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Damveld, R.A.; Kuyk, P.A. van; Arentshorst, M.; Klis, F.M.; Hondel, C.A.M.J.J. van den; Ram, A.F.J.

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Expression of *agsA*, one of five 1,3-α-D-glucan synthase-encoding genes in *Aspergillus niger*, is induced in response to cell wall stress

Robbert A. Damveld[a], Patricia A. vanKuyk[a], Mark Arentshorst[a], Frans M. Klis[b], Cees A.M.J.J. van den Hondel[a,c], Arthur F.J. Ram[a,c,*]

[a] *Clusius Laboratory, Institute of Biology, Leiden University, Wassenaarseweg 64, 2333 AL, Leiden, The Netherlands*
[b] *Swammerdam Institute for Life Sciences-Microbiology, University of Amsterdam, Nieuwe Achtergracht 166, 1018 WV, Amsterdam, The Netherlands*
[c] *TNO-Nutrition, Applied Microbiology and Genetechnology, Utrechtseweg 48, 3500 AJ, Zeist, The Netherlands*

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Abstract

1,3-α-D-Glucan is an important component of the cell wall of filamentous fungi. We have identified a family of five 1,3-α-D-glucan synthase-encoding genes in *Aspergillus niger*. The *agsA* gene was sequenced and the predicted protein sequence indicated that the overall domain structure of 1,3-α-D-glucan synthases is conserved in fungi. Using RT-PCR and Northern blot analysis, we found that expression of the *agsA* gene and to a lesser extent also of *agsE* were induced in the presence of the cell wall stress-inducing compounds such as Calcofluor White (CFW), SDS, and caspofungin. Loss of *agsA* function did not result in an apparent phenotype under normal growth conditions but rendered the cells more sensitive to CFW. The induction of 1,3-α-D-glucan synthase-encoding genes in response to cell wall stress was not limited to *A. niger*, but was also observed in *Penicillium chrysogenum*. We propose that this response to cell wall stress commonly occurs in filamentous fungi.

Keywords: 1,3-α-D-Glucan; Cell wall stress; Signalling; Cell wall remodelling; Calcofluor White; Duplex RT-PCR

1. Introduction

The cell wall of yeasts and fungi is of vital importance to the cell. It is required to resist the turgor pressure of the protoplasts to prevent cell lysis. It further protects against potentially damaging enzymes from the environment and acts as a scaffold for exposing cell wall proteins that play a role during cell–cell interactions. Composition and architecture of the fungal cell wall do not only vary among different fungal species, but also within a single species the composition and structure are highly dynamic (Klis et al., 2002; Smits et al., 1999). Developmental programs such as sporulation (Arellano et al., 2000), the response to mating pheromones (Appeltauer and Achstetter, 1989), and the dimorphic switch from yeast to hyphal growth (Borges-Walmsley et al., 2002) are all characterised by morphological changes and accompanied by alterations in cell wall structure and composition. The most abundant cell wall polymer of both yeasts and filamentous fungi is 1,3-β-D-glucan. In its mature form, 1,3-β-D-glucan is branched with 1,6-β-linkages at the branching points (Fontaine et al., 2000; Manners et al., 1973a,b). Chitin chains and cell wall 1,6-β-glucosylated mannoproteins are covalently linked to 1,3-β-D-glucan, forming a supra-molecular complex (Kollar et al., 1997). Although not present in the cell wall of *Saccharomyces cerevisiae* (Cabib et al., 1997; Lipke and Ovalle, 1998) and *Candida albicans* (Klis et al., 2001), 1,3-α-D-glucan is a prominent component in the

* Corresponding author. Fax: +31 71 5274999.
E-mail address: Ram@rulbim.leidenuniv.nl (A.F.J. Ram).

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cell walls of many fungal species, including *Schizosaccharomyces pombe* (Bacon et al., 1968; Kreger, 1954; Manners and Meyer, 1977; Sietsma and Wessels, 1990), *Aspergillus nidulans* (Bull, 1970; Zonneveld, 1971, 1972), *Aspergillus niger* (Horisberger et al., 1972; Johnston, 1965), *Aspergillus fumigatus* (Fontaine et al., 2000), *Cryptococcus neoformans* (Reese and Doering, 2003), *Histoplasma capsulatum* (James et al., 1990), *Blastomyces dermatitidis* (Hogan and Klein, 1994), and *Paracoccidioides brasiliensis* (Borges-Walmsley et al., 2002). The first 1,3-α-D-glucan synthase-encoding gene, named *ags1*, was identified and analysed in *S. pombe*. This gene was identified by complementing a temperature-sensitive mutant, that lysed at the restrictive temperature and showed reduced 1,3-α-D-glucan levels in the cell wall (Hochstenbach et al., 1998). In the genome sequence of *S. pombe*, four additional 1,3-α-D-glucan synthase-encoding genes have been described (Katayama et al., 1999). The genes of this family, *ags*1/*mok*1 and *mok*11 to *mok*14, encode large, multi-domain proteins consisting of approximately 2400 amino acids, except for *mok*14, which encodes a shorter protein lacking the NH_2-terminal domain. The different domains are proposed to synthesise 1,3-α-D-glucan, transport it over the plasma membrane and process it (Hochstenbach et al., 1998; Katayama et al., 1999). In *A. niger* two different α-glucan polymers have been identified. One of them, nigeran, was isolated as a hot-water-soluble, linear, alternating 1,4-1,3-α-D-glucan polymer (Barker et al., 1953, 1957). A second α-glucan polymer, pseudonigeran, was extracted from *A. niger* cell wall by alkaline extraction. The structure of pseudonigeran was identified as a linear 1,3-α-D-glucan polymer with some (3–10%) 1,4-α-D-linkages (Horisberger et al., 1972; Johnston, 1965). In *A. nidulans*, 1,3-α-D-glucan synthesis has been mainly studied in relation to cleistothecium formation. Zonneveld (1972, 1974) has proposed that 1,3-α-D-glucan accumulates during vegetative growth and is metabolised by an 1,3-α-D-glucanase expressed during sexual development. Surprisingly, deletion of an 1,3-α-D-glucanase that is specifically expressed during sexual development, in Hülle cells, did not affect the formation of cleistothecia (Wei et al., 2001). In *C. neoformans* 1,3-α-D-glucan has been shown to be required for the anchoring of the capsule to the cell wall (Reese and Doering, 2003).

Morphogenetic events and especially the formation of a new bud and cell separation in yeasts and the formation of new branches in filamentous fungi require drastic remodelling of the cell wall with the temporary risk of cell lysis. In addition, plants produce cell wall-degrading enzymes in response to fungal infections, which are a serious threat to fungi. To maintain the integrity of the cell wall, fungi possess a signal transduction cascade that is activated in response to cell wall stress and induces the expression of genes that prevent cell lysis. The cell wall integrity pathway has been particularly well studied in *S. cerevisiae* and is known as the Pkc1p or Slt2p/Mpk1p MAP kinase signalling pathway (Heinisch et al., 1999). Activation of the pathway is induced in response to several environmental stimuli (Klis et al., 2002). Putative sensors of the pathway are the transmembrane proteins Wsc1p–Wsc4p (Verna et al., 1997) and Mid2p (Ketela et al., 1999; Rajavel et al., 1999), which interact with a guanine nucleotide exchange factor (Rom2p) to activate the small GTPase Rho1p (Philip and Levin, 2001). Rho1p regulates multiple processes in the cell including the activation of Pkc1p (Kamada et al., 1996; Nonaka et al., 1995). Pkc1p activates the Slt2/Mpk1 MAP kinase cascade and finally results in the activation of a transcription factor Rlm1p (Dodou and Treisman, 1997; Watanabe et al., 1997). Although there is evidence in mycelial fungi for the existence of compensatory reactions in response to cell wall stress (Gooday and Schofield, 1995; Kurtz et al., 1994; Mellado et al., 2003; Sela-Buurlage, 1996), it is mainly indirect.

