Microbiology (2004), **150**, 3315–3326 DOI 10.1099/mic.0.27249-0

The cell wall stress response in *Aspergillus niger* involves increased expression of the glutamine : fructose-6-phosphate amidotransferase-encoding gene (*gfaA*) and increased deposition of chitin in the cell wall

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Perturbation of cell wall synthesis in *Saccharomyces cerevisiae*, either by mutations in cell wall synthesis-related genes or by adding compounds that interfere with normal cell wall assembly, triggers a compensatory response to ensure cell wall integrity. This response includes an increase in chitin levels in the cell wall. Here it is shown that *Aspergillus niger* also responds to cell wall stress by increasing chitin levels. The increased chitin level in the cell wall was accompanied by increased transcription of *gfaA*, encoding the glutamine : fructose-6-phosphate amidotransferase enzyme, which is responsible for the first and a rate-limiting step in chitin synthesis. Cloning and disruption of the *gfaA* gene in *A. niger* showed that it was an essential gene, but that addition of glucosamine to the growth medium could rescue the deletion strain. When the plant-pathogenic fungus *Fusarium oxysporum* and food spoilage fungus *Penicillium chrysogenum* were subjected to cell wall stress, the transcript level of their *gfa* gene increased as well. These observations suggest that cell wall stress in fungi may generally lead to activation of the chitin biosynthetic pathway.

Received 16 April 2004
Revised 27 May 2004
Accepted 3 June 2004

INTRODUCTION

Yeasts and fungi are surrounded by a cell wall that is responsible for the shape of the cell and offers protection against harmful environmental conditions. Fungal cell walls are mainly composed of mannoproteins, 1,3-β-glucan and chitin. Depending on the species, additional polymers such as 1,3-α-glucan or 1,6-β-glucan polymers may be present. Chitin, a β1,4-linked homopolymer of *N*-acetylglucosamine residues, is generally a minor component in the yeast cell wall, accounting for only 1–2 % of the cell wall dry mass (Klis, 1994; Klis *et al.*, 2002), whereas the cell walls of filamentous fungi contain higher levels of chitin, up to 10–30 % of the cell wall dry weight (de Nobel *et al.*, 2000). *Schizosaccharomyces pombe* is so far the only ascomycetous species known that seems to lack chitin in its cell wall during vegetative growth (Arellano *et al.*, 2000). In *Saccharomyces cerevisiae*, the majority of chitin is present as a ring in the bud scar, but a small amount of chitin is deposited in the lateral walls where it is linked to 1,3-β-glucan and 1,6-β-glucosylated mannoproteins (Kollar *et al.*, 1997). In both yeasts and filamentous fungi, chitin contributes significantly to the mechanical strength of the cell wall. When chitin synthesis is affected, growing hyphae tend to lyse and form pronounced bulges unless the osmolarity of the medium is increased (Benitez *et al.*, 1976; Gooday, 1990; Bago *et al.*, 1996; Specht *et al.*, 1996; Aufauvre-Brown *et al.*, 1997). Genes encoding chitin synthases have been isolated from many yeasts and filamentous fungi and have been classified into six classes according to their sequence similarity (reviewed by Roncero, 2002). Most yeasts and

Abbreviations: CFW, Calcofluor White; CM, complete medium; MM, minimal medium.

The GenBank/EMBL/DDBJ accession numbers for the sequences reported in this paper are AY594332 (AbGfaA), AY594333 (FoGfaA) and AY594334 (PcGfaA). Nucleotide sequence data reported are available in the Third Party Annotation Section (TPA) of the GenBank/EMBL/DDBJ database under accession numbers BK005223 (AnGfaA) and BK005224 (GzGfaA).

filamentous fungi contain multiple chitin synthase-encoding genes. In the yeast *S. cerevisiae* and the filamentous fungi *Aspergillus nidulans* and *Aspergillus fumigatus*, the expression and activity of different chitin synthases are highly regulated and are required during different stages of the yeast life cycle and during the different stages of fungal growth development (Orlean, 1997; Cabib *et al.*, 1997; Ichinomiya *et al.*, 2002; Mellado *et al.*, 2003 and references therein).

Chitin synthesis has also been shown to be essential in the compensatory response to cell wall stress in *S. cerevisiae*. Cell wall damage caused by mutations in cell wall-related genes results in hyperaccumulation of chitin (Ram *et al.*, 1994, 1998; Kapteyn *et al.*, 1997; Popolo *et al.*, 1997; Dallies *et al.*, 1998; Osmond *et al.*, 1999; Lagorce *et al.*, 2002). The hyperaccumulation of chitin in response to cell wall stress is not limited to *S. cerevisiae* and is also found in other yeasts such as *Kluyveromyces lactis* (Uccelletti *et al.*, 2000) and *Candida albicans* (Kapteyn *et al.*, 2000). Inability of the cells to respond to cell wall damage by increasing chitin levels, either by disrupting the major chitin synthase-encoding gene (ScChs3p) or by adding the chitin synthase inhibitor nikkomycin Z, results in cell lysis, indicating the importance of the chitin response to prevent cell death (Douglas *et al.*, 1994; el-Sherbeini & Clemas, 1995; Popolo *et al.*, 1997).

The sugar donor for the synthesis of chitin is UDP-*N*-acetylglucosamine. The metabolic pathway leading to the formation of UDP-*N*-acetylglucosamine from fructose 6-phosphate consists of five steps. The first and also rate-limiting step in this pathway is the formation of glucosamine 6-phosphate from glutamine and fructose 6-phosphate. This step is catalysed by the enzyme glutamine-fructose-6-phosphate amidotransferase (Gfa1p). The hexosamine biosynthetic pathway from fructose 6-phosphate to UDP-*N*-acetylglucosamine is conserved in lower and higher eukaryotes, as well as in bacteria.

The glucosamine : fructose-6-phosphate amidotransferase-encoding gene has been cloned from bacteria, yeasts and higher eukaryotes. Expression analysis of *GFA1* in *S. cerevisiae* has shown that its expression is strongly enhanced in response to cell wall stress-inducing conditions, both by using cell wall mutants (Lagorce *et al.*, 2002; Terashima *et al.*, 2000) and in response to the cell wall-perturbing compounds Calcofluor White (CFW) and Zymolyase (Boorsma *et al.*, 2004; Garcia *et al.*, 2004), which indicates that increased chitin synthesis requires activation of the hexosamine biosynthetic pathway by increased expression of the rate-limiting step in the pathway.

In this paper, we describe the cloning of the complete *gfaA*-encoding gene from *Aspergillus niger* and of *gfaA* fragments from *Penicillium chrysogenum* and *Fusarium oxysporum*. Using these genes as probes, we have shown that these filamentous fungi respond to various cell wall-perturbing conditions by increasing the expression level of *gfaA* mRNA. We also show that higher *gfaA* mRNA levels in *A. niger* are accompanied by increased chitin levels in the cell wall after CFW-induced cell wall stress, presenting further evidence that activation of the chitin biosynthetic pathway is a general response to cell wall stress.

