The cell wall of the filamentous fungus Aspergillus niger

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General introduction

Introduction

About 70.000 fungal species have been described and it is estimated that about 1.5 million species may exist (Hawksworth, 1991, 1995). Within the fungal group there is a large diversity in habitat preference. Some fungal species feed on dead and decaying organisms (saprophitic fungi), while others are found inside or on a living host (biotropic). Also the interaction between the fungus and the environment can be very diverse, ranging from beneficial (symbiotic) to harmful (pathogen). In our research we used the eukaryotic ascomycete *Aspergillus niger* as model organism. *A. niger* is a filamentous fungus (hyphal growth) and distributed worldwide (Abarca *et al.,* 2004). The fungus can grow on a large diversity of substrates and is considered as a common food spoilage fungus (Pitt and Hocking, 1997). Over the last decade 16 fungal genomes have been sequenced, including the genome of *A. niger*, and more then 50 are in progress (Bernal *et al*., 2001). Although most fungal genome sequences were made freely available, the genome of *A. niger* has not been published yet.

A. niger as a production host

A. niger has been used to produce citric acid for 80 years and is currently the primary source of commercial citric acid production (Magnuson and Lasure, 2003). The enormous secretion capacity of this fungus led to the use of *A. niger* as general production organism for various food enzymes such as glucose oxidase, pectinase, α-amylase, and glucoamylase. The fungus has the Generally Recognised As Safe (GRAS) status from the United States Food and Drug Administration (FDA) allowing its use in (food) enzyme production (Bigelis and Lasure, 1987).

A. niger as an opportunistic fungal pathogen

Asperillus species are very common fungi in the human environment. Airborne spores can enter the human body by inhalation and even in non-immunocompromised patients the fungus can cause infection of the lungs, sinuses and other sites. At least 20 species of *Aspergillus* have been reported to cause human disease, including *A. niger* (Denning, 1998). *A. niger* has also been reported to cause an infection of the outer ear (otomycosis) in tropical and subtropical regions (Kaur *et al*., 2000). The fungus produces many secondary metabolites. Only one secondary metabolite, ochratoxin A, which is a nephrotoxic mycotoxin produced by 3-10% of the *A. niger* strains (Abarca *et al*., 1994) is considered harmful. However *A. niger* is generally regarded as benign (reviewed by Schuster *et al*., 2002).

The fungal cell wall

The shape of the fungus is determined by its cell wall. This component is essential to the fungus. By enclosing the cell, the cell wall protects the fungus from its environment and prevents it from lysing. The general view of the cell wall has changed through time from being a rigid structure, and able to withstand the turgor pressure, to a more dynamic one, being a structure that is able to adapt to various conditions (e.g. growth, development and stress) (Smits *et al*., 1999, Klis *et al*., 2002).

Architecture and composition

The cell wall of most filamentous fungi and yeasts consists of three mayor components: chitin, glucans, and mannoproteins. The yeast cell wall composition and architecture has been studied in most detail in *Saccharomyces cerevisiae* (Klis *et al*., 1998). The cell wall of this yeast is composed of: i) glucans (~ 60 % of the cell wall dry weight) which can be divided into the main polymer, β-1,3-glucan, which makes up ~ 55 % of the cell wall dry weight (~ 20 % alkali-soluble and ~ 35 % alkali-insoluble, chitin linked), and the second polymer, β-1,6-glucan that makes up ~ 5 % of the cell wall dry weight, ii) chitin, a β-1,4-linked homopolymer of N-acetylglucosamine residues, which is a minor component in the yeast cell wall accounting for only ~ 1–2 % of the cell wall dry mass, and iii) mannoproteins (~ 40 % of the cell wall dry weight). Also the cell wall architecture is well studied. The cell wall is a layered structure as shown by electron microscopy (Osumi, 1998). The inner, most electron dense layer consists mainly of β-1,3-glucan. The β-1,3-glucan is branched with β-1,6-linkages at its branching points (Manners *et al*., 1973a and b). Both chitin chains, at the inner layer, and β-1,6-glucosylated mannoproteins at the outer layer, are covalently linked to β-1,3-glucan forming a supramolecular complex (Kollar *et al*., 1997). The chitin in *S. cerevisiae* is mainly found in bud scars and only a small amount of the total chitin content (2 %) is found in the lateral walls (reviewed by Cabib *et al*., 1993).

The cell wall architecture of filamentous fungi, when observed under the electron microscope resembles the yeast cell wall architecture. It also has a layered structure (Schoffelmeer *et al*., 1999). The inner electron dense layer is composed of chitin and glucan, which are connected to the less electron dense layer composed of mannoproteins. The cell wall composition of filamentous fungi (Fig. 1), including *A. niger*, when compared to *S. cerevisiae* is generally more chitin rich (10-15 %), and contains additional polymers like α-1,3-glucan or α-1,3-α-1,4 glucan polymers (10-35 % Fontaine *et al*., 2000, de Nobel *et al*., 2000a), components not found in *S. cerevisiae* (Lipke and Ovalle, 1998) and *Candida albicans* (Klis *et al*., 2001). The presence of the α-1,3-glucan polymer was reported in many fungal species (see for a complete list Grün, 2003).

The cell wall surface of the fungal cell wall is covered with mannoproteins, determining the surface properties of the cell wall. These mannoproteins can be divided into different classes based on their linkage and extractability: i) SDS-extractable cell wall mannoproteins, which are bound to the cell via hydrogen bonds, ii) β-mercaptoethanol/DTT-extractable cell wall mannoproteins which are attached covalently to the cell wall via disulphide-bonds (Cappellaro *et al*., 1994, 1998) or iii) cell wall mannoproteins that are covalently linked to the glucan part (β-1,3- or β-1,6-glucan) of the cell wall. For *S. cerevisiae* two different classes of glucan-linked cell wall mannoproteins have been described, the protein with internal repeats (PIR)-class and the glycosylphosphatidylinositol (GPI) linked class.

The first class of glucan-linked cell wall mannoproteins, consisting of members that belong to the protein with internal repeats (PIR)-family (Toh-e *et al*., 1993) can be liberated from the cell wall after mild-alkali treatment (Mrsa *et al*., 1997). The PIR proteins contain repeats that consist of a 19 amino acid sequence (core sequence: Q[IV][STGNH]DGQ[LIV]Q) and the number of repeats varies between the different PIR proteins (Toh-e *et al*., 1993). Additionally, all PIR proteins contain an N-terminal signal peptide, a Kex2p protease cleavage site, and a conserved cysteine motif (Klis *et al*., 2002). The Pir4p/Ccw5p contains only a single PIR motif and removal of this motif results in the loss of covalent linkage to the cell wall, indicating that this sequence is required for the covalent linkage of Pir4p/Ccw5p to the cell wall (Castillo *et al*., 2003). PIR proteins are most likely linked to the β-1,3-glucan part of the cell wall, but the exact way how PIR proteins are linked to the cell wall is still under investigation (Mrsa and Tanner, 1999, Castillo *et al*., 2003).

The second class of glucan-linked cell wall mannoproteins are attached to the cell wall through glycosylphosphatidylinositol (GPI) linkages (Lu *et al*., 1994, Montijn *et al*., 1994, Kapteyn *et al*., 1995, 1996). GPI-anchored proteins are found in all eukaryotes from fungi to mammals. The general structure of the GPI anchor is well known, and the core structure is highly conserved. It consists of subsequently linked ethanolamine phosphate, trimannoside, glucosamine and inositol phospholipids (Ferguson and Williams, 1988 and Fig. 1). However some variation exists between different species (Ikezawa, 2002, Fontaine *et al*., 2003). The GPI anchor synthesis takes place in the endoplasmic reticulum (ER) by a pathway consisting of ~ 10 reaction steps and in which ~ 20 proteins are involved (reviewed by Kinoshita and Inoue, 2000). The GPI-anchored cell wall proteins (GPI-CWPs) contain a hydrophobic sequence of 15-30 residues long, at their C-terminus that acts as a GPI-anchoring signal. GPI-anchor addition takes place in the ER where the hydrophobic domain is replaced by the pre-assembled GPI-anchor (Orlean *et al*., 1997). After transport through the secretory pathway and arrival at the plasma membrane, the GPI-anchor is processed and attached to

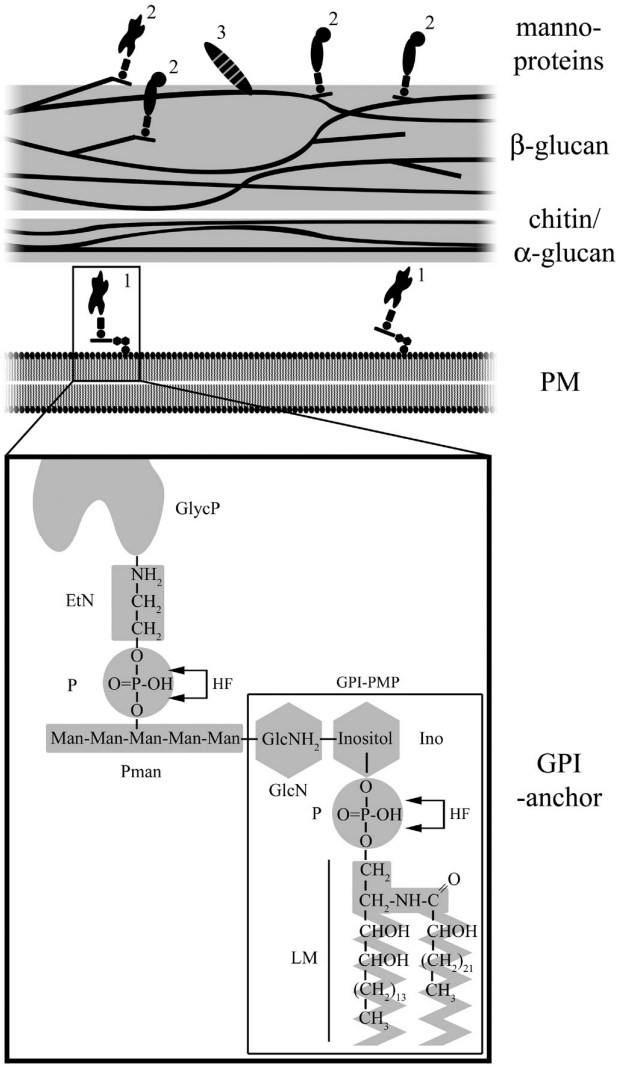

Figure 1. Schematic representation of the fungal cell wall. The cell wall is composed of chitin, glucans and mannoproteins. The proteins depicted represent: GPI-anchored plasma membrane proteins (1), GPI-anchored cell wall proteins (2), and PIR proteins (3). An enlargement of the structure of a GPI-anchored plasma membrane protein (GPI-PMP) is shown. The boxed part within the enlargement is only found in GPI-PMPs. GPI-anchored cell wall proteins (GPI-CWPs) are processed in a still unknown way, removing the part only found in GPI-PMPs and linking them to the cell wall. Abbreviations used: plasma membrane (PM), glycoprotein (GlycP), ethanolamine (EtN), hydrofluoric acid (HF), pentomannose (Pman), glucosamine (GlcN), inositol (Ino), lipid moiety (LM), phosphate (P). Among fungi some variation has been reported, like the absence of chitin or α-glucan. (Adapted from Ikezawa, 2002, Fontaine *et al*., 2003, and Grün, 2003)

β-1,6-glucan (Montijn *et al*., 1994, Kollar *et al*., 1997). GPI-CWPs are further characterized by the presence of a hydrophobic N-terminal signal sequence for import into the ER, and are often heavily O-glycosylated. The GPI-CWPS can be removed from the cell wall by enzymatic and chemical treatments. Both β-1,3- and β-1,6-glucanases liberate GPI-CWPs from the cell wall (Kapteyn *et al*., 1996). In addition, GPI-CWPs can be extracted from the cell wall by treatment with hydrofluoric acid (HF), which cleaves the phosphodiester bonds in the GPI-anchor (Kapteyn *et al*., 1996, de Groot *et al*., 2004).

Cell wall biosynthesis

Chitin synthesis is catalysed by synthases that transfer N-actetylglucosamine (GlcNAc) from UDP-N-acetylglucosamine (UDP-GlcNAc) to the newly synthesized polysaccharide. In *S. cerevisiae* three chitin synthases, encoded by *CHS1*, *CHS2*, and *CHS3*, have been identified (reviewed by Cabib *et al*., 2001). In filamentous fungi also multiple chitin synthases were identified (up to seven in *Aspergillus fumigatus*) and have been reported to be involved in lateral wall biosynthesis, septum synthesis, and spore formation (Bulawa, 1993, Munro and Gow, 2001, Reviewed by Roncero, 2002). The hexosamine biosynthetic pathway leading from fructose-6-phosphate to UDP-N-acetylglucosamine, the chitin precursor, consists of five steps and is conserved in lower and higher eukaryotes, as well as in bacteria. The rate of UDP-N-acetylglucosamine synthesis, and thereby the chitin synthesis, is determined by Gfa1p, a glutamine:fructose-6-phosphate amidotransferase, involved in the formation of glucosamine-6-P from glutamine and fructose-6-phosphate (Lagorce *et al*., 2002, Terashima *et al*., 2000).

β-1,3-glucan synthesis is catalysed by the β-1,3-glucan synthase. In *S. cerevisiae* this is performed by the subunit Fks1p and the alternate subunit Fks2p (reviewed by Douglas, 2001). Fks1p and Fks2p are large proteins with 16 putative membrane spanning domains (Mazur *et al*., 1995). These plasma membrane localized enzymes are thought to synthesise the β-1,3-glucan intracellular and facilitate the translocation of the newly synthesized β-1,3-glucan into the extracellular space (Inoue *et al*., 1996). *In vitro* synthesis of the polymer was achieved by adding UDP-glucose, GTP, glycerol and bovine serum albumin to the purified protein at pH 8.0. The polymer is present in the cell wall as 1,3-linked β-1,3-glucan with some β-1,6 linked branches. Some studies on β-1,3-glucan synthases from filamentous fungi (e.g. *Neurospora crassa*, *Aspergillus nidulans*, *Aspergillus fumigatus*, *Cryptococcus neoformans*, *Paracoccidioides brasiliensis*) have been performed (Hrmova and Seliternnikoff, 1989, Kelly *et al*., 1996, Beauvais *et al*., 1993, Thompson *et al*., 1999, Pereira *et al*., 2000). Rho1p, a small GTPase, was identified to be the key regulator of Fks1p activity (Yamochi *et al*., 1994,

Drgonova *et al*., 1996, Arellano *et al*., 1996), which in its turn can be stimulated by the addition of GTP.

The genes involved in β-1,6-glucan synthesis have been characterized in *S. cerevisiae*, based on the resistance of β-1,6-glucan muntants towards killer toxin (Roemer *et al*., 1994, Shahinian and Bussey, 2000, Levinson *et al*., 2002). However, the genes encoding proteins that could act as β-1,6-glucan synthases have not been identified yet. β-1,6-glucan is found in the walls of many fungi as a β-1,6-glucan polymer with β-1,3 branches and responsible for linking cell wall proteins to chitin and β-1,3-glucan (Kapteyn *et al*., 1999).

The α-1,3-glucan polymer is synthesized by α-1,3-glucan synthases. The presence of this polymer has been reported for many fungi among which *Aspergillus nidulans* (Bull 1970, Zonneveld 1971, Zonneveld 1972), *A. niger* (Johnston, 1965, Horisberger *et al*., 1972), *A. 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). In *A. niger* two different α-glucan polymers have been identified. One of them, nigeran, was isolated as a hot-watersoluble, linear, alternating α-1,4-1,3-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 α-1,3-glucan polymer with some (3-10 %) α-1,4-linkages (Johnston, 1965, Horisberger *et al*., 1972). In *A. nidulans*, α-1,3-glucan synthesis has been mainly studied in relation to cleistothecium formation. Zonneveld (1972, 1974) has proposed that α-1,3-glucan accumulates during vegetative growth and is metabolised by an α-1,3-glucanase expressed during sexual development. Surprisingly, deletion of an α-1,3-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* α-1,3-glucan has been shown to be required for the anchoring of the capsule to the cell wall (Reese and Doering, 2003). The genes encoding the α-1,3-glucan synthases were first identified in *S.pombe* (Hochstenbach *et al*., 1998, Katayama *et al*., 1999) and are also identified in *A. niger* (Chapter 3).

Cell wall remodeling

As mentioned above the cell wall is a highly dynamic structure (Klis *et al*., 2002). The fungus is obligated to respond to changes in its environment by altering the composition and architecture of its cell wall. Failure to adapt will result in lysis and subsequent cell death. The cell wall remodeling mechanism has been most extensively studied in the yeast *S. cerevisiae*. To maintain the integrity of the cell wall, the fungus activates a signal transduction cascade

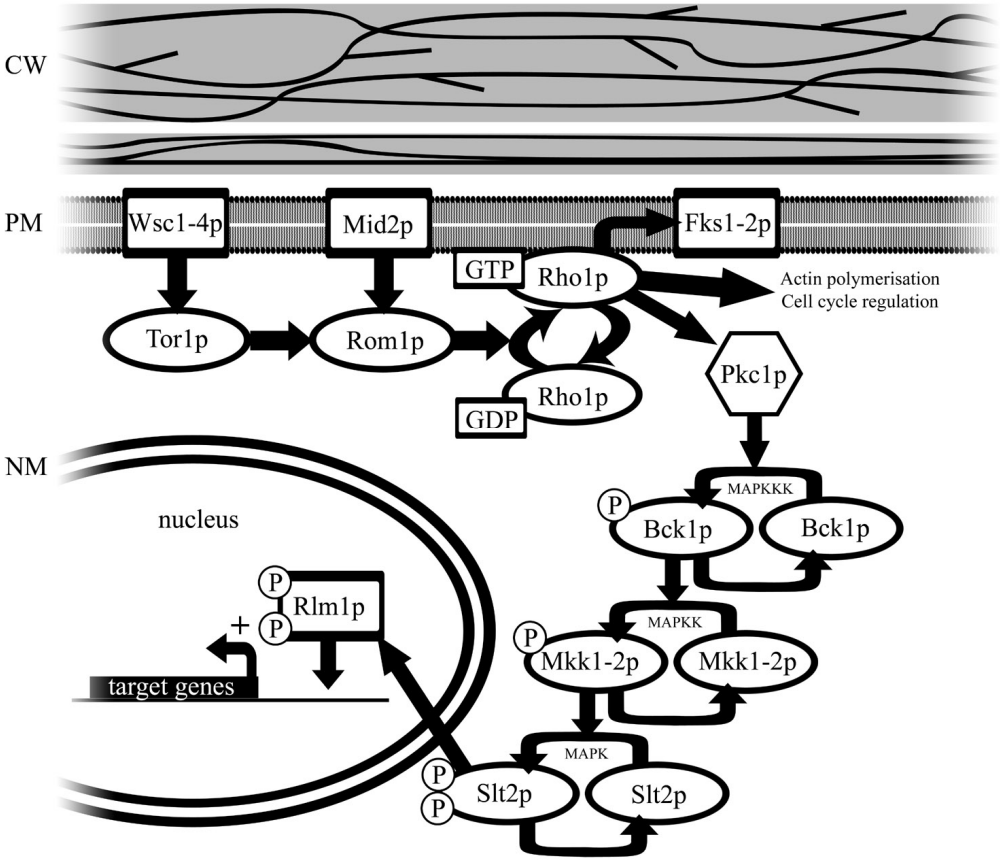

Figure 2. The fungal cell wall integrity pathway (reviewed by Heinisch *et al* .,1999). In brief, the pathway consists of plasma membrane localised sensor proteins (Wsc1-4p and Mid2), that mediate a signal through the Rho1p module and Pkc1p, resulting in the activation of the Slt2p MAPK signal transduction cascade. The MAPK Slt2p phosphorylates and thereby activates the Rlm1p transcription factor which upregulates the transcription of the Rlm1p target genes. Among these target genes are genes involved in cell wall biosynthesis. Abbreviations used: cell wall (CW), plasma membrane (PM), nuclear membrane (NM), and phosphorylation (P).

which results in the expression of genes able to alter the cell wall composition and architecture (Fig. 2.). This pathway is known as the Pkc1p, the Slt2/Mpk1p MAP kinase signaling pathway or cell wall integrity pathway (reviewed by Banuett *et al*., 1998, Smits *et al*., 1999, Heinisch *et al*., 1999). Different environmental stimuli have been reported for *S. cerevisiae* that activate the pathway: growth at elevated temperatures (Kamada *et al*., 1995), hypo-osmotic shock conditions (Davenport *et al*., 1995, Kamada *et al*., 1995), the addition of mating pheromones (Errede *et al*., 1995, Buehrer and Errede, 1997), the addition of agents

that cause cell wall stress such as Calcofluor White, Congo Red, caspofungin or β-1,3-glucanase (Ketela *et al*., 1999, de Nobel *et al*., 2000b, Reinoso-Martin *et al*., 2003, Garcia *et al*., 2004), and actin depolymerisation agents (Harrison *et al*., 2001). The pathway is also activated in mutants with impaired cell wall synthesis (Terashima *et al*., 2000, Lagorce *et al*., 2003) or in constitutively activated signaling mutants (Jung and Levin, 1999). Putative sensors of the pathway are the transmembrane proteins Wsc1p-Wsc4p (Zu *et al*., 2001) and Mid2p (Ono *et al*., 1994, Ketela *et al*., 1999, Green *et al*., 2003). The Wsc1p-Wsc4p proteins interact through Tor2p with a guanine nucleotide exchange factor, Rom2p, to activate the small GTPase Rho1p (Bickle *et al*., 1998, Sekiya-Kawasaki *et al*., 2002). Mid2p and Zeo1p also act as activators of Rho1p via a mechanism independent of Wsc1p and Rom2p (Sekiya-Kawasaki *et al*., 2002, Green *et al*., 2003). One of the functions of Rho1p is the activation of Pkc1p (Nonaka *et al*., 1995, Kamada *et al*., 1996). Pkc1p activates a linear MAPK-signaling module consisting of the MAPKKK, Bck1p, (Costigan *et al*., 1992), the redundant pair of MAPKK, Mkk1p and Mkk2p, (Irie *et al*., 1993) and the MAPK, Slt2p/Mpk1p, (Lee *et al*., 1993). Activation of the PKC-pathway results in the phosphorylation of the threonine and tyrosine residues in the TXY motif of Slt2/Mpk1p (Martin *et al*., 2000). One target of of Slt2p/Mpk1p is the MADS-box transcription factor Rlm1p. Rlm1p was identified as a gene conferring resistance to lethality of *MKK1*^{P386} overexpression (Watanabe *et al*., 1995) and belongs to the evolutionary conserved family of the MADS (Mcm1p-Agamous-Deficiens-Serum Response Factor) box transcription factor proteins (Schwarz-Sommer *et al*., 1990).

Rlm1p is most closely related to the mammalian MADS-box MEF2 transcription factors. The protein shows similar DNA-binding specificity *in vitro* CTA(T/A)$_4$TAG (Dodou and Treisman, 1997). The transcriptional activation potency of Rlm1p is regulated through phosphorylation by the protein kinase Mpk1p (Watanabe *et al*., 1997, Jung *et al*., 2002). In *S. cerevisiae*, Rlm1p is localised in the nucleus irrespective of its activation or phosphorylation status (Jung *et al*., 2002). Rlm1p and its binding sites have been shown to be required for the activation of genes involved in cell wall remodeling in response to cell wall stress (Jung and Levin, 1999, Terashima *et al*., 2000). Indeed, genome wide expression analysis of the response to different forms of cell wall stress in *S. cerevisiae* has further indicated an important role of Rlm1p in mediating the activation mechanism because of the presence of putative Rlm1p binding sites in their promoters (Roberts *et al*., 2000, Lagorce *et al*., 2003, Reinoso-Martin *et al*., 2003, Garcia *et al*., 2004, Boorsma *et al*., 2004). From these studies, it is also evident that the Pkc1p-Slt2p dependent pathway is not the only signaling pathway that contributes to cell wall remodeling in yeast. Both the calcium/calcineurin pathway (Zhao *et al*., 1998, Yoshimoto *et al*., 2002) and the Hog1p-MAPK (reviewed by Hohmann, 2002) signaling pathways are involved in the activation of genes implicated in maintaining cell wall integrity.

In addition, the Heat shock transcription factor Hsf1p, which binds to the Heat Shock Element HSE (Sorger, 1991, Fernandes *et al*., 1994) and Swi4p, a PKC1-regulated transcription factor, involved in G1/S specific gene expression was also reported to have an effect on the up-regulation of genes after cell wall stress (Igual *et al*., 1996, Madden *et al*., 1997).

Little is known about the mechanism of cell wall remodeling in filamentous fungi. Compensatory reactions in response to cell wall stress have also been observed in filamentous fungi (Gooday and Schofield, 1995, Sela-Buurlage, 1996, Kurtz *et al*., 1994, Wang *et al*., 2002, Mellado *et al*., 2003). The compensatory mechanism has become evident from morphological studies where cell wall becomes thicker and has an altered composition as observed by electron microscopy after inhibition of β-1,3-glucan synthesis activity (Kurtz *et al*., 1994). When microconidia of the filamentous fungus *Fusarium solani* were allowed to germinate in the presence of sublethal concentrations of cell wall degrading enzymes adaptation occurs since these germlings had become resistant to concentrations which for non-challenged spores were lethal (Sela-Buurlage, 1996). Mellado *et al*. provide evidence that interfering with chitin synthesis in *A. fumigatus* by deleting two chitin synthesis genes *chsG* and *chsE*, results in aberrant cell morphology and increased levels of 1,3-α-D-glucan in the cell wall of those mutants (Mellado *et al*., 2003).

The recent sequencing of many fungal genomes also revealed the presence of homologs of the cell wall integrity pathway in filamentous fungi (Table 1), however whether they perform a similar function requires further investigation. Only a small number of fungal homologs have been isolated and studied in more detail: RhoA *A. nidulans* (Guest *et al*., 2004), pkc1 *Trichoderma reesi* (Morawtz *et al*., 1996), pkcA *A. niger* (Morawtz *et al*., 1996), PCBCK1 *Pneumocystis carnii* (Vohra *et al*., 2004), MPKA *A. nidulans* (Bussink and Osmani, 1999), Mkp1 *Pneumocystis carnii* (Fox and Smulian, 1999), Mpk1 *Cryptococcus neoformans* (Kraus *et al*., 2003), Mps1 *N. crassa* (Xu *et al*., 1998, 2000). The presence of these highly homologous proteins in filamentous fungi suggests the existence of a similar cell wall integrity pathway in filamentous fungi.

Table 1. Homologs of the cell wall integrity pathway from *S. cerevisiae* found in the genomes of filamentous fungi. The annotated genomes of *A. nidulans*, *G. zea*, *N. crassa*, and *M. grisea* were

used.

S.cerevisiae protein (size bp)	(annotated) protein name	size (bp)	accesion number	organism	Score (bits)	E-value
Wsc1p (388) Wsc2p[a] (378)	AN5660.2	280	gb|EAA62753.1|	*A. nidulans*	70	4.00E-12
	FG03884.1	465	gb|EAA73352.1|	*G. zea*	54	3.00E-07
	NCU00309.1	573	gb|EAA28544.1|	*N. crassa*	48	2.00E-05
	MG09221.4	1017	gb|EAA55414.1|	*M. grisea*	47	5.00E-05
Wsc3p (556)	AN5660.2	280	gb|EAA62753.1|	*A. nidulans*	70	8.00E-12
	NCU04170.1	2076	gb|EAA31492.1|	*N. crassa*	51	3.00E-06
	FG10435.1	1763	gb|EAA68209.1|	*G. zea*	50	5.00E-06
	NCU09267.1	1105	gb|EAA35466.1|	*N. crassa*	49	1.00E-05
Wsc4p (605)	AN4674.2	304	gb|EAA60716.1|	*A. nidulans*	75	2.00E-13
	MG01466.4	356	gb|EAA55815.1|	*M. grisea*	67	7.00E-11
	FG05656.1	375	gb|EAA75227.1|	*G. zea*	62	2.00E-09
	NCU06981.1	404	gb|EAA33385.1|	*N. crassa*	52	1.00E-06
Mid2p (376)	-	-	-	-	-	-
Tor2p (2474)	AN5982.2	2371	gb|EAA57731.1|	*A. nidulans*	2128	0.0
	FG08133.1	2423	gb|EAA71932.1|	*G. zea*	2102	0.0
	NCU05608.1	2509	gb|EAA31334.1|	*N. crassa*	2004	0.0
	contig 2.1040+41	~2400	NA	*M. grisea*	1888	0.0
Rom2p (1356)	MG03064.4	1281	gb|EAA47821.1|	*M. grisea*	501	e-141
	FG08572.1	1235	gb|EAA71433.1|	*G. zea*	487	e-137
	AN4719.2	1199	gb|EAA60761.1|	*A. nidulans*	486	e-137
	NCU00668.1	1251	gb|EAA36572.1|	*N. crassa*	483	e-136
Rho1p (209)	Rho1p[(1)]	193	gb|EAA62833.1|	*A. nidulans*	297	4.00E-81
	MG07176.4	193	gb|EAA56821.1|	*M. grisea*	290	5.00E-79
	FG04400.1	195	gb|EAA72781.1|	*G. zea*	288	2.00E-78
	NCU08683.1	200	gb|EAA32796.1|	*N. crassa*	233	1.00E-61
Pkc1p (1151)	AN0106.2	1083	gb|EAA65284.1|	*A. nidulans*	479	e-135
	FG09660.1	1176	gb|EAA75979.1|	*G. zea*	462	e-130
	NCU06544.1	1142	gb|EAA33015.1|	*N. crassa*	453	e-127
	MG08689.4	1182	gb|EAA51167.1|	*M. grisea*	451	e-126
Bck1p (1478)	FG06326.1	1870	gb|EAA74943.1|	*G. zea*	323	1.00E-87
	NCU02234.1	1786	gb|EAA30411.1|	*N. crassa*	322	3.00E-87
	MG00883.4	1533	gb|EAA49225.1|	*M. grisea*	319	1.00E-86
	AN4887.2	1533	gb|EAA60965.1|	*A. nidulans*	293	1.00E-78
Mkk1p (508)	NCU06419.1	587	gb|EAA28074.1|	*N. crassa*	335	7.00E-92
	MG06482.4	515	gb|EAA56511.1|	*M. grisea*	333	2.00E-91
	FG07295.1	524	gb|EAA77528.1|	*G. zea*	328	8.00E-90
	AN4189.2	502	gb|EAA59288.1|	*A. nidulans*	325	5.00E-89
Mpk1 (484)	MpkA[(2)]	418	gb|AAD24428.1|	*A. nidulans*	527	e-148
	FG10313.1	416	gb|EAA70011.1|	*G. zea*	506	e-143
	MG04943.4	415	gb|EAA52251.1|	*M. grisea*	506	e-143
	Mps1[(3)]	454	gb|AAC63682.1|	*N. crassa*	478	e-135
Rlm1p (676)	FG09339.1	681	gb|EAA76082.1|	*G. zea*	105	1.00E-22
	AN2984.2	605	gb|EAA63555.1|	*A. nidulans*	105	2.00E-22
	NCU02558.1	625	gb|EAA36453.1|	*N. crassa*	104	4.00E-22
	-	-	-	*M. grisea*	-	-

[a] Both Wsc1p and Wsc2p gave similar results.
[(1)] Rho1p, Guest and Momany, 2004

[2] MpkA, Bussink and Osmani, 1999
[3] Mps1, Xu *et al.*, 1999
Abbreviations used: not available (NA)

Aim of the thesis

The fungal cell wall is an intriguing component of the cell. It provides the cell with the necessary support to prevent lysis, and it protects the cell from the harsh environment. Being an essential component of the cell, the cell wall is considered as an interesting target to prevent fungal growth. It has been shown that the yeast *S. cerevisiae* is able to remodel its cell wall architecture and composition in response to cell wall disturbing conditions in order to withstand cell wall threatening conditions. This remodeling mechanism has only been studied in great detail in the yeast *S. cerevisiae,* and knowledge about possible cell wall remodeling mechanisms in filamentous fungi is lacking. The goals of this thesis are (i) to provide evidence of for the existence of a cell wall remodeling mechanism in filamentous fungi and in particular *A. niger* (ii) to identify components of the signal transduction by which cell wall weakening is sensed and transduced into a transcriptional response and (iii) to develop cell wall stress reporter systems to identify new cell wall related antifungal targets and to identify cell wall related antifungal compounds.

Chapter 1 is a general introduction on the fungal cell wall. Both the composition of the fungal cell wall and the synthesis of the cell wall components are briefly discussed. The cell wall integrity pathway in *S. cerevisiae* is discussed in detail, and evidence for the existence of a cell wall integrity pathway in filamentous fungi is presented. The recently sequenced genomes of several filamentous fungi were used to provide further evidence for the existence of a cell wall integrity pathway.

Chapter 2 provides evidence for the presence of a remodeling mechanism in the filamentous fungus *A. niger*. An increased chitin content in the cell wall is observed in response to cell wall stress. An essential protein which determines the rate of chitin synthesis, named GfaA, is characterized in more detail.

In chapter 3, a family of five α-glucan synthases is studied. The expression of the genes encoding the members of this family, were monitored in response to cell wall stress. The expression of the genes encoding the two members *agsA* and *agsE* are induced in response to cell wall stress indicating that increased levels of α-1,3-glucan is part of the the cell wall remodeling mechanism.

The output of the cell wall integrity pathway is mediated via a transcription factor, named RlmA. The *in vivo* binding-site of RlmA is studied together with the function of the protein in chapter 4. We show that the transcription factor is required for proper functioning of the remodeling mechanism.

Cell wall proteins have been shown to have diverse functions. However, the function of most cell wall proteins remains unknown. Most of them are thought to have a structural role and provide the cell wall protection and determine its surface properties. In chapter 5, a putative GPI-anchored cell wall protein CwpA is studied. Biochemical evidence is provided which shows that CwpA is linked to the cell wall via its GPI-anchor.

In chapter 6 a new method for the isolation of cell wall mutants is described. The screen was set up to select for mutants with an induced *agsA* expression. Since the induction of *agsA* is proposed to be correlated with impaired cell wall integrity, it is expected to find mutants with a weakened cell wall. Secondary screens were used to select mutants most suitable for complementation. This method could provide new anti-fungal cell wall targets.

Chapter 7 describes the development of a Green Fluorescent Protein based cell wall stress reporter system. The method has been evaluated towards different known and unknown antifungal compounds and is a promising tool for the identification of new cell wall related antifungal compounds.

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Chapter 2

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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Abstract

Perturbation of cell wall synthesis in *Saccharomyces cerevisiae,* by either 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 we show 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. Our observations suggest that cell wall stress in fungi may generally lead to activation of the chitin biosynthetic pathway.

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, β-1,3-glucan, and chitin. Depending on the species, additional polymers such as α-1,3-glucan or β-1,6-glucan polymers may be present. Chitin, a β-1,4-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 wall of filamentous fungi contain higher levels of chitin, up to 10-30 % of the cell wall dry weight (de Nobel *et al.,* 2001). *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 *S. 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 β-1,3-glucan and β-1,6-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 6 classes according to their sequence similarity (reviewed by Roncero, 2002). Most yeasts and filamentous fungi contain multiple chitin synthase-encoding genes. Both in the yeast *S. cerevisiae* and the filamentous fungi *A. nidulans* and *A. fumigatus*, the

expression and activity of different chitin synthases is highly regulated and is 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 herein).

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 and 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-P 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 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 *A. niger* and of *gfaA* fragments from *P. chrysogenum* and *F. 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

Calcofluor White-induced cell wall stress, presenting further evidence that activation of the chitin biosynthetic pathway is a general response to cell wall stress.

Materials and Methods

Strains, culture conditions and fungal transformation

A. niger N402 (a *cspA1* derivative of ATCC9029; Bos *et al.,* 1988) and strain AB4.1 (van Hartingsveld *et al.,* 1987), a *pyrG* mutant derived from *A. niger* N402, were used in this study. Strains were cultivated in minimal medium (MM) (Bennett and Lasure, 1991) containing 1 % (w/v) of glucose as a 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. 10 mM uridine 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* complete medium. *Fusarium oxysporum f. sp. radicis lycopersic*i (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 four to six days of growth at 30 °C, using 0.9 % (w/v) NaCl. Conidia from *F. oxysporum* were obtained by filtering conidia from a three- to four-day-old 100 ml liquid culture. The 100 ml culture was started by inoculating 1 x 10^5 conidia ml^{-1} and grown at 30 °C on an orbital shaker at 300 rpm. *A. niger* transformations were carried out as previously described (Punt and 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^{-1} fresh weight mycelium.

