

## Class IIC $\alpha$ -mannosidase AfAms1 is required for morphogenesis and cellular function in *Aspergillus fumigatus*

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**The mammalian ER/cytosolic  $\alpha$ -mannosidase (Man2C1p), yeast vacuolar  $\alpha$ -mannosidase (Ams1p) and the *Aspergillus nidulans*  $\alpha$ -mannosidase are members of Class IIC subgroup, which is involved in oligosaccharide catabolism and *N*-glycan processing. Unlike their mammalian counterparts, the yeast Ams1p and *A. nidulans* Class IIC  $\alpha$ -mannosidase are not essential for morphogenesis and cellular function. In this study, the *Afams1*, a gene encoding a member of Class IIC  $\alpha$ -mannosidases, was identified in the opportunistic pathogen *Aspergillus fumigatus*. Deletion of the *Afams1* led to a severe defect in conidial formation, especially at a higher temperature. In addition, abnormalities of polarity and septation were associated with the  $\Delta$ *Afams1* mutant. Our results showed that the *Afams1* gene, in contrast to its homolog in yeast or *A. nidulans*, was required for morphogenesis and cellular function in *A. fumigatus*.**

**Keyword:**  $\alpha$ -mannosidase/*Aspergillus fumigatus*/cell wall/morphogenesis/polarity

### Introduction

In eukaryotes,  $\alpha$ -mannosidases play a key role in protein glycosylation, both in the modification of *N*-glycan chains prior to further elongation and in the catabolism of oligosaccharides (Moremen et al. 1994). These  $\alpha$ -mannosidases have been divided into two broad classes based on their distinctive substrate specificities, responses to inhibitors, cation requirements, protein molecular weights, subcellular localizations, and enzyme mechanisms (Daniel et al. 1994; Moremen et al. 1994; Eades et al. 1998). Class I  $\alpha$ -mannosidases are *N*-glycan processing enzymes including ER Man<sub>9</sub>-mannosidase, endomannosidase, and Golgi mannosidase I. Class II  $\alpha$ -mannosidases are a heterogeneous collection of processing and catabolic enzymes that are present in the ER, Golgi, lysosome, and cytosol (Moremen 2002).

Based on their distinct sequences and biochemical properties, Class II  $\alpha$ -mannosidases are further classified into three

subgroups (IIA, IIB, and IIC). Among these subgroups, Class IIC sequences are quite distantly related to the rest of the Class II  $\alpha$ -mannosidases and contain no cleavable signal sequence or transmembrane domain (Eades et al. 1998). Mammalian cytosolic  $\alpha$ -mannosidases (Man2C1p), yeast vacuolar  $\alpha$ -mannosidase (Ams1p), and *A. nidulans*  $\alpha$ -mannosidase are members of Class IIC subgroup (Yoshihisa and Anraku 1989, 1990; Bischoff et al. 1990; Eades et al. 1998; Costanzi et al. 2006). The rat Man2C1p is involved in oligosaccharide catabolism of mis-folded glycoproteins in the lumen of the ER which have been retro-translocated into the cytoplasm for proteolytic disposal (Bischoff et al. 1990; Grard et al. 1996; Duvet et al. 1998). A proteolytically cleaved version of the rat Man2C1p has been found in the lumen of the ER where it is believed to be involved in the early stages of glycoprotein maturation (also called ER  $\alpha$ -mannosidase II) (Weng and Spiro 1993, 1996). The role of the yeast Ams1p is to aid in recycling macromolecular components of the cell under nutrient deprivation (Hutchins and Kliensky 2001). Interestingly, after its synthesis in the cytosol, the Ams1p is translocated into the vacuole by the cytosol-to-vacuole targeting (Cvt) pathway (Hutchins and Kliensky 2001), which suggests a common feature shared by the *S. cerevisiae* Ams1p and its mammalian counterparts. However, the yeast Ams1p only participates in recycling or utilizing of oligosaccharide but not in processing of *N*-glycan. Meanwhile, although the function of the *A. nidulans*  $\alpha$ -mannosidase is not established, it is proposed that *A. nidulans*  $\alpha$ -mannosidase IIC is also involved in oligosaccharide catabolism (Eades et al. 1998). Both yeast Ams1p and *A. nidulans*  $\alpha$ -mannosidase IIC are not essential for normal cellular function, as disruption of these genes did not exhibit any visible effect on growth or morphology (Yoshihisa and Anraku 1989, 1990; Eades et al. 1998).

*Aspergillus fumigatus* is known to cause fatal invasive aspergillosis (IA) among immuno-compromised patients (Latgé 1999). The crude mortality from IA is above 90% and falls to around 50–70% if treatment is given (Steinbach et al. 2003). The main reason for patient death is the low efficiency of the drug therapies available to treat IA and the lack of an assay that can detect the fungus early during the infection. The fungal cell wall is essential for the fungal life and therefore is a unique specific target for antifungal drug development. As many glycoproteins are directly or indirectly involved in the synthesis and organization of fungal cell wall, it is of importance to assess the consequences of the catabolism of oligosaccharide in *A. fumigatus*. The development of new drugs targeting oligosaccharide processing relies on the identification and characterization of genes involved in this pathway. In this report, the *Afams1*, a gene encoding cytosolic Class IIC mannosidase, was knocked out and the phenotypes of the  $\Delta$ *Afams1* were investigated.