METHODS

Strains, culture conditions and fungal transformation. *A. niger* N402 (a *cspA1* derivative of ATCC9029; Bos *et al.*, 1988) and strain AB4.1 (van Hartingsveldt *et al.*, 1987), a *pyrG* mutant derived from *A. niger* N402, were used in this study. Strains were cultivated in minimal medium (MM) (Bennett & Lasure, 1991) containing 1 % (w/v) glucose as carbon source and 0·1 % (w/v) Casamino acids or in complete medium (CM) containing, in addition to the Casamino acids, 0·5 % (w/v) yeast extract. Uridine (10 mM) was added when required. *Penicillium chrysogenum* (ATCC 48271; Kolar *et al.*, 1988) was obtained from Dr P. Punt, TNO Nutrition, Zeist, The Netherlands, and grown in *A. niger* CM. *Fusarium oxysporum* f. sp. *radicis lycopersici* (CBS 101587; Lagopodi *et al.*, 2002) was obtained from Dr G. Bloemberg, Leiden University, The Netherlands, and grown in Czapek–Dox medium. Conidia from *A. niger* and *P. chrysogenum* were obtained by harvesting conidia from a CM plate after 4–6 days of growth at 30 °C, using 0·9 % (w/v) NaCl. Conidia from *F. oxysporum* were obtained by filtering conidia from a 3- to 4-day-old 100 ml liquid culture. The 100 ml culture was started by inoculating 1×10^5 conidia ml^{-1} and grown at 30 °C on an orbital shaker at 300 r.p.m. *A. niger* transformations were carried out as described previously (Punt & van den Hondel, 1992). For protoplast formation, lysing enzymes from *Trichoderma harzianum* (Sigma, L-1412) were used, with a final concentration of 40 mg (g fresh weight mycelium)$^{-1}$.

Cell wall stress-inducing conditions. Freshly isolated conidia from *A. niger* were inoculated into 100 ml CM at a spore density of 1×10^7 conidia ml^{-1} and grown for 5 h at 37 °C. After 5 h, CFW was added (200 μg ml^{-1}) from a freshly prepared stock solution (20 mg ml^{-1}). SDS was added from a 100 mg ml^{-1} stock solution to a final concentration of 50 μg ml^{-1}. Caspofungin (Merck) was added from a 10 mg ml^{-1} stock solution to a final concentration of 62·5 μg ml^{-1}. Freshly isolated *P. chrysogenum* conidia were used to inoculate 50 ml CM at a spore density of 1×10^7 conidia ml^{-1} and grown for 6·5 h at 30 °C before adding CFW. *F. oxysporum* conidia were inoculated at a spore density of 1×10^6 spores ml^{-1} and grown for 7 h at 30 °C before the addition of CFW. At specific time points after the addition of the antifungal compound, germlings were isolated using a gauze with a 20 μm aperture (Endecotts), frozen with liquid nitrogen and stored at −80 °C prior to isolation of cell walls or RNA. For microscopical studies, freshly harvested conidia were grown on coverslips in MM with Casamino acids at 37 °C with or without 10 mg glucosamine ml^{-1}. At specific time points, a coverslip with adherent conidia or germlings was fixed in 3·7 % (v/v) formaldehyde for 20–30 min. The coverslip was removed from the fixation buffer and placed in 7 μl mounting solution [50 % (v/v) glycerol in PBS] on a microscope slide. After sealing the coverslip with nail polish, the morphology of the germlings was analysed by viewing at least 400 conidia or germlings. Microscopic images were taken on an Axioplan 2 (Zeiss) equipped with a DKC-5000 (Sony) digital photo camera using differential interference contrast settings.

Determination of chitin content in isolated cell walls. Cell walls were isolated by grinding frozen mycelium using a pestle and mortar and rinsed three times with 1 M NaCl and three times with water. Cell walls were boiled in the presence of SDS, EDTA and β-mercaptoethanol to extract non-covalently linked cell wall components and to remove cytosolic contaminants as described by Montijn *et al.* (1994). Chitin levels were determined as described by

Tracey (1956). In brief, approximately 200 mg wet weight cell walls was freeze-dried and the dry weight was determined. Cell walls (approx. 15 mg dry wt) were subsequently hydrolysed in 6 M HCl at 100 °C for 4 h. The HCl was removed by evaporation using an air stream at 50 °C. Dried samples were resuspended in 1 ml water and centrifuged to remove insoluble material. To 0·1 ml sample, 0·1 ml solution A [1·5 N Na_2CO_3 in 4 % (w/v) acetylacetone] was added and the mixture was incubated at 100 °C for 20 min. After cooling to room temperature, 0·7 ml 96 % ethanol and 0·1 ml solution B (1·6 g *p*-dimethylaminobenzaldehyde in 30 ml concn HCl and 30 ml 96 % ethanol) was added and incubated for 1 h at room temperature. The absorbance at 520 nm was measured and compared to absorbance values from a standard curve of 0–100 µg glucosamine taken through the same reactions (Tracey, 1956; Popolo *et al.*, 1997).

DNA and RNA manipulations. Fungal chromosomal DNA was isolated as described by Kolar *et al.* (1988). Southern blot analyses were done as described by Sambrook *et al.* (1989). [α-^{32}P]dCTP-labelled probes were synthesized using the Rediprime II DNA labelling System (Amersham Pharmacia Biotech) according to the instructions of the manufacturer. RNA was extracted from mycelium, that had been flash-frozen in liquid nitrogen, using TRIzol reagent (InVitrogen). Total RNA (10 µg) was incubated with 3·3 µl 6 M glyoxal, 10 µl DMSO and 2 µl 0·1 M sodium phosphate buffer, pH 7·0, in a total volume of 20 µl for 1 h at 50 °C to denature the RNA. RNA glyoxal electrophoresis was performed in a SEA-2000 (Elchrom Scientific) at 10 °C.

PCR amplification of fungal *gfaA* fragments. Sequence alignment of Gfa1p homologues from yeasts and several higher eukaryotic species, including human, mouse and *Drosophila*, revealed several conserved stretches of amino acids. These conserved sequences were used to design two pairs of degenerated primers, to amplify fragments of *gfaA*-encoding genes from *P. chrysogenum*, *F. oxysporum* and *A. niger*. Primer set 1, PgfaP1 for (5′-cgggatcccGARTAYMGNGGNTAYGA-3′) and PgfaP2rev (5′-cggaattccgTGNGTNGCCAANCKNGT-3′) (convenient restriction sites are underlined) was used for the isolation of *gfa* fragments from *P. chrysogenum* and *F. oxysporum*. An expected PCR fragment of about 180 bp was amplified from genomic DNA from both fungi, which was cloned in pGEM-T Easy and sequenced. Primer set 2, PgfaP3for (5′-cgggatcccCAYATHAAYGCNGGNCC-3′) and PgfaP4rev (5′-cggaattccgCCYTGNARRCARTCNAC-3′) was used to isolate the *A. niger gfaA* homologue from an *A. niger* cDNA library. An expected 550 bp PCR fragment was cloned in pGEM-T Easy and sequenced.