In this study, we have isolated fragments of a family of five 1,3-α-D-glucan synthase-encoding genes (*agsA–E*). We show that expression of *agsA* is over 20-fold induced upon addition of the cell wall stress-inducing antifungal compound Calcofluor White (CFW). Deletion of the *agsA* gene renders the fungus more sensitive to CFW, suggesting that the induction of *agsA* in response to cell wall stress contributes to ensuring cell wall integrity.

2. Materials and methods

2.1. Strains, culture conditions, and transformations

Aspergillus niger N402 (*cspA1* derivative of ATCC9029, Bos et al., 1988) and the *pyrG* negative derivative of N402, AB4.1 (van Hartingsveldt et al., 1987) were used throughout this study. *Aspergillus* strains were grown in *Aspergillus* minimal medium (MM) or *Aspergillus* complete medium (CM) consisting of minimal medium with the addition of 10 g L^{-1} yeast extract and 5 g L^{-1} casamino acids (Bennett and Lasure, 1991). Growth medium was supplemented with 10 mM uridine (Serva) when required. Transformation of *A. niger* was described by Punt and van den Hondel (1992) using lysing enzymes (L1412, Sigma) for protoplast formation. *Penicillium chrysogenum* (ATCC 48271, Kolar et al., 1988) was obtained from Dr. P. Punt, TNO Nutrition, Zeist, The Netherlands, and grown in CM at 30 °C. Conidiospores from *A. niger* and *P. chrysogenum* were obtained by harvesting spores from a CM-plate after 4–6 days of growth at 30 °C, using 0.9% NaCl. The bacterial strain used for transformation and amplification of recombinant DNA was *Escherichia coli* XL1-Blue (Stratagene, La Jolla, CA). XL1-Blue was transformed using the heat shock protocol as described by Inoue et al. (1990).

2.2. Cloning of a family of 1,3-α-D-glucan synthase genes from A. niger and P. chrysogenum

Multiple sequence alignments (DNAman version 4.0) of five Ags/Mok proteins that have been described in *S. pombe* (Hochstenbach et al., 1998; Katayama et al., 1999) were performed to design degenerate primers based on conserved amino acid stretches for isolating *ags* genes from *A. niger* and *P. chrysogenum*. The primers are listed in Table 1 Nested PCRs on genomic DNA from wild-type *A. niger* and *P. chrysogenum* strains were carried out with all possible primer combinations, and PCR fragments with the expected size (ranging from 822 to 448 bp) were cloned in pGEM-T easy (Promega) and analysed by restriction enzyme digestions. After initial grouping, for both fungi, representatives of each group were sequenced. Sequence alignment of the different clones from *A. niger* revealed that we had isolated four distinct fragments that were likely to encode four different Ags proteins. Sequence analysis revealed the existence of at least three Ags homologs in *P. chrysogenum*. A fragment of the *A. niger fks1* homolog, encoding an 1,3-β-D-glucan synthase subunit, was obtained by designing degenerated primers (Table 1) against conserved amino acid sequences of Fks1p homologs, which were used in nested PCRs on genomic *A. niger* DNA. Products of the expected size (~375 bp) were cloned in pGEM-T easy and sequenced. Analysis of 10 clones revealed that the clones were all identical, suggesting the presence of a single *fks* gene in *A. niger* as also observed in other fungi. PCRs were performed in a Robocycler (Stratagene) using superTaq DNA polymerase (HT Biotechnology).

2.3. Molecular biological techniques

Chromosomal DNA of *A. niger* was isolated as described by Kolar et al. (1988). Both Southern and Northern blot analyses were carried out as described by Sambrook et al. (1989). [α-^{32}P]dCTP-labelled probes were synthesised using Rediprime II DNA labelling System (Amersham–Pharmacia Biotech) according to the instructions of the manufacturer. RNA was extracted from mycelium flash-frozen in liquid, medium using TRIzol reagent (InVitrogen). RNA glyoxal electrophoresis was performed in a SEA-2000 (Elchrom Scientific) at 10 °C. RT-PCRs were performed using the SuperScript One step RT-PCR kit from Invitrogen. In the RT-PCR, 10 ng of total RNA was used in combination with two primer sets (Table 2). Each reaction mixture contained 1× buffer, 200 μM of each dNTP, 1.5 mM $MgSO_4$, and 5 U of each reverse transcriptase and Platinum *Taq* DNA polymerase in a total volume of 50 μl. The primer concentration of the actin primer pair was fixed at 0.4 pmol μl^{-1} and a primer concentration of 0.8 pmol μl^{-1} was used for the various *ags* primer pairs. The primer concentration of the specific *ags* primers was increased to ensure similar amount of PCR product of the actin gene and the *ags* gene when genomic DNA was used as template. RT-duplex PCR cycling conditions were as follows: one cycle of cDNA synthesis at 50 °C for 30 min, one cycle of denaturation at 94 °C for 2 min, 30 cycles of denaturation for 1 min at 94 °C, annealing at 60 °C for 1 min, and extension at 72 °C for 1 min. Twenty microlitres of PCR product was separated on a 1.5% agarose gel and visualised by staining with ethidium bromide.

Table 1
Degenerated primers used in this study

Primer name	Sequence (5′–3′)	Amino acids
FKSP1for	GGN-AAY-CCN-ATH-YTN-GG	GNPILG
FKSP2rev	CC-YTT-NCC-RCA-YTG-RWA-RTA	YY/FQGKG
FKSP3for	GAY-GCN-AAY-CAR-GAY-AAY-TA	DANQDNY
FKSP4rev	CCN-GCR-WAD-ATR-TCY-TCR-TT	NEDIY/FAG
AGSAP1for	AAY-GAY-TAY-CAY-GGN-GC	NDYHGA
AGSAP2rev	WA-CCA-CCA-NCC-NGG-CAT	MPGWWF/Y
AGSAP3for	CAY-AAY-GCN-GAR-TTY-CAR-GG	HNAEFQG
AGSAP4rev	ARN-CCR-AAN-GGY-TCR-TC	DEPFGL
AGSAP5rev	CCA-NCK-NCC-NAC-RAA-NAC	VFVGRW
AGSAP6rev	AD-RTC-DAT-NCC-YTT-YTG	QKGIDL/I
AGSAP7for	TAY-CAY-RTN-AAY-GAY-TAY-CA	YHV/INDYH

Table 2
Primers and product sizes for duplex RT-PCR experiments

Primer	Sequence (5′–3′)	Product size (bp)
AbagsA-For	CCCGTCGAGACGGCTACC	258
AbagsA-Rev	CCTCATATGACCGAGTCAGAGC	
AbagsB-For	CAAATACAGGAGGTCTGCCG	277
AbagsB-Rev	CTTCAGGCTGCTTGCTCGC	
AbagsC-For	CTCAGTGCAGACGTTATGAAGC	250
AbagsC-Rev	CCTCGTTGGACTGCCATGG	
AbagsD-For	GATACTGACACTGCAAGGCG	246
AbagsD-Rev	CGATCTCACTGTCTTTCGGC	
AbagsE-For	TTGCTCCGTGTTCAATCTGG	247
AbagsE-Rev	CCTGTTCCTTGCTCCACTCAC	
AbactA-For	ATTGTCGGTCGTCCCCGTC	gDNA 387
AbactA-Rev	CCTGGATGGAGACGTAGAAGG	cDNA 313

2.4. Growth and cell wall stress-inducing conditions

Fresh spores were inoculated in 50 or 100 ml CM at a spore density of 1×10^7 spores ml^{-1} and grown for 5 h at 37 °C and 300 rpm (*A. niger*) or for 6.5 h at 30 °C and 300 rpm (*P. chrysogenum*). After the spores had germinated, germlings were treated with a cell wall stress-inducing compound (200 μg ml^{-1} CFW, 50 μg ml^{-1} SDS or 12.5 μg ml^{-1} Caspofungin) by adding the compound from a freshly prepared stock solution (20 mg ml^{-1} CFW, 100 mg ml^{-1} SDS or 10 mg ml^{-1} Caspofungin), or an equal volume of water was added as a control. At specific time points after the addition of CFW, germlings were harvested rapidly using a sieve with a 20 μm aperture (Endecotts) and frozen into liquid nitrogen prior to

the isolation of RNA. Microscopical images were taken on an Axioplan 2 (Zeiss) equipped with a DKC-5000 (Sony) digital photo camera using DIC settings.