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## Results

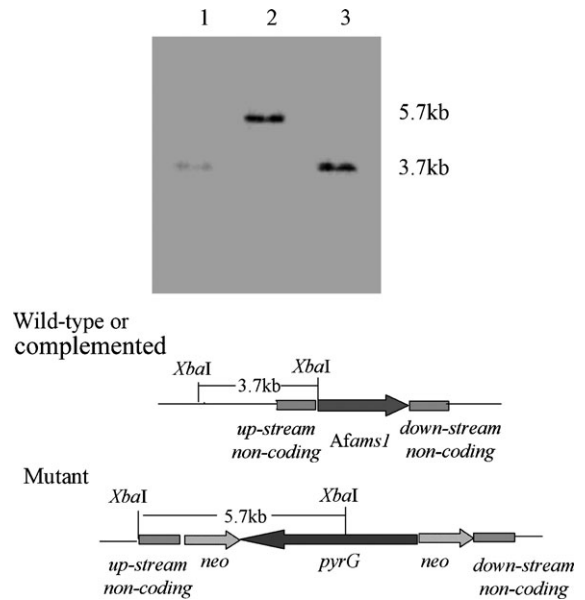
### Construction of the $\Delta Afams1$ mutant

A tBLASTn search (Altschul et al. 1997) of the *A. fumigatus* genome database was performed with the conserved amino acid sequence of *A. nidulans*  $\alpha$ -mannosidase IIC that are homologous between rat Man2C1p and *S. cerevisiae* Ams1p, and one gene, *Afams1*, was identified. The *Afams1* gene (AFUA\_3G08200) consists of a total of 3429 nt, and contains three introns. Its cDNA (AY852252) encodes a polypeptide of 1089 aa with an estimated molecular mass of 124 kDa. The predicted sequence of AfAms1 exhibits 80% amino acid identity with *A. nidulans*  $\alpha$ -mannosidase IIC, 50% with *S. cerevisiae* Ams1p, and 40% with human Man2C1p, respectively. Like other members of Class IIC  $\alpha$ -mannosidases, the predicted AfAms1 contains no cleavable signal sequence or transmembrane domain.

To investigate effects of loss of function of the *Afams1* gene in *A. fumigatus*, we created the null mutant by replacing a single copy of *Afams1* with *pyrG*. As a result, two mutants were obtained. PCR analysis demonstrated that wild-type 3.4 kb fragment of the *Afams1* was converted into a 2.5 kb fragment containing an upstream noncoding region and *neo* in the mutants (data not shown). Southern analysis of the *XbaI*-digested genomic DNA of the mutants demonstrated that the 3.7 kb *XbaI* fragment in the wild type had been converted into a 5.7 kb *XbaI* fragment (Figure 1). These results clearly demonstrated that the *Afams1* gene was replaced by a *pyrG* gene in the  $\Delta Afams1$  mutants. To ensure that all phenotypes noted for the  $\Delta Afams1$  strains were the result of specific deletion of the *Afams1*, the complemented strain was constructed by reintroduction of a wild-type copy of *Afams1* directly into the mutated locus, under the control of its own promoter. The transformation of the complemented strain was also confirmed by PCR and Southern blot (Figure 1).

### Phenotypes of the $\Delta Afams1$ mutant

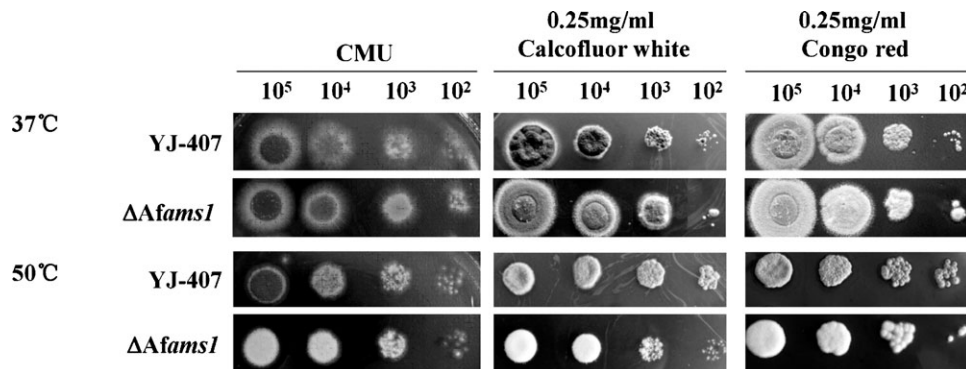
When the growth kinetics were determined, as described under *Material and methods*, the mutant mycelia grown in the complete liquid medium did not show any significant difference in the growth rate as compared with that of the wild type (data not shown). Considering that *A. fumigatus* can comfortably grow at a temperature of 30–55°C, we also tested the growth rate of the mutant at both 37°C and 50°C in the solid complete medium. As shown in Figure 2, the growth rate of the  $\Delta Afams1$  mutant



**Fig. 1.** Confirmation of the  $\Delta Afams1$  mutant and complemented strain by Southern blotting. Genomic DNA digested with *XbaI* was probed with a 2.0 kb upstream noncoding region of *Afams1*. The electrophoretic positions and sizes of DNA are indicated in the panel. Lanes: 1, wild-type; 2,  $\Delta Afams1$  mutant; 3, complemented strain. *neo*, neomycin phosphotransferase gene from transposon Tn5.

was similar to that of the wild-type strain. When the mutant was grown in the presence of Calcofluor white or Congo red, no significant difference was observed as compared with the wild-type at both 37°C and 50°C. These observations demonstrated that the cell wall integrity (CWI) was not affected in the mutant. However, the mutant grown at 50°C exhibited a white smooth colony (Figure 2), suggesting a severe defect in conidia formation. Conidia counting revealed that the mean  $\pm$  SD ( $\times 10^7$ ) conidia count of the  $\Delta Afams1$  mutant at 37°C was  $171 \pm 22$ , which was 45% of that of the wild-type strain ( $380 \pm 52$ ). The number of conidia produced by the  $\Delta Afams1$  mutant at 50°C was  $0.8 \pm 0.1$ , which was only 4.2% of that produced by the wild type ( $19 \pm 3$ ). These results confirmed a severe defect in conidiation of the mutant, especially at a higher temperature.

As compared with the wild type, the content of  $\alpha$ -glucan,  $\beta$ -glucan, and chitin in the mycelial cell wall of the  $\Delta Afams1$



**Fig. 2.** Hyphal growth and sensitivity to antifungal reagents of the mutant at 37°C and 50°C. A series of 10-fold dilutions ( $10^5$ – $10^2$  cells) of the wild-type (YJ-407) and  $\Delta Afams1$  strain were spotted on CMU agar, with and without the antifungal reagent, and cultivated at 37°C or 50°C for 24–48 h.