Cloning of the full-length *A. niger gfaA* gene. To obtain the complete sequence of the *A. niger gfaA* gene and its promoter sequence, a cosmid library containing genomic inserts of *A. niger* DNA (kindly provided by Dr F. Schuren and Dr P Punt, TNO Nutrition, The Netherlands) was ordered into 384-well microtitre plates and colonies were spotted on LB plates. After transfer of the colonies to Hybond N$^+$ filters, they were lysed using standard protocols (Sambrook *et al.*, 1989). Out of approximately 5000 colonies screened, six hybridized with the *gfaA* PCR fragment. Two cosmid clones were isolated and analysed by subcloning, Southern blot analysis and sequence analysis. Only cosmid clone *gfaA#5* contained the complete *gfaA* sequence. Two partially overlapping subclones, pPST-GFA#5 (an 8 kb *Pst*I fragment cloned in pBluescript SK) and pBAMHI-GFA#5 (a 9 kb *Bam*HI fragment cloned in pBluescript SK), were made and these were used to obtain the full-length *A. niger gfaA* sequence. Sequencing was carried out on a Perkin Elmer ABI PRISM 310 sequencer using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction kit from Applied Biosystems. All primers used in this study were obtained from Isogen.

Disruption of the *A. niger gfaA* gene and complementation of the Δ*gfaA* strain. To construct the *gfaA* gene deletion plasmid, pBAMHI-GFA#5 was digested with *Not*I and *Xho*I. A 5·9 kb fragment, containing the last 800 nt of the *gfaA*-encoding sequence, the 3′ terminator region (2·4 kb) and the pBluescript vector (2·7 kb), was isolated and used in a three-way ligation. The second fragment was obtained by digestion of pBAMHI-GFA#5 with *Not*I and *Bgl*II, and a 5·0 kb fragment containing the 5′ promoter region of *gfaA* and the first 500 nt of the *gfaA*-encoding region was isolated. The *Not*I site is present in the polylinker of pBluescript. A 3·0 kb *Bam*HI–*Sal*I fragment containing the *A. oryzae pyrG* gene was obtained from pAO4-13 (de Ruiter-Jacobs *et al.*, 1989). Ligation of the three fragments resulted in the disruption plasmid, pΔ*gfaA*. This plasmid was linearized with *Not*I and used to disrupt the *gfaA* gene.

Disruption of the *gfaA* gene in *A. niger* was confirmed by Southern blot analysis. Genomic DNA of putative Δ*gfaA* strains and a wild-type strain was isolated and digested with *Pst*I. DNA was separated on a 0·8 % agarose gel, blotted on Hybond N$^+$ and hybridized with a *gfaA* probe. A 1·2 kb *Cla*I–*Bgl*II fragment from pBAMHI-GFA#5 was used as a probe. Plasmid pBAMHI-GFA#5 was used for complementation studies.

RESULTS

Increased chitin levels in cell walls of CFW-stressed germlings of *A. niger*

The addition of sublethal concentrations of CFW to *A. niger* germlings results in the formation of swollen hyphal tips (R. A. Damveld & A. F. J. Ram, unpublished results), similar to the phenotype observed after adding Congo Red to germlings of *A. niger* (Pancaldi *et al.*, 1984). CFW and Congo Red are both known to exhibit high binding affinity to chitin and, as a consequence of this interaction, chitin microfibril assembly and probably also the formation of linkages with other cell wall components are seriously disrupted, which results in a weakening of the cell wall and subsequent swelling. Previously, it has been shown that the addition of sublethal concentrations of CFW leads to increased chitin levels in the cell wall of *Geotrichum lactis* and also *S. cerevisiae* (Roncero & Duran, 1985). In this study, we monitored the effect of CFW addition on cell wall chitin levels in *A. niger* by measuring chitin levels in CFW-treated and control germlings. As indicated in Fig. 1, chitin levels in CFW-treated germlings were consistently higher compared to control germlings. A gradual increase over time of the chitin content in the cell wall of non-CFW-treated *A. niger* germlings was observed (Fig. 1). This increase in chitin content may be due to a higher chitin level in the lateral walls in older hyphae or to an increased number of septa, which contain relatively more chitin compared to lateral cell walls.

Identification of *gfaA* homologues in fungi and the molecular cloning of the *gfaA* gene from *A. niger*

To investigate whether the increased chitin content of the cell wall after CFW stress was accompanied by an increased expression of chitin synthesis-related genes, we focussed our attention on the *gfaA* gene. This gene encodes the enzyme glutamine : fructose-6-phosphate amidotransferase, which is the first and also a rate-limiting step in the

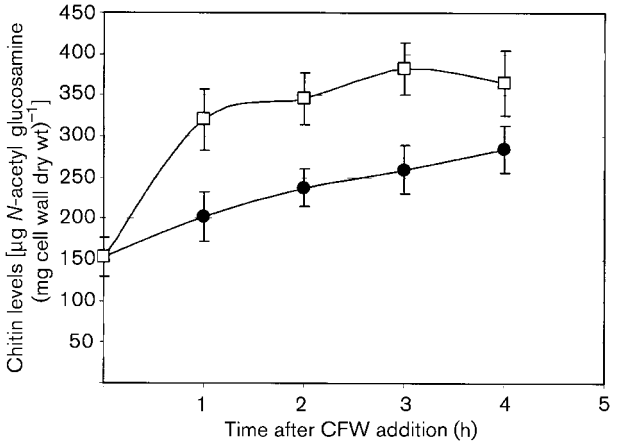

Fig. 1. Chitin content of cell walls from wild-type strain after CFW stress. Conidia were inoculated and after 5 h the germlings were treated with a sublethal concentration of 200 μg CFW ml^{-1}. Cell walls were isolated every hour after the addition of CFW and from the control cells. The amount of chitin in the cell wall was determined by the hot acid extraction method. Means ± SD were calculated from triplicate samples from two independent experiments. Filled circles, non-stressed germlings; open squares, stressed germlings.

UDP-*N*-acetylglucosamine biosynthetic pathway. We cloned fragments of the *gfaA* gene from three different fungi using degenerated primers based on conserved amino acid regions in fungal GFA proteins (Fig. 2). Specific PCR fragments of the expected size were obtained and sequenced. BLASTP analysis of the translation products deduced from the nucleotide sequences revealed that for all three fungi the gene fragments which were isolated showed high levels of amino acid identity to previously known fungal GFAs (Fig. 2). Using the *A. niger gfaA* PCR fragment as probe, a clone was isolated from an *A. niger* genomic cosmid library, which contained the complete *gfaA* ORF, including promoter and terminator regions. *gfaA* contains an ORF of 2412 bp, which is interrupted by five introns and encodes a protein of 694 aa. Comparison of the *A. niger* GfaA protein sequence to other fungal GFAs revealed a high level of identity among them (Figs 2 and 3). As expected, GFAs from bacteria, plants, insects and higher eukaryotes were more distantly related to the *A. niger* GfaA protein (Fig. 3). Searching the recently sequenced fungal genomes of *A. fumigatus*, *A. nidulans*, *Gibberella zeae* and *Neurospora crassa* revealed that these fungal genomes all contain a single putative GFAp-encoding gene, showing a high degree of identity with *A. niger* GfaAp (Fig. 3).

Expression levels of fungal GfaA-encoding genes are induced in response to cell wall stress

Studies in the yeast *S. cerevisiae* have shown that a rise in cell wall chitin levels is accompanied by higher levels of *GFA1* mRNA transcript. To determine whether the rise in chitin in the cell wall of *A. niger* was also accompanied by a higher expression of the *gfaA* gene, germlings were treated with 200 μg CFW ml^{-1}. Total RNA was isolated at different time points after adding CFW and subjected to Northern blot analysis. As shown in Fig. 4(a), the expression of *A. niger gfaA* initially increased after the addition of CFW, whereas at later time points the expression level decreased. The decrease in expression level of *gfaA* 2 and 4 h after CFW addition is probably due to inactivation of CFW and not to resistance of the germlings to CFW. When, after 4 h of initial treatment with CFW, the germlings were again treated with CFW, they stopped growing and formed swollen hyphal tips again.

