examined with a Tecnai Spirit (120 kV) TEM (FEI, USA).

Colonies for scanning electron microscopy were grown in solid CM at 37 or 50°C, fixed in phosphate-buffered glutaraldehyde followed by OsO₄, impregnated with uranyl acetate during ethanol dehydration, critical point dried, sputter coated with gold palladium and examined with a Quanta200 scanning electron microscope (Kurtz et al. 1994).
12% SDS–polyacrylamide gel electrophoresis (PAGE) and transformed to poly vinyliden fluoride (PVDF) membrane (Millipore, USA) at 300 mA for 1.5 h. The target protein was detected with the enhanced chemiluminescence substrate (Pierce, USA) by using an antibody.

The antibody against Gel1 was developed with synthesized peptide CPAKADPNWD VDNALPA (B & M Company, China). The cleavage site of the N-terminal signal of Gel2 was predicted between amino acids 21 and 22 using the sequence-analytic prediction algorithms SignalP 4.1 Server (http://www.cbs.dtu.dk/services/SignalP/). To develop the antibody against Gel2, a recombination his-gel2 was constructed by adding a His-Tag behind the encoding region of the N-terminal signal peptide of Gel2 by using overlapping PCR (gel2-N-his: 5′-CACCACCCACCACCGCTTGTTCCA TGAGGTC-3′ and 5′-CAG CATGAAGACGGCATGAAC GAGGC-3′; gel2-fusion-1: 5′-ACGAAGTCCGCTCACACC ACC-3′; gel2-fusion-2: 5′-CTTGAGCCTGTAGGAACGAC GTGTTGTTGTTGTTGG TG-3′ and gel2-fusion-3: 5′-TTA CAGCATGAAGAAGCCATG-3′). The recombination (containing the up-stream region) was amplified by primers (gel2-NotI 5′-GCGGCGCCGCAAGAAGTCGCTCACA CC-3′ and gel2-XbaI 5′-GCTCTAGATTA CAGCATGAA GAACGCCTAG-3′). The PCR products were digestion with NotI and XbaI, and then cloned into pGEM-T vector. After the construction of the whole recombination vector as we described before, the vector was linearized by digesting with NotI and then transformed into Δgel2 (pyrG- Presented by November 11, 2021

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**Conflict of interest**

None declared.

**Abbreviations**

AI, alkali-insoluble; AS, alkali-soluble; CFW, Calcoflour white; CM, complete medium; CR, Congo red; ER, endoplasmic reticulum; GPI, glycosylphosphatidylinositol; IA, invasive aspergillosis; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; TEM, transmission electron microscope; WT, wild type.

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