Fresh *A. niger* spores were diluted in CM to a final concentration of 2×10^5 spores ml^{-1}. A series of concentrations of cell wall stress-inducing compounds (CFW, Nikkomycin and Caspofungin) were prepared in 100 µl CM in a 96-well plate (Nunc, art. 164588). One hundred microlitres of spore solution ($\sim 2 \times 10^4$ spores) was added to 100 µl CM containing the stress-inducing solution. The microtitre plates were incubated at 37 °C and the OD_{590} was measured every 2 h in a Perkin–Elmer HTS-7000 Bioassay reader.

2.5. Isolation of the genomic agsA gene

To obtain the full sequence of the *agsA* gene and its promoter sequence, an existing cosmid library containing genomic inserts of *A. niger* DNA (F. Schuren and P. Punt, TNO Nutrition) was ordered into 384-well microtitre plates. *E. coli* cells were spotted on HybondN^+ filters, which were placed on LB plates. The cells were grown for 16 h at 37 °C and lysed on the filters according to standard protocols (Sambrook et al., 1989). Out of approximately 5000 cosmids screened, seven hybridised with the *agsA* PCR fragment. Two cosmids (P13P14 and P11A6) were isolated and analysed by restriction enzyme digestions and Southern blots. Overlapping subclones pRD12, a ~12 kb *Sst*I fragment containing the 5′ part of the *agsA* gene including promoter sequences from cosmid P13P14 and p*Nco*I#7, a ~13 kb *Nco*I fragment containing the 3′ end of the *agsA* gene including termination sequences from cosmid P11A6, were constructed and partially sequenced to obtain the full-length *agsA* sequence. Sequencing was carried out with a Perkin–Elmer ABI PRISM 310 sequencer using the ABI prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems). Primers for sequencing were obtained from Isogen.

2.6. Construction of the agsA::pyrG deletion strain

The *A. niger agsA* gene was disrupted by the insertion of the *Aspergillus oryzae pyrG* gene between *Eco*RI and *Xho*I sites present in the *agsA* gene thereby deleting 31 base pairs of the *agsA* promoter sequence and the sequence coding for the first 1624 of the predicted 2395 amino acids of the open reading frame. The plasmid to disrupt the *agsA* gene was made as follows: a *Not*I–*Sst*I fragment was isolated from pRD12Δ*Sst*I. In this clone, the *Sst*I site next to the *Not*I site was missing after ligation. From this plasmid the 12 kb *Not*I–*Sst*I fragment, containing 3 kb of the upstream sequence and almost the complete *agsA* coding region was cloned into pBluescript KS Δ*Xho*I–*Sal*I, from which both the *Xho*I and *Sal*I site had been previously removed by digestion and relegation, to give pRD14. This plasmid was digested with *Not*I and *Xho*I and a 4.5 kb fragment containing the plasmid backbone together with 1.5 kb of the *agsA* sequence was isolated and used in a three-way ligation. The second fragment, containing the *pyrG* gene, was obtained as an *Eco*RI–*Sal*I fragment from plasmid pAO4-13. pAO4-13 is a derivative of pAO4-2 (de Ruiter-Jacobs et al., 1989) containing the *AopyrG* gene as a 2.8 kb *Bam*HI–*Bgl*II fragment in a *Bam*HI-opened pUC19 vector. The third fragment, consisting of a 1.5 kb of 5′ *agsA* promoter sequence, was obtained from plasmid pRD2 as a *Not*I–*Eco*RI fragment. pRD2 contains a 6.0 kb *Bgl*II–*Bgl*II fragment from cosmid P13P14 with the upstream region and part of the *agsA* coding region which was cloned into the *Bam*HI site of pBluescript KS. The *Not*I site is derived from the polylinker. Ligation of the three fragments resulted in pΔagsA which was linearised with *Not*I and used to transform AB4.1. After transformation, putative *agsA* deletion mutants were purified and pools of five transformants were analysed by PCR to identify *agsA* deletion mutants. Pools that contained a potential disruption strain were further analysed by PCR to confirm successful disruption. Four putative *agsA* deletion strains were identified and further analysed by Southern blot analysis. A 0.6 kb *Bam*HI–*Bam*HI was used as a probe to detect genomic fragments after *Nco*I digestion and a 0.9 kb *Bgl*II–*Bgl*II probe from pRD9 was used to detect fragments after *Pst*I digestion. RD9 is a *Pst*I subclone from cosmid P13P14 in pBluescript.

2.7. Nucleotide sequence accession numbers

The sequence data have been submitted to the DDBJ/EMBL/GenBank databases under accession numbers: AY530786–AY530793 and AY533027: *AbagsA* (AY530786), *AbagsB* (AY530787), *AbagsC* (AY530788), *AbagsD* (AY530789), *AbagsE* (AY530790), *PcagsA* (AY530791), *PcagsB* (AY530792), *PcagsC* (AY530793), and *AbfksA* (AY533027). Nucleotide sequence data reported are available in the Third Party Annotation Section of the DDBJ/EMBL/GenBank databases under accession numbers TPA: BK004180–BK004184: *AnagsA* (BK004180), *Ncags1* (BK004181), *MgagsA* (BK004182), *CcagsA* (BK004183), and *CnagsA* (BK004184).

3. Results

3.1. Isolation of a family of 1,3-α-D-glucan synthases from A. niger

Protein sequence alignment of 1,3-α-D-glucan synthases from *S. pombe* (Hochstenbach et al., 1998; Katayama et al., 1999) revealed the presence of conserved amino acid sequences. Based on these conserved regions, seven degenerate primers were designed (Table 1 and