Northern analysis further showed that the *gfaA* gene was already highly expressed during early stages of germination. To determine whether *gfaA* induction was a specific response to CFW or was also induced by other cell wall stress-inducing compounds, *A. niger* germlings were treated with SDS or caspofungin. The presence of these compounds also resulted in increased expression levels of *gfaA* (Fig. 4b and c). The slower induction of *gfaA* after SDS treatment might be explained by a different mode of action between CFW and SDS. Whereas CFW acts directly on the assembly of the cell wall by interacting with chitin microfibrils, the effect of SDS on the cell wall might be more indirect via perturbation of the cell membrane.

We further asked the question whether the induction of *gfaA* in response to CFW was limited to *A. niger*. Therefore, *P. chrysogenum* and *F. oxysporum* germlings were treated with CFW and RNA was isolated 1 h after CFW addition. Using the *gfaA* fragments of both *P. chrysogenum* (*PcgfaA*) and *F. oxysporu*m (*FogfaA*), we observed that the levels of *gfaA* mRNA in these fungi were also increased upon CFW-induced cell wall stress (Fig. 4d and e). This indicates that the induction of *gfaA* and probably also an increased synthesis of chitin are a general compensatory mechanism to ensure cell wall integrity under cell wall stress conditions.

gfaA is essential for viability

To investigate the consequences of a loss of function of the *gfaA* gene in *A. niger*, a gene disruption vector (pΔ*gfaA*) was constructed in which an internal part (1038 bp) of the *gfaA* coding region is replaced by the *pyrG* gene from *A. oryzae* (Fig. 5a). An *A. niger pyrG*$^{-}$ strain (AB4.1), was transformed with the ~11 kb linear fragment of the disruption cassette. Transformants were selected on MM supplemented with 5 mg glucosamine ml^{-1}. In *S. cerevisiae*, *GFA1* is an essential gene and mutants can be rescued by the addition of glucosamine to the medium (Watzele & Tanner, 1989). From various transformation plates, 40 transformants were randomly picked and subjected to two rounds of purification. Next, the growth of the transformants was examined on plates with or without glucosamine to identify putative Δ*gfaA* strains, since these transformants were expected not to grow on plates without glucosamine (Fig. 5c). Glucosamine-requiring transformants were identified and

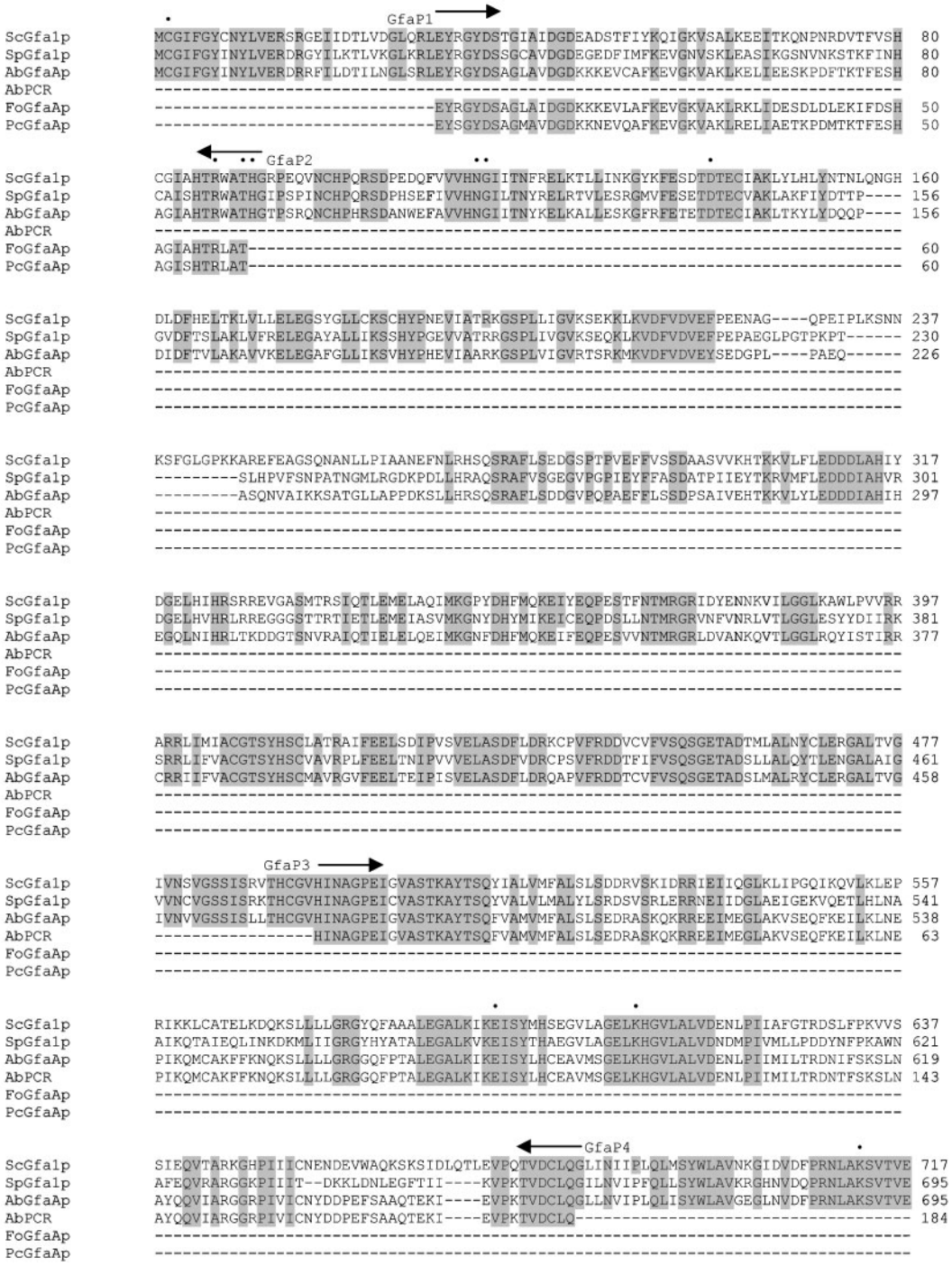

Fig. 2. Multiple sequence alignment of fungal glutamine : fructose-6-phosphate amidotransferases. Conserved amino acid regions that were used to design degenerated primers are indicated with arrows. Conserved amino acids in the glutamine-binding domain and the fructose-6-phosphate isomerase domain are indicated with a •. Identical amino acid residues are shaded. Accession numbers of the different proteins are: AbGfaA, *A. niger*, AY594332; FoGfaA, *F. oxysporum*, AY594333; PcGfaA, *P. chrysogenum*, AY594334; ScGfa1, *S. cerevisiae*, NP_012818; SpGfa1, *Schizosaccharomyces pombe*, NP_596011. Multiple sequence alignment was performed using DNAman version 4.0 using the method of Higgins & Sharp (1988).