Cell wall stress-inducing conditions

Freshly isolated conidia from *A. niger* were inoculated into 100 ml CM at a spore density of 1 x 10^7 conidia ml^{-1} and grown for five hours at 37 °C. After five hours, 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 x 10^7 conidia ml^{-1} and grown for 6.5 hours at 30 °C before adding CFW. *F. oxysporum* conidia were inoculated at a spore density of 1 x 10^6 spores ml^{-1} and grown for seven hours 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 ml^{-1} glucosamine. 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 DIC 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 (Montijn *et al.,* 1994). Chitin levels were determined as described by Tracey (1956). In brief, approximately 200 mg of wet weight cell walls were freeze-dried and the dry weight was determined. Cell walls (approximately 15 mg dry weight) were subsequently hydrolysed in 6 N HCl at 100 °C for 4 hours. 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 of solution B (1·6 g of *p*-dimethylaminobenzaldehyde in 30 ml of concentrated HCl and 30 ml of 96 % ethanol) was added and incubated for 1 hour 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 synthesised using 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 one hour 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, PgfaP1for (5'**cgggatccc**GARTAYMGNGGNTAYGA) and PgfaP2rev (5'**cggaattccg**TGNGTNGCCAANCKNGT) (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'**cgggatccc**CAYATHAAYGCNGGNCC) and PgfaP4rev **(**5'**cggaattccg**CCYTGNARRCARTCNAC) was used to isolate the *A. niger gfaA* homolog 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 microtiter plates, and colonies were spotted on LB plates. After transfer of the colonies to HybondN^+ filters, they were lysed using standard protocols (Sambrook *et al.,* 1989). Out of approximately 5,000 colonies screened, six hybridised 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 partial 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, The Netherlands.

Disruption of the *A. niger gfaA* gene and complementation of the *ΔgfaA* 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 nucleotides 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 nucleotides 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, p*ΔgfaA*. This plasmid was linearised 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 *ΔgfaA* strains and a wild-type strain was isolated and digested with *Pst*I. DNA was separated on a 0·8 % agarose gel, blotted on HybondN$^+$ and hybridised with a *gfaA* probe. As a probe an 1·2 kb *Cla*I-*Bgl*II fragment from pBAMHI-GFA#5 was used. For complementation studies, plasmid pBAMHI-GFA#5 was used.

Nucleotide sequence accession numbers

The following nucleotide and protein sequences were submitted to Genbank: AbGfaA (AY594332), FoGfaA (AY594333) and PcGfaA (AY594334). Nucleotide sequence data reported are available in the Third Party Annotation Section (TPA) of the DDBJ/EMBL/Genbank database under accession numbers: BK005223 (AnGfaA) and BK005224 (GzGfaA).

Results

Increased chitin levels in cell walls of Calcofluor White stressed germlings of *A. niger*

The addition of sublethal concentrations of Calcofluor White (CFW) to *A. niger* germlings results in the formation of swollen hyphal tips (Chapter 3), similar to the phenotype observed after adding Congo Red (CR) to germlings of *A. niger* (Pancaldi *et al.,* 1984). CFW and CR 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 is 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 CFW leads to increased chitin levels in the cell wall of *Geotrichum lactis* and also *S. cerevisiae* (Roncero and 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 in 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.

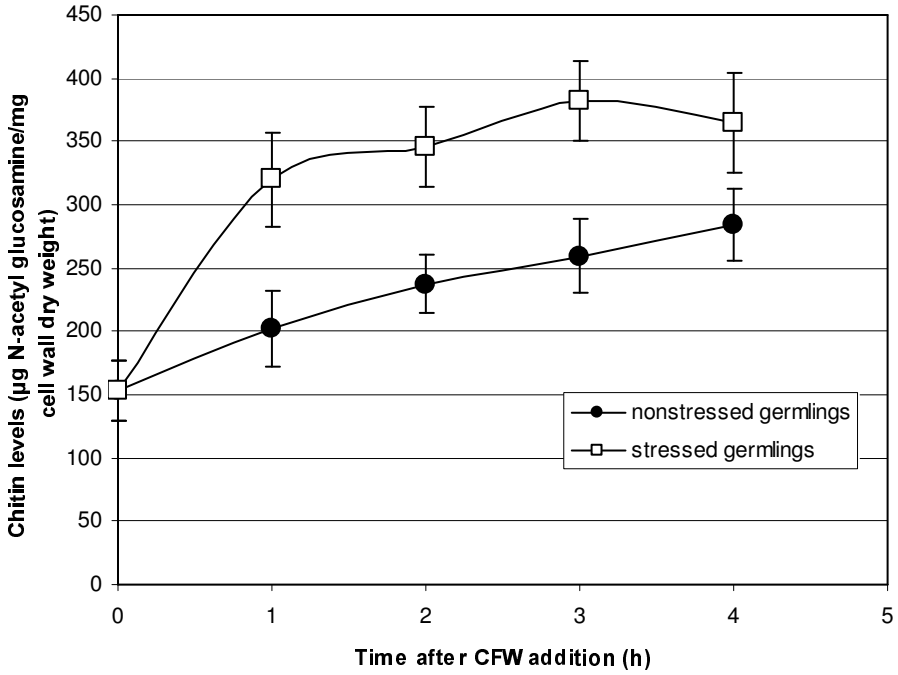

Figure 1. Chitin content of cell walls from wild-type strain after CFW stress. Conidia were inoculated and after five hours the germlings were treated with a sublethal concentration of 200 µg CFW ml^{-1}. Cell walls were isolated every hour after the addition of CFW and in the control cells. The amount 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.

Identification of *gfaA* homologs 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 rate-limiting step in the UDP-N-acetylglucosamine biosynthetic pathway. We cloned fragments of the *gfaA* gene from three

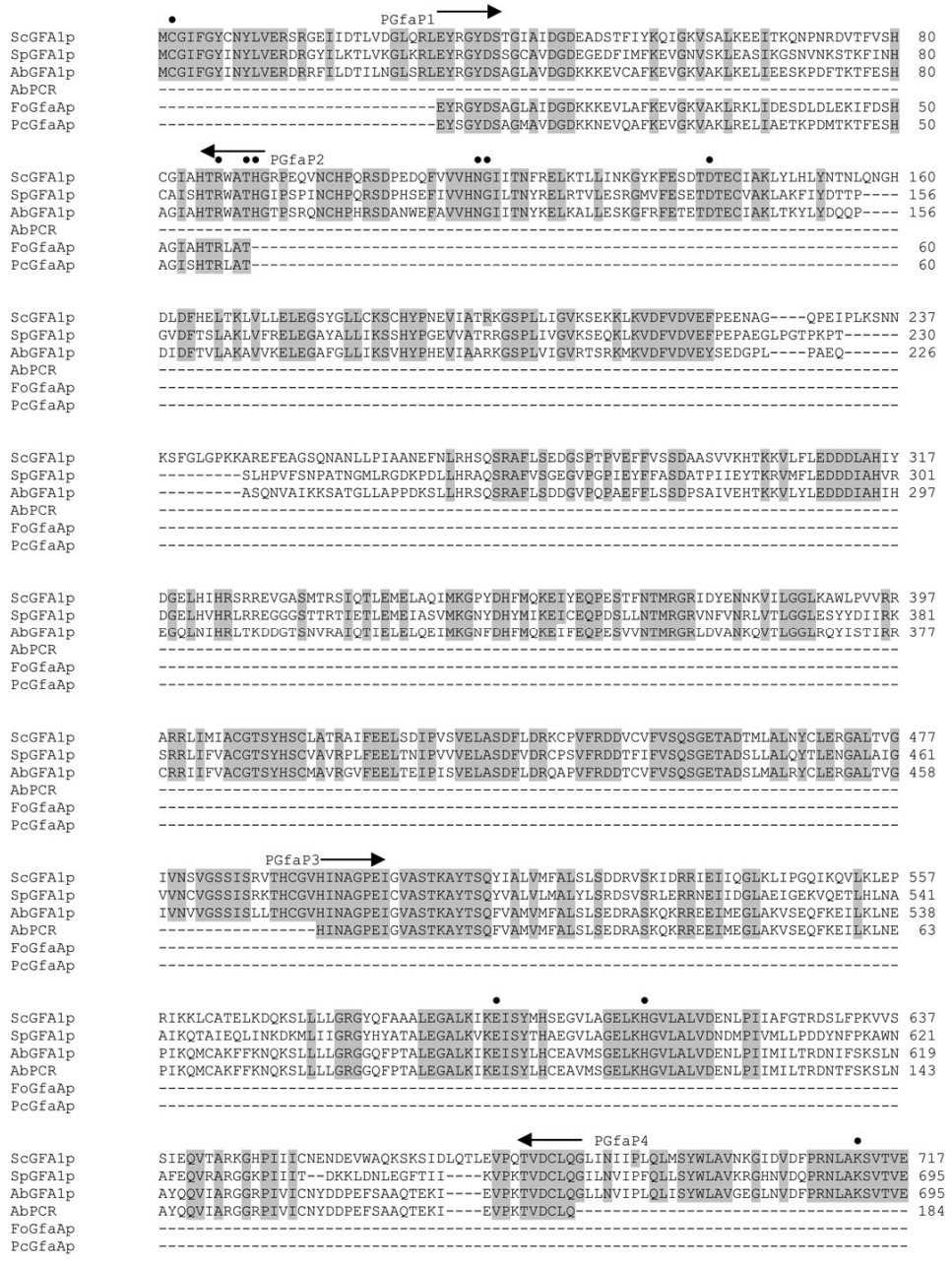

Figure 2. Multiple sequence alignment of fungal glutamine:fructose-6-phosphate amidotransferases. Conserved amino acid regions that were used to design degenerated primers are indicated with arrows. Conserved amino acids in the glutamine-binding domain and the fructose-6-phosphate-isomerase domain are 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, *S. pombe*, NP_596011. Multiple sequence alignment was performed using DNAman version 4.0 using the Higgins and Sharp method (Higgins and Sharp, 1988).

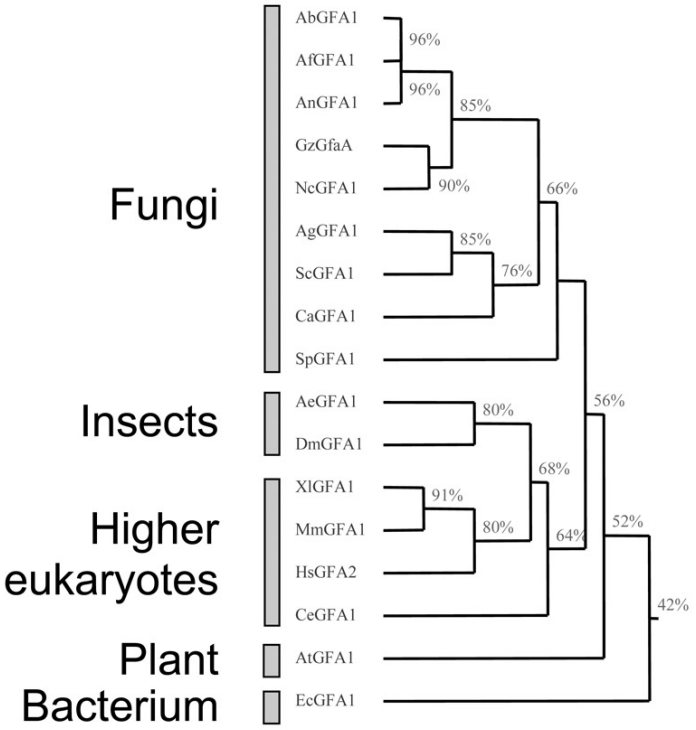

Figure 3. Phylogenetic tree of GFAs showing the clustering of fungal GFAs separate from GFAs from both prokaryotic and higher eukaryotic origin. Multiple sequence alignment was performed using DNAman version 4.0 using optimal alignment program (Feng and Doolittle, 1987; Thompson, *et al.,* 1994). % Values 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, *S. 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.

different fungi using degenerated primers based on conserved amino acid regions in fungal GFA proteins (Fig. 2). Specific PCR ragments 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 a high levels of amino acid identity to previously known fungal GFAs (Fig. 2). Using the *A. niger gfaA* PCR fragment as a probe, a clone was isolated from an *A. niger* genomic cosmid library, which contained the complete *gfaA* open reading frame, including promoter and terminator regions. *GfaA* contains an open reading frame of 2412 bp, which is interrupted by five introns and encodes a protein of 694 amino acids. Comparison of the *A. niger* GfaA protein sequence to other fungal GFAs revealed a high level of identity among them (Fig. 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 ml^{-1} CFW. Total RNA was isolated at different time points after adding CFW and subjected to Northern blot analysis. As shown in Fig. 4a, 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* after two and four hours after CFW addition is probably due to inactivation of CFW and not to resistance of the germlings to CWF. When, after four 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 4c). 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

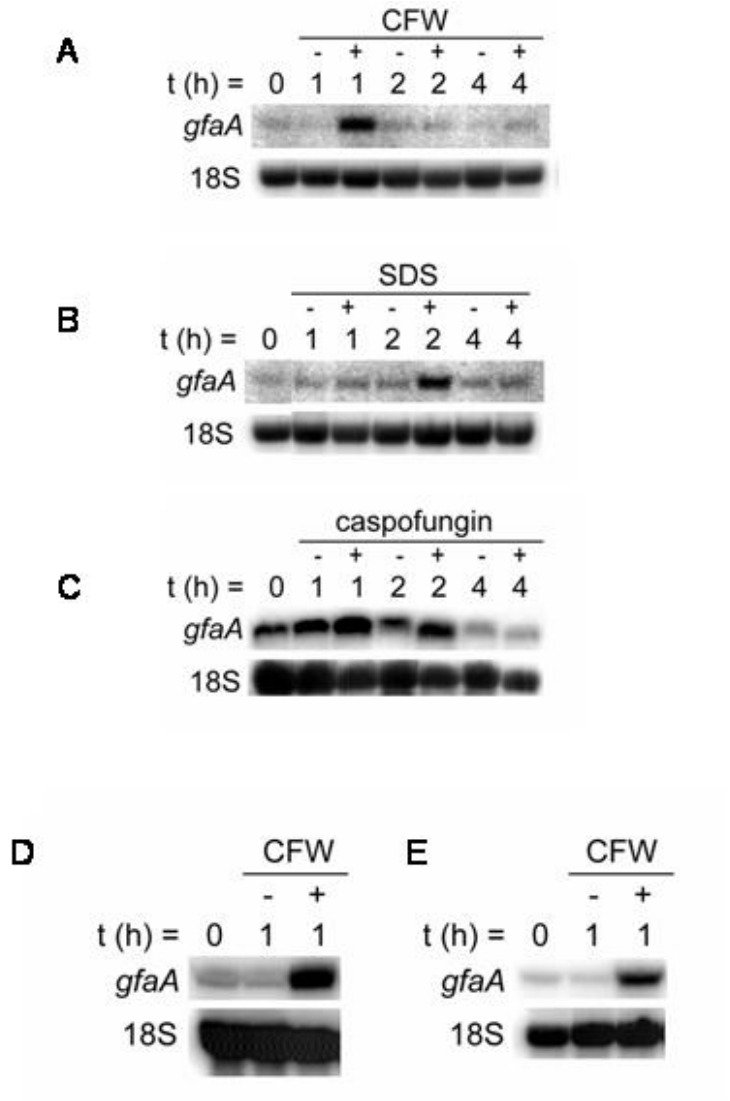

Figure 4. Induction of *gfaA* transcript levels in response to different forms of cell wall stress in different filamentous fungi. **A**) CFW-induced cell wall stress in *A. niger*; **B**) SDS-induced cell wall stress in *A. niger*; **C**) Caspofungin-induced cell wall stress in *A. niger*. Conidia were inoculated and grown for 5 hours 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 hours respectively, before adding CFW. RNA was isolated one hour 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.

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 one hour 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 4e). This indicates that the induction of *gfaA* and probably also an increased synthesis of chitin is a general compensatory mechanism in order 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 (p∆*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 minimal medium supplemented with 5 mg ml^{-1} glucosamine. In *S. cerevisiae*, *GFA1* is an essential gene and mutants can be rescued by the addition of glucosamine to the medium (Watzele and 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 *∆gfaA* strains since these transformants were expected not to grow on plates without glucosamine (Fig. 5c). Glucosamine-requiring transformants were identified and 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 that conidia from a *∆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 *∆gfaA* strain. To determine this, a fixed number of conidia from a wild-type strain and the *∆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 seven days. At all glucosamine concentrations tested, the growth of a ∆*gfaA* strain was somewhat slower than the wild-type, resulting in a smaller colony size (5 mg ml^{-1}: 3·5 ± 0·1 cm (∆*gfaA*) vs 3·7 ± 0·1 (wild-type); 10 mg ml^{-1}: 3·6 ± 0·1 vs 4·0 ± 0·1; 50 mg ml^{-1}: 4·7 ± 0·1

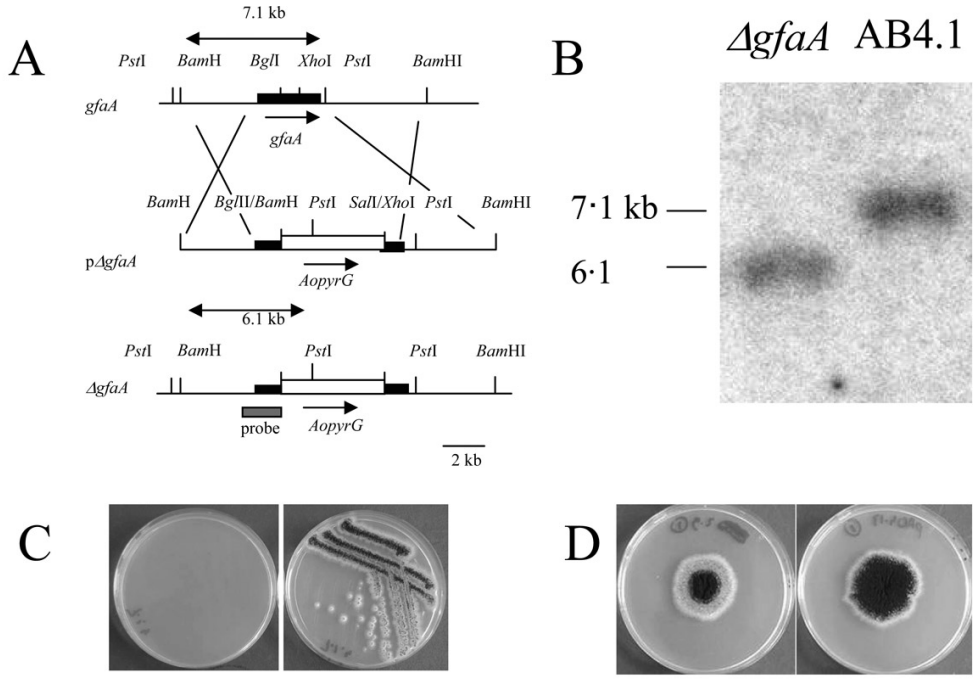

Figure 5. Disruption of the *gfaA* gene in *Aspergillus niger*. **A**) Schematic overview of the *gfaA* disruption strategy. Restriction sites and predicted sizes of genomic fragments are indicated. **B**) Southern blot analysis of the *AbgfaA* deletion mutant (*ΔgfaA*) and the recipient strain, AB4.1. The sizes of marker DNA fragments are indicated at 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 *ΔgfaA* strain requires glucosamine in the plate to support growth. Conidia from a *ΔgfaA* strain were streaked on MM (left) or on MM supplemented with 10 mg ml^{-1} glucosamine (right). **D**) The *ΔgfaA* strain (left) shows decreased levels of conidiation in the outer parts of the colonies compared to a wild-type strain (right). 100 spores were spotted in the centre of the agar plate and grown for seven days at 30 °C.

vs 5·4 ± 0·1 cm; mean ± SD; n = 2) after seven days of growth at 30 ℃. We also observed that the *ΔgfaA* 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 indeed lack of glucosamine was 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 an Δ*gfaA* strain on a plate supplemented with 10 mg ml^{-1} glucosamine. Conidia from the wild-type strain and from the

ΔgfaA strain were inoculated in glucosamine-free minimal medium containing 0.1 % casamino acids. Conidia from the wild-type strain swelled during the first four hours after inoculation and started to form a germ tube in a highly synchronised way. After six hours, greater than 95 % of the conidia had formed a germ tube. Fig. 6a shows the morphology of wild-type germlings after eight hours of inoculation. At this stage, the conidium had formed one unbranched germ tube. Inoculation of conidia from the *ΔgfaA* in medium without glucosamine resulted in severe defects in spore germination. After eight hours 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 did swell and produced a short germ tube. After thirteen hours, the wild-type strain had branched and had occasionally formed secondary germ tubes to form a mycelial network (Fig. 6c). Mircoscopical analysis of the germination process after thirteen hours 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 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 forty hours 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 eight hours 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 average size of the swollen spore of the Δ*gfaA* strain was considerable larger than the wild-type spore size (compare 6e and f). After thirteen hours 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 6c 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 glucosamimne 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 eleven hours of incubation whereas the presence of 50 mg glucosamine ml^{-1} resulted in faster germination, already observed after eight hours of growth.

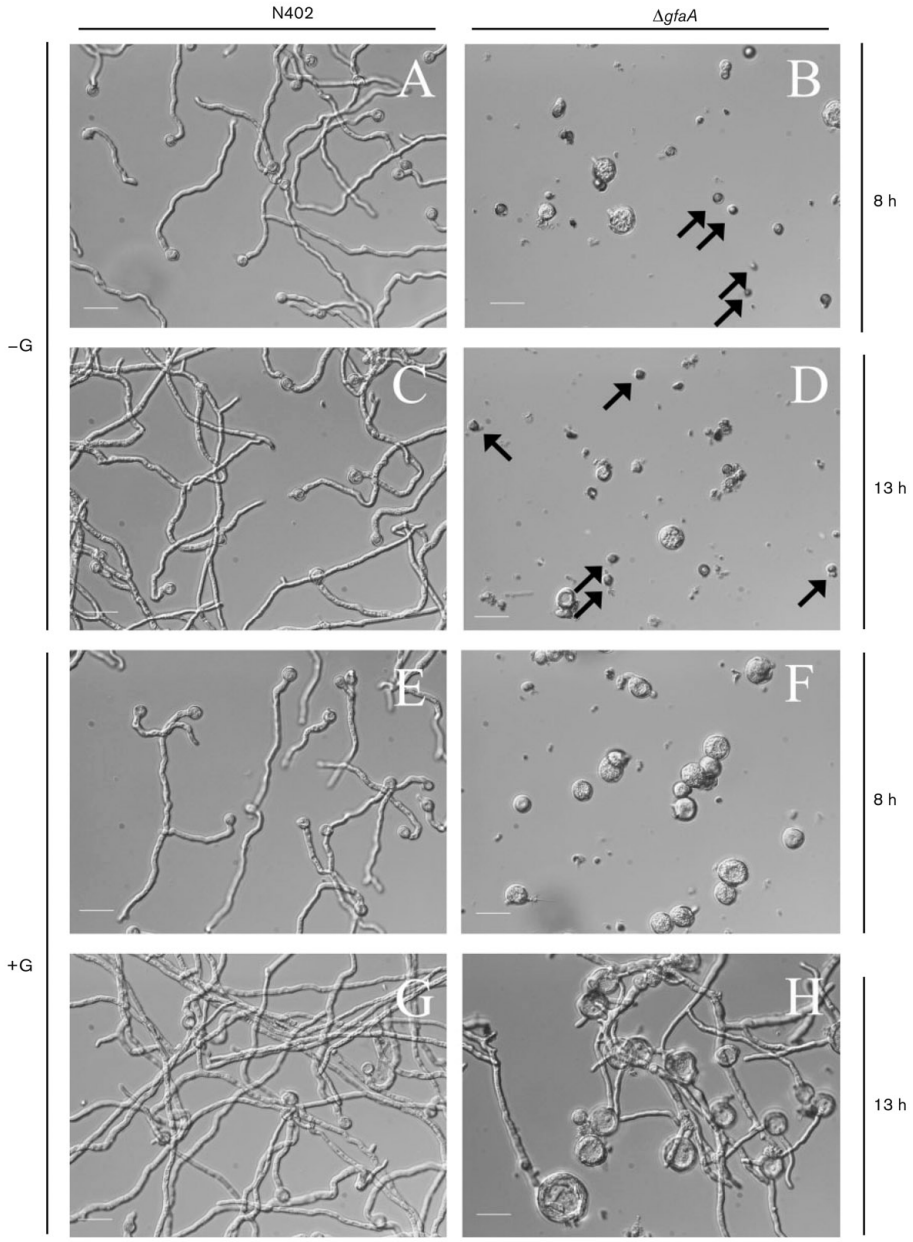

Figure 6. Addition of glucosamine to the medium can partially rescue the growth defect of the *ΔgfaA* strain. Conidia of N402 (A, C, E, G) or *ΔgfaA* (B, D, F, H) were inoculated on coverslips in liquid minimal medium lacking glucosamine (-G) (A-D) or containing 10 mg ml^{-1} glucosamine (+G) (E-H). Conidia were incubated at 37 °C for eight hours (A, B, E, F) or thirteen hours (C, D, G, H), fixed, and examined by DIC microscopy. Not swollen spores are indicated with arrows. All micrographs are at the same magnification. Bar represents 10 μm.

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 to the Δ*gfaA* strain. Protoplasts were obtained from the Δ*gfaA* strain which was pregrown in the presence of various concentrations glucosamine (1 mg ml^{-1}, 2·5 mg ml^{-1}, 5 mg ml^{-1}, 10 mg ml^{-1}, 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 concentration 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, Neurospora crassa*, and *Gibberella 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 stabilisation 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 herein).

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 in order 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 not only activated by the addition of CFW, but also after adding SDS, or caspofungin, a β-1,3-glucan synthase inhibitor (Kartsonis *et al.,* 2003). Mutations that result in cell wall weakening in *S. cerevisiae* (e.g. *Δfks1*, *Δknr4, Δgas1*, *Δkre6, Δmmn9* and *Δoch1*) 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 the 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 herein). Disruption of the *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. The 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-Rodriquea 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 (Valdiva and Schekman, 2003).

UDP-N-acetylglucosamine is not only 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 (GPI) anchor attachment. The lethality of *ΔgfaA* 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 *ΔgfaA* 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 *ΔgfaA* 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 and Lampen, 1975; Takatsuki *et al.,* 1975) and thereby blocks N-linked protein glycosylation. Because of the similar phenotype of

the *ΔgfaA* 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-acetyl glucosamine in the *ΔgfaA* 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 ΔgfaA* strain can be used as a selection marker and a similar approach seems feasible for other filamentous fungi.

Acknowledgements

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Chapter 3

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

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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.

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 (Smits *et al*., 1999, Klis *et al*., 2002). 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 (Manners *et al*., 1973a and b, Fontaine *et al*., 2000). 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 *S. cerevisiae* (Cabib *et al*., 1997, Lipke and Ovalle, 1998) and *C. albicans* (Klis *et al*., 2001), 1,3-α-D-glucan is a prominent component in the cell walls of many fungal species, including *Schizosaccharomyces pombe* (Kreger 1954, Bacon *et al*., 1968, Manners and Meyer, 1977, Sietsma and Wessels, 1990), *Aspergillus nidulans* (Bull 1970, Zonneveld 1971, Zonneveld 1972), *A. niger* (Johnston, 1965, Horisberger *et al*., 1972), *A. 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 acid, 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-watersoluble, 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 (Johnston, 1965, Horisberger *et al*., 1972). 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 remodeling 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 signaling 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

(Nonaka *et al*., 1995, Kamada *et al*., 1996). 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, Sela-Buurlage, 1996, Kurtz *et al*., 1994, Mellado *et al*., 2003), it is mainly indirect.

In this study, we have isolated fragments of a family of five 1,3-α-D-glucan synthase enocoding 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.

Materials and Methods

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.

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 PCR reactions 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 bp 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 PCR reactions on genomic *A. niger* DNA. Products of the expected size (~ 375 bp) were cloned in pGEM-T easy and sequenced. Analysis of ten 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. PCR reactions were performed in a Robocycler (Stratagene) using superTaq DNA polymerase (HT Biotechnology).

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
FKSP3 for	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

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-PCR reactions were performed using the SuperScript One step RT-PCR kit from Invitrogen.

In the RT-PCR reaction, 10 ng of total RNA was used in combination with two primer sets (Table 2). Each reaction mixture contained 1X buffer, 200 µM of each dNTP, 1.5 mM $MgSO_4$, 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 ºC for 30 min, one cycle of denaturation at 94 ºC for two minutes, 30 cycles of denaturation for one minute at 94 ºC, annealing at 60 ºC for one minute, and extension at 72 ºC for one minute. Twenty microliters of PCR product were separated on a 1.5 % agarose gel and visualised by staining with ethidium bromide.

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

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 hours at 37 ºC and 300 rpm (*A. niger*) or for 6.5 hours at 30 ºC 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 x 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). 100 µl spore solution (~ 2 x 10^4 spores) were added to 100 µl CM containing the stress-inducing solution. The microtiter plates were incubated at 37 °C and the OD_{590} was measured every 2 hours in a Perkin Elmer HTS-7000 Bioassay reader.

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 microtiter plates. *E. coli* cells were spotted on HybondN^+ filters, which were placed on LB plates. The cells were grown for 16 hrs at 37 °C and lysed on the filters according to standard protocols (Sambrook *et al.*, 1989). Out of approximately 5,000 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 SstI fragment containing the 5' part of the *agsA* gene including promoter sequences from cosmid P13P14 and pNcoI#7, a ~13 kb NcoI 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.

Construction of the *agsA::pyrG* deletion strain

The *A. niger agsA* gene was disrupted by the insertion of the *A. oryzae pyrG* gene between EcoRI and XhoI 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 NotI-SstI fragment was isolated from pRD12ΔSstI. In this clone, the SstI site next to the NotI site was missing after ligation. From this plasmid the 12 kb NotI-SstI fragment, containing 3 kb of the upstream sequence and almost the complete *agsA* coding region was cloned into pBluescript KS ΔXhoI-SalI, from which both the XhoI and SalI site had been

previously removed by digestion and religation, to give pRD14. This plasmid was digested with NotI and XhoI 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 EcoRI-SalI fragment from plasmid pAO4-13. pAO4-13 is a derivative of pAO4-2 (Ruiter-Jacobs *et al.*, 1989) containing the *AopyrG* gene as a 2.8 kb BamHI-BglII fragment in a BamHI-opened pUC19 vector. The third fragment, consisting of a 1.5 kb of 5' *agsA* promoter sequence, was obtained from plasmid pRD2 as a NotI-EcoRI fragment. pRD2 contains a 6.0 kb BglII-BglII fragment from cosmid P13P14 with the upstream region and part of the *agsA* coding region which was cloned into the BamHI site of pBluescript KS. The NotI site is derived from the polylinker. Ligation of the three fragments resulted in pΔagsA which was linearised with NotI 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 BamHI-BamHI was used as a probe to detect genomic fragments after NcoI digestion and a 0.9 kb BglII-BglII probe from pRD9 was used to detect fragments after PstI digestion. RD9 is a PstI subclone from cosmid P13P14 in pBluescript.

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), *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), *CnagsA* (BK004184).

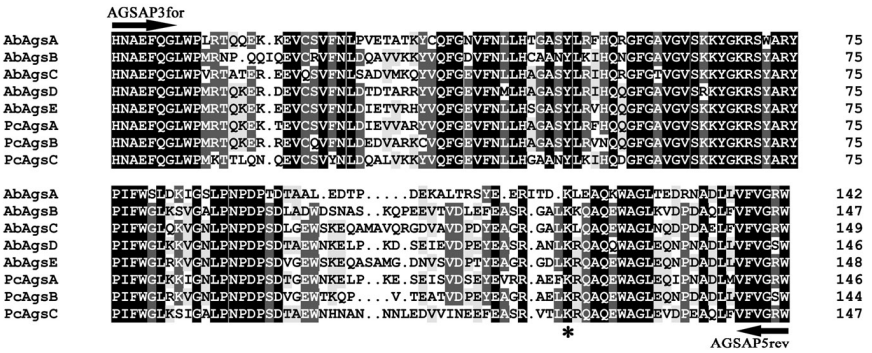
Results

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. 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 - 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 - 67 %) to Ags1 were isolated from *P. chrysogenum* (Fig. 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 *Aspergillus fumigatus*, *Aspergillus nidulans*, *Neurospora crassa*, *Magnaporthe grisea, Coprinus cinerea, Cryptococcus 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 (>10X 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* (Kelly *et al*., 1996, Beauvais *et al*., 2001, Galagan *et al*., 2003), the *A. niger* genome contains a single *fks1* gene (Dr. G. Groot, DSM, personal communication).

A


AGSAP3for
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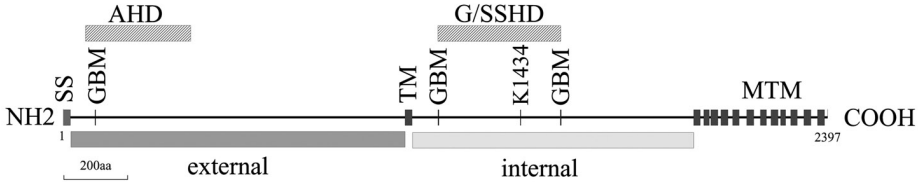
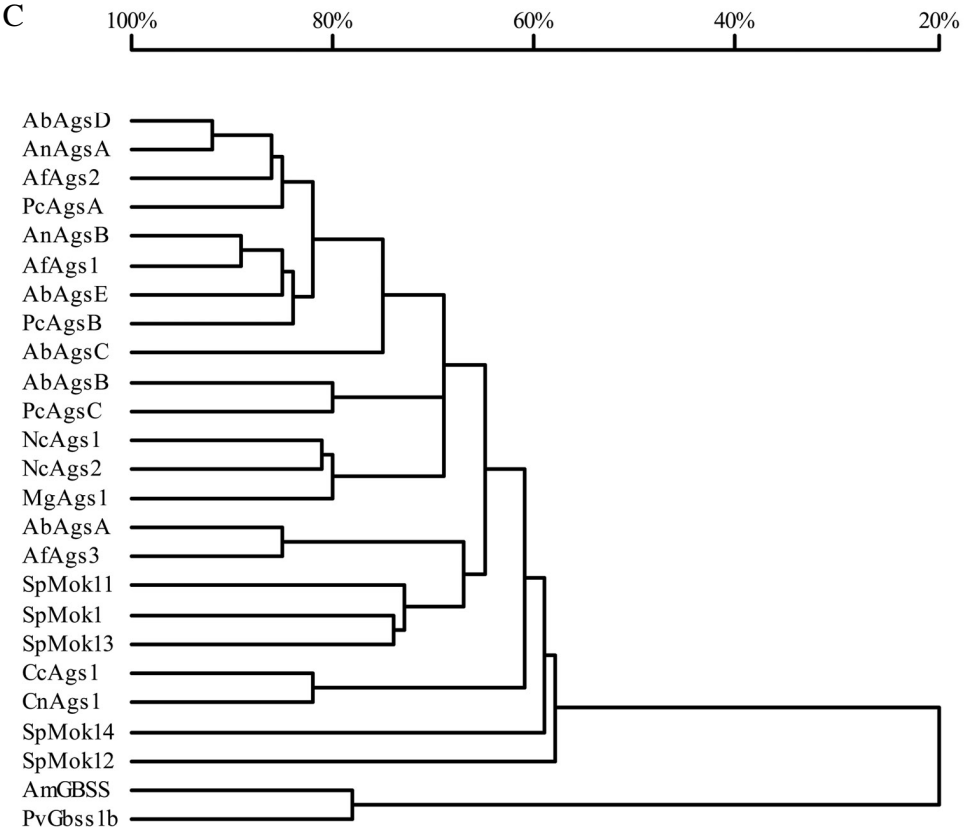
AbAgsB
AbAgsC
AbAgsD
AbAgsE
PcAgsA
PcAgsB
PcAgsC
AGSAP5rev

B


AHD
G/SSHD
SS
GBM
TM
GBM
K1434
GBM
MTM
NH2
COOH
1
2397
200aa
external
internal

C


100%
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AbAgsD
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PcAgsA
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PcAgsB
AbAgsC
AbAgsB
PcAgsC
NcAgs1
NcAgs2
MgAgs1
AbAgsA
AfAgs3
SpMok11
SpMok1
SpMok13
CcAgs1
CnAgs1
SpMok14
SpMok12
AmGBSS
PvGbss1b

Figure 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 asterix (*).