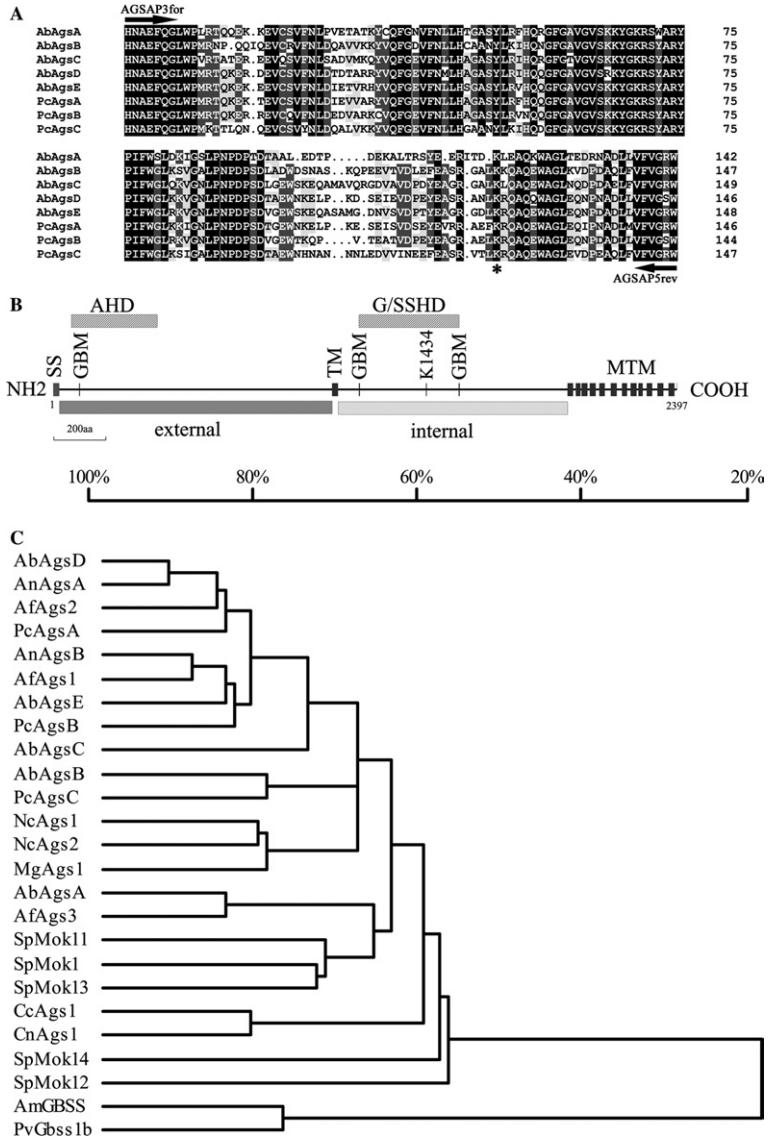

Fig. 1. (A) Alignment of eight fragments of 1,3-α-D-glucan synthase proteins from *A. niger* and *P. chrysogenum*. Amino acid residues that are identical in all protein sequences are shown in a black background. Amino acids that are ⩾75% identical are shown in dark grey background. Residues printed in a light grey background colour are identical in ⩾50% of the sequences. Protein sequence alignment was carried out using the amino acid sequences derived from PCR fragments obtained using primers AGSAP3for to AGSAP5rev (Table 1). For the *A. niger* AgsA protein this fragment corresponds to amino acids 1317–1458, which is localised in the putative internal glucan synthase domain. The conserved Lys1434 residue, which is important for the catalytic reaction in glycogen synthases (Furukawa et al., 1994) is indicated with an asterisk (∗). (B) Schematic representation of the *A. niger* AgsA protein. Protein sequence analysis revealed an organisation typical for 1,3-α-D-glucan synthase proteins. TM, transmembrane domain; MTM, multi-pass transmembrane domain; SS, secretion signal; GBM, nucleotide glucose-binding motif (R/K-X-G-G); AHD, amylase homologous domain; G/SSHD, glycogen/starch synthase homologous domain; and K1434, conserved lysine at position 1434. (C) Dendrogram of currently available 1,3-α-D-glucan synthase proteins. The tree was made based on fragments of 1,3-α-D-glucan synthase proteins, corresponding to the amino acid sequences between the AGSAP3for and AGSAP5rev fragments. As out-group two starch synthases were chosen. AbAgsA (AY530786), AbAgsB (AY530787), AbAgsC (AY530788), AbAgsD (AY530789), AbAgsE (AY530790), PcAgsA (AY530791), PcAgsB (AY530792), PcAgsC (AY530793), AfAgs1 (AAL28129), AfAgs2 (AAL18964), AfAgs3 (a_fumigatus|chr_0|Sanger.Af0121f02.plc|70 ATG on 1915297, sequence data for *Aspergillus fumigatus* were obtained from The Institute for Genomic Research website at http://www.tigr.org. Sequencing of *A. fumigatus* was accomplished with support from the National Institute of Allergy and Infectious Diseases), AnAgsA (BK004180), AnAgsB (EAA63275), NcAgs1 (BK004181), NcAgs2 (XM_328837), CnAgsA (BK004184), CcAgsA (BK004183), MgAgsA (BK004182), SpMok1 (Q9USK8), SpMok11 (Q09854), SpMok12 (Q9UUL4), SpMok13 (Q9Y719), SpMok14 (Q9Y704), AmGBSS (AAC70779), and PvGbss1b (BAC76613).

Fig. 1A) to isolate fragments of 1,3-α-D-glucan synthase-encoding genes from *A. niger* and *P. chrysogenum*. This approach resulted in the isolation of four different fragments (*agsA–D*) for *A. niger* with high sequence identity (ranging from 63 to 66%) to the *S. pombe* Ags1 protein. A fifth 1,3-α-D-glucan synthase gene was identified in the

DSM *A. niger* genome sequence (65% identity to Ags1) and named *agsE* (DSM, pers. communication). Three different fragments (*agsA–C*) with high sequence identity (between 66 and 67%) to Ags1 were isolated from *P. chrysogenum* (Figs. 1A and C). All three fragments were isolated multiple times during the isolation procedure, but formally this does not exclude the presence of additional 1,3-α-D-glucan synthase-encoding genes in *P. chrysogenum*.

Recently, several fungal genomes have become available. We have analysed the genomes of *A. fumigatus*, *A. nidulans*, *Neurospora crassa*, *Magnaporthe grisea*, *Coprinus cinerea*, *C. neoformans*, *Fusarium graminearum*, and *Ustilago maydis* for the presence of 1,3-α-D-glucan synthase genes (http://www.tigr.org and http://www.broad.mit.edu) using the *S. pombe* Ags1 protein sequence as a query sequence. Hits from BLASTP or TBLASTN searches with an *E* value of $<10E^{-20}$ were considered as putative α-1,3-D-glucan synthases. A varying number of homologs are present in the different genomes ranging from one in *M. grisae*, *C. neoformans* and *C. cinerea*, two in *A. nidulans* and *N. crassa*, and three in *A. fumigatus* (Fig. 1C). No *ags* homologs were found in the *F. graminearum* and *U. maydis* genomes. Although most genomes have been intensively sequenced (>10× coverage), the actual number of *ags* homologs might increase upon completion of the genome sequencing projects. From this analysis it is clear that *A. niger* contains a relatively large number of putative 1,3-α-D-glucan synthases compared to other filamentous fungi. The gene encoding the 1,3-β-D-glucan synthase subunit (*AbfksA*) homolog was also cloned from *A. niger*. We will refer to *A. niger* genes by including the prefix *Ab* which stands for *Aspergillus* black, to prevent confusion with *A. nidulans* genes. Like in other fungi such as *A. nidulans*, *A. fumigatus* and *N. crassa* (Beauvais et al., 2001; Galagan et al., 2003; Kelly et al., 1996), the *A. niger* genome contains a single *fks1* gene (Dr. G. Groot, DSM, personal communication).

3.2. Expression of agsA is induced upon cell wall stress

Earlier observations indicated that the synthesis of 1,3-α-D-glucan levels in the cell wall of fungi increased upon cell wall stress (Mellado et al., 2003; Seo et al., 1999). To examine the expression levels of the different *ags* genes in response to cell wall stress, spores were allowed to germinate for 5 h and challenged with the antifungal compound Calcofluor White (CFW). In *A. niger*, as in other *Aspergillus* species (Momany, 2002), spore germination starts with swelling of the fungal spore and subsequent germination. At 37 °C the swelling process lasts approximately 4 h before a small germ tube is formed. This process is highly synchronised, since over 95% of the spores have formed an equally sized germling after 5 h (Fig. 2A). Treatment of germinated spores with CFW resulted in the formation of swollen hyphae and large round hyphal tips (Fig. 2F). CFW is known to bind to glycan fibres in the cell wall and prevents their crystallisation or crosslinking to other cell wall components (Roncero and Duran, 1985). CFW is therefore thought to weaken the cell wall, resulting in swelling of the cells from internal turgor pressure as has also been described for Congo Red (Pancaldi et al., 1984). In our experiments, both the extent of hyphal tip swelling and the time that was required to resume growth were found to be dependent on the concentration of CFW. In our experimental set-up, spores were inoculated (1×10^7 spores ml^{-1} CM), grown for 5 h, and subsequently stressed with 200 μg ml^{-1} CFW. Morphologically, this resulted in an arrest of polarised growth and swelling of hyphal tips (Fig. 2F). After 3 h, polarised growth resumed, indicating that the cells could overcome the effect of CFW and that the concentration of CFW that was used here was not deleterious to the cells in the longer term (Fig. 2H). At different time points during CFW stress, RNA was isolated. The expression of the different *ags* genes was examined in germinating spores that had been treated with CFW for 1 h, using duplex RT-PCR (Fig. 3). Actin