these were analysed further by Southern blot analysis, which proved that the expected deletion of the *gfaA* gene had occurred (Fig. 5b). Whereas supplementation with 5 mg glucosamine ml^{-1} restored growth (Fig. 5b), supplementation with 1 mg glucosamine ml^{-1} did not result in the formation of colonies (data not shown). We also observed

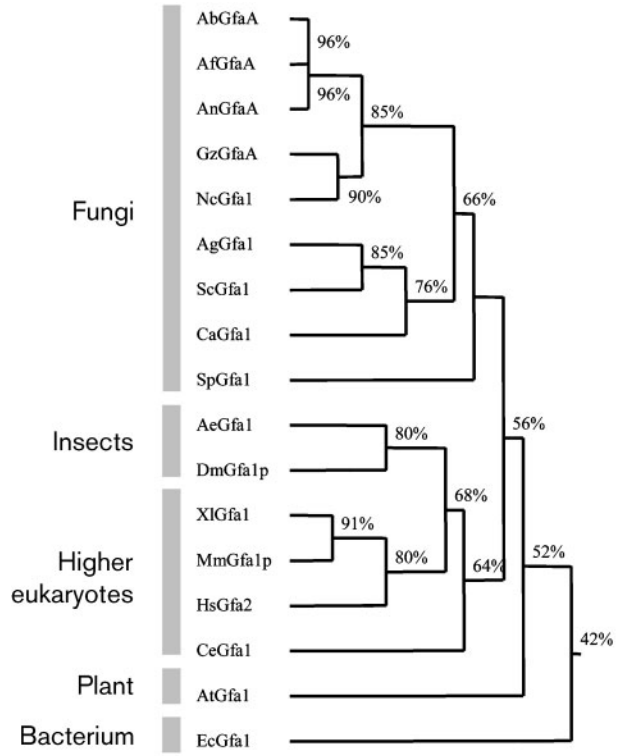

Fig. 3. Phylogenetic tree of GFAs showing the clustering of fungal GFAs separate from GFAs from both prokaryotic and higher eukaryotic origin. Multiple sequence alignment was performed using the DNAman version 4.0 optimal alignment program (Feng & Doolittle, 1987; Thompson *et al*., 1994). Percentage values represent homology calculated as the number of identical residues between sequences divided by the length of the aligned sequence minus the length of all gaps. AbGfaA, *A. niger*, AY594332; NcGfa1, *N. crassa*, XM_327652; AfGfaA, *A. fumigatus*, cosmid 13 ATG on 29524, sequence data were obtained from The Wellcome Trust Sanger Institute at http://www.sanger.ac.uk; AnGfaA, *A. nidulans*, BK005223; GzGfaA, *Gibberella zeae* (anamorph *Fusarium graminearum*), BK005224; SpGFA1, *Schizosaccharomyces pombe*, NP_596011; CaGFA1, *Candida albicans*, P53704; ScGFA1, *S. cerevisiae*, NP_012818; CeGFA-T, *Caenorhabditis elegans*, Z66511; DmGFA-T, *Drosophila melanogaster*, AAF45333; AeGfaT-1, *Aedes aegypti*, AF399921; AtGfa1, *Arabidopsis thaliana*, AP001297; HsGfaT-2, *Homo sapiens*, 094808; EcGfa1, *E. coli*, AE005604.

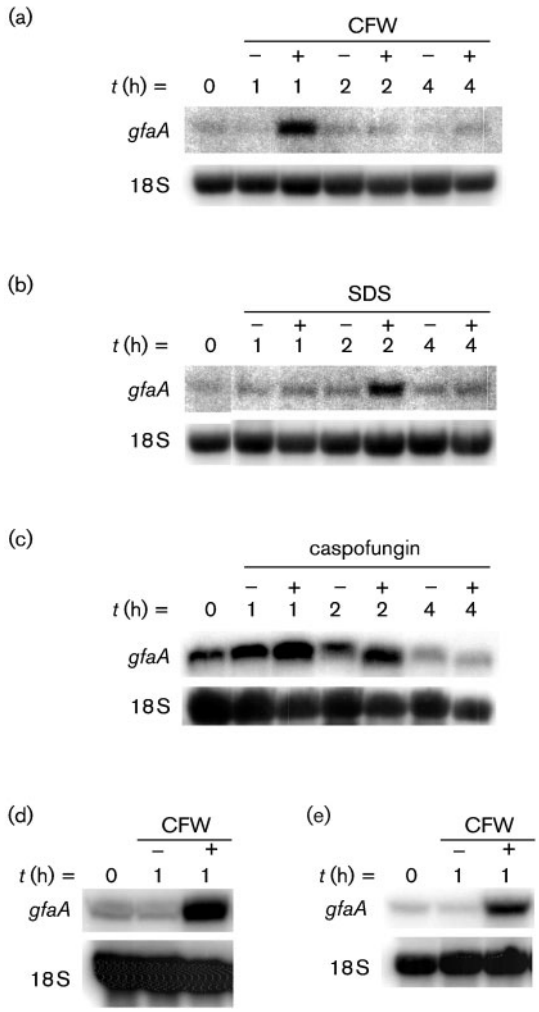

Fig. 4. Induction of *gfaA* transcript levels in response to different forms of cell wall stress in different filamentous fungi. (a) CFW-induced cell wall stress in *A. niger*; (b) SDS-induced cell wall stress in *A. niger*; (c) caspofungin-induced cell wall stress in *A. niger*. Conidia were inoculated and grown for 5 h before adding the cell wall stress-inducing compounds. RNA was extracted at various time points after addition and subjected to Northern blot analysis using the *A. niger gfaA* PCR fragment as a probe; (d) CFW-induced cell wall stress in *P. chrysogenum*; (e) CFW-induced cell wall stress in *F. oxysporum*. Conidia of *P. chrysogenum* and *F. oxysporum* were grown for 6·5 and 7 h respectively, before adding CFW. RNA was isolated 1 h later and the level of *gfaA* induction in response to stress was compared to the non-stressed situation using the *PcgfaA* and the *FogfaA* PCR fragment as probes.

that conidia from a Δ*gfaA* strain (obtained from a spore plate containing 10 mg glucosamine ml^{-1}) were as viable and able to germinate on a glucosamine-containing plate as the wild-type strain (data not shown). Next, we asked the question whether the addition of glucosamine could fully supplement the Δ*gfaA* strain. To determine this, a fixed number of conidia from a wild-type strain and the Δ*gfaA* strains were spotted in the centre of an agar plate containing different concentrations of glucosamine (5, 10 and 50 mg ml^{-1}) and the size of the colony was determined after a period of 7 days. At all glucosamine concentrations tested, the growth of a Δ*gfaA* strain was somewhat slower than the wild-type, resulting in a smaller colony size [5 mg ml^{-1}, $3{\cdot}5 \pm 0{\cdot}1$ cm (Δ*gfaA*) vs $3{\cdot}7 \pm 0{\cdot}1$ (wild-type); 10 mg ml^{-1}, $3{\cdot}6 \pm 0{\cdot}1$ vs $4{\cdot}0 \pm 0{\cdot}1$; 50 mg ml^{-1}, $4{\cdot}7 \pm 0{\cdot}1$ vs $5{\cdot}4 \pm 0{\cdot}1$ cm (mean $\pm$ SD, $n = 2$)] after 7 days of growth at 30 °C. We also