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, multipass 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; 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.p1c|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 *Aspergillus 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).

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 (Seo *et al*., 1999 and Mellado *et al*., 2003). To examine the expression levels of the different *ags* genes in response to cell wall stress, spores were allowed to germinate for five hours 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 four hours 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 five hours (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 fibers in the cell wall and prevents their crystallisation or crosslinking to other cell wall

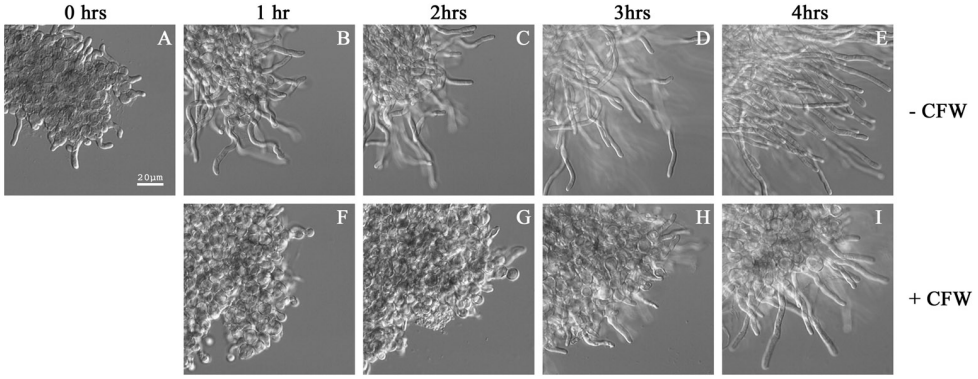

Figure 2. DIC microscope images of Calcofluor White (CFW)-stressed *A. niger* germlings. Spores were inoculated and pre-grown for 5 hours at 37 ºC (panel A). After 5 hours, the germlings were stressed with CFW (200 µg ml^{-1}, bottom panels F-I), or the same volume of water was added (top panels, B-E).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

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 x 10^7 spores ml^{-1} CM), grown for five hours, 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 three hours, 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 one hour, using duplex RT-PCR (Fig. 3). Actin 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 one hour 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* were 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

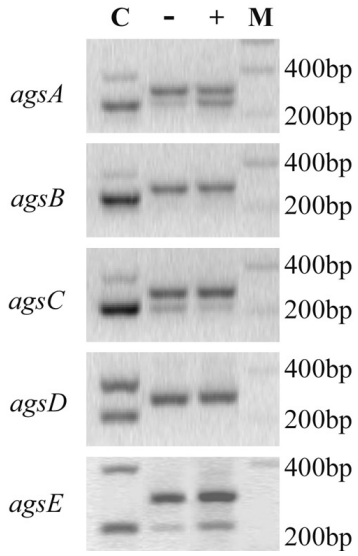

Figure 3. Expression levels of *AbagsA* and *AbagsE* are induced during CFW stress. Expression levels of the five *ags* genes were determined with RT-PCR using specific primers for each *ags* gene. As an internal control, each RT-PCR reaction also contained a primer pair specific for the *A. niger* actin gene. The RT-PCR was also performed with genomic DNA as a template to demonstrate the efficiency of primers in the RT-PCR reaction 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 hours at 37 ºC, subsequently, water (-) or 200 µg CFW ml^{-1} (+) was added. RNA was isolated after 1 hour and used in a RT-PCR reaction. Marker lane (M) showing the 200 bp and the 400 bp bands. Sizes of the different *ags* products are shown in Table 2.

levels revealed >20-fold induction of the *agsA* gene after one hour of CFW stress and a two to three-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 two hours and was back to the unstressed level after 4 hours. 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 Nobel *et al*., 2000), Delley and Hall, 1999, de Groot *et al*., 2001), also induced the expression of *agsA* and *agsE* (Fig. 4B). Caspofungin, a specific β-1,3-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 two hours after addition of SDS and Caspofungin, whereas the induction after CFW stress peaked at one hour.

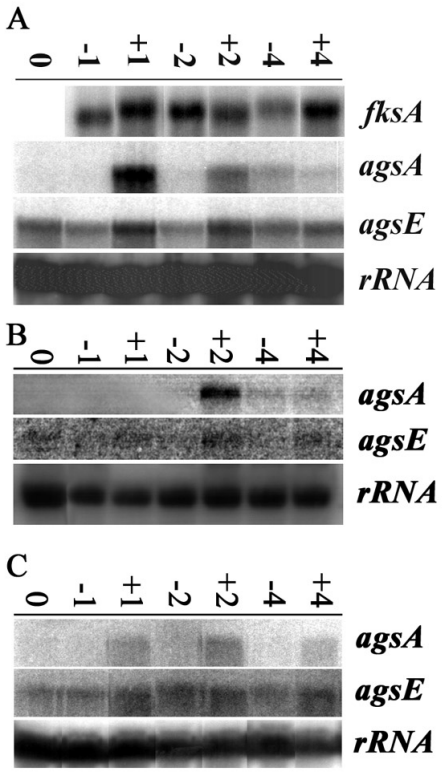

Figure 4. Expression analysis of *ags* genes in response to cell wall stress inducing agents. **A** Northern analysis of *A. niger* germlings during CFW stress. *A. niger* spores were pre-grown for 5 hours at 37 °C and water or CFW (200 µg ml^{-1}) was added to the formed germlings. RNA was isolated after 1, 2, and 4 hours. 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})

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 remodeling newly formed α-glucan or crosslinking 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.

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 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

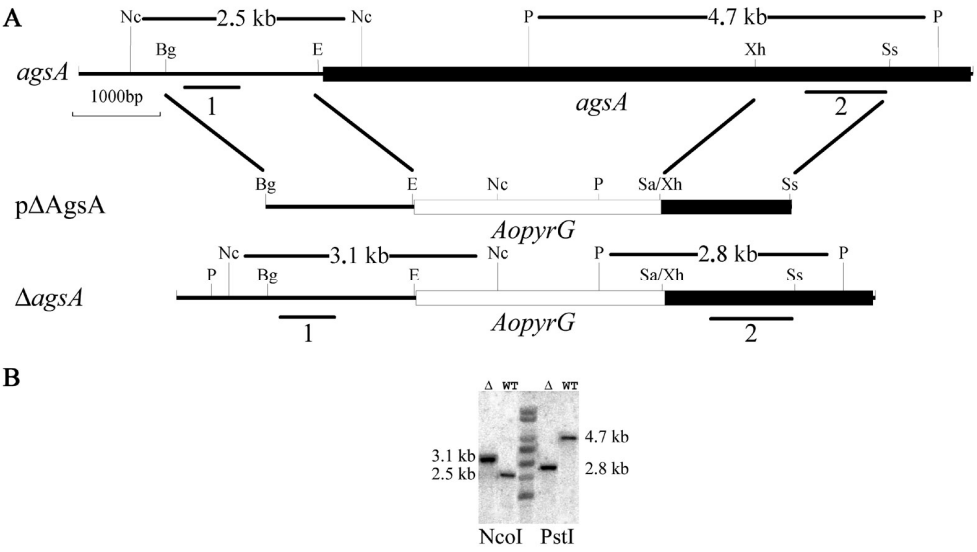

Figure 5. Disruption of the *agsA* gene in *A. niger*. **A** Schematic representation of the *agsA* wild-type locus, the plasmid pΔagsA used for disruption and the deleted locus *ΔagsA*. Abbreviations Bg, BglII; E, EcoRI; Nc, NcoI; P, PstI; Sa, SalI; Ss, SstI; Xh; XhoI; 1, BamHI probe; 2, BglII probe.
B Southern blot analysis of the *A. niger* wild-type (WT) and *agsA* deletion strain (Δ).Genomic DNA was digested with NcoI (lane 1 and 2) or PstI (lane 3 and 4). Digestion of wild-type DNA with NcoI 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 PstI 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 (NcoI digested) are probed with BamHI probe (1). The right two lanes (PstI digested) are probed with BglII probe (2).

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 ten-fold serial dilutions on CFW-containing plates (Fig. 6A). When concentrations of ≤ 50 µg 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

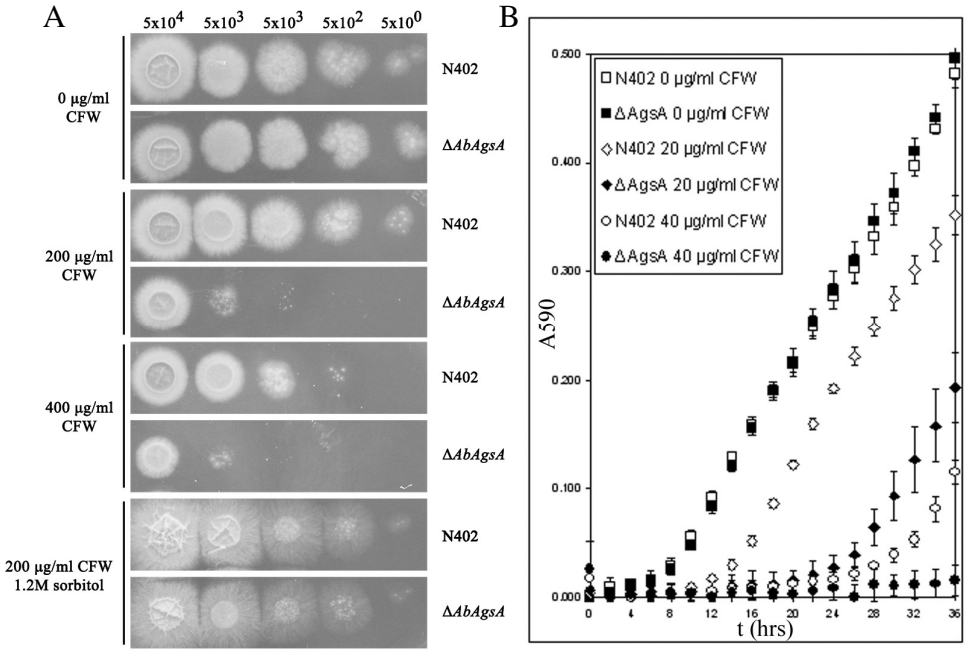

Figure 6. Deletion of *AbagsA* results in a sorbitol-remediable hypersensitivity to CFW. **A**. CFW sensitivity of strain MA22.4.4 (Δ*AbagsA*) was compared to N402 (wild-type). Spores were serially diluted and spotted on CM plates containing the indicated CFW concentrations (the total number of spores 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.

containing increasing concentrations of CFW. Figure 6B shows that both strains had the same germination kinetics in the absence of CFW. However, the presence of 20 or 30 µg 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 µg CFW ml^{-1} the wild-type strain started to grow after 24 hours 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 µg ml^{-1}. The inhibitory effect of caspofungin on growth was clearly detectable at a concentration of 0.25 µg 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 examined. However, no synergistic effect of caspofungin and nikkomycin was observed for either the wild-type strain or the *ΔagsA* strain (data not shown).

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 ℃, spores were allowed to germinate at 30 ℃ for 6.5 hours to obtain germlings. After 6.5 hours, 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 one, two and four hours. 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*.

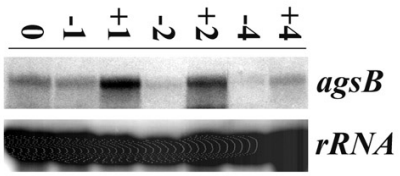

Fig. 7. Northern analysis of CFW-stressed *P. chrysogenum* germlings. Spores of *P. chrysogenum* were grown for 6.5 hours at 30 ºC in CM and subsequently treated with CFW (200 µg ml^{-1}). RNA was isolated at 1, 2, and 4 hours after the addition of CFW. Expression levels of *agsA*, *agsB* and *agsC*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

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 organization 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, remodeling 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 precense or absence of 1,3-α-D-glucan synthase encoding genes can differ within the genus of *Fusarium*. The absence of 1,3-α-D-glucan synthase genes in *Ustilago 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, a 1,3-α-D-glucan polymer with some α-1,4-linkages (Johnson, 1965a and b, Horisberger *et al*., 1972), 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*, a 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 a 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 microtiter 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 remodeling mechanism in response conditions that are likely to affect normal cell wall synthesis or assembly. We have recently shown that the cell wall remodeling response is not limited to 1,3-α-D-glucan and that the cell wall stress response in *Aspergillus 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.

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Chapter 4

The *Aspergillus niger* MADS-box transcription factor RlmA is required for cell wall remodeling in response to cell wall stress

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Submitted

Abstract

In *Aspergillus niger*, the genes coding for glutamine:fructose-6-phosphate amidotransferase (*gfaA*) and α-1,3-glucan synthase (*agsA*) are induced in response to cell wall stress. *In silico* analysis of the promoter region of the two genes revealed the presence of putative DNA binding sites for transcription factors involved in stress responses, including sites identical to the *Saccharomyces cerevisiae* Rlm1p and Msn2p/Msn4p transcription factors. A promoter deletion study indicated that the induction of the *agsA* gene in response to cell wall stress is dependent on a putative Rlm1p binding site in its promoter. Modification of the putative Rlm1p binding site in the *agsA* promoter further demonstrated the role of an Rlm1p-like transcription factor in *agsA* induction in *A. niger*. Database searches revealed the presence of *S. cerevisiae* Rlm1p homologues in most filamentous fungi examined, including *A. niger*. Deletion of the *RLM1* homologue, named *rlmA* in *A. niger*, completely eliminated the induction of *agsA* and resulted in a two-fold reduced induction of *gfaA* during Calcofluor White-induced cell wall stress and resulted in an increased sensitivity towards cell wall disturbing compounds such as CFW and SDS. Our results indicate that the RlmA transcription factor mediates transcriptional activation of genes involved in cell wall remodeling in response to cell wall stress in *A. niger*.

Introduction

The cell wall is an essential factor in maintaining the shape and the integrity of the fungal cell (de Nobel *et al*., 2000a, Klis *et al*., 2002). Both the composition and structure of the fungal cell wall have been comprehensively studied in the yeast *S. cerevisiae*. The *S. cerevisiae* cell wall is composed of β-1,3 and β-1,6-glucans (50-60 % of the cell wall dry weight), mannoproteins (40-50 %) and chitin (2 %) (Dallies *et al*., 1998). The cell wall of filamentous fungi, including *A. niger*, when compared to the cell wall of *S. cerevisiae*, is generally more rich in chitin (10-15 %), and contains α-1,3-glucan (10-35 %) (Fontaine *et al*., 2000, de Nobel *et al*., 2000a). In both *S. cerevisiae* and *Candida albicans* it has been shown that chitin chains and β-1,6-glucosylated mannoproteins are covalently linked to the β-1,3-glucan part of the cell wall (Kollar *et al*., 1997, Kapteyn *et al*., 1995). Morphogenetic events and especially the formation of a new bud in yeasts and the formation of new branches in filamentous fungi, require drastic remodeling of the cell wall with the temporal risk of cell lysis. In addition, the presence of plant produced cell wall degrading enzymes, in response to fungal infections, are a serious threat to fungi. To maintain the integrity of the cell wall, fungi posses a signal transduction cascade that is activated in response to cell wall stress and induces the expression of genes that prevent cell lysis. The so called 'cell wall integrity pathway' has been particularly well studied in *S. cerevisiae* and is also known as the Pkc1p

or Slt2p/Mpk1p MAP kinase signaling pathway (reviewed by Banuett 1998, Smits *et al*., 1999, and Heinisch *et al*., 1999). Activation of the pathway is induced in response to several environmental stimuli which include: growth at elevated temperatures (Kamada *et al*., 1995), hypo-osmotic shock conditions (Davenport *et al*., 1995, Kamada *et al*., 1995), the addition of mating pheromones (Errede *et al*., 1995, Buehrer and Errede, 1997), the addition of agents that cause cell wall stress such as Calcofluor White, Congo Red, caspofungin or β-1,3-glucanase, (Ketela *et al*., 1999, de Nobel *et al*., 2000b, Reinoso-Martin *et al*., 2003, Garcia *et al*., 2004), and actin depolymerisation agents (Harrison *et al*., 2001). The pathway is also activated in mutants with impaired cell wall synthesis (Terashima *et al*., 2000, Lagorce *et al*., 2003) or in constitutively activated signaling mutants (Jung and Levin, 1999). Putative sensors of the pathway are the transmembrane proteins Wsc1p-Wsc4p (Zu *et al*., 2001) and Mid2p (Ono *et al*., 1994, Ketela *et al*., 1999, Green *et al*., 2003). The Wsc1p-Wsc4p proteins interact through Tor2p with a guanine nucleotide exchange factor, Rom2p, to activate the small GTPase Rho1p (Bickle *et al*., 1998, Sekiya-Kawasaki *et al*., 2002). Mid2p and Zeo1p also act as activators of Rho1p via a mechanism independent of Wsc1p and Rom2p (Sekiya-Kawasaki *et al*., 2002, Green *et al*., 2003). One of the functions of Rho1p is the activation of Pkc1p (Nonaka *et al*., 1995, Kamada *et al*., 1996). Pkc1p activates a linear MAPK-signaling module consisting of the MAPKKK, Bck1p, (Costigan *et al*., 1992), the redundant pair of MAPKK, Mkk1p and Mkk2p, (Irie *et al*., 1993) and the MAPK, Slt2p/Mpk1p, (Lee *et al*., 1993). Activation of the PKC-pathway results in the phosphorylation of the threonine and tyrosine residues in the TXY motif of Slt2/Mpk1p (Martin *et al*., 2000). Three classes of targets of Slt2p/Mpk1p are known: a pair of functionally redundant High Mobility Group Hmg1p-like proteins Nhp6Ap and Nhp6Bp, the SBF transcription factor composed of Swi4p and Swi6p, and the MADS-box transcription factor Rlm1p. The Non-Histone Proteins 6A and 6B, Nhp6A/B, are chromosome associated proteins that can be activated by phosphorylation to bend the DNA with no apparent sequence specificity (Costigan *et al*., 1994, Paull and Johnson, 1995, Kolodrubetz *et al*., 2001). It has been suggested that they play a general role in promoting rearrangements of chromatin by initiating the destabilisation of core nucleosome structure (Ruone *et al*., 2003). SBF, a heterodimeric complex composed of Swi4p and Swi6p, controls the expression of cell cycle regulated genes in the G1/S phase (Sidirova and Breeden, 1993, Madden *et al*., 1997, Iyer *et al*., 2001) including genes involved in cell wall synthesis (Ram *et al*., 1995, Igual *et al*., 1996). Rlm1p was identified as a gene conferring resistance to lethality of $MKK1^{P386}$ overexpression (Watanabe *et al*., 1995). Rlm1p belongs to the evolutionary conserved family of the MADS (Mcm1p-Agamous-Deficiens-Serum Response Factor) box transcription factor proteins (Schwarz-Sommer *et al*., 1990). The superfamily consists of transcription factors containing a strong sequence homology in a ~ 90

amino acid DNA-binding domain, which is located at the N-terminus in most of the family members. Two subfamilies of MADS-box proteins have been described: the SRF (Serum Response Factor) and the MEF2-subfamily. Proteins of both families contain MADS-boxes within their core DNA binding region, predominantly located at the N-terminus of the protein, but differ within the domain following the MADS-box. Members of the MEF2 subfamily, which includes Rlm1p, contain the so-called MEF2 domain while the SRF-family members have a SAM (SRF, AG, MCM1) domain adjacent to the MADS-box. In *S. cerevisiae* four MADS-box proteins have been identified of which two (Rlm1p and Slm1p) belong to the MEF2 subfamily. The other two (Mcm1p and Arg80p) belong to the SRF-subfamily.

Rlm1p is most closely related to the mammalian MADS-box MEF2 transcription factors and shows similar DNA-binding specificity *in vitro* CTA(T/A)$_4$TAG (Dodou and Treisman, 1997). The transcriptional activation potency of Rlm1p is regulated through phosphorylation by the protein kinase Mpk1p (Watanabe *et al*., 1997, Jung *et al*., 2002). In *S. cerevisiae*, Rlm1p is localised in the nucleus irrespective of its activation or phosphorylation status (Jung *et al*., 2002). Rlm1p and its binding sites have been shown to be required for the activation of genes involved in cell wall remodeling in response to cell wall stress (Jung and Levin, 1999, Terashima *et al*., 2000). Indeed, genome wide expression analysis of the response to different forms of cell wall stress in *S. cerevisiae* has further indicated an important role of Rlm1p in mediating the activation mechanism because of the presence of putative Rlm1p binding sites in their promoters (Roberts *et al*., 2000, Lagorce *et al*., 2003, Reinoso-Martin *et al*., 2003, Garcia *et al*., 2004, Boorsma *et al*., 2004). From these studies, it is also evident that the Pkc1p-Slt2p dependent pathway is not the only signaling pathway that contributes to cell wall remodeling in yeast. Both the calcium/calcineurin pathway (Zhao *et al*., 1998, Yoshimoto *et al*., 2002) and the Hog1p-MAPK (reviewed by Hohmann, 2002) signaling pathways are involved in the activation of genes implicated in maintaining cell wall integrity. In addition, the Heat shock transcription factor Hsf1p, which binds to the Heat Shock Element HSE (Sorger, 1991, Fernandes *et al*., 1994) and Swi4p, a PKC1-regulated transcription factor, involved in G1/S specific gene expression was also reported to have an effect on the up-regulation of genes after cell wall stress (Igual *et al*., 1996, Madden *et al*., 1997).

We previously showed that the cell wall remodeling mechanism is not limited to *S. cerevisiae*, and that filamentous fungi also remodel their cell wall in response to cell wall stress. Treatment with various agents known to disturb normal cell wall assembly, such as Calcofluor White (CFW), SDS and caspofungin, lead to induced expression of an α-1,3-glucan synthase gene (*agsA*) and a glutamine:fructose-6-phosphate amidotransferase gene (*gfaA*) in *A. niger*. These responses were also found in other filamentous fungi such as *Penicillium chrysogenum* and *Fusarium oxysporum* and suggest that cell wall stress

response in fungi includes the activation of both α-1,3-glucan and chitin biosynthesis (Chapter 2 and 3).

In this study, we show that the *S. cerevisiae* Rlm1p homologue in *A. niger* (RlmA) is involved in the activation of genes in response to cell wall stress. In contrast to the disruptant in *S. cerevisiae*, deletion of the *rlmA* gene in *A. niger* renders the fungus hypersensitive to multiple cell wall stress inducing compounds. This indicates that the RlmA transcription factor has an important function during the cell wall stress response in *A. niger* to ensure cell wall integrity.

Materials and Methods

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) (Bennett and Lasure, 1991) 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. Growth medium was supplemented with 10 mM uridine (Serva) when required. Transformation of *A. niger* was performed as described by Punt and van den Hondel (1992) using lysing enzymes (L1412, Sigma) for protoplast formation. Conidiospores were obtained by harvesting spores from a CM-plate after 4-6 days of growth at 30 °C, using 0.9 % NaCl (w v^{-1}). 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.

Molecular biological techniques

Chromosomal DNA of *A. niger* was isolated as described by Kolar *et al*., 1988. Alternatively, chromosomal DNA was isolated using the FastPrep FP120 (Bio101). First, *A. niger* spores were grown in fast-prep tubes containing 1 ml CM and 0.3 g acid washed glass beads. After growth for 16 hours at 37 °C the mycelium was spun down, medium was removed, 500 µl cold extraction solution (2:2:1 v v^{-1} % mixed, TNS, 40 mM tri-isonaphtalene sulphonic acid, PAS, 0.70 M P-aminosalicylic acid, and RNB, 1.0 M Tris-HCl pH 8.5, 1.25 M NaCl, 0.25 M EDTA) and 500 µl phenol:cholorform:isoamyl alcohol (25:24:1 v v^{-1} %) was added. Vials were vigorously shaken in a FastPrep FP120 (Bio101) twice for 30 seconds at speed 6.0 and cooled for five minutes on ice between runs. 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 the instructions of the manufacturer. RNA was extracted from mycelium and snap-frozen in liquid nitrogen using TRIzol reagent (InVitrogen). Total RNA (10 µg) was incubated with 2.3 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 one hour at 50 °C to denature the RNA. RNA electrophoresis was performed in a SEA-2000 (Elchrom Scientific) at 10 °C. PCR was performed on a PTC-100 Programmable Thermal Controller (MJ Research, Inc) using Super Taq (HT Biotechnology LTD) or when required Expand High Fidelity PCR system (Roche). Primers were obtained from Isogen and are listed in Table 1. For ligation the Rapid DNA Ligation Kit (Boehringer Mannheim) was used. 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). Restriction enzymes were obtained from InVitrogen and used according to the protocol supplied by the manufacturer.

Table 1. Primers used in this study. Restriction sites are underlined

Primer Name	Sequence (5' to 3')
RlmAP1	ataagaatgcggccgcaaagtccgacccagaggctt
RlmAP2	tgctctagagggacagcggatgaacgaa
RlmAP3	tgctctagaagtggacccaatgcggaa
RlmAP4	ggggtaccccaccccttgcataaccatc
RlmAP7	ctacctgacagacagactggt
RlmAP8	tgcccagtcccttgacgtt
pAO-9	aatgtcaattccagcagcg
AgsAP5	ttagcgctctgagtgtcgg
AgsAP29	cgatgcgatccaggaagatg
AgsADRlmP1	atgacgtttcccaccgagagtagagaatga
AgsADRlmP2	ctctcggtgggaaacgtcatatcaggatagc
pAgsA-AF-F-mut-RlmA1	atatgacgtttctcggcggcgaccaccgagagtagagaatga
pAgsA-AF-R-mut-RlmA2	ctcggtggtcgccgccgagaaacgtcatatcaggatagc
pAgsA-AF-F-two-RlmA3	atatgacgtttcctaataatagccacgtttcctaataatagccaccgag
pAgsA-AF-R-two-RlmA4	ctattattaggaaacgtcatatcaggatagc
pAgsA-AF-F-three-RlmA5	atatgacgtttcctaataatagccacgtttcctaataatagccacgtttc ctaataatagccaccgag

Cell wall stress-inducing conditions and cell wall analysis

A. niger spores were inoculated in 50 or 100 ml CM at a spore density of $1 \cdot 10^7$ spores ml^{-1} and grown for 5 hours at 37 °C and 300 rpm. After the spores had germinated, germlings

were treated with a cell wall stress-inducing compound (200 µg ml^{-1} Calcofluor White, CFW) by adding the compound from a freshly prepared stock solution (20 mg ml^{-1} CFW) 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 with liquid nitrogen prior to the isolation of RNA or cell walls. Sensitivity towards various compounds was assayed in 96-well microtiter plates (Nunc, art. 164588) using a Perkin Elmer HTS-7000 Bioassay reader. A series of concentrations of stress-inducing compounds (CFW, caspofungin, hydrogen-peroxide, SDS) were prepared in 100 µl MilliQ in a 96-well plate, and 100 µl spore solution (~ 2 x 10^4 spores) in 2X complete medium was added to 100 µl stress-inducing solution. The microtiter plates were incubated at 37 °C and the OD_{590} was measured after 24 hours. To determine chitin levels in the cell walls of both wild-type and the *ΔrlmA* mutant, cell walls were isolated by grinding frozen mycelium from various time points of CFW treated and control mycelia as described (Ram *et al*., 2004). Chitin levels were determined essentially as described by Tracey (1956) with minor modifications (Ram *et al*., 2004).

Construction of the P*agsA* reporter constructs

The full length (2.0-kb) P*agsA*-GUS reporter construct was made as follows. First, an NcoI site was introduced at the ATG of the *agsA* gene by PCR during the construction of P*agsA*-*amdS*^{NcoI} (R.A. Damveld and A.F.J Ram, unpublished). The 2.0-kb SalI-NcoI *agsA* promoter region, together with cloning vector pBluescript SKII (Stratagene) was isolated from P*agsA*-*amdS*^{NcoI} as a 4.7-kb XbaI-NcoI fragment and used in a three way ligation. The *uidA* gene was isolated as an NcoI-SalI fragment from plasmid pNOM102 (accession number Z32701). The third fragment, containing the 0.8-kb *trpC* terminator region, was isolated from pAN52-1NotI (accession number Z32697) using BamHI and XbaI, first cloned into BglII-XbaI opened pUC21, and re-isolated as an XhoI-XbaI fragment. Proper ligation of the three fragments was verified by restriction enzyme digestions. To allow targeted integration of the P*agsA*(2.0-kb)-*uidA* construct at the *pyrG* locus, the *pyrG** allele (Gouka *et al*., 1995, isolated as a 3.8-kb XbaI fragment from pAN52-7*pyrG**, unpublished vector) was inserted in the unique XbaI site of P*agsA*(2.0)-*uidA* to give P*agsA*(2.0)-*uidA*-*pyrG**.

The P*agsA*(2.0-kb)-*uidA* construct was also used to construct a plasmid in which only 30 nucleotides of the *agsA* promoter were cloned in front of the *uidA* gene using an EcoRI site 30 nucleotides upstream of the ATG. A 2.6-kb EcoRI-XbaI fragment consisting of 30-bp of the *agsA* promoter, the *uidA* gene, and the *trpC* terminator region, was isolated from P*agsA*(2.0-kb)-*uidA* and ligated to the backbone of pAN56-2 which was isolated as a 2.7-kb fragment after EcoRI-XbaI digestion to give P*agsA*(30-bp)-*uidA*. Subsequently, the *pyrG**

gene was cloned into the unique XbaI site to give P*agsA*(30-bp)-*uidA-pyrG**. Other constructs containing shortened *agsA* promoter regions were made by either using appropriate restriction sites or by exo-nuclease digestion. *AgsA* promoter regions SalI-EcoRI (2.0-kb), HindIII-EcoRI (1.5-kb), BamHI-EcoRI (0.9-kb) and PstI-EcoRI (0.55-kb) fragments were isolated from RD6 (a 7.6-kb genomic subclone containing the *agsA* gene) and cloned into pUC21 (SalI-EcoRI or HindIII-EcoRI) or into pBluescript SKII (BamHI-EcoRI or PstI-EcoRI). Promoter regions were re-isolated as NotI-EcoRI fragments and inserted into a NotI-EcoRI digested P*agsA*(30-bp)-*uidA-pyrG** vector to give P*agsA*(2.0-kb-NotI)-*uidA-pyrG**, P*agsA*(1.5-kb)-*uidA-pyrG**, P*agsA*(0.9-kb)-*uidA-pyrG**, and P*agsA*(0.55-kb)-*uidA-pyrG**, respectively. The plasmid containing the HindIII-EcoRI 1.5-kb *agsA* promoter region in pUC21 was used for exo-nuclease digestion using the Erase-a-base system (Promega). The plasmid was first digested with SalI, filled with α-phosphorothioate dNTP mix using Klenow, subsequently digested with HindIII, and treated with exonuclease according to the protocol of the supplier. Time samples were taken, treated with Klenow, ligated and transformed to *E. coli.* To estimate the insert size, the *E. coli* colonies were analysed by PCR. Several colonies with different insert sizes were sequenced. One clone (#21) contained 279-bp of the *agsA* promoter and was used to make an additional reporter construct. The 279-bp promoter region was re-isolated as NotI-EcoRI fragment and inserted into NotI-EcoRI digested P*agsA*(30-bp)-*uidA-pyrG** to give P*agsA*(279-bp)-*uidA-pyrG**.

The constructs used to determine the effect of the Rlm1p box 5'-ctaataatag-3' (Rlm1p-binding site underlined) at -402-bp from the ATG in the *agsA* promoter were made by fusion PCR. For deletion of this Rlm1p element primers AgsADRlmP1 and AgsADRlmP2 were used, removing the sequence 5'-ctaataatag-3'. The Rlm1p element 5'-ctaataatag-3' was mutated into the sequence 5'-tcggcggcga-3'using primers pAgsA-AF-mut-RlmA1 and pAgsA-AF-R-mut-RlmA2. For introduction of a second Rlm1p element, adjacent to the wild-type Rlm1p element, the sequence 5'-gtttcctaataatagccac-3' was inserted using primers pAgsA-AF-F-two-RlmA3 and pAgsA-AF-R-two-RlmA4. The insertion of the sequence 5'-gtttcctaataatagccacgtttcctaataatagccac -3' containing two additional Rlm1p elements was done with primers pAgsA-AF-F-three-RlmA5 and pAgsA-AF-R-two-RlmA4. The outer primers agsAP5 and agsAP29 (Table 1) were used in combination with the four mentioned primer sets to amplify the ~ 0.9-kb fragment which was subsequently cloned in pGEMT-EASY (Promega). Inserts were checked by a double digestion with PstI/EcoRI and sequenced to confirm proper deletion, mutation, or addition of nucleotide sequences. For all four constructs, the ~ 0.6-kb PstI-EcoRI *agsA* promoter fragment was isolated from the pGEMT-EASY vector and cloned into a PstI-EcoRI opened pBLSKII vector, to enable re-isolation as a NotI-EcoRI fragment. The ~ 0.6-kb NotI-EcoRI P*agsA* fragments containing the deleted, mutated, or

(two) additional Rlm1p element(s) were ligated into a NotI-EcoRI opened P*agsA*(0.55-kb)-*uidA-pyrG** vector to give P*agsA*(0.55-kb-Δrlm)-*uidA-pyrG**, P*agsA*(0.55-kb-rlm^{mut})-*uidA-pyrG**, P*agsA*(0.55-kb-rlm^{1add})-*uidA-pyrG**, P*agsA*(0.55-kb-rlm^{2add})-*uidA-pyrG**. After transformation of the GUS reporter constructs to AB4.1, the strains were analysed for GUS activity on plates. Groups of 20 transformants were spotted on CM plates covered with Hybond-N filters. Strains were grown at 30 ℃ and after 24 hours the filters with small colonies on top were removed from the plates and subsequently frozen in liquid nitrogen and thawed twice. The filters were then incubated in Z-buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4 \cdot 7H_2O$, 2.7 µl ml^{-1} β-mercaptoethanol) 0.04% X-GlcA-Sodium salt (Duchefa, Haarlem) at 37 ℃ until blue coloration was visible (Roberts *et al*., 1989). Strains that tested positive for GUS activity on plates were purified twice and subjected to Southern analysis. To confirm single copy integration at the *pyrG* locus genomic DNA was digested with BamHI and probed with a 2.3-kb XhoI fragment containing the *pyrG* gene.

Construction of the *rlmA* deletion plasmid

The DNA sequence encoding the *A. niger* RlmA transcription factor was obtained from DSM and was used to generate a deletion construct by PCR. The 5' promoter region of *rlmA* was amplified using primers RlmAP1 and RlmAP2 (Table 1). The 3' terminator region was amplified using RlmAP3 and RlmAP4. PCR products of 1020 and 903-bp were obtained, digested with NotI and XbaI or XbaI and KpnI, respectively, and used in a three way ligation with pBluescript SKII which had been digested by NotI and KpnI to give pRLM1. The *rlmA* deletion construct was made by inserting a 2.7-kb XbaI-XbaI fragment from pAO4-13 (Ruiter-Jacobs *et al*., 1989), containing the *pyrG* gene from *A. oryzae* into the unique XbaI site of pRLM1 to give pΔRlmA. The deletion cassette was linearised with NotI and BglI followed by transformation to *A. niger pyrG*$^{-}$ strain AB4.1. To identify putative gene deletion mutants with PCR on genomic DNA isolated from fungi grown in 2 ml fast-prep tubes (Bio101, Cat # 5076-400) primers RlmAP7 and pAO-9 were used. The primer sequence of RlmAP7 (Table 1) is localised outside the 3' gene deletion construct and primer pAO-9 (Table 1) anneals on the *A. oryzae pyrG* gene. A double cross-over and thus deletion of the *rlmA* locus would result in the amplification of a 1.1-kb fragment. Genomic DNA of 11 pools each containing 20 transformants were analysed by PCR. All pools gave a 1.1-kb PCR product, indicating that they all contained at least one deletion strain. Because pool number one produced the most PCR product, genomic DNA from individual transformants of this pool was further analysed, by repeating the PCR reaction using RlmAP7 and pAO-9, and also by PCR with primer pair RlmAP7 and RlmAP8. The primer RlmAP8 is located within *rlmA* and should give a PCR product of ~ 1100-bp if the *rlmA* gene is still intact. In this pool five out of the 20 transformants

showed product with the gene deletion primer set (RlmAP7 and pAO-9). No product was found with the *rlmA* primer set (RlmAP7 and RlmAP8) indicating that those five transformants contain a deletion of the *rlmA* gene. Southern analysis was used to confirm the deletion of the *rlmA* gene.