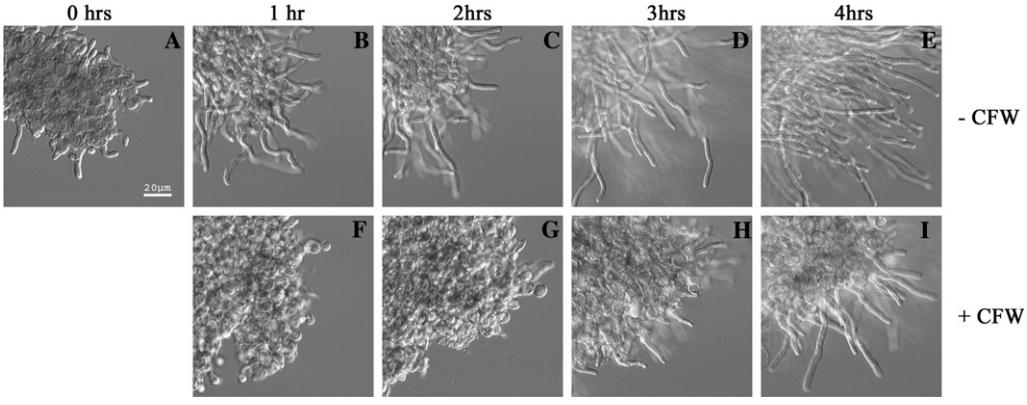

Fig. 2. DIC microscope images of Calcofluor White (CFW)-stressed *A. niger* germlings. Spores were inoculated and pre-grown for 5 h at 37 °C (A). After 5 h, the germlings were stressed with CFW (200 μg ml^{-1}, bottom panels F–I), or the same volume of water was added (top panels, B–E). Images were made after 1 h (B,F), 2 h (C,G), 3 h (D,H), and 4 h (E,I) with or without CFW.

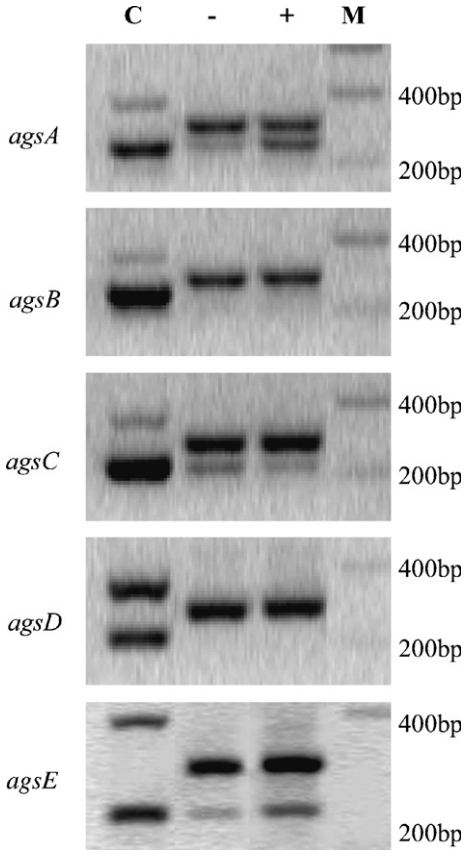

Fig. 3. Expression levels of *AbagsA* and *AbagsE* are induced during CFW stress. Expression levels of the five *ags* genes were determined with RT-PCR using specific primers for each *ags* gene. As an internal control, each RT-PCR also contained a primer pair specific for the *A. niger* actin gene. The RT-PCR was also performed with genomic DNA as a template to demonstrate the efficiency of primers in the RT-PCR and to show the absence of genomic DNA in the RNA sample (C). The product of the RT-PCR on genomic DNA is larger (387 bp) than the RNA-derived product (313 bp) because the actin primers were designed around an intron. Germlings were pre-grown for 5 h at 37 °C, subsequently, water (−) or 200 μg CFW ml^{-1} (+) was added. RNA was isolated after 1 h and used in a RT-PCR. Marker lane (M) showing the 200 and the 400 bp bands. Sizes of the different *ags* products are shown in Table 2.

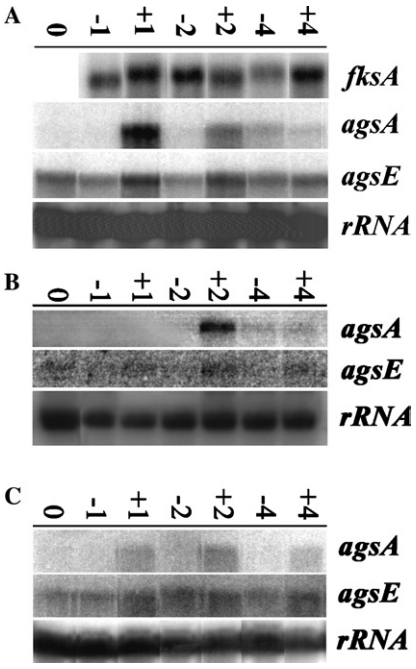

Fig. 4. Expression analysis of *ags* genes in response to cell wall stress-inducing agents. (A) Northern analysis of *A. niger* germlings during CFW stress. *A. niger* spores were pre-grown for 5 h at 37 °C and water or CFW (200 μg ml^{-1}) was added to the formed germlings. RNA was isolated after 1, 2, and 4 h. The messenger levels of *AbfksA* (1,3-β-D-glucan synthase), *AbagsA*, and *AbagsE* were determined. rRNA hybridisation was performed to control the loading. (B) Northern analysis of *A. niger* germlings subjected to SDS stress (50 μg ml^{-1}). (C) Northern analysis of *A. niger* germlings subjected to Caspofungin (12.5 μg ml^{-1}).

was used as a control for the amount of mRNA present in the sample and as a marker for genomic contamination. The actin primer pair was selected around an intron region. Whereas amplification from genomic DNA would result in the amplification of a 387 bp fragment, amplification from cDNA would result in a fragment of 313 bp in size. The RT-PCR results showed that the expression of *agsA* was strongly induced 1 h after the addition of CFW. Also the expression of *agsE* was induced, but to a lesser extent. The expression level of *agsC* seems to decline after CFW addition. (Fig. 3). No expression of *agsB* and *agsD* was detected in these RNA samples. The results from the RT-PCR experiment were confirmed by Northern blot analysis to quantify the induction of expression. Quantification of messenger levels revealed >20-fold induction of the *agsA* gene after 1 h of CFW stress and a 2- to 3-fold induction of the *agsE* gene (Fig. 4). The expression of *agsC* could not be detected by Northern blot analysis, probably because of its low expression level. Additional Q-RT-PCR experiments did not reveal a significant reduction of *agsC* expression in response to CFW (data not shown). The level of *agsA* mRNA decreased again after 2 h and was back to the unstressed level after 4 h. The expression level of the 1,3-β-D-glucan synthase subunit *fksA* was not affected by the presence of CFW (Fig. 4A).

Induction of *agsA* and *agsE* was not limited to CFW. The addition of SDS, which is also known to destabilise the cell wall at low concentrations (de Groot et al., 2001; Delley and Hall, 1999; de Nobel et al., 2000), also induced the expression of *agsA* and *agsE* (Fig. 4B). Caspofungin, a specific 1,3-β-D-glucan inhibitor also gave an increase in both *agsA* and *agsE* messenger levels (Fig. 4C). It should be noted that the induction reached its highest level 2 h after addition of SDS and Caspofungin, whereas the induction after CFW stress peaked at 1 h.