**Table I.** Cell wall components of the mutant

Temperature	Strain	Alkali-soluble					
		Mannoprotein				Alkali-insoluble	
		Protein ( $\mu\text{g}$ )	Gal ( $\mu\text{g}$ )	Man ( $\mu\text{g}$ )	$\alpha$ -glucan ( $\mu\text{g}$ )	Chitin ( $\mu\text{g}$ )	$\beta$ -glucan ( $\mu\text{g}$ )
37°C	YJ-407	43 $\pm$ 3	0.9 $\pm$ 0.1	4.1 $\pm$ 0.5	552 $\pm$ 45	143 $\pm$ 15	1399 $\pm$ 135
	$\Delta Afams1$	22 $\pm$ 2	1.3 $\pm$ 0.1	6.0 $\pm$ 0.5	770 $\pm$ 62	172 $\pm$ 16	1970 $\pm$ 132
50°C	YJ-407	77 $\pm$ 7	1.3 $\pm$ 0.2	2.6 $\pm$ 0.3	715 $\pm$ 31	155 $\pm$ 12	1617 $\pm$ 65
	$\Delta Afams1$	112 $\pm$ 14	3.2 $\pm$ 0.2	12.4 $\pm$ 1.1	688 $\pm$ 48	177 $\pm$ 19	2008 $\pm$ 73

Conidia were inoculated into a 100 mL complete liquid medium at a concentration of  $10^6$  conidia  $\text{mL}^{-1}$  and incubated at 37°C or 50°C with shaking (200 rpm.) for 24 h. The mycelium was then harvested and lyophilized, and three aliquots of 10 mg dry mycelium were used as independent samples for the analyses of unbound cell wall proteins and water-soluble sugars, as described under *Material and Methods*. The experiment was repeated twice. The values shown are  $\mu\text{g}$  cell wall component per 10 mg dry mycelium ( $\pm\text{SD}$ ).

mutant grown at 37°C was increased by 40%, 41%, and 20%, respectively. When the mutant strain was grown at 50°C, the  $\Delta Afams1$  showed a 4% reduction in  $\alpha$ -glucan, a 14% increase in chitin, and a 24% increase in  $\beta$ -glucan as compared with the wild type grown at the same temperature (Table I). Although protein in the mycelial cell wall of the  $\Delta Afams1$  mutant grown at 37°C was reduced by 41%, galactose and mannose released from these proteins were increased by 40%. When culture temperature was elevated to 50°C, the protein content was increased by 45%, whereas galactose and mannose released from these proteins were increased more as compared with the wild type. These results might suggest an important role of the *Afams1* in mannoprotein synthesis. In addition, it appears that the increase in  $\alpha$ -glucan,  $\beta$ -glucan, and chitin in cell wall components can compensate the loss of mannoprotein in the mutant at 37°C, while the increase in mannoprotein can strengthen the mutant cell wall at higher temperatures.

Taking these results together, we concluded that the *Afams1* gene was not essential for the growth of *A. fumigatus*; however, the  $\Delta Afams1$  mutant exhibited a defect in conidia formation and a change in cell wall polysaccharides.

#### Morphogenesis of the $\Delta Afams1$ mutant

During asexual reproduction, a filamentous fungus initiates its life cycle from conidial germination and terminates it with conidiation. When the conidia break dormancy, nuclear division is accompanied by a series of ordered morphological events, including the switch from isotropic to polar growth, the emergence of second germ tubes from the conidia, and septation. As shown in Figure 3, when incubated at 37°C in rich medium containing glucose as the carbon source, the wild-type conidium germinated in a typical bi-polar pattern at an angle of 180°, and the second germ tube and the first septation occurred after four rounds mitosis (7–8 h). The septum formed at the neck site of the first germling. In comparison with the wild type, the earliest emergence of the second germ tube occurred in the  $\Delta Afams1$  mutant after the second mitotic division (6 h), and the third germ tube, or branching of the germling, was found after the third or fourth nuclear division (7–8 h). After four rounds of mitotic division, some of germinated conidia of the  $\Delta Afams1$  mutant were not able to form a septum, while for those conidia that could form a septum, it was usually formed at the neck site of the newly emerged germ tube or germling, instead of the neck site of the first germling as in the wild-type strain. Moreover, the

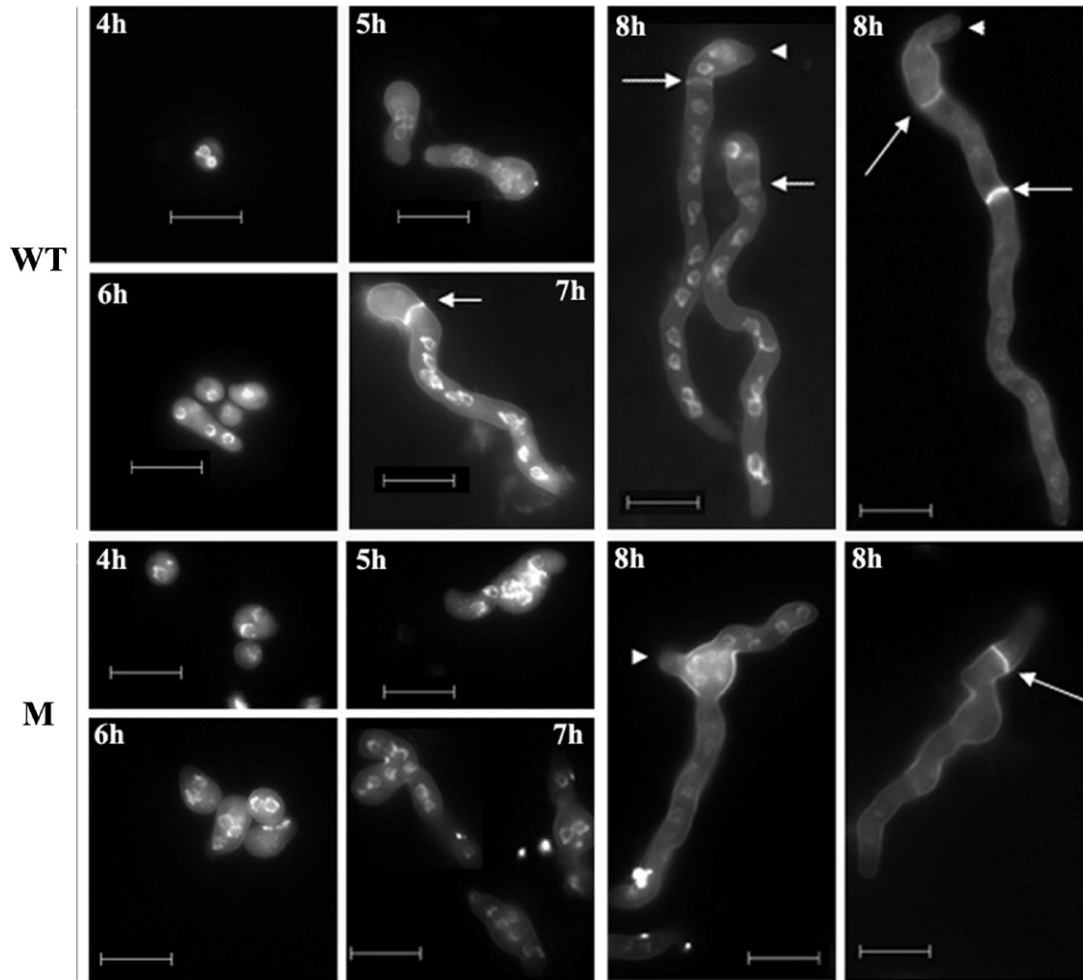
**Table II.** Statistics of germination of the mutants