Nucleotide sequence accession numbers

The *A. niger rlmA* sequence was obtained from DSM (Dr. N. van Peij and Dr. G. Groot, pers. communication). The sequence data have been submitted to the DDBJ/EMBL/GenBank databases under accession numbers: AY704272 *rlmA* and AY704271 *actA* probe HindIII-SalI fragment.

Results

The transcriptional activation of the *agsA* gene in response to CFW requires a putative Rlm1-like transcription factor binding site in its promoter

We previously reported that both the *agsA* and *gfaA* genes, encoding an α-1,3-glucan synthase and a glutamine:fructose-6-phosphate amidotransferase respectively, are induced in response to different forms of cell wall stress (Damveld *et al*., 2005, Ram *et al*., 2004, Chapter 2 and 3). To obtain information about how this induction is regulated at the transcriptional level, both promoter sequences were analysed *in silico*. The analysis revealed the presence of several putative transcription factor binding sites. For our search we used consensus sequences corresponding to binding sites of fungal transcription factors encoded by *MSN2/4*, *RLM1*, *HSF1*, *CRZ1*, *SWI4/6, stuA* and *AnCP/AnCF* genes (Table 2). The results

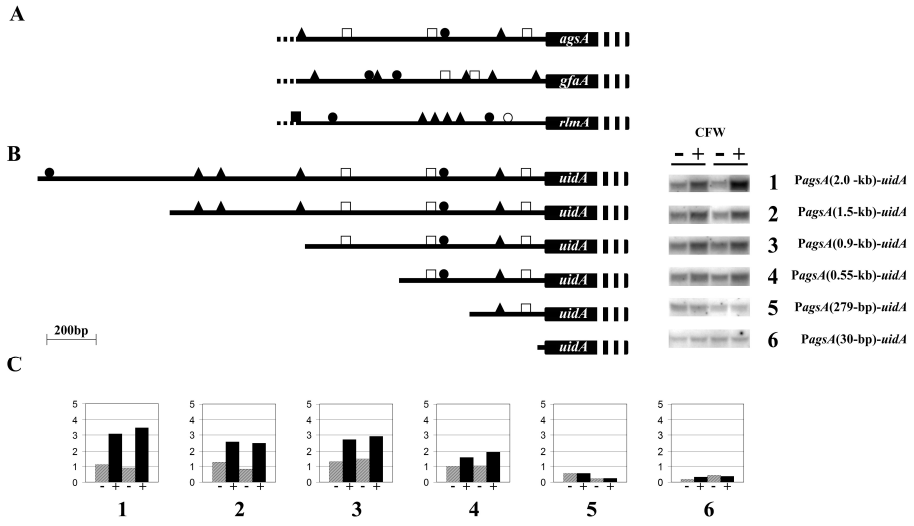


Figure 1. **A**. Schematic representation of the promoter elements found in the promoters of *agsA, gfaA*, and *rlmA*. Putative transcription factor binding sites are indicated as follows: ▲Msn2/4p, ■ Crz1p, ● Rlm1p, ○ StuA, □ AnCP/AnCF. **B**. Northern analysis of the *agsA* promoter deletion series. Schematic representation of the promoter deletion constructs. The GUS messenger levels in two individual transformants are shown with (+) and without (-) CFW stress. **C**. Quantification of the Northern blots. The amount of messenger present in each lane was corrected for differences in loading based on hybridisation with an 18S ribosomal probe. The messenger levels in the graphs are in relative units. The EcoRI-NcoI 1.1-kb fragment of the GUS gene from pNOM102 was used as a probe.

Table 2. *In silico* anaysis of three cell wall stress induced promoters.

Promoter element	Sequence	Position in			Protein name	Org.[1]	Reference
		P*agsA*	P*gfaA*	P*rlmA*			
Stress response element (STRE)	CCCCT	-184 -960	-47 -211 -309 -682 -939	-353 -404 -441 -507	Msn2/4p	*S. c.*	Schüller *et al*., 1994 Treger *et al*., 1998
Heat shock element (HSE)	GAANNTCC				Hsf1p	*S. c.*	Sorger, 1991 Fernandes *et al*., 1994
Calcineurin-dependent response element (CDRE)	AGCCTC			-1007	Crz1p	*S. c.*	Yoshimoto *et al*., 2002 Lagorce *et al*., 2003
Rlm1p promoter element	$TA(W)_4TAG$	-406	-607 -709	-231 -862	Rlm1p	*S. c.*	Jung and Levin, 1999
SCB (Swi4/6p cell cycle box) binding factor (SBF)	CGCGAAAA				Swi4/6p	*S. c.*	Iyer *et al*., 2001
StuA promoter element	NWWCGCG WNM			-161	StuA	*A. n.*	Dutton *et al*., 1997
HAP like element	CCAAT	-73 -436 -792	-291 -413		AnCP/ AnCF	*A. n.*	Kato *et al*., 1998

[1] The organism from which the consensus of the promoter element originates. *S.c.* = *S. cerevisiae*, *A.n.* = *A. nidulans*

of the analysis are schematically represented in Figure 1A. In both the 1-kb *agsA* and 1-kb *gfaA* promoter sequences putative binding sites for the Msn2/4p, Rlm1p, and AnCP/AnCF transcription factors are present.

Since the *agsA* gene showed the strongest induction (around 20-fold) in response to CFW stress, the *agsA* promoter was studied in more detail. The *agsA* promoter region contains two putative stress response elements at positions -184 and -960 and a single putative Rlm1-binding site at position -406 (Table 2). To determine the role of the putative transcription factor binding sites in the *agsA* promoter during cell wall stress, a promoter deletion study was performed. Six constructs were made that contained different lengths of the *agsA* promoter (Fig. 1B). These promoter fragments were fused to the β-glucuronidase (*uidA*) reporter gene (Roberts *et al*., 1989). All constructs were targeted to the *pyrG* locus of strain AB4.1 using the *pyrG** gene (Gouka *et al*., 1995) to allow comparison of expression levels between the different transformants. For each reporter construct, two independent transformants with a single copy integration of the construct at the *pyrG* locus were selected and subjected to further analysis. Attempts to measure GUS activity of the transformants after CFW stress failed because CFW inhibited the GUS activity assay (data not shown). Since we were unable to measure GUS activities directly, Northern analysis of the reporter strains was performed using a fragment of the GUS gene as a probe (Fig. 1B and C). The results of the Northern analysis show that 1-kb of the *agsA* promoter is sufficient to give the same expression before and after cell wall stress when compared to the 1.5-kb and 2-kb promoter constructs. Transformants containing the 550-bp promoter fusion still resulted in the induction of the *uidA* mRNA after cell wall stress. However, both the basal level of expression and the maximum level of induction were less. In the transformants containing the 300, and 30-bp promoter regions fused to the GUS reporter, no induction after cell wall stress was observed. These results suggest that the regulatory element(s) responsible for the induction of the *agsA* gene after cell wall stress are located between -550 to -300-bp in the *agsA* promoter region. Within this region (at position -406), a single putative Rlm1p transcription factor binding site is present, which suggests a possible role for the putative Rlm1p-binding site in the induction of *agsA* in response to CFW.

To confirm the role of the putative Rlm1p binding element in the *agsA* promoter, the element was deleted and mutated. In transformants containing the *agsA* promoter-*uidA* construct with the Rlm1p-binding site deleted or mutated (P*agsA*(0.55-kb-Δrlm)-*uidA* and P*agsA*(0.55-kb-rlm^{mut})-*uidA*) induction of the *uidA* mRNA after CFW stress was abolished, indicating that this Rlm1p element is important for the up-regulation of the *agsA* gene in response to CFW stress (Fig. 2). The effect of additional Rlm1p elements adjacent to the wild-type Rlm1p-element was also investigated in transformants carrying constructs with one or two additional Rlm1p elements in the *agsA* promoter, P*agsA*(0.55-kb-rlm^{1add})-*uidA* and P*agsA*(0.55-kb-rlm^{2add})-*uidA*. The addition of a single Rlm1p element adjacent to the wild-type Rlm1p element was sufficient to give a ~ 2.5-fold higher induction after CFW stress

compared to the wild-type construct carrying a single Rlm1p element. When two additional Rlm1p elements were introduced into the reporter construct a ~ five-fold induction after CFW stress was observed after CFW stress for one transformant. The other transformant gave an induction comparable to that of the reporter construct carrying one additional Rlm1p element (Fig. 2).

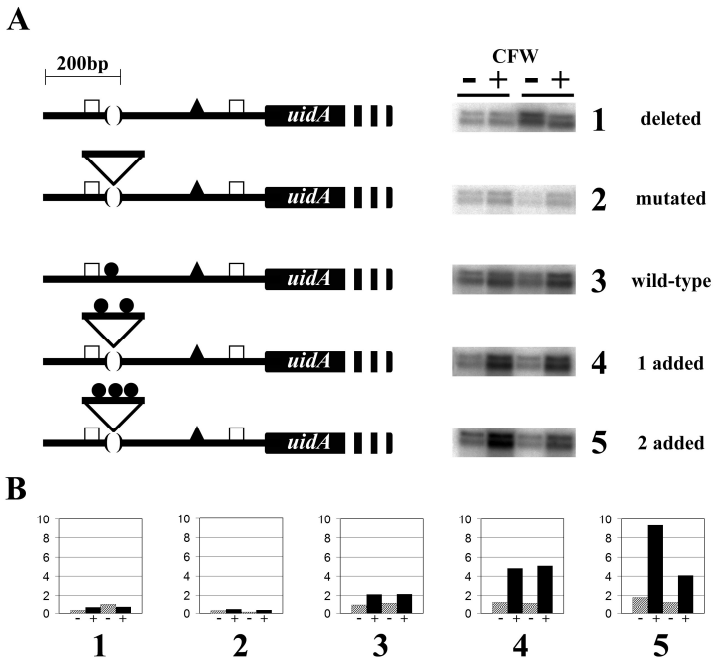


Figure 2. The role of the RlmA-element in the *agsA* promoter. **A**. Schematic representation of the P*agsA*-GUS constructs. The putative transcription factor binding sites in the *agsA* promoter are indicated as follows: ▲Msn2/4p, ● Rlm1p, □ AnCP/AnCF. The brackets ( ) represent the removal of promoter sequence. The fragment above the brackets indicates what fragment was inserted at that position, if applicable. To the right of a GUS fusion construct the corresponding Northern blot is depicted. GUS messenger levels in two individually obtained transformants were analysed with and without CFW stress. **B**. Quantification of the Northern blots. The messenger levels were corrected for differences in loading based on hybridisation with an 18S ribosomal probe. The messenger levels in the graphs are in relative units.

Identification of the *A. niger* MADS-box transcription factor homologue RlmA

The previous results indicate that the induction of *agsA* and possibly also the *gfaA* gene in *A. niger* in response to cell wall stress seems to be mediated by a Rlm1p-like transcription factor. Therefore, we searched in the *Aspergillus niger* genome sequence for

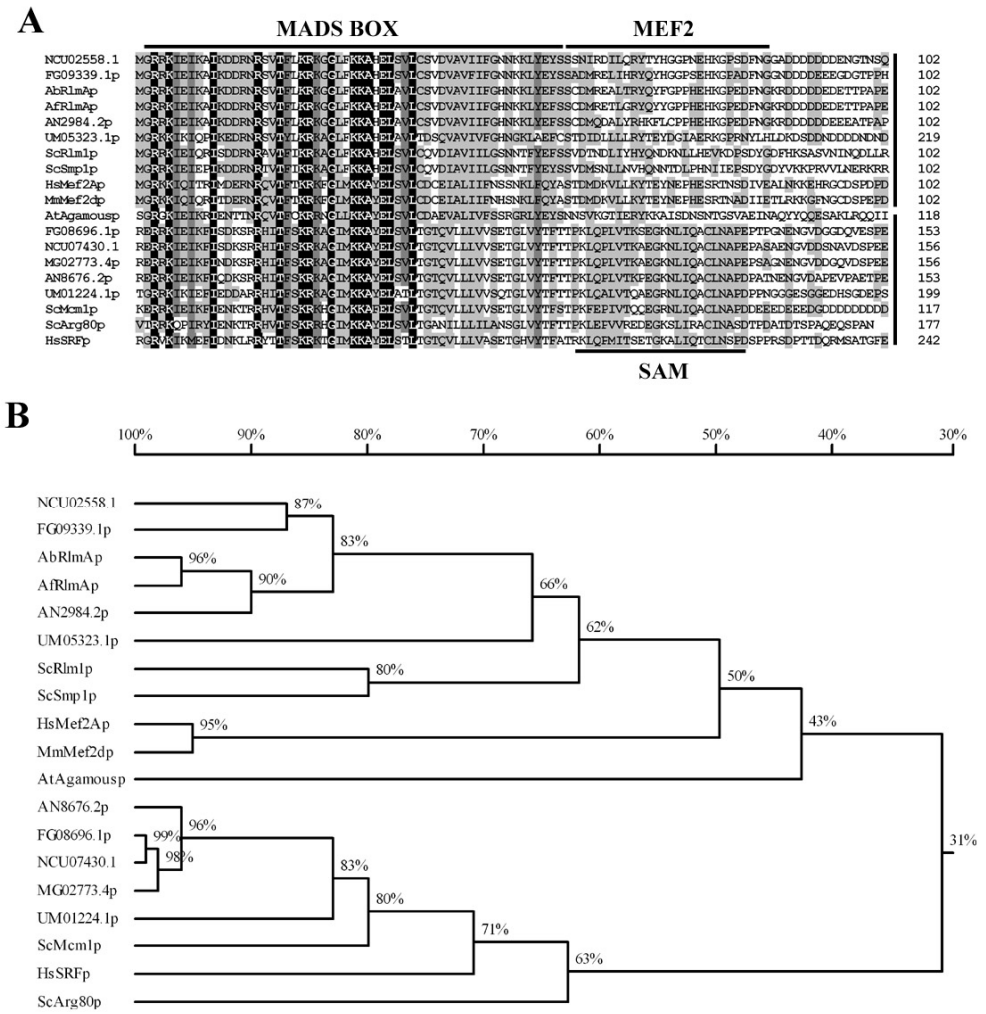


Figure 3A. Alignment of the 102-amino acid fragment containing the MADS-box and MEF2/SAM domain of MADS-box transcription factors. Amino acid residues that are identical in all protein sequences are shown in a black background. Residues printed in a light- or dark-grey background are ≥ 50 % or ≥ 75 % identical respectively. Proteins aligned are: *Aspergillus nidulans* (AN2984.2, EAA63555 and AN8676.2, EAA60098), *Giberelia zeae* (anamorph *Fusarium graminearum*) (FG09339.1, EAA76082 and FG8696.1, EAA70796), *Neurospora crassa* (NCU02558.1, EAA36453 and NCU07430.1, EAA35381), *Ustilago maydis* (UM05323.1, EAK86572 and UM01124.1, EAK81831), *A. niger* (RlmA, AY704272), *Magnaporthe grisea* (MG02773.4, EAA47530), *A. fumigatus* (AfRlmA, a_fumigatus|chr_0|TIGR.5237|59 ATG at 1893428), *Saccharomyces cerevisiae* (ScSmp1p, CAA85143, ScRlm1p, AAB68210, ScMcm1p, CAA88409, ScArg80p, CAA88408), *Homo sapiens* (HsSRF, AAH48211, HsMef 2A, AAH13437), *Mus musculus* (MmMef2D, AAH11070) and *Arabidopsis thaliana* (AtAgamous, NP_567569). Proteins were aligned in DNAMAN. Indicated with black lines are: the 57 amino acid MADS-box region, required for DNA binding, the 28 amino acid MEF2 domain and the 24 amino acid SAM domain.

B. Homology tree of the 102-amino acid fragment containing the MADS-box and MEF2/SAM domain of MADS-box transcription factors. The tree was created with DNAMAN using default settings for gap and extension penalties.

Rlm1p homologues. Two putative MADS-box proteins were identified, one of them being homologous to Rlm1p. The *A. niger* Rlm1p homologue, RlmA, groups with the MEF2-subfamily of MADS-box proteins. Significant homology (57%) between the *A. niger* RlmA protein (AbRlmAp) and the *S. cerevisiae* Rlm1 protein (ScRlm1p) is only found in the MADS-box and MEF2 region (Fig. 3B). The overall homology between the full length AbRlmAp and ScRlm1p is only 19.7 %. MADS-box transcription factors were also identified in the *A. nidulans* and *A. fumigatus* genome sequences. Two MADS-box proteins were found in the genome of *A. nidulans* and one MADS-box protein in the genome of *A. fumigatus*. Sequence alignment of the two MADS-box proteins from *A. nidulans* with other MADS box proteins, revealed that both a MEF2-like and a SRF-like MADS-box transcription factor were present. The single MADS-box protein of *A. fumigatus* belonged to the MEF2-subfamily (Fig. 3A). Other available fungal genomes were analysed for the presence of MADS-box proteins. For most fungi, two MADS-box proteins were identified in the genome, belonging in all cases to both subfamilies. Exceptions so far are in *A. fumigatus*, as described above, and *M. grisae* in which only a SRF-member was found (Fig. 3A). However, conclusive remarks about the number of MADS-box proteins in these fungi require complete sequencing of their genomes.

The *A. niger rlmA* gene is required for the induced expression of *agsA* in response to CFW induced cell wall stress

To examine the role of RlmA during the induction of *agsA* and *gfaA,* the *rlmA* gene was deleted. A deletion cassette containing the *pyrG* gene from *A. oryzae* flanked by ~ 1-kb promoter and ~ 1-kb terminator region of *rlmA* was constructed as described in materials and methods and is shown in Figure 4A. After transformation of the linearised construct, putative *rlmA* deletion strains were first identified by PCR. Correct deletion of the *rlmA* gene in PCR positive transformants by a double cross-over event, was confirmed with Southern blot analysis (Fig. 4B). To determine the role of *A. niger* Rlm1p in the activation of *agsA* in response to cell wall stress, the expression of *agsA* was analysed in both the wild-type strain (N402) and the *rlmA* deletion strain (*ΔrlmA*) after CFW treatment (Fig. 5). N402 was grown for five hours and the *ΔrlmA* strain was grown for 5.5 hours until both strains had formed a small germ tube. Both strains were treated with 200 µg ml^{-1} CFW. RNA was isolated after 0, 15, 30, 45, and 60 minutes from cultures treated with and without CFW. In the wild-type strain the expression level of the *agsA* gene was already induced 15 minutes after CFW addition,

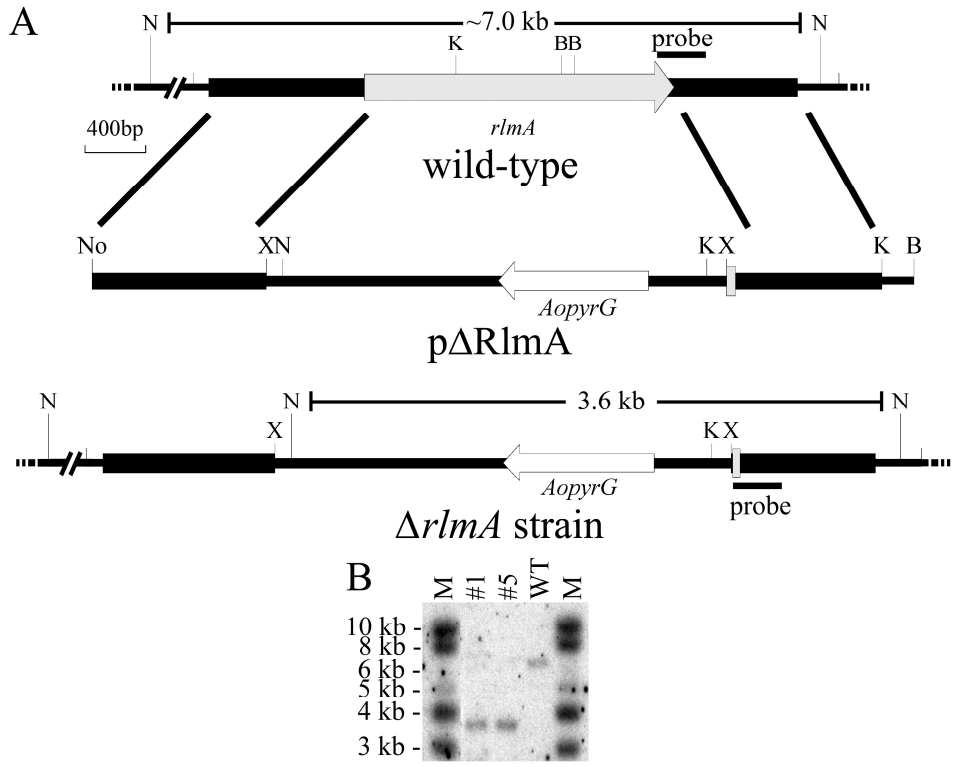


Figure 4. Deletion of the *rlmA* gene in *A. niger*. **A**. Schematic representation of: the *rlmA* wild-type locus (top), the plasmid, pΔRlmA, used for deletion (middle), and the deleted locus of the *ΔrlmA* strain (bottom). Abbreviations: BglI (B), KpnI (K), NdeI (N), NotI (No), XbaI (X), probe (1). **B**. Southern blot analysis of the wild-type (WT) and *rlmA* deletion strains (#1 and #5). Genomic DNA was digested with NdeI. Digestion of wild-type DNA is predicted to result in a 7.0-kb fragment, whereas digestion of genomic DNA from a deletion strain should result in a 3.6-kb fragment. The blot was probed with an approximately 300-bp XbaI-NcoI Tr*lmA* fragment as indicated in the figure.

indicating a rapid transcriptional response to the presence of CFW. The expression level of *agsA* remained high for one hour. After one hour the expression of *agsA* gradually decreased to reach the unstressed level after four hours (data not shown, Damveld *et al*., 2005, Chapter 3). Since no *agsA* mRNA could be detected in the *ΔrlmA* strain after treatment with CFW, the induction of *agsA* seems dependent on the RlmA transcription factor (Fig. 5). This result provides further evidence for an important role of an Rlm1p dependent signal transduction cascade in *A. niger* which mediates the cell wall remodeling response. No significant alteration in expression of the actin gene was detected after cell wall stress. The *gfaA* gene has been shown to be induced in response to CFW (Ram *et al*., 2004). As shown previously,

the *gfaA* mRNA level in the wild-type is four-fold induced in response to CFW and visible 15 minutes after CFW addition. Induction of the *gfaA* mRNA after CFW addition was still observed in the *ΔrlmA* strain, but only a two-fold induction was observed after quantification, and correction for the 18S signal, of the hybridisation signals (Fig. 5 and data not shown). This result shows that the induction of *gfaA* in response to CFW is partially dependent on the RlmA transcription factor.

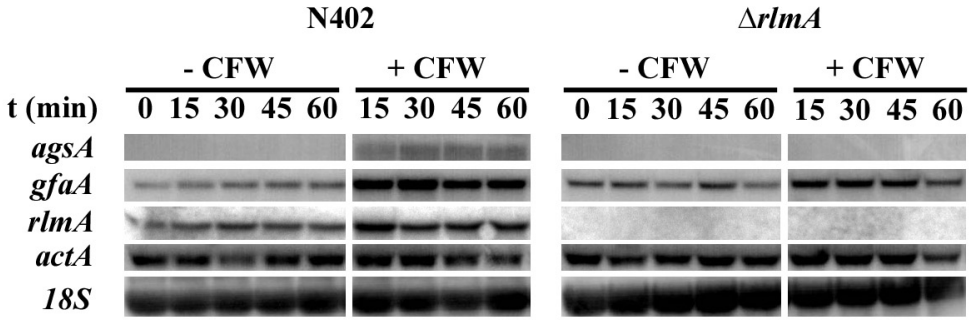


Figure 5. Cell wall integrity response in the wild-type (N402) and the *ΔrlmA* strain. Northern analysis of the *the agsA, gfaA, rlmA, actA, and 18S messenger levels*. Strains were grown in shake flask until small germ tubes were formed. Subsequently CFW (+) or an equal volume of water (-) was added to the cultures. RNA was extracted at the timepoints indicated above the Northerns, t = time in minutes. The probes used: an ~ 1.3-kb EcoRI fragment containing the ORF of *rlmA* was isolated from pBlue-rlmA (unpublished vector), for *gfaA* a ~ 1.3-kb ClaI-BglII fragment from pGfaA-BamHI (Ram *et al*., unpublished vector) was obtained, for *agsA* a ~ 0.6-kb EcoRI *agsA* fragment was isolated from pGEMT-AgsA (Damveld *et al*, unpublished vector), *actA* was obtained as a 1.1-kb HindI-SalI fragment, and the 18S ribosomal probe was isolated as a 2-kb BglI fragment from pMN1 (Borsuk *et al*., 1982.)

In silico analysis of the 1-kb *A. niger rlmA* promoter sequence (Fig. 1A) revealed the presence of four stress response elements (at -353-bp, -404-bp, -441-bp and -507-bp) and two putative Rlm1p binding sites (at -231-bp and -862-bp). To determine whether *rlmA* might enhance its own transcription in response to cell wall stress, as has been shown for the *S. cerevisiae RLM1* gene (Boorsma *et al*., 2004, Garcia *et al*., 2004, Lagorce *et al*., 2004), the expression of *rlmA* after CFW stress was examined (Fig. 5). The *rlmA* mRNA levels were quantified and corrected for differences in loading based on hybridisation with an 18S ribosomal probe. The analysis showed a ~ 2-fold induction of the *rlmA* gene 15 minutes after the addition of CFW. The induction is transient, 30 minutes after the addition of CFW, the *rlmA* mRNA levels were comparable to the control cells. As expected no hybridisation signal was observed in the Δ*rlmA* strain.

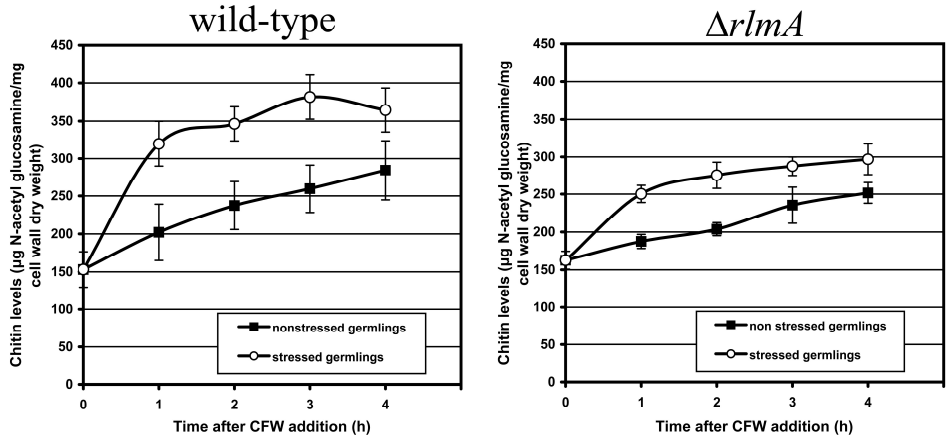


Figure 6. Chitin content of cell walls from wild-type and *rlmA* deletion strain after CFW stress. Conidia were inoculated and after five hours the germlings were treated with a sublethal concentration of 200 µg CFW ml^{-1}. Cell walls were isolated every hour after the addition of CFW and in the control 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.

The effect of a lower induction of *gfaA* in the *ΔrlmA* strain on the chitin content of the cell wall was examined by comparing chitin levels of wild-type and the *ΔrlmA* strain (Fig. 6). The chitin levels in the cell walls of both strains were comparable after five hours of growth. After the addition of CFW however, the amount of chitin in the cell walls of the *ΔrlmA* strain did not increase to the same level as in the wild-type strain. As increased chitin levels require a higher flux through the hexosamine pathway to generate the sugar donor for chitin biosynthesis, UDP-N-acetylglucosamine, a lower induction of the *gfaA* gene in the *ΔrlmA* strain might result in limiting amounts of UDP-N-acetylglucosamine and decreased deposition of chitin in the cell wall of a *ΔrlmA* strain.

Hypersensitivity of the *ΔrlmA* strain towards cell wall related antifungal compounds.

Having shown that the RlmA transcription factor is involved in the induction of cell wall related genes in *A. niger* in response to cell wall stress, the morphological consequences of a loss of function of *rlmA* after cell wall stress were examined. As shown previously (Damveld *et al*., 2005, Chapter 3), the addition of CFW to germinated wild-type spores results in an inhibition of polarised growth and swelling of the hyphal tips (Fig. 7). After four hours of growth, the germlings resumed polarised growth (Fig. 7). The *ΔrlmA* strain was also examined and a slight germination delay was observed. Whereas in wild-type cells it took five

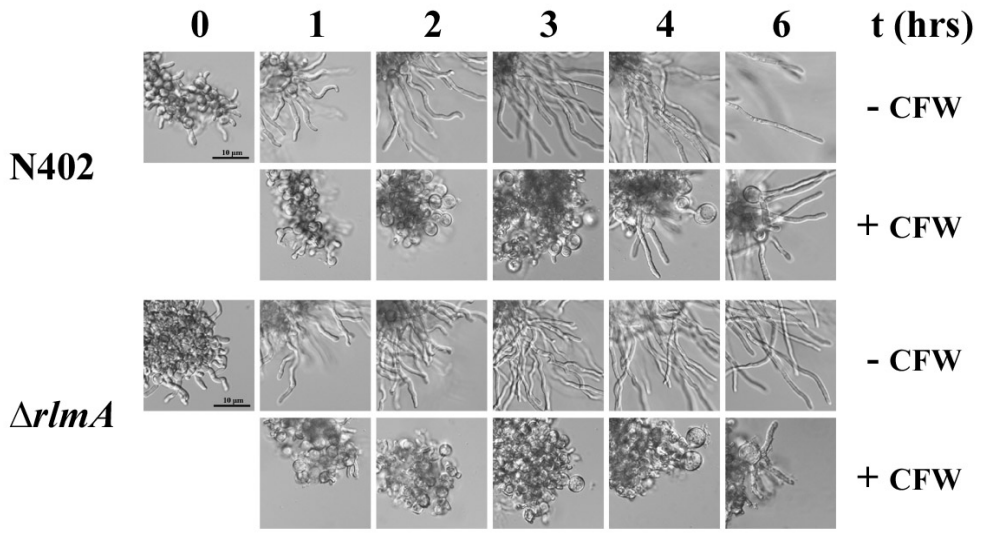
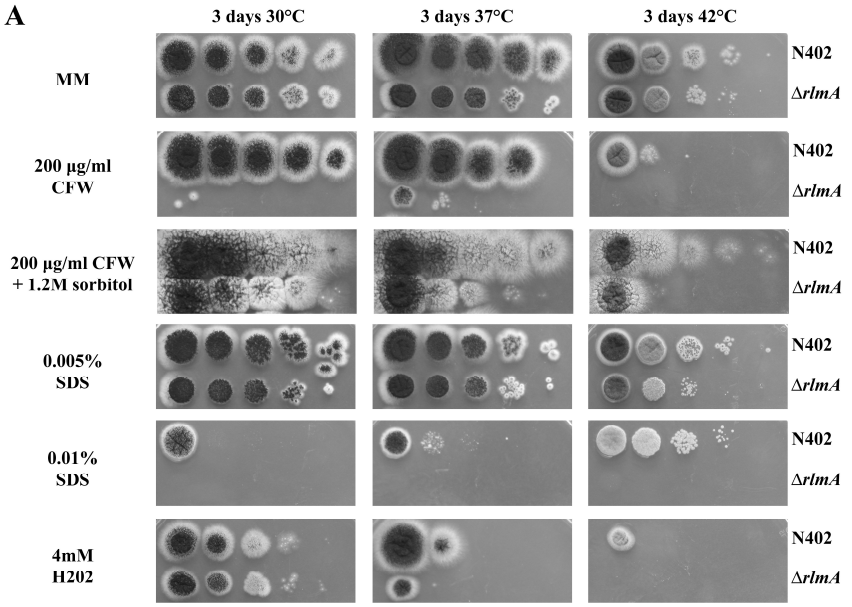


Figure 7. Microscopic images of both wild-type (N402) and the *rlmA* deletion strain, with (+) and without (-) the addition of the cell wall stress inducing compound CFW. Strains were grown in shake flask cultures at 37 °C until small germ tubes were visible (t=0). Subsequently cultures were stressed with CFW (200 µg ml^{-1} final concentration) or an equal volume of water was added. Images were taken after 0, 1, 2, 3, 4, and 6 hours using DIC settings. All images were taken at the same magnification. The scale bar indicates 10 µm.

hours at 37 ℃ to form a small germ tube, the *ΔrlmA* strain had to be grown for 5.5 hours before a small germ tube was formed. No obvious morphological differences were observed between the germlings of wild-type and those of the *ΔrlmA* strain grown under standard growth conditions. Treatment of the *ΔrlmA* strain with CFW also resulted in cessation of polarised growth but the effect was more pronounced and led to more aberrant looking germlings. Occasionally, germlings of the *ΔrlmA* strain lysed after one hour of incubation with CFW, a phenotype that was not observed for wild-type germlings. Furthermore, the time to resume polarised growth after CFW stress, seemed to be prolonged (more then four hours) for the *ΔrlmA* strain. Both observations indicate that the *ΔrlmA* germlings were more sensitive to CFW stress.