3.3. Molecular cloning of the agsA gene

Using the *agsA* PCR fragment as a probe, genomic clones were isolated from a cosmid library of the *A. niger* strain N402 and the complete sequence of *agsA* was

determined. The gene contains an open reading frame of 7393 bp which is interrupted by three introns and encodes a 2397-amino acid protein with a calculated MW of 268.9 kDa. Comparison of the AgsA protein sequence with the Ags proteins described in *S. pombe* reveals several conserved features. As in the SpAgs1 protein sequence, three distinct domains can be identified in the AbAgsA sequence (Fig. 1B). The first domain is flanked by two hydrophobic amino acid sequences. The first N-terminal 23 amino acids represent a putative secretion signal (Nielsen et al., 1997). The second hydrophobic region (aa1073–1095) is strongly predicted to form a transmembrane domain (Krogh et al., 2001). The region between these two hydrophobic sequences, which is predicted to be located extracellularly, shows significant sequence similarities to α-amylases belonging to family 13 of the glycosyl hydrolases (Henrissat, 1991) and is denoted as an amylase homologous domain (AHD). Therefore, it has been suggested that this domain contains transglucosylation activity and is involved in remodelling newly formed α-glucan or cross-linking it to the existing cell wall (Hochstenbach et al., 1998). This domain shows 39% sequence identity to the same domain from the SpAgs1 protein. The second domain (aa1096–1976) is also bordered by two hydrophobic sequences (aa1073–1095 and aa1977–1999) and has 42% sequence identity to the same domain from SpAgs1. This part of the protein is predicted to be intracellular and contains a glycogen or starch synthases homologous domain (G/SSHD). Therefore, it is likely that it encodes the glucan synthase domain of AbAgsA. The third domain (aa2000–2397) is predicted to span the membrane 11 times. This multi-pass transmembrane region (MTM) is thought to be involved in the transport of the α-glucan chain across the plasma membrane. The Lys1434, which has been reported to be important for the catalytic reaction in bacterial glycogen synthases (Furukawa et al., 1994), is also present in AbAgsA. This MTM domain has 44% sequence identity to the MTM domain from SpAgs1. Other characteristics are three glucose-binding motifs (GBM) at residues 100, 1176, and 1559 which match the consensus (R/K-X-G-G).

The domain structures of 1,3-α-D-glucan synthases identified in the genomes of other fungi (Fig. 1B) are also highly conserved. In all Ags proteins the three glucose-binding motifs are conserved. The AbAgsA Lys1434 amino acid residue is conserved in most fungi, except in MgAgsA and NcAgs1 and NcAgs2 where the lysine residue is substituted by an arginine residue at this position.

3.4. AgsA expression and induction is not required for survival

To investigate the effect of loss of function of the *agsA* gene in *A. niger*, a disruption plasmid (pΔagsA) was constructed as described in Materials and methods. Integration of the linearised plasmid into the genome at the *agsA* locus is predicted to result in the deletion of 31

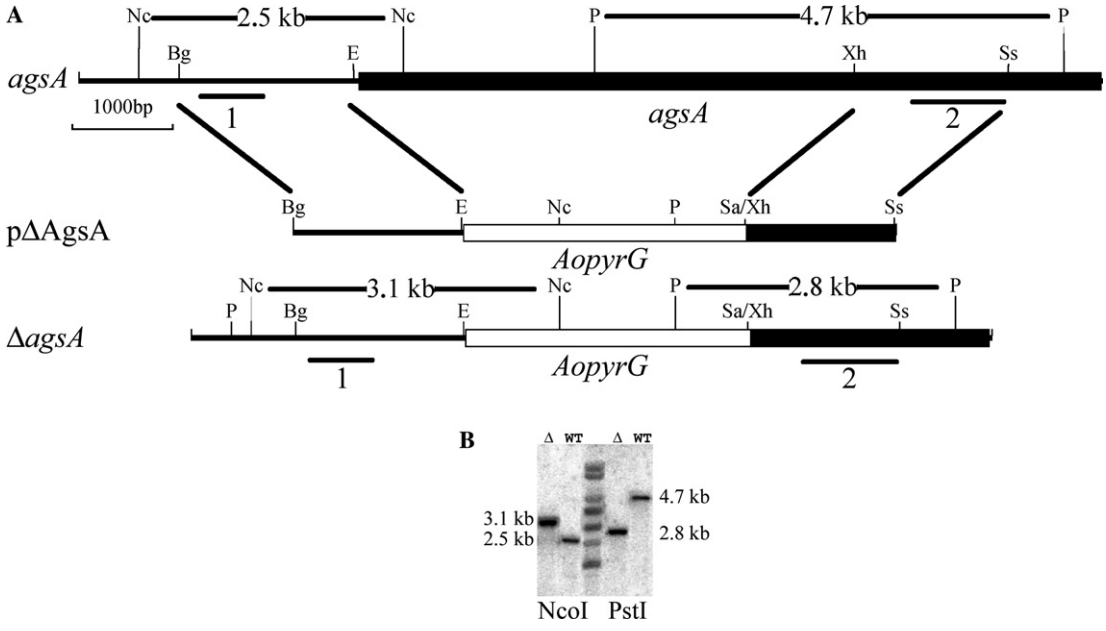

Fig. 5. Disruption of the *agsA* gene in *A. niger*. (A) Schematic representation of the *agsA* wild-type locus, the plasmid pΔagsA used for disruption and the deleted locus *ΔagsA*. Abbreviations Bg, *Bgl*II; E, *Eco*RI; Nc, *Nco*I; P, *Pst*I; Sa, *Sal*I; Ss, *Sst*I; Xh; *Xho*I; 1, *Bam*HI probe; and 2, *Bgl*II probe. (B) Southern blot analysis of the *A. niger* wild-type (WT) and *agsA* deletion strain (Δ).Genomic DNA was digested with *Nco*I (lanes 1 and 2) or *Pst*I (lanes 3 and 4). Digestion of wild-type DNA with *Nco*I should result in a 2.5 kb fragment and in an *agsA* deletion mutant a fragment of 3.1 kb was expected. Digestion of wild-type genomic DNA with *Pst*I should result in a 4.7 kb fragment and in the *agsA* deletion mutant a fragment of 2.8 kb is expected. Left two lanes (*Nco*I digested) are probed with *Bam*HI probe (1). The right two lanes (*Pst*I digested) are probed with *Bgl*II probe (2).