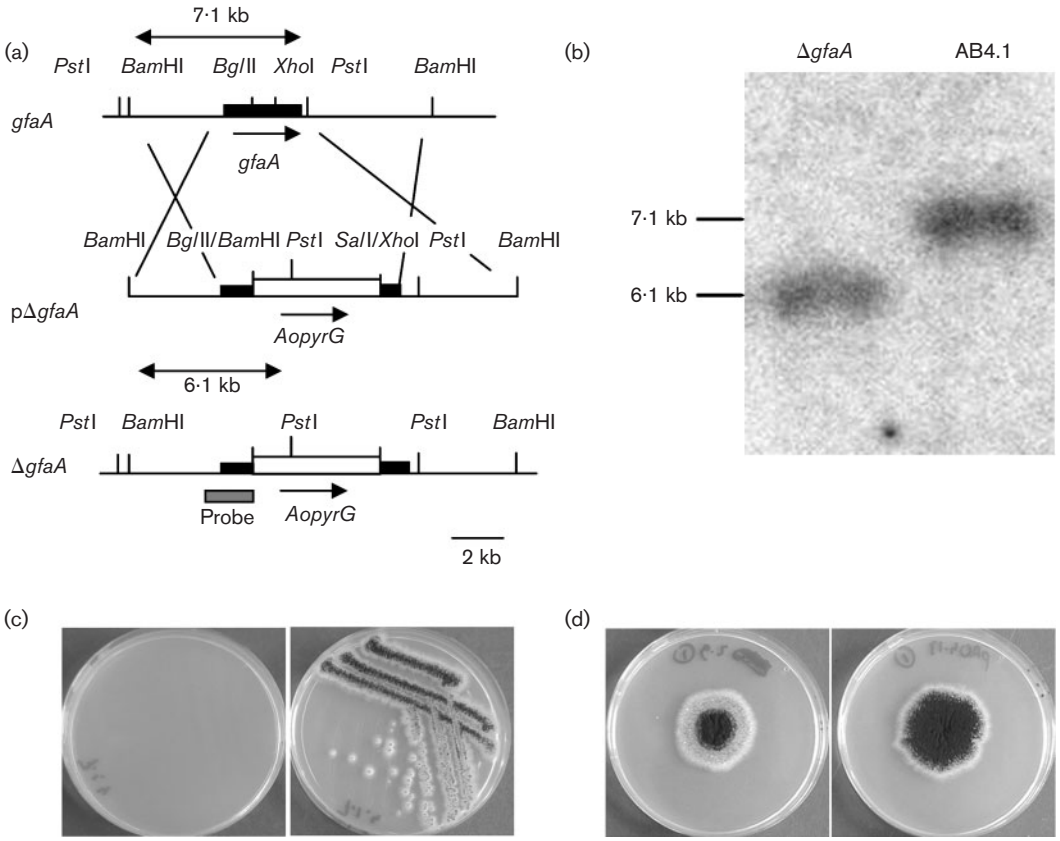

Fig. 5. Disruption of the *gfaA* gene in *A. niger*. (a) Schematic overview of the *gfaA* disruption strategy. Restriction sites and predicted sizes of genomic fragments are indicated. (b) Southern blot analysis of the *AbgfaA* deletion mutant (Δ*gfaA*) and the recipient strain, AB4.1. The sizes of marker DNA fragments are indicated on the left. A 7·1 kb fragment is present in the wild-type strain whereas a predicted 6·1 kb fragment is present in the disruptant strain. (c) The Δ*gfaA* strain requires glucosamine in the plate to support growth. Conidia from a Δ*gfaA* strain were streaked on MM (left) or on MM supplemented with 10 mg glucosamine ml^{-1} (right). (d) The Δ*gfaA* strain (left) shows decreased levels of conidiation in the outer parts of the colonies compared to a wild-type strain (right). One hundred spores were spotted in the centre of the agar plate and grown for 7 days at 30 °C.

observed that the Δ*gfaA* strain conidiates poorly on a plate containing 10 mg glucosamine ml^{-1} (Fig. 5d). This was most pronounced at the outer rim of the colony where hardly any conidiophores were formed. Most likely, the glucosamine in the plate is depleted after vegetative growth and the lack of glucosamine inhibits conidiation. Addition of more glucosamine to the plate (50 mg ml^{-1}) resulted in a less pronounced effect on conidiation (data not shown), indicating that lack of glucosamine was indeed causing this effect.

To determine in more detail the effect of the *gfaA* deletion on spore germination in the absence of glucosamine, conidia were isolated after growth of a Δ*gfaA* strain on a plate supplemented with 10 mg glucosamine ml^{-1}. Conidia from the wild-type strain and from the Δ*gfaA* strain were inoculated in glucosamine-free MM containing 0·1 % Casamino acids. Conidia from the wild-type strain swelled during the first 4 h after inoculation and started to form a germ tube in a highly synchronized way. After 6 h, more than 95 % of the conidia had formed a germ tube. Fig. 6(A) shows the morphology of wild-type germlings after 8 h of inoculation. At this stage, the conidium had formed one unbranched germ tube. Inoculation of conidia from the Δ*gfaA* in medium without glucosamine resulted in severe defects in spore germination. After 8 h the majority of the conidia (74 %) had not swelled in the absence of glucosamine (Fig. 6B). The remaining conidia (25 %) had started to swell. Only 1 % of the conidia swelled and produced a short germ tube. After 13 h, the wild-type strain had branched and had occasionally formed secondary germ tubes to form a mycelial network (Fig. 6C). Microscopical analysis of the germination process after 13 h of incubation of Δ*gfaA* in the absence of glucosamine revealed that most of the conidia were unable to swell (69 %), some were swollen without a germ tube (28 %) and 3 % of the conidia were swollen and had produced a germ tube (Fig. 6D). Prolonged incubation of the Δ*gfaA* strain in the absence