The consequences of the loss of function of *rlmA* were also determined by comparing growth of the wild-type strain to growth of the *ΔrlmA* strain on agar plates (Fig. 8A). At 30 ℃, the growth of the *ΔrlmA* was identical to the growth of the wild-type strain. As observed in the previous experiment, the *ΔrlmA* strain displayed a small growth delay when grown at 37℃. At 42 ℃, the growth of the wild-type was also effected. The growth delay at 42 ℃ was slightly more pronounced in the *ΔrlmA* strain. To test whether the *A. niger* RlmA is specifically required for survival during exposure to different cell wall disturbing compounds,


A
3 days 30°C
3 days 37°C
3 days 42°C
MM
200 µg/ml CFW
200 µg/ml CFW + 1.2M sorbitol
0.005% SDS
0.01% SDS
4mM H202
N402
ΔrlmA

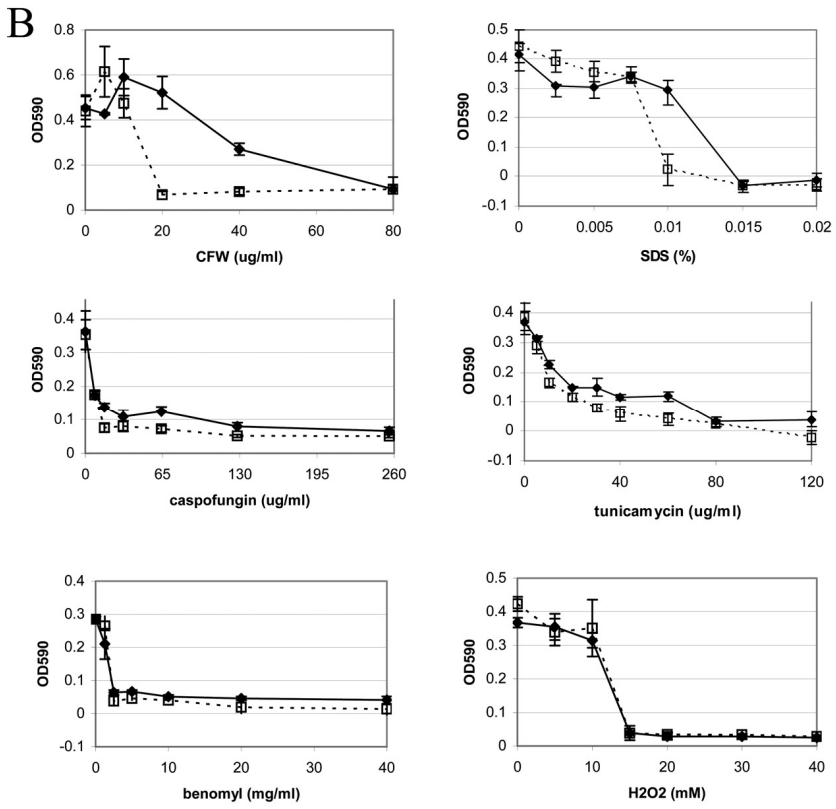

B
OD590
CFW (ug/ml)
SDS (%)
caspofungin (ug/ml)
tunicamycin (ug/ml)
benomyl (mg/ml)
H2O2 (mM)

Figure 8. Sensitivity of the *ΔrlmA* strain towards different compounds. **A**. Ten-fold dilutions, starting at $5 \cdot 10^4$ spores as the highest concentration, from spores of wild-type (N402) and *ΔrlmA* were spotted on CM plates containing various compounds, as listed on the left, and grown as indicated. **B**. Growth curves of wild-type (N402), indicated with ● and the *ΔrlmA* strain indicated with □ .Spores were grown in CM for 24 hrs at 37 °C. Data are represented as the mean and standard error of the mean obtained from four replicates.

the sensitivity of the *ΔrlmA* strain was tested on plates containing different compounds and compared to the sensitivity of the wild-type strain. CFW and SDS are two compounds which are known to induce the cell wall stress response pathway and the *ΔrlmA* strain displayed a hypersensitive phenotype towards these compounds at the different temperatures tested (Fig. 8A). Interestingly, some growth of the *ΔrlmA* strain at 37 °C in the presence of CFW was observed, whereas no growth is observed at 30 °C. This suggests that at 37 °C, an RlmA independent pathway might be activated which helps to overcome the deleterious effect of CFW. The effect of the cell wall disrupting compound CFW could be partially remediated by the addition of 1.2 M sorbitol (Fig. 8A) and to a lesser extent by the addition of 0.6 M KCl to the plates (data not shown). Caffeine and vanadate are inducers of the *S. cerevisiae* Slt2p/Mpk1p MAPK pathway (Martín *et al*., 2000). Plate assays using various concentrations of vanadate and caffeine indicated that the Δ*rlmA* strain is not hypersensitive to these compounds (data not shown). When grown at 30 °C the *ΔrlmA* strain also did not show hypersensitivity to towards oxidative stress conditions (4 mM H_2O_2) (Fig. 8A). At higher temperatures, a slight but significant increase in sensitivity was observed. A possible explanation might be that the effects of increased temperature and oxidative stress in relation to cell wall stress are additive.

The Δ*rlmA* strain did not show an altered sensitivity towards high osmolarity stress inducing conditions (1.2 M sorbitol, 0.6 M KCl, 0.6 M $CaCl_2$) (data not shown), alltogether indicating that RlmA is specifically required in response to cell wall stress and not in response to other stress conditions. The sensitivity of the wild-type and the *ΔrlmA* strains towards various compounds were also measured by determining fungal growth in a microtiter plate based growth assay (Fig. 8B). The *rlmA* deletion strain displayed a strong hypersensitive phenotype towards several cell wall disturbing compounds, such as CFW and SDS. Sensitivity of the *ΔrlmA* strain was only slightly enhanced towards the cell wall biosynthesis related compounds caspofungin, tunicamycin or the microtubules inhibitor benomyl in comparison to the wild-type strain. Under these growth conditions, the *ΔrlmA* strain displayed no hypersensitive phenotype towards H_2O_2 (Fig. 8B).

Discussion

Cell wall remodeling is an important mechanism used by fungi to escape from cell wall threatening conditions such as the presence of cell wall lytic enzymes secreted by plant cells or cell wall ihibitors secreted by fungi or bacteria. The fungal response to the presence of such compounds consists of alterations in both composition and architecture of the cell wall. These changes include (i) increased deposition of certain cell wall mannoproteins in the outer layer of the cell wall, probably aimed to limit the access of e.g. plant secreted glucanases and chitinases to the underlying glucan/chitin part of the cell wall (Narasimhan *et al*., 2003, Klis *et al*., 2002), (ii) increased deposition of cell wall chitin (Popolo *et al*., 1997 Kapteyn *et al*., 1997, Ram *et al*., 2004), (iii) different cross-linking of β-1,6-glucosylated mannoproteins to the cell wall (Kapteyn *et al*., 1997), (iv) increased expression of β-1,3-glucan synthase in the case of *S. cerevisiae* (de Nobel *et al*., 2000b, Terashima *et al*., 2000) or the increased expression of α-1,3-glucan synthase encoding genes in the filamentous fungi *A. niger* and *Penicillium chrysogenum* (Damveld *et al*., 2005, Chapter 3). The signal transduction cascade sensing, mediating, and triggering the transcriptional reaction in response to cell wall stress has been elucidated in detail in *S. cerevisiae* (see for reviews Heinisch *et al*., 1999, Smits *et al*., 1999), and is known as the Pkc1p cell wall integrity pathway. Mutants in the pathway display a cell lysis defect which can be prevented by providing osmotic support. Activation of the pathway results in dual phosphorylation and activation of a MAP kinase (Slt2p/Mpk1p). The transcriptional response in *S. cerevisiae* that leads to cell wall remodeling after Slt2p activation is mainly mediated by the Rlm1p transcription factor (Jung and Levin, 1999, Terashima *et al*., 2000, Boorsma *et al*., 2004).

We show here that induced expression of genes involved in cell wall remodeling in the filamentous fungus *A. niger* in response to cell wall stress is also mediated via an Rlm1p-like MADS-box transcription factor protein, called RlmA. Whereas the induction of *agsA* seems completely dependent on RlmA, the induction of *gfaA,* in a Δ*rlmA* strain, was only two-fold lower during CFW stress, indicating that other signaling networks are also involved in the transcriptional response after CFW treatment. Nevertheless, our findings suggest an important role for RlmA in regulating and activating gene expression in response to cell wall stress and further suggest that signaling via the cell wall integrity pathway and the Rlm1p transcription factor is conserved among fungi. Indeed, members of the Rlm1p-like MADS-box transcription factors are present in all fungal genomes (Fig. 3). The highest homology between Rlm1p-like proteins was found in the MADS-box and the MEF2 regions, which are required for DNA binding and dimerisation of the transcription factor. Outside these two regions, no obvious homology could be detected. The *S. cerevisiae* Rlm1 protein contains an acidic region (aa121-134) and an asparagines-rich region (aa567-599) (Watanabe *et al*.,

1995). In all fungal MEF2-homologues an acidic region is also present at a similar position in the protein with the exception of the *S. cerevisiae* Smp1 protein. The asparagines-rich region is not conserved among the different MADS-box proteins. The function of either the acidic region or the asparagines-rich region is not known. The mammalian MEF2A, MEF2C and the *S. cerevisiae* Rlm1p transcription factors are phosphorylated and activated by their upstream MAP kinases (Han *et al*., 1997, Yang *et al*., 1999, Watanabe *et al*., 1997, Jung *et al*., 2002). Three phosphorylation sites in MEF2C and two phosphorylation sites in Rlm1p have been shown to be required for activation *in vivo* (Han *et al*., 1997, Jung *et al*., 2002). The *A. niger* RlmA protein contains fifteen Ser/Thr-Pro phosphorylation motifs. Because of the lack of overall homology between the *A. niger*, the *S. cerevisiae*, and the mammalian MEF-box proteins, it is difficult to predict the putative phosphorylation sites of RlmA. Sequence comparison between the Rlm1p from *S. cerevisiae* and the mammalian MEF2A/C MADS-box proteins revealed the presence of a conserved MAP kinase docking site (Jung *et al*., 2002). This site is important for the activation of the transcriptions factor by their MAP kinase but could not be detected in the *A. niger* RlmA protein.

The *S. cerevisiae* Rlm1p has been shown to be important for the induction of many genes that are induced in response to different forms of cell wall stress (Jung *et al*., 2002). Many of the cell wall stress induced genes contain putative Rlm1p binding sites in their promoter regions (Lagorce *et al*., 2003, Boorsma *et al*., 2004). The consensus sequence of MEF2-subfamily of MADS-box transcription factor binding-sites ($CTA(T/A)_4TAG$) is conserved between mammals and *S. cerevisae* (Pollack and Treisman, 1991, Dodou and Treisman, 1997). Our results confirm the important role of this consensus sequence during transcriptional activation, indicating further conservation of the mechanism by which MADS-box proteins bind to DNA and activate transcription.

Despite the fact that the *S. cerevisae* Rlm1 protein is necessary for the induction of many cell wall related genes, disruption of *rlm1* does not lead to a clear cell wall mutant related phenotype. Cell wall mutants often display higher sensitivity towards either CFW or Congo Red (Ram *et al*., 1994, Lussier *et al*., 1997, Garcia *et al*., 2004) or a more sensitive to Zymolyase (van der Vaart *et al*., 1995). Surprisingly, the *S. cerevisiae rlm1* deletion strain did not show hypersensitivity towards either CFW or Congo Red; instead they become more resistant to CFW and Zymolyase (Dodou and Treisman, 1997, Garcia *et al*., 2004), nor does it display higher sensitivity towards Zymolyase (Dodou and Treisman, 1997). We have tested the sensitivity of the *A. niger rlmA* deletion strain towards several antifungal compounds. It displayed a strong Calcofluor White hypersensitive phenotype, indicating that the RlmA transcription factor plays an important role during the induction of genes that collectively help to ensure the integrity of the cell wall. The hypersensitivity of the *ΔrlmA* strain was not limited

to CFW, but was also found for other cell wall stress inducing compounds such as SDS and to a lesser extent caspofungin, and tunicamycin. In *S. cerevisiae*, the *⊿rlm1* strain displays hypersensitivity towards caffeine, indicating that Rlm1p is required for the induction of genes that overcome the inhibiting effect on growth of those compounds (Watanabe *et al*., 1995). Our finding that the *A. niger ΔrlmA* is not hypersensitive to caffeine or vanadate (data not shown) suggests that a potential transcriptional response to this compound is not mediated by RlmA in *A. niger*.

Over the past few years evidence for the existence of a MAPK-dependent cell wall pathway in filamentous fungi has been accumulating. Mizutani *et al*. (2004) have shown that the disruption of *kexB*, a subtilisin-like processing enzyme in *A. oryzae*, resulted in an increased expression of several cell wall related genes. Furthermore, they showed that the activation of gene expression was accompanied by high levels of phosphorylated MpkA and an increased level of *mpkA* transcript as observed in *S. cerevisiae* (Jung and Levin, 1999, de Nobel *et al*., 2000b, Boorsma *et al*., 2004, Garcia *et al*., 2004). In *Cryptococcus neoformans*, a MAPK homologue (CnMpk1) has been identified and characterised. Perturbations of cell wall biosynthesis by adding antifungal agents that disturb cell wall biosynthesis resulted in induced phosphorylation of CnMpk1. *C. neoformans* mutants lacking Mpk1 do not grow at elevated temperatures and are hypersensitive to agents that affect cell wall biosynthesis (Kraus *et al*., 2003). Also in *Magnaporthe grisea*, an Mpk1p homologue (MgMps1) has been described and the deletion of this MAPK increased the sensitivity of *M. grisea* to cell wall degrading enzymes. The inability of the *M. grisea Δmps1* strain to penetrate the host cell, suggests an important role for this gene in the remodeling of the cell wall during appressorium formation (Xu *et al*., 1998). A deletion mutant of the Slt2p MAPK homologue has also been described in *A. nidulans*. The deletion mutant (*ΔmpkA*) displayed an osmosensitve phenotype with abnormal swellings at the hyphal tips, indicating cell wall related defects (Bussink and Osmani, 1999). In *Pneumocystis carinii*, an opportunistic fungal pathogen, two members of a MAP kinase signaling cascade have been described that are involved in the cell wall integrity pathway, the MAPK Mkp1 and the MAPKKK Bck1. Both proteins are able to complement the functions of their homologues in *S. cerevisiae* (Fox and Smulian, 1999, 2000, Thomas *et al*., 2003). Finally, a Rho1 homologue has recently been characterised in *A. nidulans.* The thickened cell walls and increased chitin levels in the cell wall of an *A. nidulans* strain expressing a constitutively activated allele of RhoA (RhoAG14V) suggests a constitutive activation of the cell wall response pathway (Guest *et al*., 2004). Taken together, these and our own observations, supported by the fact that homologs of the different components of the cell wall integrity pathway are widely conserved and present in

publicaly available fungal genomes (data not shown), indicates that the cell wall integrity pathway and molecular mechanism of activation is conserved among fungi.

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Chapter 5

Characterisation of CwpA, a putative glycosylphosphatidylinositol anchored cell wall mannoprotein in the filamentous fungus *Aspergillus niger*

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Submitted

Abstract

Glycosylphosphatidylinositol (GPI)-anchored proteins in fungi are found at the cell surface, either attached to the plasma membrane (GPI-PMPs) or attached by a remnant of the GPI-anchor to the cell wall. In the yeasts *Saccharomyces cerevisiae* and *Candida albicans*, it has been shown that GPI-anchored cell wall mannoproteins (GPI-CWPs) become attached to the β-1,3-glucan or chitin part of the cell wall through a flexible β-1,6-glucan moiety. GPI-CWPs can be extracted from the fungal cell wall by treatment with hydrofluoric acid (HF), which cleaves the phosphodiester bond that is present in the remnant of the GPI-anchor. The filamentous fungus *Aspergillus niger* contains at least five HF-extractable cell wall mannoproteins. One gene encoding an HF-extractable cell wall mannoprotein, *cwpA,* was cloned and further characterized. The protein sequence of CwpA indicated the presence of two hydrophobic signal sequences both at the N-terminus and C-terminus of the protein, for entering the ER and the addition of a GPI-anchor, respectively. A CwpA specific antiserum was raised and in combination with fractionation experiments, we confirmed that this protein was abundantly present as a HF-extractable protein in the cell wall and hardly present in the membrane fraction. The *ΔcwpA* strain displayed however a more sensitive phenotype towards the cell wall disturbing compound Calcofluor White, indicating that CwpA might have a structural role in maintaining cell wall integrity.

Introduction

The cell wall of a fungus is an intriguing component of the fungal cell. It not only determines the shape of the cell, it also protects the cell from the harsh external environment and is the site at which the fungus to physically interacts with its environment. Electron microscopic images of fungal cell walls have revealed a layered structure. The outer, electron dense layer is composed of cell wall mannoproteins (Zlotnik *et al*., 1984, Hazen and Hazen, 1992). The inner, electron transparent layer is composed mainly of glucan, (β-1,3- and β-1,6-glucans) and chitin which acts as a scaffold for exposing cell wall mannoproteins (Zlotnik *et al*., 1984, Kollar *et al*., 1997). The cell walls of filamentous fungi consist of the same basic components as the cell walls of yeasts (mannoproteins, β-glucans and chitin), but generally contain more chitin (10-15 % chitin instead of 1-2 % chitin in the yeast cell wall) and can contain additional polymers such as α-1,3-glucan or α-1,3-α-1-4 glucan (Schoffelmeer *et al*., 1999, Fontaine *et al*., 2000). The composition and structure of the cell wall are not static but are modified in response to changes in the environment (Klis *et al*., 2002). The surface properties of fungi are primarily determined by the presence of cell wall mannoproteins. Cell wall mannoproteins are grouped in different classes based on their extractability and

proposed linkage: i) SDS-extractable cell wall mannoproteins which are bound to the cell wall by hydrogen bonds, ii) β-mercaptoethanol/DTT-extractable cell wall mannoproteins which are attached covalently to the cell wall by disulphide-bonds (Cappellaro *et al*., 1994, 1998) or iii) cell wall mannoproteins that are covalently linked to the glucan part of the cell wall. Two different classes of glucan-linked cell wall mannoproteins have been described for *S. cerevisiae*. The first class of glucan-linked cell wall mannoproteins consist of members that belong to the protein with internal repeats (PIR)-family (Toh-e *et al*., 1993). These proteins can be liberated from the cell wall after mild-alkali treatment (Mrsa *et al*., 1997). The repeats consist of a glutamine-rich consensus sequence (Q-I/V-X-D-G-Q-I/V/P-Q) and the number of repeats varies between the different PIR proteins (Toh-e *et al*., 1993). Pir4p/Ccw5p contains only a single PIR motif and it has been shown that this sequence is required for the covalent linkage of Pir4p/Ccw5p to the cell wall (Castillo *et al*., 2003). PIR proteins are most likely linked to the β-1,3-glucan part of the cell wall. However, the nature of this linkage has not yet been established (Mrsa and Tanner 1999, Castillo *et al*., 2003). The second class of glucan-linked cell wall mannoproteins are attached to the cell wall through glycosylphosphatidylinositol (GPI) linkages (Lu *et al*., 1994, Montijn *et al*., 1994, Kapteyn *et al*., 1995, 1996). GPI-anchored cell wall proteins (GPI-CWPs) contain a hydrophobic sequence at their C-terminus that acts as a GPI-anchoring signal. GPI-anchor addition takes place in the endoplasmic reticulum (ER) were the hydrophobic domain is replaced by the pre-assembled GPI-anchor (Orlean *et al*., 1997). After transport through the secretory pathway and arrival at the plasma membrane the GPI-anchor is processed and attached to β-1,6-glucan (Montijn *et al*., 1994, Kollar *et al*., 1997). GPI-CWPs are further characterized by the presence of a hydrophobic N-terminal signal sequence for import into the ER, and are often heavily O-glycosylated. GPI-CWPS can be removed from the cell wall by enzymatic and chemical treatments. Both β-1,3- and β-1,6-glucanases liberate GPI-CWPs from the cell wall (Kapteyn *et al*., 1996). In addition, GPI-CWPs can be extracted from the cell wall by treatment with hydrofluoric acid (HF), which cleaves the phosphodiester bonds in the GPI-anchor (Kapteyn *et al*., 1996, de Groot *et al*., 2004).

Cell-cell or cell-surface interactions of the fungal cell are often determined by the presence of GPI-CWPs. In *S. cerevisiae* flocculation and agglutination properties are mediated via the flocculins and agglutinins respectively, and these proteins are exposed and bound to the cell surface of the cell wall through their GPI-anchor (Teunissen and Steensma, 1995, Bony *et al*., 1997, Lu *et al*., 1994). ALS proteins in *C. albicans* and Epa1p in *Candida glabrata* are GPI-anchored cell wall proteins proteins that are important for mediating the adhesion properties of the *Candida* cell to human epithelial cells (Cormack *et al*., 1999, Hoyer *et al*., 2001, Klis *et al*., 2001, Frieman *et al*., 2002, Sundstrom, 2002). In addition to a few

examples of cell wall proteins with a clear biologically relevant function, numerous GPI-CWPs have been identified whose function is not yet clear (Klis *et al*., 2002). In general, they are considered to be cell wall proteins with a structural role. Interestingly, the transcription of genes encoding GPI-CWPs is highly regulated in response to internal and external factors. Progression through the cell cycle is accompanied by a regulated expression of several genes encoding GPI-CWPs (Caro *et al*., 1998, Spellman *et al*., 1998, Smits *et al.,* 1999). Expression of GPI-CWPs encoding genes is also highly regulated in response to different forms of environmental stress. Cell wall stress inducing conditions (Ram *et al*., 1998a, Jung and Levin, 1999, Terashima *et al*., 2000), high osmolarity (Posas *et al*., 2000, Rep *et al*., 2000, Gasch *et al*., 2000), extreme pH (Kapteyn *et al*., 2001, Lamb *et al*., 2001), anaerobic growth conditions (Abramova *et al*., 2001), and entering the stationary growth phase (Shimoi *et al*., 1998, Puig *et al*., 2000), all have been shown to increase the expression level of a number of GPI-CWP encoding genes. The induced expression of certain GPI-CWPs (like Cwp1p) is not limited to a specific growth condition indicating that their expression might be under the control of different transcriptional activating networks. The genes encoding members of the PIR protein family are also induced by a wide range of environmental stimuli (Toh-e *et al*., 1993, Jung and Levin, 1999, Lagorce *et al*., 2003).

GPI-CWPs have been identified and characterised most extensively in *S. cerevisiae* and *Candida ssp*., but also recently in *Yarrowia lipolytica* (Jaafar and Zueco, 2004). For filamentous fungi, the presence of a GPI-CWP (Fem1p) has only been reported in the cell wall of the plant-pathogenic fungus *Fusarium oxysporum* (Schoffelmeer *et al*., 1999, 2001). In this paper we have cloned a gene encoding an HF-extractable cell wall mannoprotein and named the gene *cwpA*. As expected for an HF-extractable cell wall mannoprotein, the protein contains a putative GPI-anchor addition signal and demonstrates further conservation of the presence of GPI-linked cell wall mannoproteins in filamentous fungi.

Materials and Methods

Strains, culture conditions and transformations

The *A. niger* strains used in this study are N402 (a *cspA1* derivative of ATCC9029, Bos *et al*., 1988) and the *pyrG* negative derivative of N402, AB4.1 (van Hartingsveldt *et al*., 1987). Strains were grown in *Aspergillus* minimal medium (MM) (Bennett and Lasure, 1991) or *Aspergillus* complete medium (CM) which contains the same compounds as minimal medium with the addition of 10 g l^{-1} yeast extract and 5 g l^{-1} casamino acids. When required the growth medium was supplemented with 10 mM uridine (Serva). Transformation of *A. niger* was carried out as described by Punt and van den Hondel (1992) using lysing enzymes (L1412, Sigma) for the formation of protoplasts. Conidiospores were obtained by harvesting

spores from a CM-plate after 4-6 days of growth at 30 °C, using a 0.9 % NaCl solution. The bacterial strain used for transformation and amplification of recombinant DNA was *Escherichia coli* XL1-Blue or DH5α. Bacterial transformations were performed according to the heat shock protocol as described by Inoue *et al*., 1990.

Molecular biological techniques

Small scale chromosomal DNA isolations for PCR screening of transformants were performed using a FastPrep FP120 (Bio101). *A. niger* spores were transferred to Fast-prep tubes containing 1 ml of CM and 0.3 g acid washed glass beads (∅ 400-600 µm, Sigma). After growth for 16 hours at 37 °C the mycelium was spun down and the medium was removed. Subsequently, 500 µl cold extraction solution (2:2:1 mixed, TNS, 40 mM tri-isonaphtalene sulphonic acid, PAS, 0.70 M P-aminosalycilic acid, and RNB, 1.0 M Tris-HCl pH 8.5, 1.25 M NaCl, 0.25 M EDTA) and 500 µl phenol:chloroform:isoamyl alcohol (25:24:1 v/v %) were added. Vials were closed carefully and vigorously shaken two times for 30 seconds at speed 6.0 and cooled five minutes on ice between runs. When larger quantities of chromosomal DNA were required, as for Southern analysis, genomic DNA was isolated as described by Kolar *et al*., 1988. RNA was extracted from mycelium flash-frozen in liquid nitrogen using TRIzol reagent (Invitrogen). Both Southern and Northern blot analyses were carried out as described by Sambrook *et al*., 1989. Electrophoresis of RNA was performed as described (Damveld *et al*., 2005). [α-^{32}P]dCTP-labelled probes were synthesised using Rediprime II DNA labelling System (Amersham Pharmacia Biotech) according the instructions of the manufacturer. PCR was performed on a PTC-100 Programmable Thermal Controller (MJ Research, Inc) using Super Taq (HT Biotechnology LTD) or when required Expand High Fidelity PCR system (Roche). Primers were obtained from Isogen and are listed in Table 1. For ligation the Rapid DNA Ligation Kit (Boehringer Mannheim) was used. 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). Restriction enzymes were obtained from Invitrogen and used according to the protocol supplied by the manufacturer.

Isolation of the *cwpA* gene from *A. niger*

We identified three cDNAs in the DDBJ/EMBL/GenBank databases with accession numbers: BE759683, BE758883, and BE759871 (Tsang and Storm, unpublished data) which displayed a significant homology to the mannoproteins from *Penicillium marneffei* Mp1 (E-value 4e-09) and *A. fumigatus* Mp1 (1e-12). Using these cDNA sequences two primers were designed, AnCWPP1for and AnCWPP2rev (Table 1), which in combination with the vector

primers F1590 and F1591 (Table 1), were used to amplify two overlapping parts of the *cwpA* gene from an *A. niger* cDNA library in pEMBLyex4 (Veldhuisen *et al*., 1997). The PCR fragments obtained were 0.4 kb (F1591 and AnCWPP2rev) and 1.2 kb (AnCWPP1for and F1590) in size and were cloned into pGEM-T Easy (Promega) and sequenced. Southern blot analysis on genomic DNA from *A. niger* showed that the *cwpA* gene was located on a 3 kb BglII fragment (data not shown). Genomic DNA was digested with BglII and fragments of approximately 3 kb were isolated from gel to ligate into a BamHI opened pBluescript II SK vector (Stratagene). This BglII mini-library was transformed to *E. coli* and white colonies were transferred to new LB plates. Using the colony lift protocol from Hybond N+ (Amersham, see suppliers manual) approximately 200 colonies were lysed on Hydond N+ filters and subsequently subjected to Southern analysis using the *cwpA* cDNA fragment as a probe. One hybridizing clone was identified and named pCwpA3.0 and sequenced. The *A. niger cwpA* nucleotide sequence data has been submitted to the DDBJ/EMBL/GenBank databases under accession number AY704270.

Table 1. Primers used in this study.

Primer name	Sequence 5‘ to 3‘[a]
AnCWPP1for	taataacatacctaccactc
AnCWPP2rev	gagttgatctccgagacc
pyrGAO-NarI-F	atggcgccgttgctcggtagctgatta
pyrGAO-NarI-R	atggcgcccgatggataattgtgccg
Rev	ggaaacagctatgaccatg
Uni	gtaaaacgacggccagt
F1590	gctatcatttcctttgatattgg
F1591	aaattaccggatcaattcgagc
cwpAp9	aacaacaatattcaggcctc
pAO9	aatgtcaattccagcagcg
cwpA-Rec1	ttttttggatccatggccaaccccaccaag
cwpA-Rec2	ttttttctcgagagccacgtccttgta
rodA1dw	ggagcactcctggaagagac
rodA1up	tcaccgctgctgttcttg
brlAdw	gcatgagatcttgggacttgg
brlAup	cagcttctcacccatggaatc

[a] Restriction sites are underlined

Construction of the *cwpA::AopyrG* deletion plasmid

To construct a plasmid for deletion of the *cwpA* gene, the *A. oryzae pyrG* gene was amplified using primers pyrGAO-NarI-F and pyrGAO-NarI-R to introduce NarI sites at both sides of the *pyrG* gene using pAO4-13 (de Ruiter-Jacobs *et al*., 1989) as a template. After amplification, the expected 1.7 kb fragment was cloned in pGEM-T Easy and transformed to *E. coli*. To confirm the functionality of the *pyrG* selection marker, the DNA obtained from small scale DNA isolations was transformed to *A. niger* strain AB4.1. Transformation of plasmid pGEMT-PyrGAO-NarI#2 resulted in the formation of uridine prototrophic transformants indicating that this plasmid contains a functional *AopyrG* gene. The *AopyrG* gene was isolated from pGEMT-PyrGAO-NarI#2 as a 1.7 kb NarI fragment and cloned into the NarI digested pCwpA3.0. Digestion of pCwpA3.0 with NarI removed a 990 bp fragment, containing 610 bp of the *cwpA* promoter sequence and 380 bp of the *cwpA* coding region, which was then replaced with the 1.7 kb NarI fragment containing the *AopyrG* gene, to give plasmid pΔ*cwpA*. The orientation of the insert was determined by EcoRV digestion. Constructs with both orientations were obtained and a plasmid pΔcwpA#2, with the *AopyrG* gene oriented so that the coding strand was in the opposite direction to that of the *cwpA* gene was used for transformation to *A. niger*. Before transformation to *A. niger* AB4.1, pΔcwpA#2 was linearised by a BglI digestion. Transformation of 25 µg linearised plasmid yielded 220 transformants. Transformants were screened by PCR using primers cwpAp9, located outside and downstream of the deletion cassette, and pAO9. Only transformants with a targeted deletion of the *cwpA* gene should result in the amplification of a 0.7 kb PCR fragment. PCR positive transformants were analysed by Southern blot analysis. Genomic DNA of two PCR positive transformants was digested with BglII and probed with a genomic *cwpA* fragment to confirm single copy, targeted integration of the deletion cassette (see also fig 4).

Growth conditions and developmental probes

To analyse the expression of *cwpA* in shake flask cultures, RNA samples were isolated from 50 ml CM cultures that were inoculated with 1 x 10^6 spores ml^{-1} and grown for 8, 16, 24, 32 and 64 hours at 30 ℃ using Trizol reagent (Invitrogen). Parallel cultures were used for biomass determination and cell wall isolations. Mycelium was harvested using a sieve with a 20 µm aperture (Endecotts) and stored at -80 ℃ prior to RNA or cell wall isolation. The biomass of the parallel cultures was determined by harvesting the spores over myracloth filter (Omnilabo) and drying the mycelium at 70 ℃.

A. niger sequences encoding BrlA and RodA proteins that were highly homologous to the *A. nidulans* orthologs (E-values of e-159 and 1e-27 respectively), were kindly provided by DSM. The *A. niger brlA* and *rodA* homologs were isolated by PCR from *A. niger* with primers

pairs brlAdw-brlAup and rodA1dw-rodA1up (Table 1) and cloned into pGEM-T Easy. The isolated fragments were confirmed by sequence analysis and the partial coding sequences were submitted to the DDBJ/EMBL/GenBank databases under accession numbers AY817176 (*brlA*) and AY817177 (*rodA*). The probes used are a 300 bp EcoRI fragment containing the 5' region of the *cwpA* gene, an 1147 bp EcoRI fragment from pGEMT-brlA containing the *brlA* gene, a 314 bp EcoRI fragment from pGEMT-rodA containing a part of the *rodA* open reading frame, and an 18S ribosomal probe as loading control, isolated as a 2 kb BglI fragment from pMN1 (Borsuk *et al*., 1982).

Cell wall extractions

Cell walls were isolated by grinding frozen mycelium using a pestle and mortar, breakage (> 95 %) was confirmed by phase-contrast microscopy. Ground mycelium was lyophilised, weighed and resuspended in 25 µl Tris buffer (0.05 M Tris-HCl, pH 7.8) mg^{-1} dry weight. In all cases approximately 40 mg dry weight mycelium was used for extractions. The cytosolic fraction was separated from the cell walls and membranes by centrifugation at 13.000 rpm for 10 min at 4 °C. This fraction was stored at -20 °C as the cytosolic protein fraction (cyt). The ground mycelium was extracted again with 25 µl Tris buffer mg^{-1} dry weight and pelleted as described above. To remove residual cytosolic contaminants, membrane proteins, and di-sulphide linked cell wall proteins, the pellets were boiled three times in 25 µl SDS-extraction buffer (50 mM Tris-HCl, pH 7.8, 2 % w v^{-1} SDS, 0.1 M Na-EDTA, and 1.6 µl β-mercaptoethanol) mg^{-1} dry weight. The cell walls were pelleted after extractions as described above, and the supernatants were stored as SDS fractions (SDS1-3) (Montijn *et al*., 1994). Cell walls were washed six times with MilliQ, lyophilised, and weighed. Freeze dried cell walls were incubated with 10 µl HF-pyridine mg^{-1} dry weight for three hours at 0 °C (de Groot *et al*., 2004). After centrifugation, the supernatant containing the HF-extracted proteins, was collected (in 100 µl aliquots) and proteins were precipitated by the addition of 9 volumes 100 % methanol buffer (100 % v v^{-1} methanol, 50 mM Tris-HCl, pH 7.8) and subsequently incubated at 0 °C for two hours. Precipitated proteins were collected by centrifugation (13.000 rpm, 10 min, at 4 °C). The pellet was washed three times with 90 % methanol buffer (90 % v v^{-1} methanol, 50 mM Tris-HCl, pH 7.8) and lyophilised. The HF-extracted proteins were dissolved in 500 µl 1 X sample buffer. Prior to loading on gel both cytosolic- and SDS-fractions were mixed in a 1:1 ratio with 2 X sample buffer.

Western analysis

Protein samples (10 µl) were loaded on a 4-15 % Tris-HCl SDS-PAGE gel (Bio-Rad, Art# 161-1104) and run on a Mini-Protean II Electrophoresis cell (Bio-Rad), according to the

supplier's manual. Each 10 µl protein sample that is loaded on SDS-PAGE corresponds to the amount of protein released by an extraction of 200 µg dry weight mycelium (comparable to 75 µg dry weight cell walls). Proteins were transferred overnight to polyvinylidene-difluoride membrane (PVDF, Immobilon-P, Millipore) using a Mini Trans-Blot Electophoretic Transfer cell (Bio-Rad), according the supplier's manual using 25 mM Tris-HCl, 192 mM glycine, 20 % v v^{-1} methanol, pH 8.3 as a blotting buffer. The PVDF membranes were blocked with 3 % w v^{-1} BSA (Sigma-Aldrich, Buchs, Switzerland) dissolved in PBS with 0.1 % v v^{-1} Tween-20. For immunological detection of CwpA, an antiserum was raised against recombinant CwpA (see below). Blots were incubated for two hours with 500-fold diluted CwpA antiserum in PBS containing 3% w v^{-1} BSA and 0.1 % v v^{-1} Tween-20. After incubation with the primary antibody, blots were washed twice in PBS with 0.1 % v v^{-1} Tween-20 for 15 minutes. Subsequently, the blots were incubated for two hours with a 2000-fold dilution of goat-anti-rabbit peroxidase linked antibody (GARPO, Dako, Denmark) in PBS containing 3% w v^{-1} BSA and 0.1 % v v^{-1} Tween-20. Alternatively, for the detection of mannoproteins the blots were incubated for two hours with peroxidase conjugated concanavalin A (conA-PO) which was diluted 1000-fold in PBS containing 2.5 mM $CaCl_2$, 2.5 mM $MnCl_2$, 3% w v^{-1} BSA and 0.1 % v v^{-1} Tween-20. All blots were washed twice in PBS with 0.1 % v v^{-1} Tween-20 for 15 minutes prior to detection of peroxidase activity using an ECL-plus kit (Amersham, Pharmacia Biotech, UK).

Raising antibodies against CwpA

For the generation of a CwpA specific antiserum, part of CwpA (aa1-168) was expressed as a GST-fusion protein in *E. coli* strain BL21 (DE3) and cloned into pLysS. Two primers (cwpA-Rec1 and cwpA-Rec2) were designed to amplify a part of the CwpA gene using pCwpA3.0 as a template. The construction of plasmids, the expression and purification of GST-CwpA was performed by Fusion Antibodies Ltd, Belfast. Polyclonal antisera were obtained by the immunisation of a rabbit with the purified recombinant CwpA using a standard immunization protocol from Harlan Sera-Lab Ltd, Leicestershire, UK. To remove non-specific antibodies, 1 ml of the antiserum was pre-absorbed with acetone powder, obtained from a protein extract of the *cwpA* deletion strain according to standard protocols (Harlow and Lane, 1988).