nucleotides upstream of the *agsA* start codon and the sequence coding for the first 1624 of the 2397 amino acids of the AgsA protein sequence. After transformation of pΔagsA to AB4.1, *pyrG*$^{+}$ transformants were purified and screened with PCR for putative deletion mutants. These putative mutants were further examined by Southern blot analysis to verify the deletion of the *agsA* gene (Fig. 5B). Based on the Southern blot analysis all four transformants were disrupted in the *agsA* gene and transformant MA22.4.4 was used for further analysis. The growth phenotype of an *agsA* disruption strain was compared to a wild-type strain. The *agsA* disruption strain grew similar to the wild-type strain at different temperatures (25, 30, and 42 °C), and also conidiophore formation and hyphal morphology were indistinguishable from the wild type (data not shown). Since the expression of *agsA* was significantly induced in response to CFW stress, the CFW sensitivity of *ΔagsA* germlings was determined by two methods. First, CFW sensitivity was determined by inoculating spores in 10-fold serial dilutions on CFW-containing plates (Fig. 6A). When concentrations of $\leqslant 50\,\mu g\,CFW\,ml^{-1}$ were used, no effect on growth for both the wild-type strain and the *ΔagsA* was visible. At higher CFW concentrations, the deletion strain showed a CFW-hypersensitive phenotype compared to the wild-type strain. The CFW-hypersensitive phenotype was completely remediable by the addition of the osmostabiliser sorbitol, and to a lesser extent KCl. Second, spores from wild-type and *ΔagsA* strains were inoculated in a 96-well plate containing increasing concentrations of CFW. Fig. 6B shows that both strains had the same germination kinetics in the absence of CFW. However, the presence of 20 or $30\,\mu g\,CFW\,ml^{-1}$ in the growth medium resulted in more pronounced growth retardation of the *ΔagsA* strain compared to the wild-type strain. At $40\,\mu g\,CFW\,ml^{-1}$ the wild-type strain started to grow after 24 h whereas the *ΔagsA* strain did not grow, even after prolonged incubation. From these results, it is concluded that the increased expression of *agsA* is contributing to withstand the deleterious effect of CFW on fungal growth. Using the same assay, it was also examined whether the *ΔagsA* strain was more sensitive to nikkomycin, an inhibitor of chitin synthesis, and caspofungin, an inhibitor of 1,3-β-D-glucan synthesis in fungi, compared to the wild-type strain. Both *ΔagsA* and the wild-type strain were insensitive to nikkomycin up to concentrations of $124\,\mu g\,ml^{-1}$. The inhibitory effect of caspofungin on growth was clearly detectable at a concentration of $0.25\,\mu g\,ml^{-1}$. No difference in sensitivity was observed between the *ΔagsA* and the wild-type strain (data not shown). Finally, a possible synergistic effect of adding caspofungin and nikkomycin was exam-

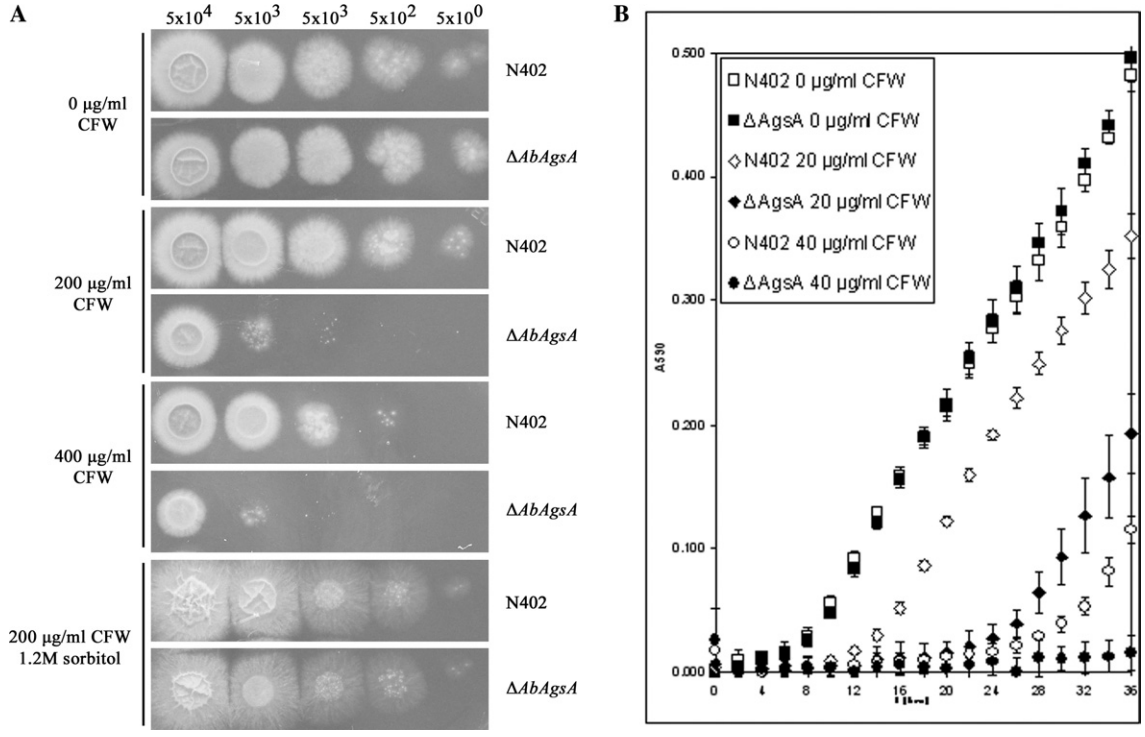

Fig. 6. Deletion of *Ab agsA* results in a sorbitol-remediable hypersensitivity to CFW. (A) CFW sensitivity of strain MA22.4.4 (Δ*AbagsA*) was compared to N402 (wild-type). Spores were serially diluted and spotted on CM plates containing the indicated CFW concentrations (the total number of spores that were spotted is indicated above the figure). Images were taken after 3 days at 37 °C. The Δ*AbagsA* strain revealed a CFW-hypersensitive phenotype which could be fully rescued by the addition of the osmostabiliser sorbitol. (B) Effect on spore germination and growth of CFW addition to wild-type and the Δ*AbagsA* strain. Results represent data from four independent experiments. Error bars in the figure represent the standard deviation.

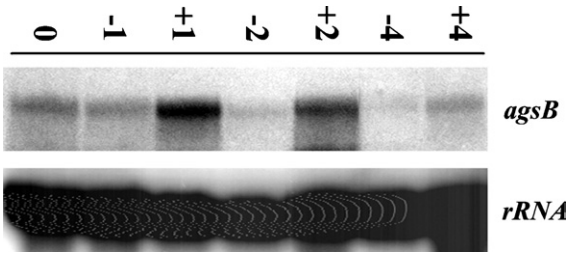

Fig. 7. Northern analysis of CFW-stressed *P. chrysogenum* germlings. Spores of *P. chrysogenum* were grown for 6.5 h at 30 °C in CM and subsequently treated with CFW (200 μg ml^{-1}). RNA was isolated at 1, 2, and 4 h after the addition of CFW. Expression levels of *agsA*, *agsB*, and *agsC* were determined using Northern blot analysis. Only messenger levels of *agsB* were detectable and these rose strongly after the addition of CFW.

ined. However, no synergistic effect of caspofungin and nikkomycin was observed for either the wild-type strain or the *ΔagsA* strain (data not shown).

3.5. Induction of 1,3-α-D-glucan synthase transcription during CFW stress in other fungi

To investigate whether the induction of an 1,3-α-D-glucan synthase-encoding gene by CFW is a general response among fungi or specific for *A. niger*, gene fragments of three 1,3-α-D-glucan synthases from *P. chrysogenum* were isolated and named *agsA*, *agsB*, and *agsC*. Since *P. chrysogenum* does not germinate at 37 °C, spores were allowed to germinate at 30 °C for 6.5 h to obtain germlings. After 6.5 h, CFW was added to a final concentration of 200 μg ml^{-1}, which led to a similar morphological response of the *P. chrysogenum* germlings as observed for *A. niger* (data not shown). After the addition of CFW, RNA was isolated at 1, 2, and 4 h. Northern analysis indicated that *PcagsB* was temporarily induced during CFW stress (Fig. 7). No messenger was detectable for *PcagsA* and *PcagsC* (data not shown). The results suggest that induced expression of an 1,3-α-D-glucan synthase-encoding gene in fungi is a general response mechanism and not restricted to *A. niger*.