Time	Wild-type				$\Delta Afams1$			
	Number of germ tube				Number of germ tube			
	0	1	2	3 or more	0	1	2	3 or more
5 h	33 $\pm$ 2	67 $\pm$ 4	0	0	44 $\pm$ 1	48 $\pm$ 4	8 $\pm$ 2	0
6 h	10 $\pm$ 1	90 $\pm$ 2	0	0	21 $\pm$ 2	42 $\pm$ 2	36 $\pm$ 3	1 $\pm$ 1
7 h	2 $\pm$ 1	87 $\pm$ 3	7 $\pm$ 1	4 $\pm$ 0	3 $\pm$ 1	31 $\pm$ 2	52 $\pm$ 3	13 $\pm$ 2
8 h	1 $\pm$ 0	77 $\pm$ 5	20 $\pm$ 2	2	1 $\pm$ 1	32 $\pm$ 3	55 $\pm$ 5	12 $\pm$ 2

$10^7$  freshly harvested conidia were poured into a Petri dish containing a glass coverslip and incubated in 10 mL of complete liquid medium at 37°C. The coverslips with adhering germlings were removed and counted under microscope. For each independent experiment, 100 conidia were counted and three independent experiments were carried out. All data are statistically significant ( $P < 0.05$ ).

germling of the mutant was more swollen than that of the wild type (Figure 3). As summarized in Table II, after three rounds of mitotic division (6 h), 36% of the conidia of the  $\Delta Afams1$  mutant formed a second germ tube, while about 13% were found with a third germ tube after four rounds of mitosis (7 h). These results demonstrated that deletion of the *Afams1* gene led to random budding and septation at an early stage of germination.

Although the growth rate of the mutant was similar to that of the wild type, the hyphae of the mutant was found to be swollen (the mean  $\pm$  SD hyphae width ( $\mu\text{m}$ ) of the wild-type and  $\Delta Afams1$  mutant was  $3.1 \pm 0.3$  and  $4.1 \pm 0.4$ , respectively) ( $P < 0.05$ ) and contained more nuclei in each basal cell (the mean  $\pm$  SD nuclei counting of the wild-type and  $\Delta Afams1$  mutant was  $5 \pm 1$  and  $8 \pm 3$  in each basal cell, respectively) ( $P < 0.05$ ), and no septum was formed in hyphal tip after four rounds of mitotic division (Figure 4), suggesting a reduced polar growth and a lagged septation during hyphal elongation. Since conidiation is the preparation for the beginning of the next life cycle and is thus also an important event of development, we also examined the morphogenesis of the mutant at an early stage of conidiation. As shown in Figure 4, the mutant was able to form conidiophore vesicles; however, these vesicles contained more nuclei (the mean  $\pm$  SD nuclei counting of the wild-type and  $\Delta Afams1$  mutant was  $23 \pm 3$  and  $32 \pm 4$  in each conidiophore vesicle, respectively) ( $P < 0.05$ ) and formed less metulae than those of the wild type (the mean  $\pm$  SD metulae counting of the



**Fig. 3.** Germination and septation of the  $\Delta Afams1$  mutant. Freshly harvested conidia ( $10^7$ ) were poured into a Petri dish containing a glass coverslip and incubated in a 10 mL complete liquid medium at  $37^\circ\text{C}$ . The coverslips with adherent germings were removed, fixed in fixative solution, and stained with Calcofluor white and DAPI, as described in *Material and methods*. Typical photographs are shown. The second germ tube and the septum are marked with an arrowhead and an arrow, respectively. WT, wild-type; M,  $\Delta Afams1$  mutant. Bar: 10  $\mu\text{m}$ .

wild-type and  $\Delta Afams1$  mutant was  $26 \pm 3$  and  $20 \pm 3$  on each vesicle, respectively) ( $P < 0.05$ ), which is consistent with the reduced conidiation associated with the mutant and suggests aberrant polarity and septation at early stage of conidiation.