Results

HF-pyridine extraction of the cell wall of *A. niger* results in the release of putative GPI-CWPs

Extraction of fungal cells walls with aqueous HF or HF-pyridine has been shown to be a reliable method for the release of GPI-anchored cell wall mannoproteins (Kapteyn *et al*., 1996, Schoffelmeer *et al*., 2001, de Groot *et al*., 2004). HF-pyridine specifically cleaves phosphodiester bonds, through which GPI-CWPs are linked to the β-1,6-glucan component of the cell wall. Extensively SDS/β-mercaptoethanol extracted cell walls of *A. niger* were incubated with HF-pyridine for three hours on ice. HF-extracted proteins were detected by Western analysis using the lectin concanavalin A (Fig. 1A). At least seven distinct HF-pyridine extractable proteins could be identified with molecular masses of approximately 180, 130, 120, 75, 65, 55 and 40 kDa (Fig. 1A). In mock experiments, in which HF-pyridine was

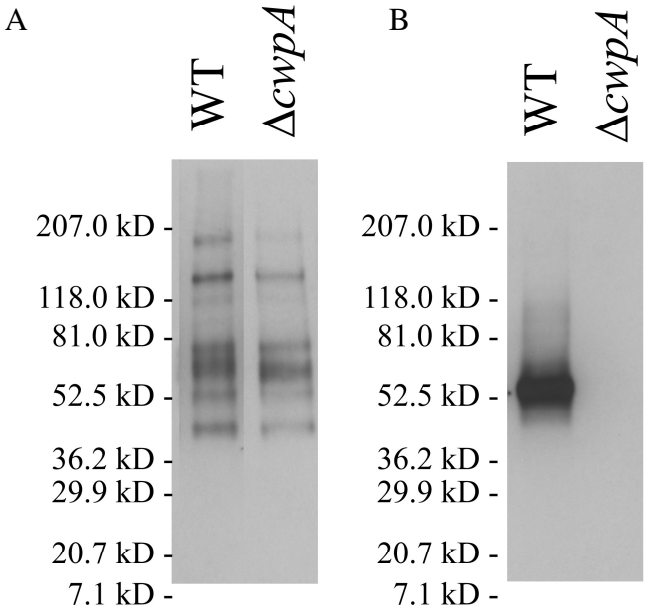

Figure 1. Western analysis of HF-pyridine extracted proteins from SDS- extracted cell walls of the wild-type (N402) and the *cwpA* deletion strain (Δ*cwpA*). **A**) Glycosylated proteins were detected with concanavalin-A conjugated peroxidase. **B**) Proteins detected with a CwpA specific antibody. In each lane the extracted proteins from 200 µg of dry weight cells were loaded. Molecular weight markers (kDa) are indicated.

replaced by MilliQ, no significant amount of protein was released (data not shown). To clone any of the HF-pyridine extractable proteins N-terminal sequencing was performed using the same method that was previously used to obtain the Fem1p N-terminal amino acid sequence (Schoffelmeer *et al*., 2001). An amino acid sequence of the 40 kDa protein was obtained (W-

V-T-E-T-N-G-D-L), but attempts to clone the gene encoding this putative cell wall mannoprotein by PCR using degenerated primers were not successful and therefore an alternative approach was used to clone a gene encoding a cell wall protein from *A. niger*.

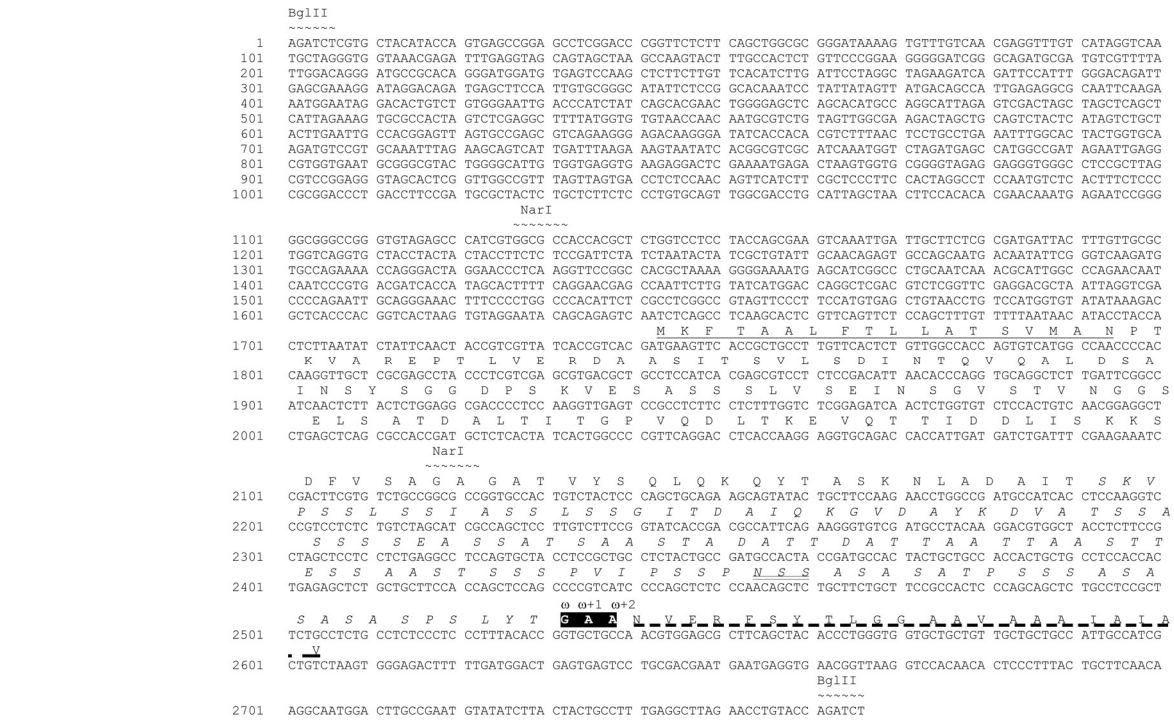

Figure 2. The sequence of the 2.8 kb genomic BglII subclone containing the *cwpA* gene. The predicted protein sequence of 288 residues is shown above the open reading frame. Some features shown are characteristic for GPI-anchored cell wall proteins: a 17 amino acid N-terminal cleavable signal sequence (single underlined); a potential N-glycosylation site, at position 238 (double underlined); serine/threonine rich region (italics) the ω, ω+1, and ω+2 sites (black background); and a C-terminal cleavable GPI signal peptide (dashed underlined).

Isolation of the *cwpA* gene

HF-extractable cell wall mannoproteins obtain a GPI-anchor in the endoplasmic reticulum were the hydrophobic C-terminal part of the precursor protein is replaced by a preassembled GPI-anchor. Putative cell wall mannoproteins have been identified in *P. marneffei* Mp1, *A. fumigatus* Mp1, and *F. oxysporum* FEM1p (Cao *et al*., 1998, Yuen *et al*., 2001, Schoffelmeer *et al*., 2001) which contain a GPI-anchor addition signal at their C-terminus. We identified in a collection of about 2000 *A. niger* cDNAs (Tsang and Storms

2000, unpublished), three almost identical partial clones which displayed a significant homology to the mannoproteins Mp1 from *P. marneffei* (E-value 4e-09) and Mp1 from *A. fumigatus* (1e-12). Using the available cDNA sequences we designed two primers to obtain the complete cDNA (see materials and methods). Translation of the obtained cDNA fragment (Fig. 2) was shown to encode a protein homologous (56 % identity) to the *A. fumigatus* MP1 homologue, and was named CwpA. The cDNA fragment was used to obtain a genomic clone carrying the full length gene. The plasmid containing the *cwpA* gene was completely sequenced and appeared to contain the full length *cwpA* gene, flanked by 1.7 kb upstream sequence and 0.2 kb of downstream sequence (Fig. 2).

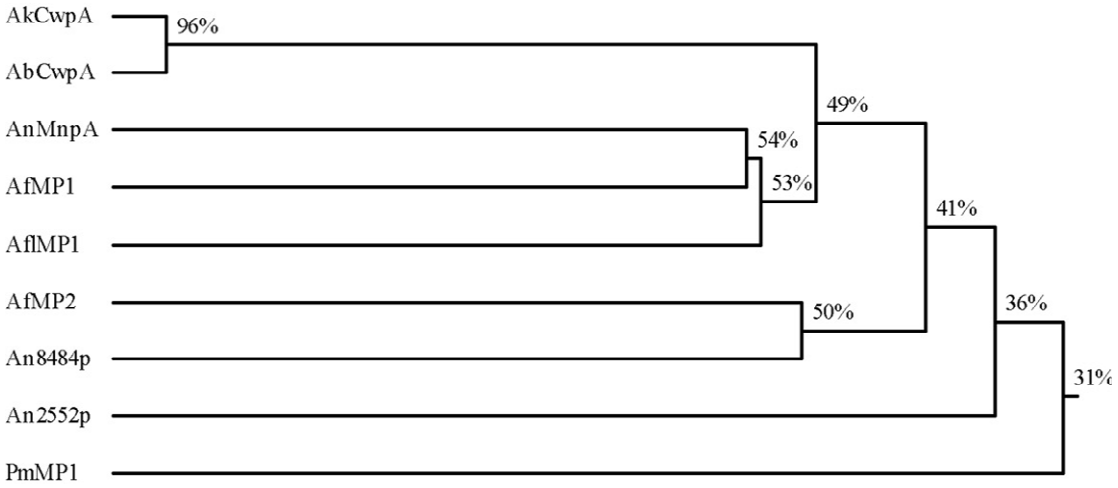

Figure 3. Phylogenetic tree of putative GPI-CWPs from filamentous fungi. Protein sequences were aligned using DNAMAN (Lynnon BioSoft, v4.0) with standard gap and extension penalties. AkCwpA, AB109764.1 *Aspergillus kawachii* CwpA; AbCwpA, AY704270 *Aspergillus black* = *Aspergillus niger* CwpA; AnMnpA, AF497720.1 *Aspergillus nidulans* MnpA; AfMP1, AY007312.1 *Aspergillus fumigatus* MP1; AflMP1, AF461762.1 *Aspergillus flavus* MP1; AfMP2, AY460182.1 *Aspergillus fumigatus* MP2; AN8484p, XM_412621.1 *Aspergillus nidulans* AN8484.2p; AN2552p, XM_406689.1 *Aspergillus nidulans* AN2552.2p; PmMP1, AF009957.1 *Penicillium marneffei* MP1. % indicate identical amino acids residues.

CwpA encodes a putative GPI-CWP

The genomic 2766 bp BglII subclone was analysed and the sequence revealed that the *cwpA* gene has an uninterrupted open reading frame of 864 nucleotides encoding for a 288 residues protein with a predicted molecular weight of 28.2 kDa. Phylogenetic analysis revealed that CwpAp has high homology with several putative cell wall proteins

mannoproteins from various filamentous fungi (Fig. 3). The protein sequence of CwpA, revealed the presence of both a hydrophobic signal sequence at the N-terminus (residues 1-17) that directs the protein to the endoplasmic reticulum and a C-terminal hydrophobic sequence that meets the criteria required for it to be cleaved off and to be replaced by a GPI-anchor (de Groot *et al*., 2003, Eisenhaber *et al*., 2004). The most probable GPI-anchor attachment site (ω-site) in the protein sequence is the glycine at position 264. The two amino acids following the ω site (ω +1 and ω+2 sites) have also been shown to be critical for GPI-anchor attachment, and the alanines at position 265 and 266 (ω +1 and ω+2 sites) allow GPI-anchoring. The ω-region (GAA) is conserved in all CWP homologs given in Figure 3 (data not shown). The ω-region is followed by a rather short spacer of four amino acids and a hydrophobic tail of 18 amino acids (Fig. 2). CwpAp contains one N-glycosylation site (N-X-S/T with X≠ P) at residue 238, and many potential O-glycosylation sites (33 % of the mature protein consist of serine or threonine residues). The O-glycosylation sites are predominantly located in the C-terminal half of the protein (aa150-263) where 50 % (56 of the 114) amino acids are serine or threonine residues.

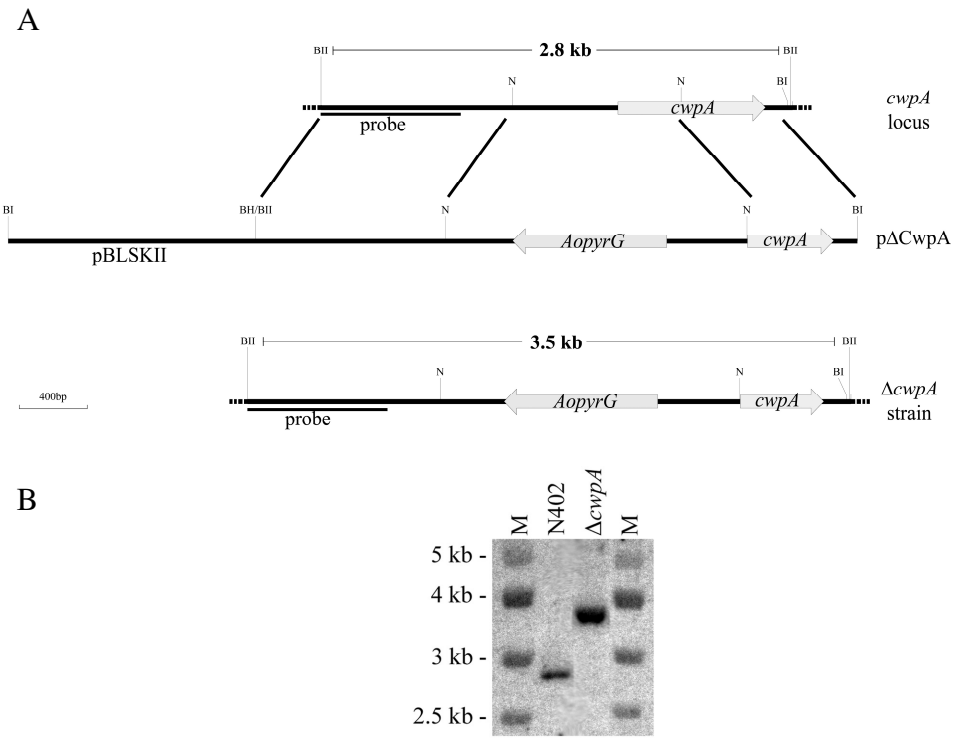

Figure 4. Deletion of *cwpA*. **A**) Schematic representation of the *cwpA* deletion strategy. The wild-type *cwpA* locus, the linear BglI fragment from the deletion plasmid pΔcwpA#2 used to transform to *A. niger*, and the *cwpA* locus after correct integration of the deletion cassette are shown. Abbreviations: (BH)

BamHI, (BI) BglI, (BII) BglII, (N) NarI. The indicted probe fragment represents a 781 bp XbaI fragment from pΔcwpA#2. Regions between diagonal lines are homologous. Scale bar represents 400 bp. **B**) Southern analysis of wild-type (N402) and the *cwpA* deletion strain (*ΔcwpA*). Genomic DNA was digested with BglI and probed with the fragment as indicated in fig. 4A. The DNA size markers (M) are shown on both sides of the blot.

Deletion of *cwpA*

To determine whether CwpA is one of the HF-extractable cell wall mannoproteins identified in *A. niger* (Fig. 1), a *cwpA* deletion strain was constructed. The deletion plasmid (*cwpA::pyrG#2*) was transformed to the *A. niger pyrG*$^{-}$ strain AB4.1 and uridine prototrophs were selected. Transformants were screened for possible deletion of the *cwpA* gene by PCR (see materials and methods). Southern analysis of BglII digested genomic DNA showed a shift in molecular size from 2.8 kb in the wild-type to the anticipated 3.5 kb hybridising fragment in the *ΔcwpA* strain indicating proper deletion of *cwpA* (Fig. 4).

To investigate whether CwpA is linked to the cell wall by a GPI-anchor, cell wall proteins were isolated by HF-pyridine treatment from the *ΔcwpA* strain and compared to the wild-type HF-extract. In the HF-extract of the *ΔcwpA* mutant, the same set of proteins with identical molecular weights was identified as in the wild-type after immunodetection using with concanavalin-A conjugated peroxidase (Fig.1A). To detect CwpA specifically, an antiserum against CwpA was raised. Immunodetection of HF-extracts from the wild-type and the *ΔcwpA* strain with the CwpA antiserum revealed the presence of the CwpA protein, in the HF-pyridine extract of wild-type (N402) cell walls (Fig. 1B). No signal was found in HF-pyridine extracts of cell walls from the *cwpA* deletion strain (*ΔcwpA*), indicating the specificity of the antiserum. The estimated molecular weight based on the Western result of CwpA is around 55 kDa (Fig. 1, fig. 5). Re-examining the HF-extractable proteins from the wild-type and the *ΔcwpA* strain after detection with the concanavalin-A peroxidase suggests that there is another glycosylated protein at 55 kDa which is detected in *ΔcwpA* (Fig. 1A). The difference in molecular weight between the predicted size of the mature protein (24 kDa) and the observed size (55 kDa) is most likely due to protein glycosylation. In the protein fractions containing predominantly cytosolic proteins (Tris-extract) or predominantly membrane proteins (SDS-extract) minor amounts of CwpA-reactive material was present (Fig. 5). The proteins that reacted with the CwpA antiserum in the Tris- and SDS-extract were specific, since no reaction with the CwpA antiserum was found in similar extracts from the *ΔcwpA* strain (data not shown). From these results we conclude that CwpA is a HF-extractable cell wall mannoprotein which is most probably anchored to the cell wall through processing of the GPI-anchor and subsequent linkage to the glucan part of the cell wall.

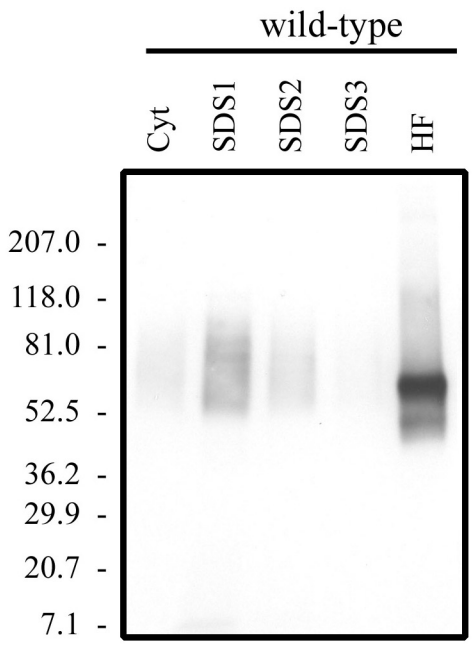

Figure 5. CwpA is predominantly localised in the cell wall. Cell walls from the wild-type strain (N402) were isolated and extracted with Tris-buffer (Cyt-extract), three times with SDS-buffer (SDS-extracts 1-3) and HF-pyridine (HF-extract). Proteins were detected with a CwpA antiserum. Molecular weight markers (kDa) are indicated on the left side of the blot. In each lane the equivalent of 200 µg of dry weight cells was loaded.

Phenotypic analysis of *cwpA* deletion strain shows an increased sensitivity towards CFW

The *cwpA* deletion strain did not show a difference in growth rate or conidiation on CM-plates when compared to wild-type at either 30 or 37 °C. To detect cell wall related phenotypes, the *ΔcwpA* strain was screened for altered sensitivity towards various compounds. Spores of wild-type and the *ΔcwpA* strain were spotted in ten-fold dilutions on CM-plates containing different concentrations of Calcofluor White (CFW), SDS, lysing enzymes (Sigma), caffeine, hydrogen peroxide, KCl, sorbitol or vanadate (Fig. 6 and data not shown). An increase in sensitivity towards CFW was observed when high concentrations (200, 400, or 1000 µg ml^{-1}) were used (Fig. 6). The increased sensitivity could be remediated by the addition of 1.2 M sorbitol. Sensitivity of the wild-type and the *ΔcwpA* mutant towards the other compounds tested did not reveal any difference in growth or sensitivity between the two strains.

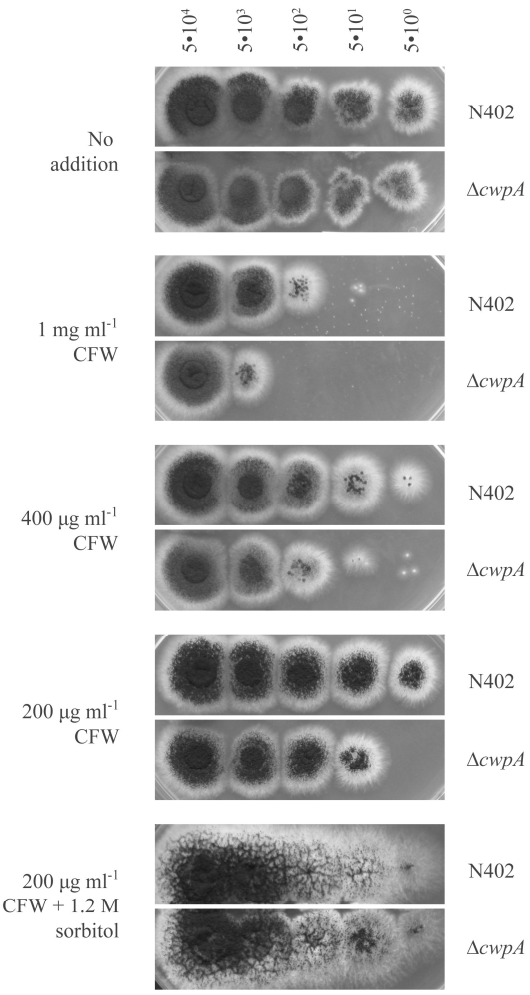

Figure 6. Phenotype of the *cwpA* deletion strain. Ten-fold serial dilutions of spores of wild-type (N402) and the *cwpA* deletion strain (Δ*cwpA*) were spotted on complete medium plates containing CFW, indicated at the left side of the plates. Pictures were taken after incubation at 37 °C for three days.

Expression analysis of *cwpA*

Expression of cell wall proteins in *S. cerevisiae* has been shown to be highly regulated in response to growth conditions and in response to environmental changes (Smits *et al.,* 1999). We examined the expression of *cwpA* under normal growth conditions in shake flask. Spores were inoculated in complete medium and grown at 30 °C or 37 °C for 8, 16, 24, 32, or 64 hours. The expression of *cwpA* seems to peak around 24 to 32 hours independent of the

growth temperature used (Fig. 7A and data not shown). When the 8 and 32 hour time points are compared, a more than 100-fold induction is observed for *cwpA* expression at both temperatures (Fig. 7A and data not shown). As expected, no signal was detected in RNA samples from the *cwpA* deletion strain. The growth curve of these culture conditions showed that maximum biomass level (µg dry weight mycelium ml^{-1} culture) was reached after 24 hours, and remained constant for at least 8 hours before declining (Fig. 7B). We conclude that the induction of *cwpA* coincides with the later stages of exponential growth and/or with the entrance into stationary phase. At 64 hours, the biomass of the culture decreased, which is indicative for cell lysis and is accompanied by a decrease in *cwpA* messenger levels. The induced expression of *cwpA* in the shake flask culture is probably not caused because of conidiation. The expression of conidiation specific markers (*brlA* and *rodA*) was only observed at the 64 h time point (Fig. 7A). The results suggest that *cwpA* expression is dependant on the growth phase of the fungus and induced during the later phases of growth.

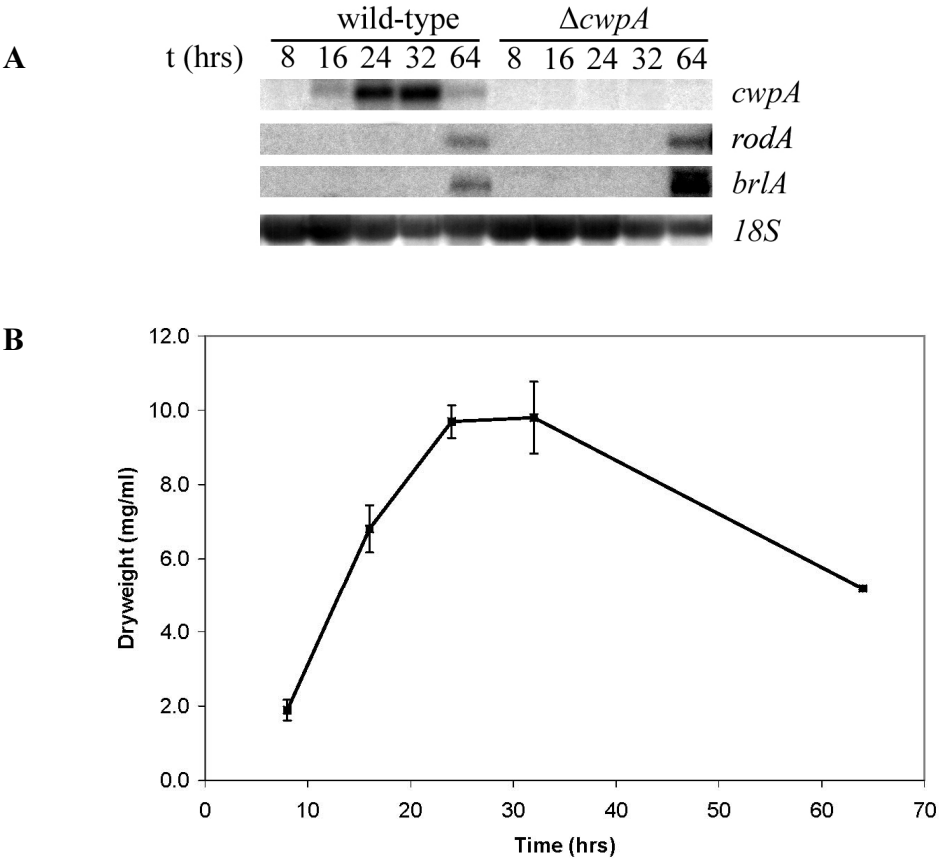

Figure 7. Analysis of *cwpA* expression. **A**) Northern analysis of *cwpA*, *rodA* and *brlA* expression levels under normal growth at 30 °C in shake flask CM cultures for both wild-type and the *cwpA* deletion strain.

Total RNA was isolated after 8, 16, 32, and 64 hours. Northerns were probed with an 18S ribosomal probe as a loading control. **B**) Growth curve of the wild-type strain grown as described above.

Discussion

In this study, we present the identification and characterisation of the *cwpA* gene from *A. niger*. The *cwpA* gene encodes a protein of 228 amino acids which contains a putative signal peptide and a putative GPI-anchor attachment signal. In fungi, GPI–anchored proteins are found at the cell surface, either attached to the plasma membrane (GPI-PMPs) or attached by a remnant of the GPI-anchor to the cell wall and are referred to as GPI-cell wall proteins (GPI-CWPs). Studies in *S. cerevisiae* have shown that GPI-PMPs remain attached predominantly to the plasma membrane, whereas GPI-CWPs become attached to β-1,3-glucan or the chitin part of the cell wall through a flexible β-1,6-glucan moiety (Kapteyn *et al*., 1995, 1996, Kollar *et al*., 1997). Upon arrival at the plasma membrane, GPI-CWPs are liberated from the plasma membrane by a still unknown mechanism. Subsequently, a remnant of the GPI-anchor reacts with the β-1,6-glucan part of the cell wall and results in the cross-linking of GPI-CWPs to the cell wall. Whether a GPI-anchored protein is a GPI-PMP or GPI-CWP can be determined by a simple fractionation experiment (Frieman and Cormack, 2003). In their studies, Frieman and Cormack, show that GPI-PMPs are present in the SDS-soluble (membrane) fractions, whereas GPI-CWPs are SDS-resistant and found in the cell wall fractions. GPI-CWPs can either be liberated from the cell wall after β-1,3- or β-1,6-glucanse digestion or by HF-treatment. In this study, we have used a similar fractionation method to show that CwpA is a GPI-CWP. CwpA is hardly detectable in the SDS (membrane) fraction, and it is abundantly present in the HF-extract of SDS-extracted cell walls (Fig. 5). To our knowledge, this is the first time that the comparison between SDS- and HF-extractability of a putative cell wall protein has been made for a filamentous fungus. Previously, the most convincing evidence for the presence of GPI-anchored cell wall proteins in filamentous fungi was the report of FEM1p in *F. oxysporum* (Schoffelmeer *et al*., 2001). However, one could still argue that the amount of FEM1p detected in the HF-extract is only a small part of the total protein present since the amount of FEM1p extracted by SDS has not been reported.

The sorting signal that is determining whether GPI-anchored proteins are GPI-PMPs or GPI-CWP is still under investigation. Based on *in silico* analysis of GPI-CWPs in *S. cerevisiae*, Caro *et al.* (1997) predicted that a dibasic motif in the four amino acids upstream of the GPI-anchor attachment site results in the retention of the protein to the plasma membrane. This prediction has been experimentally confirmed by Frieman and Cormack (2003) who have shown that the introduction of a dibasic motif upstream of the ω-site of Cwp2 alters its localisation dramatically from the cell wall to the plasma membrane. In

addition, Hamada *et al*. (1999) predicted that hydrophobic amino acids present at positions ω-2, ω-4, and ω-5 favour the localization to the cell wall. However, this prediction was not an absolute requirement for cell wall localization but such amino acids probably increase the efficiency by which a GPI-CWP is incorporated into the cell wall (Frieman and Cormack, 2003). The absence of a dibasic motif in the amino acids upstream of the predicted ω-site in CwpA is in accordance with the cell wall localisation of CwpA.

Homologues of CwpA have been previously described in *P. marneffei* (Cao *et al*., 1998), *Aspergillus fumigatus* (Yuen *et al*., 2001, Chong *et al*., 2004) *Aspergillus nidulans* (Jeong *et al*., 2003) and *Aspergillus flavus* (Woo *et al*., 2003). All these CwpA homologs are predicted to contain a GPI-anchor attachment signal, and although cell wall localisation has been reported from immuno-EM studies (Cao *et al*., 1998, Yuen *et al*., 2001), biochemical evidence that these proteins are covalently bound to the cell wall by their GPI-anchor moiety is still lacking. Based on our observations, we propose that the CwpA homologues will be incorporated into the cell wall in a similar manner to that of the *A. niger* CwpA.

The HF-extractions of extensively SDS-extracted cell walls of *A. niger* revealed that at least five HF-pyridine extractable proteins could be detected using peroxidase labelled concanavalin-A with CwpA being one of the less abundant. Recently, a rapid and simple method to identify those proteins by LC/MS/MS was successfully used to identify HF-extractable cell wall mannoproteins from *C. albicans* (de Groot *et al*., 2004), and this method could also be used to identify these proteins in other fungi including *A. niger*.

The protein band corresponding to CwpA in the HF-extract is somewhat smaller and more distinct in size compared to the protein detected in the SDS-extract (Fig. 5). This could be due to the cleavage of additional HF-sensitive linkages which include phosphomannans by HF-pyridine present in N- and O-glycosidic chains as has been shown in the yeast *S. cerevisiae* (Jigami and Odani, 1999).

No obvious function of CwpA could be deduced from its knock out phenotype. The *ΔcwpA* strain did not show an apparent difference in growth and/or conidiation under normal growth conditions. However, the *ΔcwpA* strain showed an increased sensitivity towards Calcofluor White (CFW). CFW is known to interfere with normal cell wall assembly and hypersensitivity towards CFW is indicative for a cell wall integrity defect (Ram *et al*., 1994, Lussier *et al*., 1997). At the moment we favour the idea that CwpA has a structural role and that the absence of CwpA leads to alterations in the cell wall which makes the fungus more sensitive to CFW. Sorbitol was shown to fully suppress the CFW hypersensitive phenotype, which is in agreement with the proposed cell wall integrity defect of the *ΔcwpA* strain. In *S. cerevisiae*, deletion of *cwp1* or *cwp2* or the simultaneous deletion of both genes leads to

hypersensitivity towards Congo Red (CR) (van der Vaart *et al*., 1995). Congo Red binds, like CFW, to chitin fibrils in the cell wall and disturbs proper cell wall assembly.

Certain GPI-anchored cell wall proteins in *S. cerevisiae* have been shown to be specifically induced in response to various cell wall stress inducing conditions (Jung and Levin, 1999, Terashima *et al*., 2000, Lagorce *et al*., 2003, Garcia *et al*., 2004). CFW induced cell wall stress in *A. niger* did not result in an increased expression of *cwpA* (Damveld and Ram, unpublished data). The expression of *cwpA* is most abundant during the late exponential or stationary phase of growth and resembles the expression of Sed1p, a cell wall protein of *S. cerevisiae* involved in lytic enzyme resistance (Shimoi *et al*., 1998). Deletion of Sed1p had also an effect on the cell wall since *ΔSED1* cells were more sensitive to Zymolyase (β-1,3-glucanase) digestion (Shimoi *et al*., 1998). To correlate the expression data of *cwpA* with CwpA localisation, a GFP-CwpA fusion was constructed. The GFP was inserted at similar position as has been previously successfully used to analyse the localization of GFP-CWPs in *S. cerevisiae* (Ram *et al*., 1998b). However, insertion of GFP did not result in successful transport of GFP-CwpA to the cell surface and it suggests that the GFP-CwpA fusion protein is not transported through the secretory pathway (Damveld, Wierckx and Ram, unpublished results). No further attempts have been undertaken to try to insert GFP at different sites in CwpA protein.

It has been shown for *S. cerevisiae* and *C. albicans* that the GPI-CWPs are linked to β-1,6-glucans through a remnant of the GPI-anchor (Lu *et al*., 1994, 1995, Kapteyn *et al*., 1995). An intriguing point of interest, which requires further investigation, is the identification of the exact linkage between the GPI-CWPs and the cell wall in filamentous fungi. The presence of β-1,6-glucosylated cell wall proteins in *A. niger* and *F. oxysporum* have been reported (Brul *et al*., 1997, Schoffelmeer *et al.,* 1996) which indicates that a similar incorporation mechanism might exist in yeasts and filamentous fungi. However, β-1,6-glucan, as it is in present in *S. cerevisiae,* seems to be absent in the filamentous fungus *A. fumigatus* (Bernard and Latge, 2001) and detailed studies for other fungi are missing. The way in which GPI-CWPs are attached to the cell wall and to which extent a similar incorporation mechanism exists in all filamentous fungi is not known. This, in addition to the identification of enzymes involved in these processes in *A. niger* is the subject of our current research. The generation of a highly specific antiserum against CwpA will greatly facilitate future studies and will also open the way for a genetic screen to identify mutants disturbed in the incorporation of cell wall mannoproteins in *A. niger*.

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Chapter 6

A novel screening method for the identification of genes involved in cell wall biosynthesis in *Aspergillus niger*

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Abstract

We previously showed that the cell wall integrity response of *Aspergillus niger* to cell wall stress includes a 20-fold induction of *agsA* which encodes an α-1,3-glucan synthase. We further showed that the cell wall integrity transcription factor RlmA was required for this induced expression. In this study we present a novel positive screening method for the isolation of cell wall mutants by cloning the *agsA* promoter region in front of a selectable marker (*amdS*). The rationale of this screen is that a mutation in a cell wall biosynthetic gene is expected to result in cell wall weakening and as a result will trigger the expression of the *amdS* gene from the *agsA* promoter, allowing growth on acetamide as the sole N-source. A reporter strain was constructed in which the *agsA* promoter was fused both to the acetamidase (*amdS*) selection marker and to the nuclear targeted GFP (H2B-GFP) reporter construct, allowing the selection for trans-acting mutations that activate the cell wall integrity response and thus give a constitutively increased *agsA* promoter activity. The primary screen yielded 240 *mia* mutants (mutant with induced *agsA* promoter activity) that were subjected to various secondary screens (e.g. osmotic remediable temperature sensitivity, and Calcofluor White-, and SDS-sensitivity). Based on the results of the secondary screens, four osmotic remediable and CFW- and/or SDS-hypersensitive mutants, *miaA-D*, were selected for complementation. All four mutants were successfully complemented. Two mutants, *miaA* and *miaB* are complemented by cosmids with overlapping inserts indicating that their mutations are possibly allelic. Further subcloning experiments are underway to identify the mutated genes in the various mutants. Complementation of the mutants and identification of the genes involved is expected to lead to the discovery of new antifungal targets related to cell wall biosynthesis.