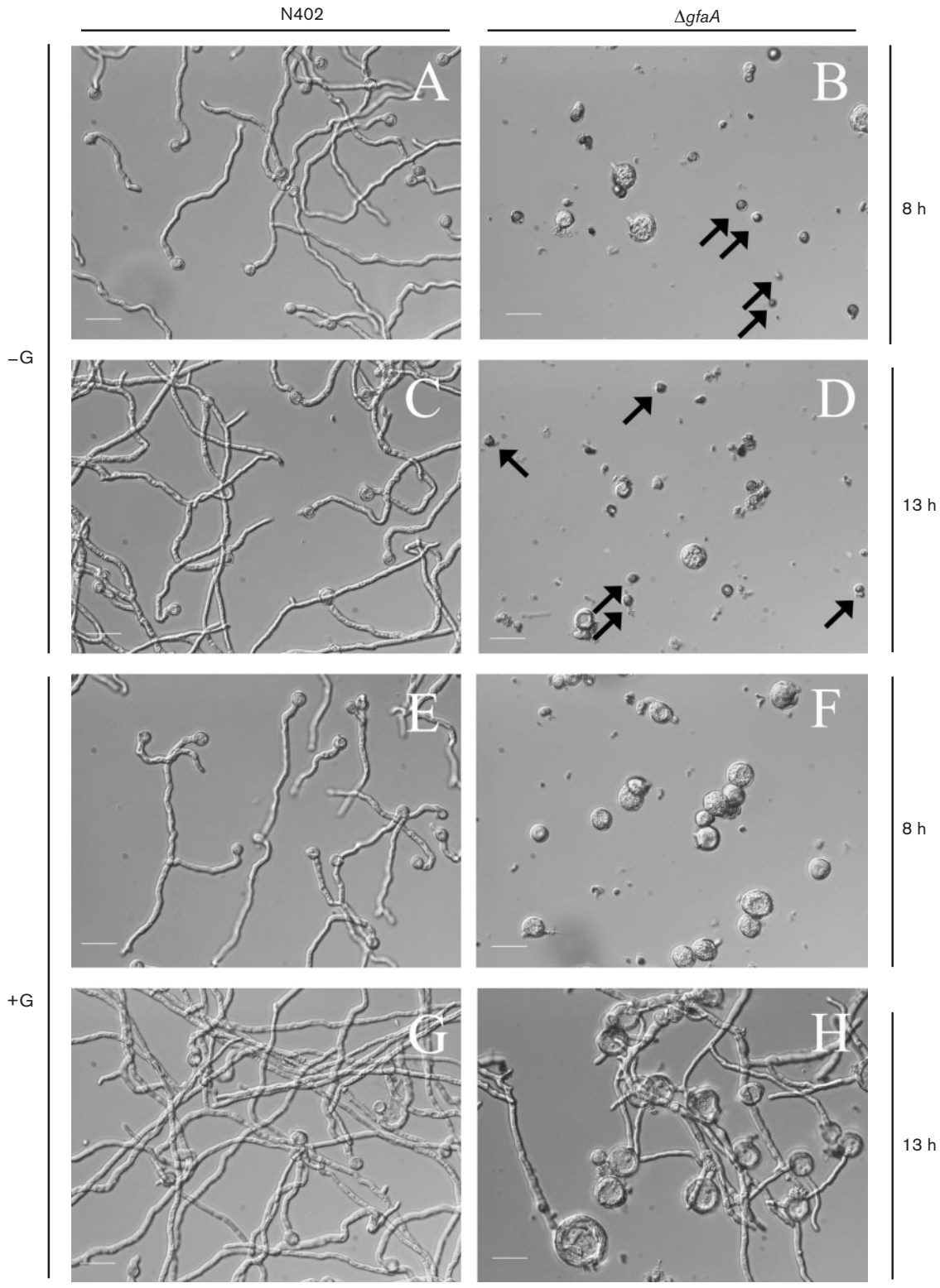

Fig. 6. Addition of glucosamine to the medium can partially rescue the growth defect of the Δ*gfaA* strain. Conidia of N402 (A, C, E, G) or Δ*gfaA* (B, D, F, H) were inoculated on coverslips in liquid MM lacking glucosamine (−G) (A–D) or containing 10 mg glucosamine ml^{-1} (+G) (E–H). Conidia were incubated at 37 °C for 8 (A, B, E, F) or 13 h (C, D, G, H), fixed and examined by differential interference contrast microscopy. Non-swollen spores are indicated with arrows. Bars, 10 μm.

of glucosamine further exaggerated this phenotype. Conidia that had started to swell continued to expand isotropically, resulting in extremely large cells with a diameter of up to 40 μm. Occasionally, the giant cells produced a germ tube with a diameter comparable to a wild-type germ tube. After 40 h many of those giant cells started to shrink indicating cell death.

Supplementation of the Δ*gfaA* strain with glucosamine did not result in complete restoration of wild-type growth behaviour at both low (10 mg ml^{-1}) and high (50 mg ml^{-1}) glucosamine concentrations. After 8 h in the presence of 10 mg glucosamine ml^{-1}, the majority of the Δ*gfaA* conidia (86 %) were swollen, but only very few of them were also able to form a small germ tube (3 %) (Fig. 6F). The mean size of the swollen spore of the Δ*gfaA* strain was considerable larger than the wild-type spore size (compare Fig. 6E and F). After 13 h of growth, 91 % of the conidia were swollen and the majority (58 %) had formed a normally sized germ tube (Fig. 6H). The addition of 10 mg glucosamine ml^{-1} to the growth medium did not have an effect on the growth of the wild-type strain (compare Fig. 6A to E or C to G).

Addition of high concentrations (50 mg ml^{-1}) glucosamine also did not restore wild-type morphology. However, the swelling of the *gfaA* deletion strain in presence of 50 mg glucosamine ml^{-1} was less pronounced than at 10 mg glucosamine ml^{-1} (data not shown). We also observed that in the presence of 10 mg glucosamine ml^{-1}, germination of swollen conidia started after 11 h of incubation whereas the presence of 50 mg glucosamine ml^{-1} resulted in faster germination, already observed after 8 h of growth.

gfaA as a selection marker

The inability of the Δ*gfaA* strain to grow on medium without glucosamine prompted us to investigate whether the Δ*gfaA* strain, in combination with the *gfaA* gene, could be used as a selection marker for fungal transformation. Therefore, subclone pBAMHI-GFA#5, which contains the complete *gfaA* ORF, including promoter and terminator regions, was transformed into the Δ*gfaA* strain. Protoplasts were obtained from the Δ*gfaA* strain which was pregrown in the presence of various concentrations of glucosamine (1, 2·5, 5, 10 and 50 mg ml^{-1}). All cultures gave similar amounts of biomass ranging from 2·7 to 3·5 g fresh weight per 250 ml CM, but the number of protoplasts decreased at the higher glucosamine concentrations, indicating that it is more difficult to produce protoplasts from Δ*gfaA* cultures after growing them at high concentrations of glucosamine. After transformation, the protoplasts were plated out on MM plates. Analysis of the transformation plates revealed some background growth on the negative control plates (no DNA added). However, no colonies were formed that were able to produce conidiophores. On the transformation plates on which protoplasts had been plated out that were transformed with the *gfaA*-containing plasmid, sporulating colonies were readily obtained after 5 days of growth against a background of non-sporulating mycelia. Further purification showed that the sporulating colonies had become glucosamine-prototrophic. Southern blot analysis of 16 randomly chosen transformants from different transformation plates revealed that the sporulating colonies contained the disrupted allele of the *gfaA* gene and a wild-type copy of the *gfaA* gene in their genome (data not shown).

DISCUSSION

Using degenerate primers, which were based on conserved amino acid sequences in glutamine : fructose-6-phosphate amidotransferase enzymes in various organisms, three fungal GFAs or fragments thereof were isolated. In addition, GFA homologues were identified in recently sequenced fungal genomes of *A. fumigatus*, *A. nidulans*, *N. crassa*, and *G. zeae*. GFAs are composed of two domains: the glutamine-binding domain and the fructose 6-phosphate-binding isomerase domain. Both domains are linked by a non-conserved amino acid sequence of variable length. Structure–function analysis of the *E. coli* GFA protein has identified amino acid residues involved in glutamine binding and hydrolysis. The Cys2 residue (amino acids are numbered according to the *A. niger* protein sequence) participates directly in glutamine hydrolysis and residues Arg87 and Asp140, together with Thr89, His90, Asn115 and Gly116, are involved in binding and stabilization of the glutamine molecule. All these amino acids are conserved in the GFA homologues identified in fungi (Fig. 2) and in higher eukaryotes (not shown), suggesting a conserved molecular mechanism of glutamine binding and hydrolysis. The catalytic site of the isomerase domain consists of three residues: Glu574, His491 and Lys690, all of which are conserved (Fig. 2) (Milewski, 2002 and references therein).