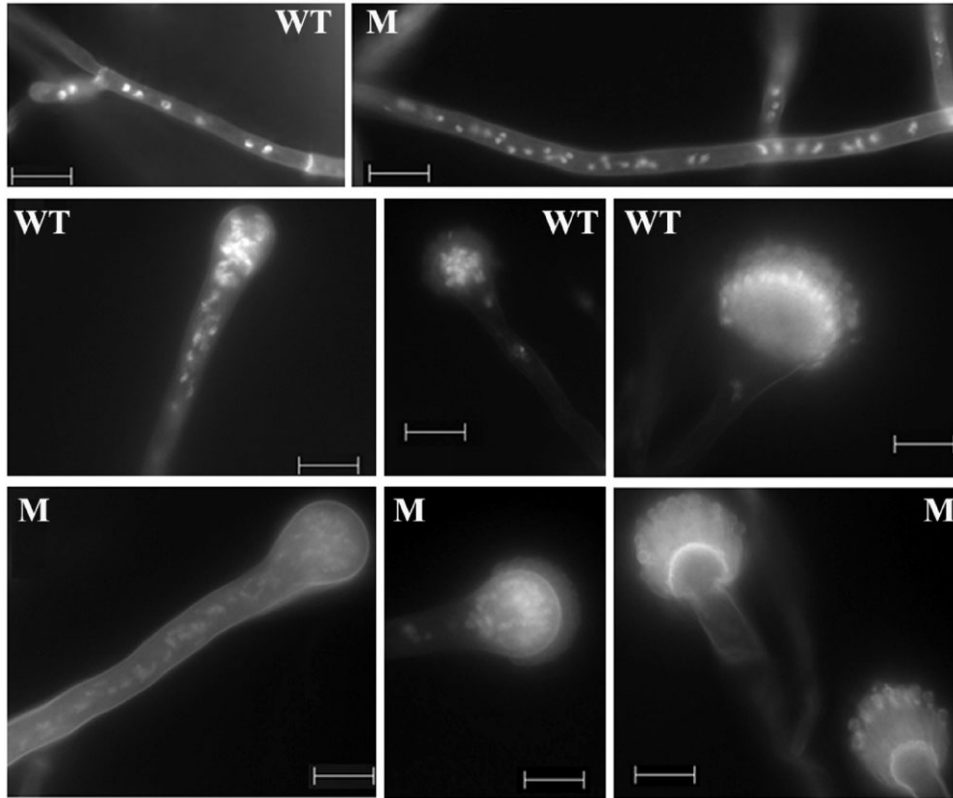
#### *Actin staining of the $\Delta Afams1$ mutant*

Since actin cytoskeleton is both necessary and sufficient for cell polarization and polarized hyphal growth in filamentous fungi (Heath et al. 2000), we also monitored the actin cytoskeleton of the mutant by staining the actin with an anti-actin antibody. As shown in Figure 5, during early stage of germination (7 h), the actin patches accumulated in the isotropic swollen conidium and the expanding tip of germ tube in a polarized manner in the wild type. The actin ring also formed at the neck site of the first germ tube, where the first septation occurred, while in the case of the mutant the actin patches were mainly visualized in the isotropic swollen conidium and no obvious accumulation was found in the first and second germ tubes, especially at their growing tips. These observations strongly suggest a defective polarized localization of the actin cytoskeleton in the mutant.

#### **Discussion**

In mammalian cells free oligosaccharides (fOS) are generated by oligosaccharyltransferase (OST)-mediated hydrolysis of  $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2\text{-PP-dolichyl}$  in the lumen of the ER or peptide *N*-glycanase (PNGase)-mediated de-*N*-glycosylation of newly synthesized glycoproteins either in the ER or the cytosol. fOS that are liberated in the lumen of the ER can be transported into the cytosol, where they are trimmed by an endo- $\beta$ -D-*N*-acetylglucosaminidase H (endo H)-like enzyme and the  $\alpha$ -mannosidase Man2C1p in order to yield an oligosaccharide,  $\text{Man}_5\text{GlcNAc}$ , that can be imported directly into lysosomes to be degraded (Bischoff et al. 1990; Weng and Spiro 1993, 1996; Grard et al. 1996; Duvet et al. 1998; Suzuki et al. 2006; Chantret and Moore 2008).

In yeast, the cytosolic  $\alpha$ -mannosidase Ams1p, a homolog of the Man2C1p, is also involved in the processing of fOS. Since the yeast Png1p is mainly localized to the cytosol, it is proposed that the Png1p generated fOS may both be generated and processed in the cytosol (Chantret et al. 2003). Unlike its mammalian counterpart, the yeast Ams1p only participates in recycling or utilizing of oligosaccharide but not in processing

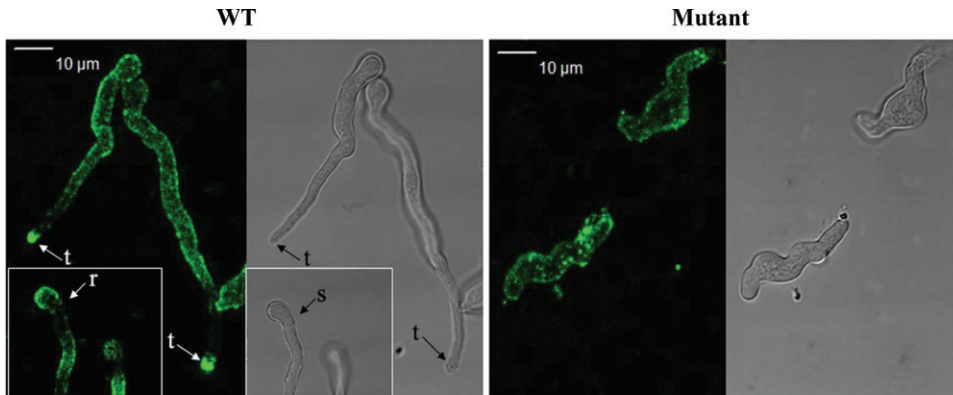


**Fig. 4.** Hyphal growth and conidia formation of the mutant. A 100 mL volume of complete liquid medium was inoculated with  $10^6$  conidia and incubated with shaking (200 rpm.) at 37°C for 17 h. The mycelium was removed and placed on a glass coverslip, which was then put in a Petri dish containing two layers filter paper saturated with a complete liquid medium. After incubation at 37°C for 2–8 h, the coverslip was removed and stained, as described under *Material and methods*. The arrow indicates the septum. Representative micrographs of the mycelium and conidiophore vesicle are shown. WT, wild-type; M,  $\Delta$ Afams1 mutant. Bar: 5  $\mu$ m.

of *N*-glycan (Chantret et al. 2003). Similarly, *A. nidulans*  $\alpha$ -mannosidase IIC is also proposed to be involved in oligosaccharides catabolism (Eades et al. 1998). Both *A. nidulans*  $\alpha$ -mannosidase IIC and *S. cerevisiae* Ams1p are not essential for normal cellular function since disruption of these genes did not

have any visible effect on growth or morphology (Yoshihisa and Anraku 1989, 1990; Eades et al. 1998).