Introduction

Cell wall remodeling in response to cell wall stress is of great importance to yeasts and filamentous fungi. Inability of the cell to respond to cell wall threatening conditions might result in cell lysis and cell death. The fungal cell wall integrity pathway is best studied in the yeast *Saccharomyces cerevisiae* and consists of a signal transduction network that is able to sense cell wall weakening. This weakening of the cell wall generates a signal resulting in the activation of the transcription factor Rlm1p, required to induce the expression of genes that encode proteins which help to withstand cell wall threatening conditions. The response to cell wall stress is mediated by the so called Pkc1-Slt2 cell wall integrity pathway (reviewed by Heinisch *et al*., 1999). Mutants in this signaling network display a cell lysis defect which can be remediated by the addition of an osmostabiliser to the growth medium (Paravicini *et al*., 1992, Costigan *et al*., 1992, Borgia and Dodge, 1992, Irie *et al*., 1993, Lee *et al*., 1993). Activation of the signaling pathway can be achieved either by compounds that interfere with cell wall biosynthesis or assembly (de Nobel *et al*., 2000, Garcia *et al*., 2004), but is also constitutively activated in mutants that are defective in cell wall biosynthesis (Lagorce *et al*., 2003). In *S. cerevisiae,* genome wide analyses have indicated that the cell wall remodeling response consists of a number of alterations in the cell wall. First, a higher expression of certain GPI-anchored cell wall mannoproteins which are thought to have a structural role in the cell wall and by their higher abundance increase the strength of the cell (Terashima *et al*., 2000). Alternatively, since these proteins are incorporated mainly at the outside of the cell wall surface they might protect the underlying glucan/chitin layer from being attacked by e.g. glucanases and/or chitinases. Secondly, increased chitin synthesis has been shown to be an important compensatory response to cell wall stress. Both the addition of cell wall disturbing compounds and in cell wall mutants increased chitin levels in the cell wall have been reported (Ram *et al*., 1994, Popolo *et al.,* 1997, Dallies *et al.,* 1998, Osmond *et al.,* 1999, Lagorce *et al.,* 2002). The increased chitin synthase activity is accomplished by activation of chitin synthases at a post-translational level (Valdivia and Schekman, 2003). The *GFA1* gene, encoding the enzyme glutamine:fructose-6-phosphate amidotransferase, is required for the synthesis of UDP-N-acetylglucosamine, the sugar donor for the synthesis of chitin is induced 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 (Boorsma *et al.,* 2004, Garcia *et al.,* 2004). The induced expression of the *GFA1* gene in response to cell wall stress is not limited to the yeast *S. cerevisiae* but has also been shown to occur in filamentous fungi such as *Aspergillus niger*, *Penicillium chrysogenum* and *Fusarium oxysporum* (Ram *et al*., 2004). A third remodeling response specific for filamentous fungi consists of activation of genes encoding α-1,3-glucan synthases has recently been

described for the filamentous fungi *A. niger* and *P. chrysogenum*. *S. cerevisiae* lacks α-1,3-glucan in its cell wall and also lacks the genes encoding the α-1,3-glucan synthases. *A. niger* contains a family of five *ags* genes. *AgsA* and to a lesser extend *agsE* are induced in response to CFW induced cell wall stress (Damveld *et al*., 2005, Chapter 3). The activation of P*agsA* is dependent on RlmA, a homolog of the Rlm1p transcription factor in *S. cerevisiae*, indicating that a similar cell wall integrity pathway exists in filamentous fungi. In this study we have used the activation of the *agsA* promoter to identify genes involved in cell wall synthesis.

Material and Methods

Strains, transformations, and growth conditions

A. niger N402 (a *cspA1* derivative of ATCC9029, Bos *et al*., 1988) and AB4.1 (van Hartingsveldt *et al*., 1987) a *pyrG*$^-$ derivative of N402 were used in this study. Strains were grown on minimal medium (MM) (Bennett and Lasure, 1991) containing 1 % (w v^{-1}) glucose and 0.1 % (w v^{-1}) casamino acids or on complete medium (CM), containing 0.5 % (w v^{-1}) yeast extract in addition to MM. When required, plates were supplemented with uridine (10 mM) or hygromycin (100 µg ml^{-1}). Conidia were isolated with 0.9 % (w v^{-1}) NaCl from CM plates after growth for 4-6 days at 30 °C. MM agar plates containing acetamide as a sole nitrogen source were made as described (Kelly and Hynes, 1985). Transformation of *A. niger* was performed as described by Punt and van den Hondel (1992), using 40 mg lysing enzymes (Sigma, L-1412) per g fresh weight mycelium. For co-transformations using the hygromycin selection marker, pAN7-1 (accession number Z32698) was used. *Escherichia coli* strain DH5α (Invitrogen) was transformed by electroporation, according to the suppliers manual, for the propagation and amplification of cosmids. XL1-Blue (Stratagene, La Jolla, CA) was transformed using the heat shock protocol as described by Inoue *et al*. (1990) and used for the amplification of plasmids. *E. coli* was grown in LB as described in Sambrook *et al*. (1989), with the addition of 50 µg ml^{-1} ampicillin when required.

Molecular techniques

Fungal chromosomal DNA was isolated as described by Kolar *et al*. (1988). [α-^{32}P]dCTP labeled probes were synthesized using the Rediprime II DNA labeling system (Amersham Pharmacia Biotech) according to the instructions of the manufacturer. Southern blot analyses were performed as described by Sambrook *et al*. (1989) using HybondN+ (Amersham Pharmacia Biotech), and hybridization signals were detected using a Phosphor Imager (Molecular Dynamics). Restriction enzymes were obtained from Invitrogen and used according to the supplier's manual. The ligation of DNA fragments was performed with the

Rapid DNA ligation kit (Roche). When required, fragments were dephosphorylated with Shrimp Alkaline Phosphatase (Roche). Sequencing was performed by ServiceXS (Leiden, Netherlands).

Construction of recombinant plasmids

For the construction of plasmid P*agsA*-*amdS*-T*amdS*, a 2010-bp SalI-EcoRI fragment containing the *agsA* promoter region was isolated from pRD12 (Damveld *et al*., 2005, Chapter 3, SstI subclone of cosmid P13P14 containing the complete *agsA* ORF with ~ 4-kb 5' and ~ 2-kb 3' sequence) and ligated into an SalI-EcoRI opened pBluescipt II SK (Stratagene) vector. The resulting plasmid was opened with EcoRI-XbaI yielding the first fragment for a three-way ligation. The second fragment, a 587-bp EcoRI-BglI fragment containing 30-bp of the *agsA* promoter and ~ 0.56-kb of the 5` sequence of the *amdS* gene, was created by fusing PCR using primers AmdS-agsAP1 (5`-cacagaattcctggtaccacacgccgcttgccatcatgcctcaatcctgggaag-3`, EcoRI site and ATG of *amdS* underlined) and AmdS-agsAP2 (5`-gccatgagatgtagcccattg-3`), using p3SR2 (Corrick *et al*., 1987) as a template. The resulting PCR product was cloned in pGEM-T Easy (Promega) and verified by sequence analysis. The 587-bp fragment was isolated after digestion with EcoRI and BglI. The third fragment was obtained by digestion of p3SR2 with BglI and XbaI. The 1544-bp fragment, containing the 3' part of the *amdS* gene and the *amdS* terminator sequence was isolated and ligated with the other two fragments to give vector P*agsA*-*amdS*-T*amdS*. After ligation, this vector was opened with XbaI to introduce the *pyrG** gene (Gouka *et al*., 1995), isolated as a 3.8-kb XbaI fragment from pAN52-7pyrG* (Damveld, unpublished vector) to give P*agsA*-*amdS*-T*amdS*-*pyrG**.

The plasmid P*agsA*-H2B-GFP-T*trpC* was constructed by a three way ligation. First a 0.6-kb EcoRI-NcoI fragment containing the 30-bp promoter fragment of *agsA* fused to H2B was generated by PCR using primers AgsAH2BP1(5'-cacagaattcctggtaccacacgccgcttgccatcatgcctcccaaagctgccg-3', the EcoRI site in the *agsA* promoter and the ATG of the H2B are underlined in the sequence) and AgsAH2BP2 (5'-ctcaccatgggatcgaactcgatgggtttggc-3', removing the EcoRI site from H2B) using pH2BG (Maruyama *et al*., 2001) as a template. The PCR product was cloned in pGEM-T Easy and verified by sequencing. The second fragment containing GFP-T*trpC* and the pUC18 backbone sequence was isolated as a NcoI-NotI fragment from P*gpdA*-H2B-GFP-T*trpC* (M. Arentshorst and A.F.J. Ram, unpublished vector). The third fragment containing ~ 2-kb of the *agsA* promoter sequence was obtained after ligation of a ~ 2-kb SalI-EcoRI fragment from pRD12 into a SalI-EcoRI opened pUC21 and subsequent re-isolation after NotI-EcoRI digestion. The three fragments were ligated together to give P*agsA*-H2B-GFP-T*trpC*. The

unique XbaI site was used to introduce the *pyrG** gene (Gouka *et al*., 1995), isolated as a 3.8-kb XbaI fragment from pAN52-7pyrG* to give P*agsA*-H2B-GFP-T*trpC-pyrG**.

Mutagenesis and the primary mutant screen

The strains used for mutagenesis were constructed as follows. The AB4.1 (*pyrG*$^{-}$) strain was transformed with P*agsA-amdS*-T*amdS-pyrG** or P*agsA*-H2B-GFP-T*trpC-pyrG**. For both constructs, transformants were selected that had a single copy of the construct integrated on the *pyrG* locus based on Southern analysis (data not shown), and were named RD1.7 and RD5.43 respectively. Next, strain RD1.7 was co-transformed with P*agsA*-H2B-GFP-T*trpC* and strain RD5.43 was co-transformed with P*agsA-amdS*-T*amdS*, using pAN7-1 in both cases. Transformants were analysed by PCR for the presence of the complete reporter constructs, yielding strains RD6.13 and RD6.47 (containing P*agsA-amdS*-T*amdS* targeted to the *pyrG* locus and P*agsA*-H2B-GFP-T*trpC* co-transformed) and strains RD15.4 and RD15.8 (containing P*agsA*-H2B-GFP-T*trpC* targeted to the *pyrG* locus and P*agsA-amdS*-T*amdS* co-transformed). All four strains were subjected to UV mutagenesis. Freshly harvested spores were diluted to $1x10^7$ spores ml^{-1} and 15 ml spore solutions were mutagenised in a Bio-Rad cross linker (maximum energy output at λ = 254 nm, UV dose 60 J s^{-1} m^2) for 0-100 seconds with 10 seconds intervals. Survival rates at the different time-points were determined by plating out dilutions of the mutagenised conidia suspensions on CM-plates. The conidia from spore suspensions with a ~ 66% survival rate were used for the primary screen. For each of the four strains, 60 MM-plates with acetamide as the sole nitrogen source were inoculated with $1x10^4$ conidia and incubated at 30 ºC. After five days a single fast growing colony from each plate was transferred to CM-plates and purified two times, yielding 240 primary mutants.

Secondary screens

Growth on acetamide. The purified mutants were re-tested for their ability to grow on acetamide plates at 30 ºC. Equal amounts of conidia (~ $5x10^2$) were spotted on MM-plates, with acetamide as the sole nitrogen source and images were taken after 3 days.

Nuclear GFP levels. For microscopical images, conidia were grown on coverslips in MM with casamino acids at 30 ºC for 18 hours. The coverslips with adherent conidia were placed on microscope slides and microscopic GFP images were taken on an Axioplan 2 (Zeiss) equipped with a DKC-5000 (Sony) digital photo camera using a fixed exposure time of one second. As a negative control N402, as a positive control P*gpdA*-H2B-GFP (+), and for the basal level the non-mutagenised parental strains (P*agsA*-H2B-GFP) were used. GFP images were analysed using Qwin Pro (LEICA, v2.2) In brief, the green channels of the

images were analysed by selecting all green pixels with a value > 130, which corresponded as expected to the nuclei. The average GFP values (Mean Grn) and the maximum GFP values (Max Grn) were determined for these selections and compared to the non-mutagenised values. Mutants in which the average or maximum GFP values were higher when compared to non-mutagenised strains were scored as mutants with increased GFP expression from the *agsA* promoter.

Temperature sensitivity. Mutant strains were grown at both 30 °C and 42 °C on MM-plates for three days. The colony morphology (e.g. sporulation and diameter) was compared between the two plates.

Osmotic remediability at 30 °C and *42 °C.* The effect of the addition of the osmotic stabilizer sorbitol was examined. Therefore strains were grown on MM with or without the addition of 1.2 M of sorbitol at 30 °C or 42 °C for three days.

Sensitivity towards SDS and CFW. MM plates containing 0.005 % (w v^{-1}) SDS, 0.1 mg ml^{-1} CFW or 0.01 mg ml^{-1} CFW were inoculated with the mutant strains and grown at 30 °C for three days. Colony size and morphology were compared to MM-plates without additives grown under the same conditions.

Complementation of the cell wall mutants

Four mutants were selected for complementation and their *pyrG*$^-$ derivatives were obtained by plating out $1x10^5$ spores on MM-plates in which the $NaNO_3$ was replaced by 10 mM prolin as N-source. To allow selection for uridine auxotrophs 10 mM uridine and 0.75 g l^{-1} 5-fluoro-orotic acid (5-FOA, USBiological) was added. Conidiating colonies were purified twice on MM plates supplemented with 10 mM uridine and analysed based on their phenotype on MM-plates containing 0.005 % (w v^{-1}) SDS with and without uridine. Hence, mutations in both *pyrE*, an orotate phosphoribosyl transferase (OPRTase; EC 2.4.2.10) and *pyrG*, an orotidine-5'-monophosphate decarboxylase (OMPdecase; EC 4.1.1.23) can confer resistance towards 5-FOA (Boeke *et al*., 1984, Takeno *et al*., 2004), the resistant strains were transformed with plasmids containing either *pyrE* (pMApyrE) or *pyrG* (pAB4.1) and analysed for complementation based on growth without uridine. 5-FOA resistant strains that could only be complemented by *pyrG* were used for further analysis. The sequence of *pyrE* was submitted to DDBJ/EMBL/GenBank databases with accession number AY840014.

The *pyrG*$^-$ mutants were transformed with a genomic cosmid library (kindly provided by Dr. F. Schuren and Dr. P. Punt, TNO Nutrition, The Netherlands). Transformants were selected on transformation plates based on the ability to grow without uridine (*pyrG* complementation). Complementation of the mutant phenotype was analysed by screening for strains that had obtained the parental SDS sensitivity at 42 °C. After transformation with the genomic cosmid

library, spores were isolated from transformation plates, transferred to plates containing minimal medium with 0.005 % (w v^{-1}) SDS, and grown for four days at 42 °C. Cosmids from the putative complemented *A. niger* strains were isolated using the protocol for isolation of genomic DNA (Kolar *et al*., 1988). The cosmids were transformed to *E. coli* (DH5α) and grown on LB plates with ampicillin. Subsequent cosmid isolations from 40 ml of overnight cultures were performed using small scale DNA isolation method as described by Sambrook *et al*. (1989). Primers cosT7 (5'-gcttatcgatgataagcggtc-3') and cosUL (5'-aaagcttgcatgcctgcaggtcg-3') were used for sequencing the ends of the inserts.

Results

Rationale of the cell wall mutant screen

To identify genes involved in the synthesis of the fungal cell wall, we designed a positive screening procedure for the isolation of mutants disturbed in cell surface assembly. The screen is based on our previous observation that the *agsA* gene, encoding an α-1,3-glucan synthase, is transcriptionally induced in response to different forms of compound induced cell wall stress (Damveld *et al*., 2005, Chapter 3). The induced expression of *agsA* in response to cell wall disturbing compounds like Calcofluor White (interferes with cell wall assembly, or caspofungin (inhibitor of β-1,3-glucan synthase activity) has been shown to be dependent on the RlmA transcription factor (Damveld *et al*., submitted, Chapter 4). We also found sequences in the publicly available fungal genome databases that are homologous to the genes of the cell wall integrity pathway in yeast (Chapter 1). Based on these observations, and the observations of Mizutani *et al*. (2004), we propose the existence of a cell wall integrity pathway (Fig. 1) in *A. niger*, similar to the cell wall pathway in *S. cerevisiae* (Gustin *et al*., 1998). Our cell wall mutant screen is based on the hypothesis that a mutation in a gene, which effects cell wall biosynthesis would result in a disturbed or weakened cell wall. As a consequence of the weakening of the cell wall, P*agsA* is expected to be transcriptionally activated via the cell wall integrity pathway, thereby mimicking the effect of e.g. CFW (Fig. 1). To select positively for mutants with a constitutively activated cell wall integrity pathway, the *agsA* promoter region was cloned in front of the *A. nidulans amdS* selection marker. The *amdS* gene encodes an acetamidase and it has been shown that high expression of the gene, generated by multiple copy integrations, is necessary to allow *A. niger* to grow on acetamide plates (Kelly and Hynes 1985, Hanegraaf *et al*., 1991, Verdoes *et al*., 1993). Therefore the reporter strain, containing the *PagsA-amdS* fusion construct, is expected to grow on acetamide plates only after activation of the *agsA* promoter by the loss of function of an important gene involved in cell wall biosynthesis (Fig. 1). In addition to the

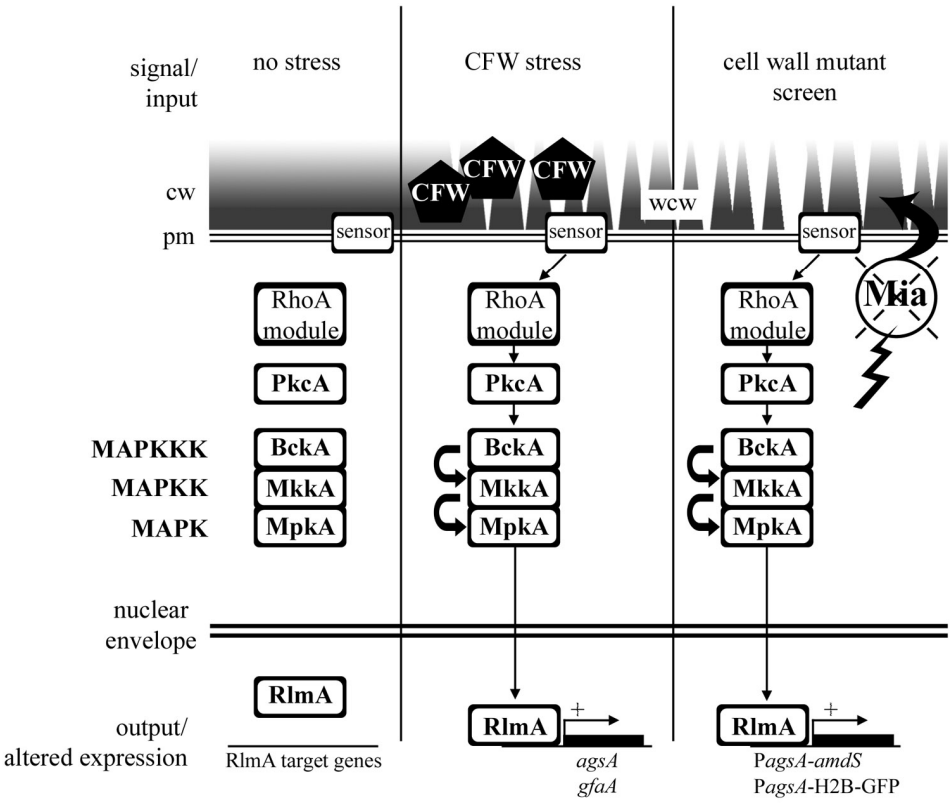

Figure. 1. The rationale of the screen. The left part of the figure shows the fungal cell wall integrity pathway under normal growth conditions (no stress), in which some basal expression of RlmA target genes might exist. The middle part of the figure shows the assumed triggering of the cell wall integrity pathway by a cell wall assembly disturbing compound (CFW stress), resulting in the increased expression of *agsA* and *gfaA*. The right part of the figure shows the rationale of the cell wall mutant screen. We envisioned that a mutation in a gene encoding a protein which is involved in cell wall biosynthesis would result in a weakened cell wall which would activate the *agsA* promoter activity. The use of the P*agsA-amdS* reporter construct allows the positive selection for mutants with an induced *agsA* promoter activity (*mia*). Abbreviations used: cell wall (cw), plasma membrane (pm), Calcofluor White (CFW), and weakened cell wall (wcw). The + marks represent an increased promoter activity and the gaps in the layer representing the cell wall indicate an altered cell wall integrity.

PagsA-amdS reporter construct, a second reporter construct was made in which the *agsA* promoter region was fused to a nuclear targeted version of GFP (H2B-GFP, Maruyama *et al*., 2001). The second reporter was introduced to discriminate between cis- and trans-acting mutations. The presence of both constructs as single-copy integrants was confirmed by

Southern analysis (data not shown). When the parental strain was inoculated on MM-agar plates containing acetamide as sole nitrogen source, only poor growth was observed, indicating that the basal activity of P*agsA* is not sufficient to allow growth on acetamide (Fig. 2A). Also the use of the *pyrG* gene was examined as a selection marker for the mutant screen instead of the *amdS* gene, but apparently the basal activity of the *agsA* promoter supplies the cell with sufficient amount of *pyrG* protein since the transformant (*pyrG*$^{-}$, P*agsA*-*pyrG*) could grow on plates without uridine (data not shown). Having shown that the basal level of acetamidase expressed from the *agsA* promoter is not enough to allow growth

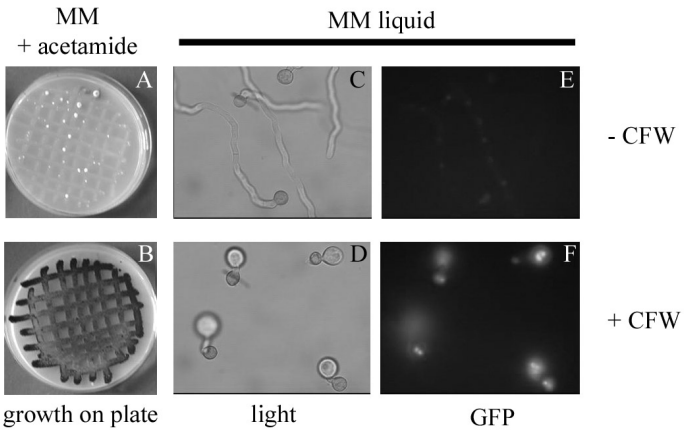

Figure 2. The reporter strain RD6.47, containing both the nuclear targeted GFP (H2B-GFP) and acetamidase (*amdS*) reporters under control of the *agsA* promoter, was grown for three days at 30 ℃ on plates containing acetamide as the sole nitrogen source under normal growth conditions (A) and with the addition of 400 µg ml^{-1} CFW giving an induction of the reporter (B). The reporter strain was also allowed to germinate on submerged coverslides for five hours at 37 °C, subsequently 200 µg ml^{-1} CFW (D and F) or an equal amount on MilliQ (C and E) was added and the strains were incubated for one more hour at 37 ℃.

on acetamide plates, we next examined whether the addition of CFW to these plates would activate the *agsA* promoter. As shown in Fig. 2B, the addition of 0.5 mg ml^{-1} CFW to the plates resulted in growth and even sporulation of the reporter strain. The response of the second reporter to CFW induced cell wall stress was also examined. Therefore, the parental strains were grown in liquid minimal medium. As expected from the low basal level of the *agsA* gene under normal growth conditions (no stress), the fluorescence signal from the nuclear targeted H2B-GFP fusion protein was low and no growth abnormalities were observed (Fig. 2C and E). The addition of CFW (0.2 mg ml^{-1}) to the liquid minimal medium resulted in the formation of swollen hyphal tips and induction of *agsA* expression since the

nuclear targeted GFP was clearly visible (Fig. 2D and F). Using the P*agsA*-H2B-GFP reporter strain, we were able to show that the induction of *agsA* was not limited to CFW, but that the induction was also achieved by adding other cell wall disturbing compounds such as caspofungin and tunicamycin. In addition, the induction is specific for cell wall stress, since other forms of stress (high osmolarity stress, oxidative stress or temperature stress) did not result in activation of the *agsA* promoter (Chapter 7). All together, these results indicate that the reporter strain is suited to identify mutants with increased levels of *agsA* expression which is likely derived from a mutation that affects the integrity of the cell wall.

Isolation and phenotypic characterization of the putative cell wall mutants

Spore suspensions of the four parental strains were mutagenised as described in material and methods. Mutagenised spores with a ~ 66 % survival rate were plated out on acetamide containing selection plates. For each strain, 60 acetamide plates were inoculated with ~ 7000 viable spores per plate and incubated at 30 ℃. After five days, between one and five fast growing colonies were present on the plates. The fastest growing colony from each plate was transferred to a CM-plate and subjected to two rounds of purification, yielding 240 mutants from this primary screen. All mutant strains were stored in 50 % glycerol (v v^{-1}) at -80 ℃. After purification the 240 mutants were retested for the ability to grow well on acetamide. Out of the 240 mutants, 161 mutants still grew well on acetamide (Fig. 3). Potentially, these mutants could have a mutation in a gene involved in cell wall biosynthesis, but the growth on acetamide might also derive from a mutation in the *agsA* promoter, which would result in constitutive expression, or from mutations that activate the *A. niger* endogenous acetamidase gene (J. Visser, personal communication). To discriminate between potential cell wall mutants and other mutants, the 161 mutants were analysed for the presence of increased nuclear GFP levels using fluorescence microscopy. Only three mutants failed to show increased nuclear GFP levels compared to the parental strain and were not studied further. For a more detailed identification of potential cell wall-related genes, each mutant was analysed using phenotypic assays which are indicative of mutations leading to a defective cell wall. The mutants were tested for increased sensitivity towards elevated temperatures, Calcofluor White, and SDS. In addition, the temperature-sensitive (ts) mutants were tested for osmotic remediability of the ts-phenotype. As indicated before, CFW is a cell wall assembly disturbing compound that has been used to identify mutants with a weakened cell wall (Ram *et al*., 1994, Lussier *et al*., 1997, de Groot *et al*., 2001). SDS is known to affect membrane stability and hypersensitivity towards SDS is also indicative for alterations in the cell wall (Shimizu *et al*., 1994, Igual *et al*., 1996, Bickle *et al*., 1998, de Groot *et al*., 2001).

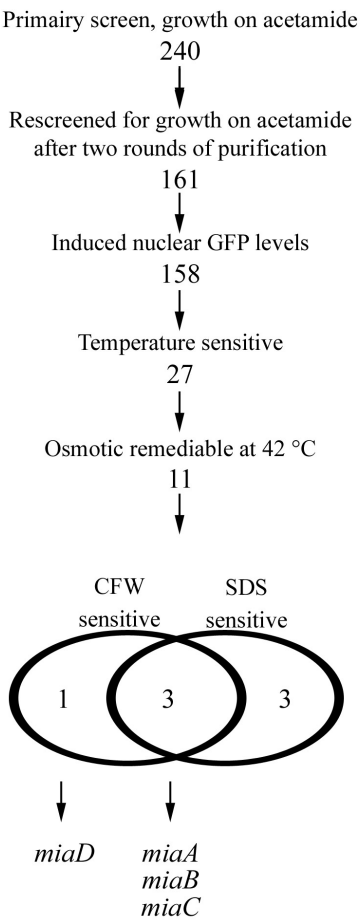

Figure 3. Overview of the results from the cell wall mutant screen. The figure shows the sequence and result of the different secondary screens (see also materials and methods) to which the 240 primary mutants were subjected.

Finally, many cell wall related mutations lead to a temperature-sensitive growth defect which is suppressed by the addition of sorbitol to the medium as an osmotic stabilizer (Paravicini *et al*., 1992, Costigan *et al*., 1992, Borgia and Dodge, 1992, Irie *et al*., 1993, Lee *et al*., 1993). Out of the 158 mutants, 27 mutants displayed a temperature-sensitive growth defect at 42 °C. Growth of 11 of the ts-mutants was improved by the addition of sorbitol to medium, indicating an osmotic remediable temperature-sensitive phenotype. Although we have determined both CFW- and SDS- sensitivity for all 158 mutants (12 mutants showed a CFW-hypersensitive phenotype and 32 mutants showed an SDS-hypersensitive phenotype with an overlap of six mutants displaying both hypersensitivity to CFW and SDS), we have further focussed on the

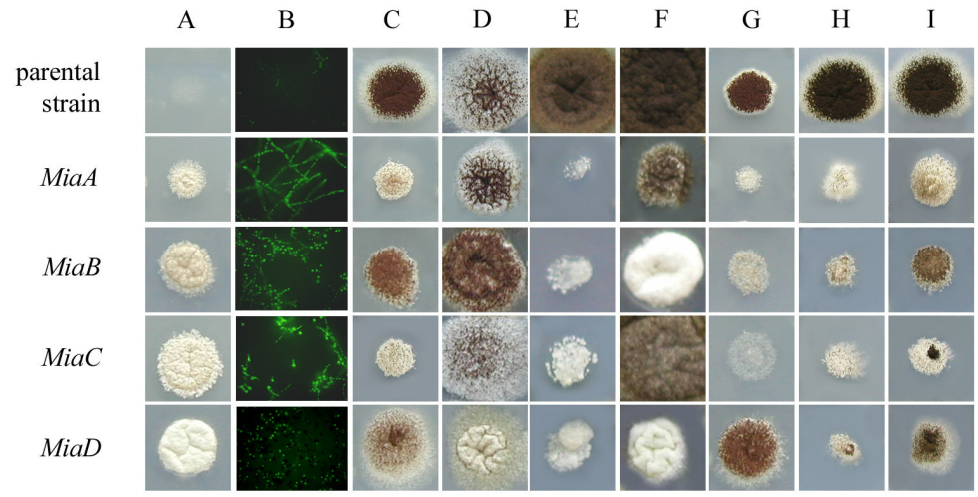

Figure 4. Phenotypical analysis of four selected *mia* mutants. Equal amounts of spores were spotted on different types of media under different conditions to determine the phenotype of the mutants. (A) Agar plate containing MM with acetamide as sole N-source, (B) Liquid MM, (C) MM, (D) MM with sorbitol, (E) MM (42 °C), (F) MM with sorbitol (42 °C), (G) MM with 0.005 % w v^{-1} SDS, (H) MM with 0.1 mg ml^{-1} CFW, (I) MM with 0.01 mg ml^{-1} CFW. All growth experiments shown are performed at 30 °C and grown for three days unless indicated differently.

ts-osmotic remediable mutants. Four of these mutants displayed a higher sensitivity towards CFW and six of those mutants were more sensitive towards SDS. The CFW hypersensitive phenotype was coupled to a higher sensitivity towards SDS in three of the mutants. We selected the four ts-osmotic remediable CFW-hypersensitive mutants for further complementation analysis. These mutants were named *miaA-D* (mutant with induced *agsA* promoter activity). As shown in figure 4, all four mutants grow well on acetamide plates (Fig. 4A), show induced nuclear GFP levels (Fig. 4B), and are temperature sensitive (compare Fig. 4C and E) which can be (partially) remediated by the addition of an osmostabiliser (compare Fig. 4E and F). Also all four mutants have an increased CFW sensitivity (compare Fig. 4C and H/I). *MiaA, miaB* and *miaC s*howed an increased sensitivity towards SDS (compare Fig. 4C and G).

Complementation of *miaA-D*

The available genomic cosmid library contained the *pyrG* gene as a selection marker. It was therefore necessary to obtain *pyrG*$^{-}$ derivatives of the *miaA-D* mutants. Attempts to directly complement the temperature sensitive phenotype of the mutants were not successful. Neither was it possible to determine the amount of transformants that were obtained when a

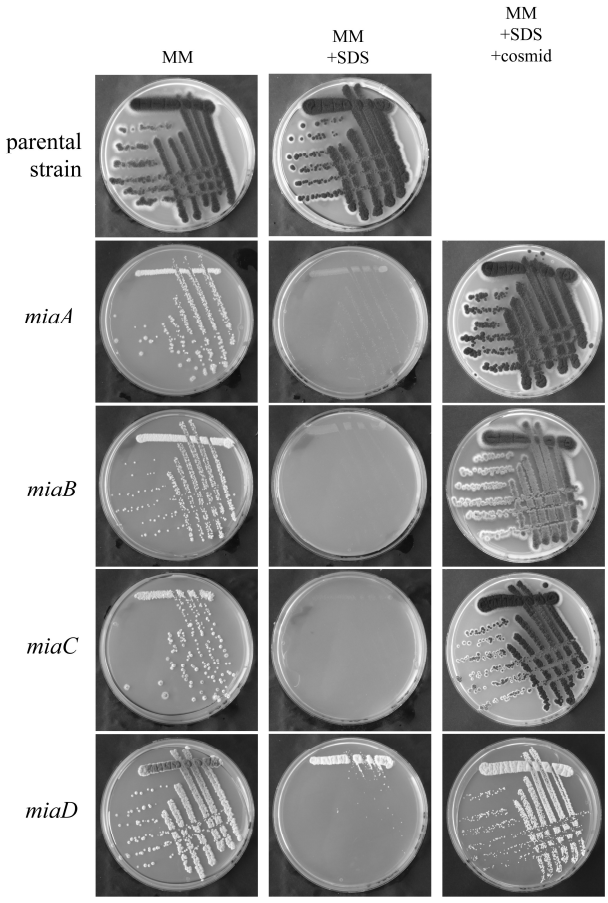

Figure 5. Complementation of the *miaA-D* mutant with the isolated cosmids. The *miaA-D* mutants were re-transformed with the cosmids isolated. In total nine cosmids were transformed back to the *mia* mutants. The result of one complementing cosmid for each *mia* mutant is shown. The two cosmids for *miaB* and three cosmids *miaC* gave comparable results (data not shown). Transformants originating by transformation with a single cosmid were pooled and grown at 42° C for 3 days on MM-plates containing 0.005 % (w v^{-1}) SDS which were supplemented with uridine. The SDS- and temperature-sensitivity was used to screen for complementing cosmids.

functional *pyrG* gene was present in the mutant. *PyrG*$^{-}$ derivatives were made of mutants selected for complementation by growing the mutant strains on MM plates containing 5-FOA and uridine (see materials and methods). Putative auxotrophic, uridine requiring mutants that grew on the 5-FOA plates were purified. 5-FOA resistance can be caused by mutation in either the *pyrG* or the *pyrE* gene and to discriminate between these two possibilities, 5-FOA resistant strains were transformed with either the *A. niger pyrG* or the *A. niger pyrE* gene.

Strains that could only be complemented with *pyrG* (pAB4.1) were selected. The phenotypes of the *miaA-D pyrG*$^-$ derivatives were also analysed and no differences were observed when compared to their parental strains (data not shown). The *miaA-D pyrG*$^-$ strains were transformed with the genomic cosmid library, yielding between 400 and 7500 transformants per strain. Spores originating from one transformation plate (~ 200 individual transformants) were pooled and subsequently analysed for complementation of both the temperature sensitive phenotype and the SDS sensitive phenotype. Cosmids of putative complemented transformants were isolated by transformation to *E. coli* by electroporation. After amplification in *E. coli*, the cosmids were subjected to restriction analysis (data not shown). Of each independently obtained complemented transformant, the restriction pattern of at least four cosmids was obtained. Non-identical cosmids were transformed back to their corresponding *mia* (*pyrG*$^-$) strain and analysed for their ability to complement the mutation, based on restoring the wild-type temperature-sensitivity and SDS-sensitivity (Fig. 5 and data not shown). Based on restriction pattern and the complementation test, a single complementing cosmid was found for *miaA*. Three different, but overlapping

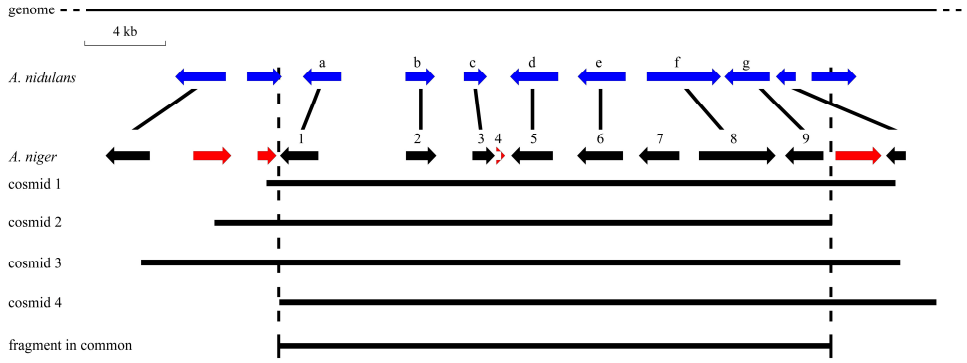

Figure 6. Schematic map of the genomic cosmid inserts that were able to complement *miaA* (cosmid 1) or *miaB* (cosmid 2-4). The region that is present in all four cosmids is indicated between the two vertically dashed lines. The nine ORFs present on this shared region, are numbered (1-9) in the *A. niger* map. Analysis of this region in *A. nidulans* revealed 7 homologous ORFs (a-g). Homology between an *A. niger* and an *A. nidulans* gene is indicated with a black line connecting the genes. The annotation of the genes is shown in table 1.

cosmids were obtained that complemented the *miaB* mutant. The restriction pattern of *miaA* and *miaB* were similar indicating that they might be overlapping. Four complementing cosmids were obtained for *miaC*. Unfortunately these *miaC* cosmids were resistant to restriction enzyme digestions and end-sequencing (see below) which hampered the further analysis. Finally, for the *miaD* mutant, a single complementing cosmid was obtained. The

possible overlap found by restriction analyses of *miaA* and *miaB* complementing cosmids was confirmed by sequencing of the ends of the insert of the cosmids (Fig. 6). Comparison of the cosmids to the *A. niger* genome sequence and to each other showed that the *miaA* complementing cosmid and the three *miaB* complementing cosmids shared a 35 kb region containing at least 9 predicted ORFs (Fig. 6). No obvious gene was present that encodes a protein which is directly involved in cell wall biosynthesis. Since the cosmids complementing the *miaC* mutant were recalcitrant to further analysis these cosmids are not yet studied further. The cosmid complementing the *miaD* mutant was also end-sequenced. Comparison of the ends with the *A. niger* genome sequence indicated that the ends were 1.6 Mbp separated from each other, which did not match with the estimated insert size of ~ 40 kb deduced from the digestion pattern. The latter results indicate that either in N402, of during the construction of the library a rearrangement had occurred which hampered the proper analysis. In the future, we will focus on the identification of the gene(s) complementing the *miaA* and *miaB* mutants.