We have extended the finding in the yeast *S. cerevisiae* that cell wall weakening or cell wall stress activates a compensatory mechanism that includes the increased deposition of chitin in the cell wall to ensure cell wall integrity. We have shown that the addition of the cell wall-disturbing compound CFW to germlings of the filamentous fungus *A. niger* results in an increased level of cell wall chitin. Increased chitin synthesis also demands a higher flux through the hexosamine metabolic pathway to generate UDP-*N*-acetylglucosamine, the sugar donor for chitin biosynthesis. In *S. cerevisiae*, the higher flux is at least partially achieved by an increased expression of *GFA1*, encoding the glutamine : fructose-6-phosphate amidotransferase activity, which is the rate-limiting step in the pathway (Lagorce *et al.*, 2002). In this paper, we show that in *A. niger* an increased chitin level in the cell wall in response to cell wall stress is also accompanied by increased transcription levels of *gfaA*, further indicating that both yeasts and filamentous fungi respond to cell wall stress by activating the chitin biosynthetic pathway. We have used the induction of *gfaA* to obtain evidence that this response is a general fungal mechanism to cope with cell wall stress, since a

similar induction was observed in *P. chrysogenum* and *F. oxysporum*. Furthermore, we have shown that the induction of *gfaA* is activated not only by the addition of CFW, but also after adding SDS or caspofungin, a 1,3-β-glucan synthase inhibitor (Kartsonis *et al.*, 2003). Mutations that result in cell wall weakening in *S. cerevisiae* (e.g. Δ*fks1*, Δ*knr4*, Δ*gas1*, Δ*kre6*, Δ*mmn9* and Δ*och1*) all display increased chitin levels in their cell wall, which is accompanied by higher *GFA1* transcript levels and higher GFA activities (Lagorce *et al.*, 2002; Bulik *et al.*, 2003). This relationship between an increased level of chitin in the cell wall and a mutant in which cell wall biosynthesis is affected (RhoA) has recently also been found for *A. nidulans* (Guest *et al.*, 2004).

In *S. cerevisiae*, it is well established that increased chitin synthesis is carried out by only one of the three chitin synthase genes, namely *CHS3*. Deletion of *CHS3* in combination with a mutation that affects cell wall biosynthesis results in a synthetic lethal phenotype (Popolo *et al.*, 1997; Osmond *et al.*, 1999). It will be of interest to identify the chitin synthase gene in *A. niger* (and in other filamentous fungi) that is responsible for the increased chitin level. Chitin synthase genes have not yet been described in *A. niger*, but chitin synthesis has been studied in detail in both *A. fumigatus* and *A. nidulans*. The chitin synthase family in *A. fumigatus* consists of at least seven different *CHS* genes (*AfChsA–AfChsG*) and six *CHS* genes have been identified in *A. nidulans* (*AnChsA*, *B*, *C*, *E* and *AnCsmA*/*ChsD*) (Mellado *et al.*, 2003 and references therein). Disruption of *AnCsmA* resulted in a strain that is highly sensitive to CFW (Fujiwara *et al.*, 1997; Horiuchi *et al.*, 1999), which may suggest that this gene is involved in the induction of chitin synthesis in response to cell wall stress. CsmAp is a unique chitin synthase in which the chitin synthase domain is fused with a myosin tail and both domains are required for its function (Horiuchi *et al.*, 1999). Possibly, the function of the myosin domain in CsmAp is used to rapidly translocate the protein, using the actin cytoskeleton, to places that require additional chitin deposition in response to cell wall stress.

Although chitin levels in *S. cerevisiae* can increase dramatically under some cell wall stress-inducing conditions, transcription levels of *CHS3* increase to a lesser extent (Lagorce *et al.*, 2003). Chs3 protein levels have not been shown to vary significantly (Popolo *et al.*, 1997; Valdivieso *et al.*, 2000; Garcia-Rodriquez *et al.*, 2000), indicating that post-translational events are responsible for increasing Chs3 activity in response to cell wall stress. Indeed, such a mechanism to recruit Chs3p from internal stores (chitosomes) to the plasma membrane has recently been identified (Valdivia & Schekman, 2003).

UDP-*N*-acetylglucosamine is incorporated into chitin, but the nucleotide sugar donor also serves as a donor for the biosynthesis of two essential modifications of secretory proteins: *N*-linked glycosylation and glycosylphosphatidylinositol anchor attachment. The lethality of Δ*gfaA* is therefore not simply due to the inability to synthesize chitin, although it is generally assumed that also in filamentous fungi chitin biosynthesis is essential for viability. The majority of conidia (70 %) of the Δ*gfaA* strain do not swell in the absence of glucosamine. This indicates that most conidia contain only a relative small pool of UDP-*N*-acetylglucosamine or its precursors in the cell. The addition of glucosamine to the medium allows spore germination, indicating that glucosamine can be taken up by the cell and be converted, probably into glucosamine 6-phosphate, thereby circumventing the need for GfaAp. The finding that higher concentrations of glucosamine (50 mg ml^{-1}) accelerated spore germination compared to a lower concentration (10 mg ml^{-1}) suggests that the uptake of glucosamine is limiting at low glucosamine concentrations. It further suggests that the glucosamine is taken up by the fungus with a low affinity transport mechanism, but the mechanism by which glucosamine is taken up is not known.

Although most Δ*gfaA* conidia do not swell in the absence of glucosamine, some conidia do swell and form large cells. The phenotype of those cells resembles the phenotype of *A. niger* conidia that germinate in the presence of tunicamycin (Katoh *et al.*, 1976). Tunicamycin inhibits the transfer of *N*-acetylglucosamine 1-phosphate from UDP-*N*-acetylglucosamine to dolicholmonophosphate (Tkacz & Lampen, 1975; Takatsuki *et al.*, 1975) and thereby blocks *N*-linked protein glycosylation. Because of the similar phenotype of the Δ*gfaA* conidia and tunicamycin-treated conidia, one might suggest that the inability to form *N*-chains is the primary effect of the absence of UPD-*N*-acetylglucosamine in the Δ*gfaA* strain. Alternatively, tunicamycin is also known as an inducer of the Unfolded Protein Response (UPR) in *A. niger* (Mulder *et al.*, 2004). The presence of tunicamycin might therefore result in accumulation of cell wall-synthesizing enzymes in the ER which are normally transported to the cell surface via the secretory pathway, and thereby causing defects in cell wall biosynthesis.

Finally, we have shown that the *A. niger gfaA* gene, in combination with the *A. niger* Δ*gfaA* strain, can be used as a selection marker and a similar approach seems feasible for other filamentous fungi.

ACKNOWLEDGEMENTS

We thank Peter Punt and Frank Schuren for providing us with the *A. niger* cosmid library and *P. chrysogenum*, Guido Bloemberg for providing *F. oxysporum*, Hans de Nobel and Hans Sietsma for sharing ideas and helpful advice. This work was supported by a grant from STW (Dutch Foundation for Technical Research).

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