In this study, we have deleted the gene encoding a cytosolic/vacuolar form of  $\alpha$ -mannosidase in *A. fumigatus*. The molecular weights of proteins, such as MsdS, AfChiB1, and Alg1, secreted



**Fig. 5.** Actin-staining of the mutant at early stage of germination. A 10 mL volume of complete liquid medium was inoculated with  $10^7$  freshly harvested conidia, poured into a Petri dish containing glass coverslips, and incubated at 37°C for 7–24 h. At the specified times, coverslips with adhering germings were removed and fixed as described under *Material and methods*. The coverslips were incubated for 1 h at room temperature with mouse anti-actin C4 monoclonal (MP Biomedicals) at a 1:400 dilution in PBS-BSA containing 0.5% Nonidet P-40 and then stained for 1 h in the dark with an Alexa Fluor® 488 goat anti-mouse IgG antibody (invitrogen) at a 1:400 dilution in PBS-BSA. Finally, the coverslips were examined under a LeICA TCS SP2 microscope. WT, wild-type; t, hyphal tip; s, septation site; r, actin ring.

by the  $\Delta Afams1$  mutant were identified to be the same as their counterparts secreted by the wild-type or the complemented strain (data not shown). Western blotting revealed that only one AfChiB1 band was detected in the intracellular and secreted preparations of the  $\Delta Afams1$  mutant, and this band exhibited the same molecular size as the one from the wild-type or the complemented strain (data not shown). These results indicate that deletion of the *Afams1* did not affect protein secretion in *A. fumigatus*.

In contrast to its counterpart in yeast or *A. nidulans*, the AfAms1 was required for normal cellular function in *A. fumigatus*. Although the  $\Delta Afams1$  mutant did not show any change in the growth rate and CWI, a reduction in mannoprotein was observed in the mutant at 37°C, which appeared to be compensated by an increase in  $\alpha$ -glucan,  $\beta$ -glucan, and chitin content, while an increase in mannoprotein can strengthen the mutant cell wall at 50°C. In addition to the changes in cell wall components, the mutant displayed a deficiency in polarized growth during morphogenesis. Furthermore, the severe defect in conidial formation associated with the mutant could also be explained by the defective polarity since the mutant appeared partially lost its ability to form metulae, a polarized cell emerged on conidiophore vesicle. The involvement of the *Afams1* gene in polarized growth demonstrates that the processes involved in fOS regulation are important for *A. fumigatus*. Therefore, the  $\Delta Afams1$  mutant could serve as a simple model to investigate the mechanism of  $\alpha$ -mannosidosis.

In yeast, the defect of cell wall requires the cells to induce the CWI pathway to survive and the compensatory mechanism featured with increased chitin content is triggered (Carotti et al. 2002). Upon environmental stimuli or cell wall stress, the CWI pathway is activated, which then regulates a variety of outputs involved in cell wall biogenesis, actin organization, and polarized secretion (Levin 2005). In this study, we have shown that the mutant was not able to reorganize its actin skeleton. Although the investigation of the CWI pathway has not been initiated in *A. fumigatus*, analysis of Rho protein modules in *Ashbya gossypii* suggests a similar CWI pathway in filamentous fungi (Wendland and Philipsen 2001). Thus, it is likely that the AfAms1 is involved in cell wall synthesis and hence polarity through a similar pathway. Of course, our future challenge is to identify the substrate proteins of the *Afams1* gene, which are located upstream of the CWI pathway or responsible for synthesis of cell wall components.

## Material and methods

### *Strains and growth conditions*

*Aspergillus fumigatus* strain YJ-407 (China General Microbiological Culture Collection Center, CGMCC0386) was maintained on potato glucose (2%) agar slant (Xia et al. 2001). *A. fumigatus* strain CEA17 (Weidner et al. 1998), a kind gift from C. d'Enfert, Institute of Pasteur, France, was propagated at 37°C on YGA (0.5% yeast extract, 2% glucose, 1.5% Bacto-agar), complete medium (Cove 1966), or minimal medium with 0.5 mM sodium glutamate as a nitrogen source (Cove 1966). Uridine and uracil were added to the medium at a concentration of 5 mM when CEA-17 or complemented strain was grown. Mycelium was harvested from strains grown in the complete liquid medium at 37°C with shaking at 250 rpm. At the spec-

ified culture time point, mycelium was harvested and washed with distilled water, and then frozen in liquid N<sub>2</sub> and ground by hand. The powder was stored at -70°C for DNA, RNA, and protein extraction. Conidia were prepared by growing *A. fumigatus* strains on solid complete medium with uridine and uracil (CMU) for 48 h at 37°C. The spores were collected, washed twice with 0.01% Tween-20 in physiological saline, and resuspended in 0.01% Tween-20 in saline, and its concentration was confirmed by hemocytometer counting and viable counting. Vectors and plasmids were propagated in *Escherichia coli* DH5 $\alpha$  (BRL).

### *Molecular cloning of A. fumigatus Afams1*

The *Afams1* genomic sequence was identified in the search of the *A. fumigatus* genome database (<http://www.tigr.org/tdb/e2k1/afu1/>), using a tBLASTn program to search for sequences corresponding to the conserved amino acid sequence of *A. nidulans*  $\alpha$ -mannosidase IIC that were homologous between rat Man2C1p and *S. cerevisiae* Ams1p. A 3.4 kb genomic DNA fragment was found to contain the entire open reading frame. Based on the nucleotide sequence, the forward primer (5'-ATGTGGAAGTGGCTGCAG-3') and the reverse primer (5'-CTACCGAGGCTTCCGCGT-3') were designed for cloning the cDNA of the *Afams1* by PCR. The PCR products were subcloned into a pGEM-T easy Vector (Promega, Madison, WI) and the resulting plasmid T-Afams1 was sequenced. The position of the intron was determined by comparing the cDNA with genomic sequence.