4. Discussion

A family of five putative 1,3-α-D-glucan synthase genes in *A. niger* and of three 1,3-α-D-glucan synthase genes in *P. chrysogenum* have been identified. The amino acid sequence of AbAgsA revealed a similar multi-domain organisation of the protein as previously described for the Ags1 protein of *S. pombe* (Hochstenbach et al., 1998). The different domains of the 1,3-α-D-glucan synthases are predicted to be involved in synthesis, transport, remodelling, and crosslinking of 1,3-α-D-glucan to the existing cell wall. Inspection of publicly available fungal genomes revealed that some fungi have only a single *ags* gene or seem to lack any 1,3-α-D-glucan synthase-encoding gene. No *ags* homologous genes were identified in the published genome sequences of *F. graminearum* and *U. maydis*, suggesting that these fungi do not contain 1,3-α-D-glucan in their cell walls. However, 1,3-α-D-glucan has been identified in a different *Fusarium* species, *Fusarium oxysporum f.* sp. *lycopersisi* (Schoffelmeer et al., 1999), indicating that the genes have not been sequenced yet in the ongoing sequencing project, or that the presence of absence of 1,3-α-D-glucan synthase-encoding genes and the absence of presence of 1,3-α-D-glucan can differ within the genus of *Fusarium*. The absence of 1,3-α-D-glucan synthase genes in *U. maydis* is consistent with the observation that no 1,3-α-D-glucan has been identified in the Ustilaginomycetes. The occurrence of single *ags* genes is not limited to the basidiomycetes *C. neoformans* and *C. cinerea*, but is also found in the genome of the ascomycete *M. grisae.* Currently, it is not clear why some ascomycetes have only a single *ags* gene (e.g., *M. grisae*), while others contain multiple genes (e.g., *P. chrysogenum* and *A. niger*).

What might be the reason for some fungi to have multiple 1,3-α-D-glucan synthases? In general, the existence of multiple gene families can have several reasons: (i) the cell or organism wants to regulate the process precisely and therefore is able to induce or repress the expression of specific members according to internal or environmental triggers, families of multiple genes that encode proteins with similar activities allow such a precise regulation, (ii) the proteins encoded by the various genes make related products with different properties, (iii) combination of these two reasons. In this study, we show that the expression of *AbagsA* is specifically induced in response to cell wall stress. The expression of *AbagsA* is hardly detectable under normal growth conditions, but increases significantly in case of cell wall stress. We therefore believe that the *AbagsA* geneproduct acts as a kind of cell wall repair enzyme in response to cell wall damage. In addition to the induction of *AbagsA*, we also observed an induction of *AbagsE* in response to CFW stress. We consider *AbagsE* as the major 1,3-α-D-glucan synthase gene expressed during vegetative growth. The function of *AbagsE* both during vegetative growth and in response to cell wall stress is currently being investigated by constructing an *AbagsE* deletion strain. Expression of *AbagsB*, *AbagsC*, and *AbagsD* was not detectable on Northern blot during vegetative growth. The use of the more sensitive RT-PCR method showed that *AbagsC* is expressed at a low level. Expression analysis of the *ags* genes during conidiophore development showed that *AbagsD* is induced during conidiophore forming conditions, suggesting a specific role for *AbagsD* during asexual conidiophore development (data not shown). It therefore seems likely that the different *ags* genes are expressed differentially during the fungal life cycle and in relation to environmental conditions.

Do the different Ags proteins make different forms of 1,3-α-D-glucan? *A. niger* is known to synthesise both pseudonigeran, an 1,3-α-D-glucan polymer with some α-1,4-linkages (Horisberger et al., 1972; Johnston, 1965), and nigeran, which consists of alternating α-1,3- and α-1,4-linked glucose residues (Barker et al., 1953, 1957). It is currently unknown whether any of the five *ags* genes identified in this study are responsible for the synthesis of one of either polymer. Pseudonigeran is most similar to the 1,3-α-D-glucan identified in *S. pombe*, which might suggest that the *ags* genes are at least involved in the synthesis of this polymer. Whether the *ags* genes are involved in the synthesis of nigeran is not known and awaits further study.

Cell walls of submerged grown *A. niger var. awamori* contain approximately 4% of their dry weight as hot water-extractable nigeran. Transfer of the mycelium to nitrogen free medium resulted in a dramatic increase in nigeran content of the cell wall reaching 28% of the dry weight (Bobbitt et al., 1977), indicating that the synthesis of 1,3-α-D-glucan is dependent on growth conditions. In *A. oryzae*, an 1,3-α-D-glucan synthase gene was identified of which the expression was specifically present when the fungus was growing in submerged culture, but absent in RNA isolated from solid-medium culture (Akao et al., 2002). Expression of an 1,3-α-D-glucan synthase has also been shown to be linked with hyphal morphology. Based on these observations it is very likely that fungi are using the various *ags* genes under different growth conditions and that this is one of the reasons to have multiple genes. Whether the different Ags proteins are involved in the synthesis of different forms of α-glucan remains to be seen and will require the systematic disruption of the various genes in combination with biochemical analysis of both the α-glucan content in the cell wall and its structure.

The function of α-glucan in the fungal cell wall is controversial. In *S. pombe*, disruption of a single 1,3-α-D-glucan synthase gene (*ags1* or *mok1*) is lethal (Hochstenbach et al., 1998; Katayama et al., 1999). The phenotype of the *ags1* temperature-sensitive mutants strongly suggests that α-glucan has an important function in determining cell shape and protects the fungal cell from lysis, indicating that α-glucan is essential for fission yeast (Hochstenbach et al., 1998). In *A. nidulans*, complete inhibition of α-glucan synthesis by adding 2-deoxyglucose does not have a severe growth effect, but abolishes the formation of cleistothecia, indicating that α-glucan synthesis is predominantly required for cleisthothecium development (Zonneveld, 1973). Indeed, *A. nidulans* mutants lacking 1,3-α-D-glucan in their cell walls are viable (Polacheck and Rosenberger, 1977), indicating that under normal growth conditions α-glucan is not an essential component of the cell wall in *A. nidulans*. In *C. neoformans* 1,3-α-D-glucan is required to anchor the polysaccharide capsule to the cell wall. Using RNA interference it was shown that a 93% reduction of *ags1* mRNA levels resulted in an acapsular phenotype, but also strongly affected the growth rate, especially at higher temperatures (Reese and Doering, 2003). In *A. fumigatus*, disruption of two of the three *ags* genes also resulted in a reduction of growth rate (Bernard and Latge, 2001). In both cases, residual α-glucan synthase activity might be present, which at this moment makes it difficult to conclude whether α-glucan synthesis is essential in these fungi.

Disruption of the *AbagsA* gene does not have an affect on growth under normal conditions. Since the gene is induced upon the presence of compounds that affect cell wall integrity we examined whether *AbagsA* is required under those conditions. Therefore, the sensitivity towards CFW was measured using two methods. In both methods, the use of agar plates with increasing concentrations CFW and a microtitre plate-based growth assay revealed that the *AbagsA* disruption stain is more sensitive to CFW than its parental strain. As mentioned, we also observed an induced expression of the *AbagsE* gene in response to the presence of CFW. Using Q-RT-PCR we have examined the expression level of *AbagsE* in a Δ*AbagsA* background in order to determine whether the fungal cell is compensating for the loss of *AbagsA* via a higher expression of *AbagsE*. The results so far, indicate that the expression levels of *AbagsE* are not further induced in the absence of *AbagsA*, even under cell wall stress-inducing conditions (vanKuyk and Ram, unpublished).

In this study, we have shown that the expression of *agsA*, encoding a putative 1,3-α-D-glucan synthase gene, is induced in response to different forms of cell wall stress, suggesting that increased 1,3-α-D-glucan deposition in the fungal cell wall is part of the fungal cell wall remodelling mechanism in response conditions that are likely to affect normal cell wall synthesis or assembly. We have recently shown that the cell wall remodelling response is not limited to 1,3-α-D-glucan and that the cell wall stress response in *A. niger* also involves an increased deposition of chitin in the cell wall (Ram et al., 2004). Our observations suggest that cell wall stress in filamentous fungi generally leads to the activation of both chitin and 1,3-α-D-glucan biosynthesis.

Acknowledgments

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