### *Construction of the Afams1 null mutant and complemented strains*

To delete *Afams1*, a deletion construct was designed to replace the entire coding region of *Afams1* with a *pyrG* cassette by homologous recombination (d'Enfert 1996). PCR primers were designed to amplify a 1.8 kb upstream noncoding region of the *Afams1* before the ATG start codon (5' primer pair 5'-GGGTTACCGC GGCCGCAGACCATCGCGATCCTTAG AG-3' and 5'-GGAATTCCGTTAACTTGTGCCATGACAGCATTGC C-3'; the *KpnI*, *NotI*, *EcoRI*, and *HpaI* restriction sites are underlined, respectively) and a 1.8 kb downstream noncoding region of the *Afams1* after the stop codon (3' primer pair 5'-GGAATTC CGTTAACTTGTCTCATGGTGTCCATAATG-3' and 5'-GCTCTAGAACCCTCGTAACCTGCT GTCAAG-3'; the *EcoRI*, *HpaI*, and *XbaI* restriction sites are underlined, respectively). These PCR fragments were cloned into the relevant sites of pBlueScript II SK. The *pyrG* blaster cassette (8.6 kb) released by the digestion of pCDA14 (d'Enfert 1996) with *HpaI* was cloned into the site between the up- and downstream noncoding regions of the *Afams1*, to yield the deletion construct pCDA16-59. At a unique *NotI* site, the linearized pCDA16-59 was transformed into strain CEA17 by protoplast transformation (Yelton et al. 1984) and screened for mutants with uridine and uracil autotrophy. The deletions in the mutants were confirmed by PCR and Southern blotting. For PCR confirmation, a forward primer 5'-ATGTGGAAGTGGCTGCAG-3' and a reverse primer 5'-CTACCGAG GCTTCCGCGT-3' were used to amplify the 3.4 kb coding region of *Afams1*; a pair of primers (u59: 5'-CTTGTTTCGTATATCTCCCCG-3' and p1: 5'-CTTCGGACAATTGACTGG CA-3') was used to generate a 2.5 kb DNA fragment containing an upstream noncoding region of *Afams1* and partial *neo* (a Tn5 neomycin phosphotransferase

gene) in the  $\Delta Afams1$  mutant (Prentki and Krisch 1984; d'Enfert 1996).

The complemented strain was constructed by the replacement of *pyrG* with a wild-type copy of *Afams1* in the  $\Delta Afams1$  mutant. *Afams1*, with its 1.8 kb upstream and 1.8 kb downstream noncoding regions, was amplified by PCR (primer pair 5'-AGACCATCG CGATCCTTAGAG-3' and 5'-ACCCCTCGTAACCTGCTGTCAAG-3'), and the product was cloned into a pGEM-T easy vector and sequenced. The resulting plasmid T-long59 was then linearized with *PstI/HindIII*, before transformation into the  $\Delta Afams1$  null mutant. The transformants were chosen by PCR, and then the transformation was confirmed by Southern blot analysis, using the upstream noncoding region as a probe. The probe was labeled by following the protocol of the DIG-labeled hybridization kit (catalogue no. 1093657; Roche Applied Science, Indianapolis, IN).

#### Western blotting of *AfChiB1*

Chitinase *AfChiB1*, secreted by *A. fumigatus*, was induced and purified as previously described (Xia et al. 2001). An anti-*AfChiB1* antibody developed in mouse was used in Western blotting. Proteins in cell lysate or culture supernatant were run on a 12% SDS-PAGE gel and transferred to PVDF (Bio-Rad) at 300 mA for 1.5 h. The anti-*AfChiB1* mouse serum was diluted at 1:5000. Protein was detected with the enhanced chemoluminescence substrate (Pierce, Rockford, IL) and autoradiography on film.

#### Phenotypic analysis of the mutant

Growth kinetics of *A. fumigatus* strains was assayed as follows. A 100 mL slurry of spores ( $1 \times 10^9 \text{ mL}^{-1}$ ) was inoculated into a 100 mL liquid CMU medium. After incubation at 37°C with shaking (200 rpm), three 1 mL aliquots of liquid were taken for each strain at set time intervals, and dried and weighed. The mean weight was used to plot the growth kinetics. The experiment was repeated three times.

To test the sensitivities of the mutant to anti-fungal reagents, conidiospores were collected from the wild-type, the mutant, and the complemented strain, and, for each strain, similar numbers of conidiospores were spotted on CMU plates in the presence of 100  $\mu\text{g}$  Calcofluor white  $\text{mL}^{-1}$ , 250  $\mu\text{g}$  Congo red  $\text{mL}^{-1}$  or 60  $\mu\text{g}$  SDS  $\text{mL}^{-1}$ . After incubation at 37°C or 50°C for 24–48 h, the plates were taken out and photographed.

For examination of conidial germination, a 20 mL complete liquid medium was inoculated with  $10^7$  freshly harvested conidia, poured into a Petri dish containing a glass coverslip, and incubated at 37°C for the time indicated in each experiment. At the specified times, the coverslips with adhering germlings were removed, and spore germination was observed and counted under differential interference contrast microscopy.

For examination of nuclei, septa, and cell wall at the germination stage, the coverslips with adherent germlings were removed and fixed in a fixative solution (4% formaldehyde, 50 mM phosphate buffer, pH 7.0, and 0.2% Triton X-100) for 30 min. Coverslips were then washed with PBS, incubated for 15 min with 1 mg 4',6-diamidino-2-phenylindole (DAPI)  $\text{mL}^{-1}$  (Sigma), washed with PBS, and then incubated for 5 min with a 10 mg  $\text{mL}^{-1}$  solution of fluorescent brightener 28 (Sigma), washed again, and germlings were photographed using a microscope.

For examination of nuclei, septa, and cell wall at the conidiation stage, a 100 mL complete liquid medium was inoculated with  $10^6$  conidia and incubated with shaking (200 rpm) at 37°C for 17 h. The mycelia were taken and placed on a glass coverslip. The glass coverslip was then put in a Petri dish containing two layers filter paper saturated with the complete liquid medium. After incubation at 37°C for 2–8 h, the coverslip was removed and stained with DAPI and fluorescent brightener. The hyphae width, nuclei number in each conidiospore vesicle, and metulae number on vesicle were calculated from 20 individual hyphae or vesicle from each strain. The *P* value < 0.05 was considered statistically significant.

A 100 mL volume of complete liquid medium was inoculated with  $10^8$  freshly harvested conidia and incubated at 37°C or 50°C with shaking (200 rpm) for 12 h. Mycelia were harvested by filtering the culture through two layers of Miracloth (Calbiochem), washed twice with distilled water, and streaked on minimal medium agar. After incubation at 37°C or 50°C for 2, 4, 6, and 8 h, mycelia were suspended in 5 mL distilled water, and conidial production was expressed as the mean number of conidia per volume (milliliter).

Conidia or mycelia produced on the solid complete medium were fixed with 2.5% glutaraldehyde and 1% osmium tetroxide, and then examined under a Quanta 200 scanning electron microscope (FEI, Hillsboro, OR). Conidia were fixed in 2.5% glutaraldehyde in a 0.1 M phosphate buffer, pH 7.0, for 4 h, or overnight at 4°C. Hyphal cells were fixed in 2.5% glutaraldehyde in 0.1 M phosphate, washed three times with 0.1 M phosphate, postfixed in 1% osmium tetroxide, incubated for 2–4 h in 0.1 M phosphate, and then treated for 15–20 min in each of 30, 50, 70, 85, 95, and 100% methanol and postfixed in 2% uranyl acetate/30% methanol. Cells were rinsed, dehydrated, and embedded in Epon 812 by the floating sheet method. The sections were examined under an H-600 electron microscope (Hitachi, Tokyo, Japan).

#### Chemical analysis of cell wall

Conidia were inoculated into a 100 mL complete liquid medium at a concentration of  $10^6$  conidia  $\text{mL}^{-1}$  and incubated at 37°C with shaking (200 rpm) for 24 h. The mycelium was harvested by filtering the culture through two layers of Miracloth, washed with deionized water, and frozen at  $-80^\circ\text{C}$ . To isolate cell walls, 10 mg of dry mycelial pad was added to a tube containing 50 mM  $\text{NH}_4\text{HCO}_3$  at pH 8.0 and 0.2 g of glass beads (diameter 1 mm). The mycelium was disrupted by successively shaking the tube with a Disruptor Genie (5 min every time, five times) (Scientific Industries, Bohemia, NY). The cell homogenates were then centrifugated and washed several times. Three independent samples of lyophilized mycelial pad were used as for cell wall analysis, and the experiment was repeated twice.

The cell walls were boiled for 5 min in 1 mL 2% SDS in the 50 mM Tris/HCl buffer supplemented with 100 mM Na-EDTA, 40 mM  $\beta$ -mercaptoethanol, and 1 mM PMSF to remove noncovalently bound proteins and membrane fragments. Cell walls were collected by centrifugation, extracted for a second time, and washed three times with deionized water. After washing, cell walls were treated in 1 M KOH and incubated at 70°C for 30 min under  $\text{N}_2$  to release glycoprotein and  $\alpha$ -glucan. The alkaline-soluble materials were acidified with acetic acid to pH 5.0, and the precipitated  $\alpha$ -glucans were collected by

centrifugation and washed with water. The glycoprotein in supernatant was precipitated with two volumes of ethanol, washed twice with 64% ethanol, and dissolved in distilled water. The glycoprotein was determined using the Bradford assay (Bradford 1976). The monosaccharides were liberated from glycoprotein by acid hydrolysis (6 N HCl at 100°C for 2 h) and separated on a CarboPac PA1 anion-exchange column (Dionex, Sunnyvale, CA), equipped with an Amino Trap guard column (Dionex). Elution was performed at room temperature at a flow rate of 1 mL min<sup>-1</sup> with 18 mM NaOH. Detection of sugars was performed with a pulsed amperometric detector PAD equipped with a gold electrode according to the manufacturer's manual (Dionex).

The alkali-insoluble materials were washed with water for several times and digested in 6 N HCl at 100°C for 2 h to release monosaccharides from  $\beta$ -glucan and chitin. After digestion, HCl was evaporated and the residues were dissolved in 0.2 mL distilled water (Elorza et al. 1985; Hearn and Sietsma 1994; Schoffemeer et al. 1999).  $\alpha$ -Glucan and  $\beta$ -glucan were estimated by measuring the released glucose using the phenol-sulfuric acid method (Dubois et al. 1956). Chitin was determined by measuring the released *N*-acetylglucosamine after digestion using the method described by Lee et al. (2005).

#### Proteins secreted by the mutant

The *A. fumigatus* strains were incubated in the complete liquid medium at 37°C at 250 rpm for 60 h. The secreted proteins in the culture medium were precipitated from the culture filtrates by the addition of 4 volumes icecold acetone, and the culture filtrates were kept at 4°C overnight. The pellet was then recovered by centrifugation, resuspended in water, and separated by PAGE. After staining with Coomassie brilliant blue R-250, the protein bands of interest were cut, and analyzed by liquid chromatography/tandem MS (LC-MS/MS) (ThermoFinnigan, San Jose, CA).

#### Cytoskeletal staining

A 10 mL volume of complete liquid medium was inoculated with 10<sup>7</sup> freshly harvested conidia, poured into a Petri dish containing glass coverslips, and incubated at 37°C for the time indicated in each experiment. At the specified times, coverslips with adhering germlings were removed and fixed. Briefly, coverslips were transferred to 3.7% formaldehyde in PBS and incubated for 30 min at room temperature, and then washed twice. For cell wall digestion, coverslips were overlaid for 40 min at room temperature with a solution of 10 mg Novozym 234 per mL PBS containing 2% BSA (Esnault et al. 1999). After three washes, coverslips were immersed in absolute ethanol at -20°C for 10 min and then washed several times in PBS. The coverslips were incubated for 1 h at room temperature with mouse anti-actin C4 monoclonal (MP Biomedicals) at a 1:400 dilution in PBS-BSA containing 0.5% Nonidet P-40. After four 10 min washes with PBS, coverslips were then stained for 1 h in the dark with an Alexa Fluor<sup>®</sup> 488 goat anti-mouse IgG antibody (Invitrogen) at a 1:400 dilution in PBS-BSA. Finally, the coverslips were washed for 10 min with PBS, mounted on glass slides in 25% glycerol, and examined under a Leica TCS SP2 microscope (Carl Zeiss, Oberkochen, Germany).

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#### Conflict of interest statement

None declared.

#### Abbreviation

Cvt, cytosol-to-vacuole targeting pathway; CWI, cell wall integrity; fOS, free oligosaccharides; IA, invasive aspergillosis.

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