Discussion

To identify proteins involved in fungal cell wall assembly, we have designed a novel screening method for the identification of cell wall mutants. The screen is based on the observation that the *A. niger agsA* gene, which encodes an α-1,3-glucan synthase, is strongly induced in response to different forms of cell wall stress (Damveld *et al*., 2005, Chapter 3). We reasoned that if the *agsA* is transcriptionally activated in response to cell wall disturbing compounds, the *agsA* gene might also be constitutively induced in mutants in which cell wall synthesis was disturbed. By fusion of the *agsA* promoter region to a selectable marker, in this case the *A. nidulans amdS* gene, we set up a positive screening method for mutants in which the *agsA* promoter is constitutively expressed thereby allowing growth on acetamide plates. A second reporter protein, consisting of a fusion of the *agsA* promoter to the H2B-GFP fusion protein, was also included in our reporter strain to identify trans-acting mutations. For the mutagenesis, a low doses UV-irradiation was used (66 % survival), to minimize the possibility of multiple mutations. The imperfect nature of *A. niger* (e.g. no sexual cycle) hampers proper genetic analysis to show that the ability to grow on acetamide was caused by a single gene mutation. In our screen, the approximately 7000 viable, mutagenised spores per plate yielded around one to five colonies that were able to grow on acetamide. We were not surprised by the easiness by which these mutants were acquired. Cell wall biosynthesis is an extremely well controlled process, which requires integration of several cellular processes. Interference with of one of these processes might already lead, directly or indirectly, to cell wall

biosynthetic defects. Even small alterations might be sensed by the cell and might result in the activation of P*agsA*.

In our mutant collection, we expect to find mutants in which genes are mutated that encode cell wall biosynthetic enzymes or genes directly involved in cell wall biosynthesis. We also expect to find other genes whose products encode proteins that are clearly more indirectly linked to cell surface assembly. This class of mutants might include genes related to function in the secretory pathway, cytoskeleton organization and proteins with a regulatory function in fungal morphogenesis. Studies in the yeast *S. cerevisiae* have already indicated that also mutations in other cellular processes can result in cell wall related phenotypes (Lussier *et al*., 1997, de Groot *et al*., 2001) and often the direct link of the mutated gene to cell wall biosynthesis is not clear. Examples of the latter category includes genes involved in primary metabolism, related to mitochondrial function, involved in DNA synthesis and repair and RNA polymerase activity (Lussier *et al*., 1997, de Groot *et al*., 2001). We have analysed our primary collection of 161 mutants by several secondary screens to classify and group the mutants according to their phenotypes. The screens we have used consist of simple sensitivity assays that are indicative of cell wall defects. Although a significant number of mutants had cell wall related phenotypes (45/158) in combination with the increased *agsA* expression level, the majority of the mutants (113/158) did not display additional phenotypes. However, our list of secondary screens was limited and additional secondary screens are currently considered. Just recently, we obtained a specific antiserum against an *A. niger* GPI-anchored cell wall protein (CwpA) (Damveld *et al*., submitted, Chapter 5). With this antibody we are able to screen through our *mia*-mutants for mutants in which the incorporation of CwpA is effected. By using this screen we are able to identify mutants that are specifically disturbed in the incorporation mechanism of cell wall proteins. This class of proteins might be important as a potential target for the development of new antifungal drugs.

Complementation of the *mia* mutants is a time consuming and sometimes difficult task. In principle, it is possible to select for complementing cosmids by growing transformants on fluoroacetamide containing plates. Transformants in which the cell wall disturbing mutation is complemented by the cosmid, should no longer induce *amdS* expression and thus give raise to growth in the presence of fluoroacetamide. However, we have not yet used this procedure to clone any of our mutants. In this study we have selected temperature and CFW and/or SDS- sensitive mutants and selected complementing transformants based on complementation of these phenotypes. Although successful, it will take a huge effort to identify the genes affected in the mutants. With the rapid progress of the sequencing of fungal genomes, including the genome of *A. niger*, we plan to repeat the isolation of mutants, but perform the mutagenesis by using the *Agrobacterium* T-DNA gene tagging method to

generate mutants (Michielse *et al*, submitted for publication). Identification of the mutated genes will be relatively easy and straightforward using plasmid rescue techniques. This procedure circumvents the need for genetic complementation and enables large numbers of new *A. niger* genes involved in cell wall biosynthesis to be identified.

Complementation analysis of the *miaA* and *miaB* mutants indicated that the two mutants are complemented by cosmids which contain overlapping regions. Although not demonstrated, it is most likely that both mutants contain mutations in the same gene giving raise to similar phenotypes. Currently, subcloning experiments are carried out to identify the complementing gene from this 35 kb region. From DSM we obtained the sequence map of this 35 kb region. The region contains at least 9 putative open reading frames. Interestingly, the genome sequence of *A. nidulans* revealed a highly conserved region which contains seven homologs of the *A. niger* genes (Fig. 6). Since we do not have the complete *A. niger* DNA sequence for this region, we can not further compare the *A. niger* and *A. nidulans* genome organization. The annotation of the *A. niger* and *A. nidulans* ORFs indicate the presence of among others, sugar transporter-like proteins and a RNA polymerase III general transcription factor (Table 1). Transposon insertions in similar genes have been shown to result in a CFW hypersensitive phenotype and are likely to have an effect on cell wall biosynthesis (Lussier *et al*., 1997).

Large-scale functional studies on sequenced genomes in filamentous fungi are in their infancy. On one hand, systematic gene disruption approaches are extremely powerful tools to allow functional genomics. In *S. cerevisiae* such an approach is feasible since gene disruption strategies require short homologous flanks to allow homologous recombination and disruption of a certain locus. In filamentous fungi, simple and efficient PCR-based disruption protocols are not (yet) available, which limits at the moment the possibilities to start large scale disruption studies. Alternatively, the use of simple screening methods, as described in this study, together with the *Agrobacterium* mediated tagging mutagenesis, allows rapid identification of mutant genes by retrieving part of the genomic sequence adjacent to the tag. Together with the genome information this can lead to a rapid identification of fungal genes required for cell wall biosynthesis. These genes are potentially interesting as antifungal targets for the development of new antifungal drugs.

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Chapter 7

A novel GFP-based reporter method for the identification of cell wall integrity disturbing antifungal compounds

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Abstract

Activation of the fungal cell wall integrity pathway is a mechanism used by fungi to escape from cell wall threatening conditions. In *A. niger*, we have previously shown that the *agsA* gene, encoding an α-1,3-glucan synthase, is transcriptionally activated in response to cell wall stress, and that this response is mediated via a RlmA dependent signal transduction pathway. We have used the *agsA* promoter region to set up a GFP-based reporter system to identify compounds that activate the *agsA* expression by fusing the P*agsA* to the cytosolic GFP or to a nuclear targeted version of GFP. The reporter strains confirmed earlier observations that the *agsA* gene is activated in response to the presence of sub-lethal concentrations of various cell wall disturbing compounds such as CFW, caspofungin, and tunicamycin. Other forms of stress (e.g. oxidative stress or high osmolarity stress) did not activate *agsA* gene expression, indicating that the induction of *agsA* is cell wall stress specific and not a general stress response. We have used the cell wall stress reporter system to analyse the effect of 33 putative antifungal compounds in relation to the cell wall remodeling.

Introduction

The cell wall of fungi is an essential component of the fungal cell. By interfering with the synthesis or assembly of the fungal cell, the cell will lyse and die, which makes the cell wall an ideal antifungal target. Furthermore, a useful and effective antifungal compound must be toxic to the fungus, but not to its host. Since the cell wall is unique to the fungal pathogen, it is likely that antifungal compounds with a mode of action against the fungal cell wall will be specific for the fungus. Finally, an antifungal compound that interferes with the synthesis or assembly of the cell wall at the outside of the cell is highly preferable, since the antifungal compound does not need to be transported across the plasma membrane. This transport might be a bottleneck for the antifungal activity. In addition, the fungal cell posseses several mechanisms to remove antifungal from the cell by exporting them via plasma membrane localised transporters which also decrease the efficiency by which an antifungal can act (Zwiers *et al*., 2002 and reviewed by Balkis *et al*., 2002). In conclusion, an antifungal agent that interferes with fungal cell wall biosynthesis and acts at the outside of the cell is highly preferable. Likewise, successful antibacterial therapies often take advantage of interfering with the synthesis of the bacterial cell wall e.g. penicillin.

Antifungals used to treat fungal infections can be grouped into six classes based on their site of action: i) azoles (eg. fluconazole, itraconazole, ketoconazole, ravuconazole, posaconazole and voriconazole) inhibit ergosterol synthesis by bocking cytochrome P450 14-α-demethylation (Kelly *et al*., 1995). This step is important in the sterol pathway that leads from lanosterol to ergosterol, the main fungal sterol, ii) allylamines (eg. terbinafine, butenafine and amorolfine) also inhibit the ergosterol synthesis like the azoles, but have different molecular targets. Terbinafine and butenafine act on the squalene epoxidase (Favre and Ryder, 1996) leading to accumulation of squalene, which is toxic to the cells. Amorolfine interferes with ergosterol biosynthesis at two steps, the sterol-Δ^{14}-reductase and the sterol-Δ^{7}-Δ^{8} isomerase (Polak, 1992), iii) polyenes (eg. amphotericin B and nystatin) which bind to fungal membrane sterol, resulting in the formation of aqueous pores through which essential cytoplasmic materials leak out (Coutinho *et al*., 2004), iv) flucytosine, an antimetabolite, is a DNA synthesis substrate analog (Polak and Scholer, 1975) interfering with correct DNA synthesis, v) griseofulvin acts on the microtubules, thereby disrupting the mitotic spindle (Gull *et al*., 1973), vi) candins (eg. caspofungin, micafungin, anidulafungin and pupulacandin B), which function by inhibiting the synthesis of β-1,3-glucan, the major structural polymer of the cell wall.

Only compounds of the the latter class, the candins, are antifungals that specifically inhibit cell wall biosynthesis. The fungal cell wall contains several classes of macromolecules, including β-1,3-glucan, β-1,6-glucan, chitin, cell wall mannoproteins and in some cases β-1,3

or β-1,3-β-1,4-glucan (Cabib *et al*., 1997, Klis *et al*., 1998, Fontaine *et al*., 2000). Both the presence of these components and the crosslinking of the different components to each other to form a rigid cell wall are essential. Thus, antifungals that interfere with the synthesis of one of these components or antifungals that interfere with the crosslinking of those compounds are interesting as antifungal agents. Although the class of candins consists of interesting and potential valuable antifungal drugs the effects concerning possible resistance mechanisms at the long term are still lacking, due to its recent introduction in clinical trials. However, laboratory experiments using *S. cerevisiae* have shown that mutants resistant to candins can spontaneously arise (Douglas *et al*., 1997). Furthermore, candins display a poor antifungal activity towards some fungi, eg. *C. neoformans*, and its activity towards non-Aspergillus molds have not been established today. Finally, tolerance against candins has been reported through activation of the PKC1 signaling cascade which offers the fungal cell a pathway to become resistant to candins (Reinoso-Martin *et al*., 2003). Therefore it is clear that there is a need for additional antifungals.

A recent antifungal discovery program at Lilly Research Laboratories was aimed at the identification of antifungals that inhibit Pkc1p, an important component of the cell wall integrity pathway by *in vitro* assays. Although the screen has identified cercosporamide as a potent inhibitor the Pkc1p activity, this screening method was very laborious and it has the disadvantage that the *in vitro* results do not always translate directly to *in vivo* applications. In this study we present a novel straightforward screening method for the identification of antifungals that are affecting cell wall biosynthesis. We have previously shown that the expression of a gene encoding an α-1,3-glucan synthase (*agsA*) is strongly induced in response to different forms of cell wall stress (Damveld *et al*., 2005). By fusing the *agsA* promoter (P*agsA*) to the GFP reporter protein (either cytosolic expressed GFP, or nuclear targeted (H2B) GFP), we have constructed two reporter strains in which the effect of an antifungal on the expression of *agsA* can be monitored by determining the GFP fluorescence, thereby monitoring the activation of the cell wall integrity pathway in response to the addition of different antifungal compounds *in vivo*.

The screen has been set up in glass bottom microtiter plates, which allows rapid screening of compounds at various concentrations. We show that the P*agsA* activation is specific for stress that weakens the cell wall. No induction was observed after oxidative, high osmolarity, and high temperature stress, or by treatment of the fungus with antifungals that do not affect the cell wall biosynthesis directly.

We have validated our cell wall stress reporter system by determining the effect on P*agsA* activity of 33 putative antifungals. Known cell wall disturbing compounds such as CFW, caspofungin and tunicamycin resulted in an induced GFP expression indicating

activation of the cell wall integrity pathway. In addition the antifungals, spiroxamine, fenpropiomorph, terbinafine, fludioxonil, and cyprodinil were identified as antifungals that strongly activated P*agsA*, indicating that the mode of action of these antifungals might involve the inhibition of cell wall biosynthesis.

Material and Methods

Strains and Media

The wild-type *Aspergillus niger* strain used in this study is N402 (cspA1 derivative of ATCC9029, Bos *et al*., 1988) and the *pyrG*$^{-}$ derivative AB4.1 (van Hartingsveldt *et al*., 1987). The *Aspergillus* strains were grown in *Aspergillus* minimal medium (MM) (Bennett and Lasure, 1991) 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. Growth medium was supplemented with 10 mM uridine (Serva) when required. For plasmid isolation and propagation *Escherichia coli* XL1-Blue (Stratagene, La Jolla, CA) was used and transformed according to Inoue *et al*., 1990. Transformations using PEG/$CaCl_2$ were performed according to Punt and van den Hondel, 1992. Transformants were selected based on complementation of the uridine auxotrophy. *Agrobacterium* mediated transformations were performed as described by de Groot *et al*., 1998 with minor modifications (Michielse *et al*., 2004), using *Agrobacterium* strain LBA1100 (Beijersbergen *et al*., 1992). *Agrobacterium* derived transformants were selected based on hygromycin resistance.

Construction of reporter strains

The reporter strain containing the PagsA-H2B-GFP-TtrpC reporter construct (RD6.47) has been described previously (Damveld *et al*, in preparation). The reporter strain with the cytoplasmatic expressed GFP from the *agsA* promoter (P*agsA*-GFP-T*trpC*) was made as follows. The plasmid P*agsA*-GFP-T*trpC* was constructed by ligation of a 2-kb SalI-NcoI fragment, containing P*agsA* from P*agsA*-*uidA*-T*trpC* (Damveld *et al*., submitted, Chapter 4), into an SalI-NcoI opened P*agsA*-H2B::GFP-T*trpC* vector, thereby removing the P*agsA*-H2B and replacing it with P*agsA* to give pJD1. For the *Agrobacterium* mediated transformation the P*agsA*-GFP-T*trpC* construct was inserted into a binary vector. The ~ 3-kb HindIII fragment containing P*agsA*-GFP-T*trpC* from pJD1 was cloned into a HindIII opened pTAS5 vector to give pTAS5-P*agsA*-GFP-T*trpC*. The pTAS5 vector consists of the binary vector pSDM14 (Offringa *et al*., 1990) with the hygromycin expression cassette from pAN7.1 (Punt *et al*., 1987) inserted between the borders. pTAS5-P*agsA*-GFP-T*trpC* was transformed to *A. niger* strain N402 using the *Agrobacterium* strain LBA1100. Hygromycin resistant transformants were subjected to Southern analysis to confirm complete integration. Genomic DNA was

isolated according to Kolar *et al*. (1988) and digested with PstI or SstII to determine copy-number. Strain JvD1.1. containing multiple copies (≥ 2) integrated in the genome (data not shown), was selected as reporter strain.

GFP reporter based screening of antifungal compounds in glass bottom microtiter plates

Conidia ($2x10^4$) from reporter strains RD6.47 or JvD1.1 were inoculated in each well of 96-well optical glass bottom microtiter plates (Nunc art# 164588) in 100 µl 2X CM and grown for 6 hours at 37°C. After germination of the spores, 100 µl of a two-fold dilution series for each antifungal compound was added to individual wells. The effect of each compound was tested for at least 7 different concentrations. After adding the antifungal solution, the microtiter plates were incubated for 3 more hours at 37 °C. After discarding the medium by inverting the microtiter plate, germlings that were adherent to the bottom of each well were observed. Both light and fluorescence images were taken on an Axioplan 2 (Zeiss) equipped with a DKC-5000 (Sony) digital photo camera. For GFP images a fixed exposure of two seconds was used. As a positive control, strain MA26.1, containing P*gpdA*-H2B-GFP-T*trpC* single copy at the *pyrG* locus (M. Arentshorst, unpublished strain) was used. Strain N402 was used as a negative control and a dilution series with the compound CFW was included on every microtiter plate. Acquired images were analysed for both growth and GFP levels manually.

Results and Discussion

Construction of the reporter strains

We used the *agsA* promoter to monitor the activation of the cell wall integrity signaling cascade. It was shown previously that the *agsA* promoter is activated upon cell wall stress inducing conditions and that the transcription factor RlmA plays an important role in the activation (Damveld *et al*., submitted, Chapter 4). By fusion of the *agsA* promoter to GFP we were able to monitor the expression level of the cell wall integrity pathway *in vivo*. Two reporter strains were constructed, named RD6.47 (Damveld *et al*., in preparation, Chapter 6) and JvD1.1 (this study). The construction of the strains is described in materials and methods. In brief, the strains carry either the H2B-GFP (RD6.47) or the GFP (JvD1.1) reporter under control of P*agsA*. RD6.47 has single copy integration and JvD1.1 has more than one copy integrated in the genome.

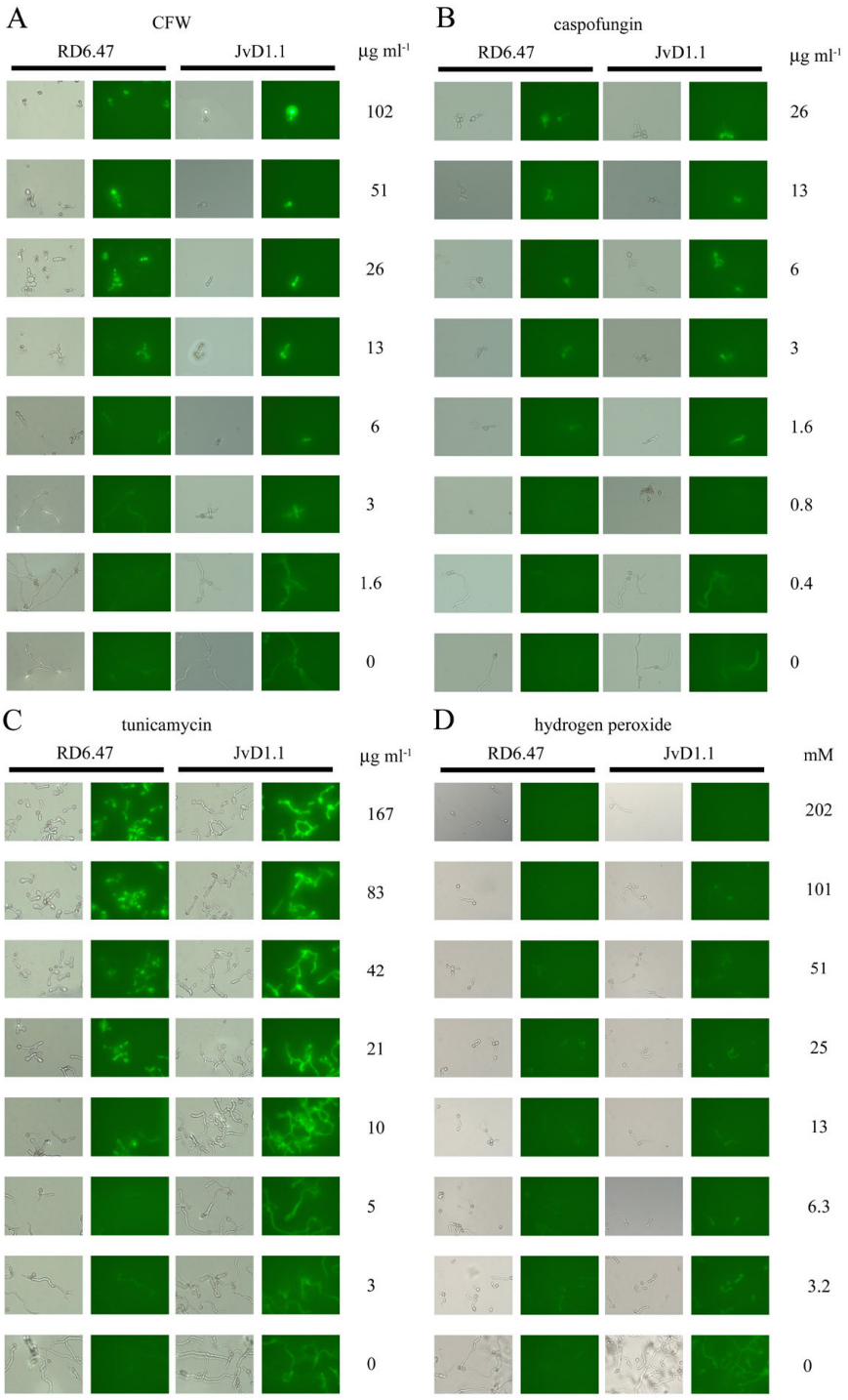
A
CFW
RD6.47
JvD1.1
µg ml-1
102
51
26
13
6
3
1.6
0
B
caspofungin
RD6.47
JvD1.1
µg ml-1
26
13
6
3
1.6
0.8
0.4
0
C
tunicamycin
RD6.47
JvD1.1
µg ml-1
167
83
42
21
10
5
3
0
D
hydrogen peroxide
RD6.47
JvD1.1
mM
202
101
51
25
13
6.3
3.2
0

Figure 1. Microtiter plate based screening of compounds by microscopical analysis of the morphology and GFP levels of the reporter strains RD6.47 and JvD1.1. The strains were pre-grown for 6 hours and subsequently stressed for 3 hours at 30 ℃ as described in materials and methods. Different compounds (CFW, caspofungin, tunicamycin, and hydrogen peroxide) were used, panel A, B, C, and D respectively. The compounds were 2-fold serial diluted. The final concentration of the compounds is indicated to the right of each image.

Response of the reporter to four well known compounds

We used several compounds (Calcofluor White (CFW), hydrogen peroxide, caspofungin, and tunicamycin) of which the targets are known in order to test our reporter system.

The first compound assayed, CFW, is a negatively charged dye that interferes with proper cell wall assembly causing an induction of cell wall biosynthesis related genes (Boorsma *et al*., 2004). CFW has been used to identify mutants with a weakened cell wall (Ram *et al*., 1994, Lussier *et al*., 1997, de Groot *et al*., 2001). We previously showed by Northern analysis that CFW induces the *agsA* expression (Damveld *et al*., 2005, Chapter 3). When the reporter strains were grown in glass bottom microtiter plates (as described in materials and methods) and subsequently stressed with different CFW concentrations (2-fold dilution series 3-205 µg ml^{-1}), the light microscopy images revealed that at concentrations of ≥ 13 µg ml^{-1} the fungal growth was affected and at concentrations of ≥ 51 µg ml^{-1} aberrant hyphal morphology (e.g. swelling at the tip) was observed (Fig. 1A). Fluorescent microscopy of the same samples showed a clear induction of the GFP level for both nuclear (H2B) and cytoplasmic GFP containing reporter strains (Fig. 1A). The second compound, caspofungin, is a potent inhibitor of β-1,3-glucan synthesis, causing a weakened cell wall (Franzot and Casadevall, 1997, Ullmann, 2003). Northern analysis has indicated that also the addition of caspofungin resulted in an increased expression of *agsA* (Damveld *et al*., 2005, Chapter 3). The induction of *agsA* in response to the presence of caspofungin was also observed in the reporter strains, indicating that the reporter constructs confirmed the previous Northern expression data (Fig. 1B). The third compound tested was tunicamycin, an inhibitor of protein N-glycosylation. N-glycosylation mutants in *S. cerevisiae* have been shown to have defects in cell wall integrity (Ram *et al*., 1994, Lagorce *et al*., 2003). Therefore we predicted that the addition of tumicamycin would result in weakening of the cell wall and activation of the cell wall integrity pathway and thus in induced *agsA* expression. Indeed, when the reporter strains were stressed with tunicamycin, swollen hyphae were visible and the GFP reporter showed a high induction (Fig. 1C). The possible effect of tunicamucin on the cell wall intergrity pathway has also been observered in *S. cerevisiae*. Tunicamycin-induced stress caused upregulation of many genes, including genes involved in cell wall biogenesis (Travers *et al*., 2000).

Finally, the effect of the addition of hydrogen peroxide (H_2O_2) was tested. H_2O_2 is an inducer of oxidative stress and does not have a direct effect on the integrity of the cell wall (Gasch *et al*., 2000, Causton *et al*., 2001, Moye-Rowley, 2003, Imlay, 2003). When a series of hydrogen peroxide concentrations 0.63-0.01 % (v v^{-1}) was assayed using glass bottom microtiter plates, no induction of GFP was observed (Fig. 1D) although light microscopy revealed a significant growth inhibition of the fungus by hydrogen peroxide. The analysis of the four compounds (CFW, caspofungin, tunicamycin and hydrogen peroxide) with our GFP-based reporter method showed that the induction of the reporter seems to be correlated with compounds that are thought to have an effect on the integrity on the cell wall (CFW, caspofungin, and tunicamycin) and no induction was found for the oxidative stress inducing compound hydrogen peroxide.

Screening of compounds with the GFP-based reporter method

In the next set of experiments we have further analysed the effect of the addition of antifungal compounds on the expression of the *agsA* gene. The purpose of these experiments was two-fold. Some of the tested compounds have a poorly defined mode of action and by testing them we provide information whether or not the compound affects cell wall biosynthesis. In addition, by testing additional antifungal compounds with a defined mode of action we were eager to show that our reporter system would be specifically induced in response to cell wall related stress.

To analyse the effect of different compounds with known and unknown functions on the cell wall reporter, the strains were grown in the presence of 1 of 33 putative antifungal compounds as listed in Table 1. It should be noted that no effect on growth or on GFP expression was found when the solvents DMSO or ethanol were used (data not shown). We devided the compounds into four groups based on their effect on growth and P*agsA*-(H2B)-GFP expression.

The first group, which consists of: citric acid, nystatin, antimycin, carboxin, myriocin, nikkomycin, and tricyclazole. These compounds did not have an effect on either growth or GFP expression at the concentrations used. *A. niger* has been used to produce citric acid for 80 years and is currently the primary source of commercial citric acid production (Magnuson and Lasure, 2003). It is therefore not surprising that the addition of citric acid to the fungus does not have an effect at the concentrations used. The compound nikkomycin, of the class of cell wall synthesis disturbing compounds, was shown to interfere with cell wall

Table 1. Compounds tested on the reporter strains.

Compound (chemical class)	proposed action	reference	concentration range [a]	GFP expression level [b] / growth inhibition [c]						
Food preservatives										
cinnamic acid	cell membrane perturbation	Burt, 2004	1.5-93.7 mM	1	1	1	0	0	0	0
				0	0	1	2	2	2	2
cinnamaldehyde	inhibition of amino acid decarboxylase	Burt, 2004	1.0-63.1 mM	0	0	0	0	0	0	0
				1	1	1	2	2	2	2
cinnamic alcohol	cell membrane perturbation	Burt, 2004	1.0-63.1 mM	1	1	1	0	0	0	0
				0	0	1	2	2	2	2
sorbic acid	disruption of pH homeostasis	Plumridge *et al*., 2004	1.2-74.3 mM	0	1	1	0	0	0	0
				0	0	1	2	2	2	2
acetic acid	not fully known	Rocken, 1996	9-583 mM	1	0	0	0	0	0	0
				1	2	2	2	2	2	2
citric acid	not fully known	Beuchat, 1988	0.3-20.8 µg ml^{-1}	0	0	0	0	0	0	0
				0	0	0	0	0	0	0
Mebrane pertubants										
SDS	detergent effecting membrane stability	de Groot *et al*., 2001	0.003-0.167 % w v^{-1}	1	1	0	0	0	0	0
				1	1	2	2	2	2	2
chitosan	disturbance of cell membrane integrity	Rabea *et al*., 2003	5-310 µg ml^{-1}	1	1	1	1	1	1	1
				1	1	1	2	2	2	2
nystatin (polyenes)	membrane pertubation	Coutinho *et al*., 2004	1.6-104.2 µg ml^{-1}	0	0	0	0	0	0	0
				0	0	0	0	0	0	0
Respiratory chain inhibitors										
boscalid (carboxamides)	respiratory chain inhibitor	endurafungicide.com	1.6-104.2 µg ml^{-1}	0	0	0	0	0	1	1
				0	1	1	1	2	2	2
antimycin	respiratory chain inhibitor	Izzo *et al*., 1978	1.6-104.2 µg ml^{-1}	0	0	0	0	0	0	0
				0	0	0	0	0	0	0
carboxin (carboxamides)	respiratory chain inhibitor	Ito *et al*., 2004	1.6-104.2 µg ml^{-1}	0	0	0	0	0	0	0
				0	0	0	0	0	0	0
pyraclostrobin (carbamates)	respiratory chain inhibitor targeting the ubiquinol ox.	Fisher *et al*., 2004	1.6-104.2 µg ml^{-1}	0	0	0	0	0	0	0
				1	1	1	1	1	2	2

1,10 phenanthroline	damages mitochondrial function	Coyle *et al*., 2003	1.6-104.2 µg ml^{-1}	0	0	0	0	0	1	1
				0	0	0	1	2	2	2
Disturbing lipid synthesis											
epoxiconazole (triazoles)	effects sterol biosynthesis by inhibition of C14-demethylase	Oestreich *et al*., 1997	1.6-104.2 µg ml^{-1}	0	0	0	1	1	1	1
				0	0	0	0	1	1	1
fenhexamide	effects sterol biosynthesis by inhibition of C3-ketoreductase	Priestel and Grupp, 2003	1.6-104.2 µg ml^{-1}	0	0	0	0	0	0	0
				0	0	0	0	0	1	2
spiroxamine (spiroketalamine)	effects sterol synthesis by inh. of C-14-reductase and C-8 – C-7 isomerase	Fourie and Zahn, 2001	1.6-104.2 µg ml^{-1}	1	2	2	2	2	2	1
				0	1	1	1	2	2	2
fenpropimorph (morpholines)	effects ergosterol synthesis by inh. of C-14 reductase and C-8 isomerase.	Marcireau *et al*., 1990	1.6-104.2 µg ml^{-1}	0	2	2	2	2	2	2
				1	1	1	1	1	2	2
terbinafine (allylamines)	effects ergosterol synthesis by inhibition of squalene epoxidase	Leber *et al*., 2003	1.6-104.2 µg ml^{-1}	2	2	2	2	2	2	2
				1	1	1	1	1	1	1
myriocin	effects sphingolipid synthesis by inh. of serine polmitiol transferase	Miyake *et al*., 1995	1.6-104.2 µg ml^{-1}	0	0	0	0	0	0	0
				0	0	0	0	0	0	0
Tubulin assembly inhibitors											
carbendazim (benzimidazoles)	binds to the colchicine-binding site of tubulin	Kiso *et al*., 2004	1.6-104.2 µg ml^{-1}	0	0	0	0	0	0	0
				2	2	2	2	2	2	2
benomyl (benzimidazoles)	binds to the colchicine-binding site of tubulin	Kiso *et al*., 2004	1.6-104.2 µg ml^{-1}	0	0	0	0	0	0	0
				0	0	1	1	1	2	2
Cell wall synthesis disturbing compounds											
nikkomycin	competitive inhibitor of chitin synthase	Cabib, 1991	1.6-104.2 µg ml^{-1}	0	0	0	0	0	0	0
				0	0	0	0	0	0	0
caspofungin (pneumocandin)	inhibits the synthesis of β-1,3-glucan	Franzot and Casadevall, 1997	0.4-25.6 µg ml^{-1}	0	1	2	2	2	2	2
				0	1	2	2	2	2	2
calcofluor white	inhibits chitin assembly	Roncero *et al*., 1988	1.6-102.4 µg ml^{-1}	0	0	0	0	1	1	2
				0	1	1	2	2	2	2
Inhibitors of cell signaling											
fludioxonil (phenylpyrroles)	(hyper)activation of MAP kinase in osmotic signal transduction	Kojima *et al*., 2004	1.6-104.2 µg ml^{-1}	2	2	2	2	2	2	2
				0	1	1	2	2	2	2
quinoxyfen	proposed target is G-prot in early signaling	Cabras *et al*., 2000	1.6-104.2 µg ml^{-1}	1	1	1	0	0	0	0
				1	2	2	2	2	2	2

Compounds inhibiting melanin synthesis, methionine synthesis, or with a multi site activity											
tricyclazole	Effects melanin synth by inh. of a reductase in this pathway	Romero-Martinez *et al.*, 2003	1.6-104.2 µg ml^{-1}	0	0	0	0	0	0	1
				0	0	0	0	0	0	0
cyprodinil (anilino-pyrimidines)	interferes with methionine biosynth. and secretion of hydrolytic enzymes	Fritz *et al.*, 1997	1.6-104.2 µg ml^{-1}	2	2	2	2	2	2	2
				1	1	1	1	1	1	1
tunicamycin	inhibits intracellular N-glycosylation of proteins	Lehle and Tanner, 1976	2.6-166.7 µg ml^{-1}	0	0	2	2	2	2	2
				1	2	2	2	2	2	2
hydrogen peroxide	redox imbalance, toxic damage to lipids, proteins, and DNA	Imlay, 2003	3.2-202 mM	0	0	0	0	0	0	0
				0	2	2	2	2	2	2
cycloheximide	inhibits protein synthesis	Lin and Hsu, 2000	1.6-104.2 µg ml^{-1}	0	0	0	0	0	0	0
				1	1	1	1	2	2	2
chlorthalonil (antgraquiones)	multi-site activity	Kim *et al.*, 2004	1.6-104.2 µg ml^{-1}	0	0	0	0	0	0	0
				0	0	0	0	0	0	1

[a] The concentration range of the antifungal compounds which were 2-fold serial diluted giving 7 different concentrations. The value of the highest and lowest concentration is shown.

[b] Schematic representation of the average GFP levels in both reporter strains (RD6.47 and JvD1.1) grown in the presence of different concentrations of putative antifungal compounds. The left column represents the lowest antifungal concentration used. The numbers 0, 1, and 2 represent low/basal, intermediate, and high GFP levels respectively.

[c] The effect on growth based on the hyphal length in the microscope images. The numbers 0, 1, and 2 represent no, intermediate, and high growth inhibition.

biosynthesis by inhibition of chitin synthase in *S. cerevisiae* (Cabib, 1991). Li and Rinaldi (1999) showed that *A. niger* together with other *Aspergilli* was not sensitive to nikkomycin concentrations (MIC > 64 µg ml^{-1}). These results are consistent with our finding that nikkomycin had no effect on either growth or GFP expression.

The second group of compounds consisting of: cinnamaldehyde, pyraclostrobin, fenhexamide, carbendazim, benomyl, hydrogen peroxide, cycloheximide, and chlorthalonil showed an inhibition of growth at one ore more concentrations used but showed no induction of the GFP expression. None of the compounds of this group was reported to have a direct or indirect effect on cell wall biosynthesis.

The third group of compounds consists of: cinnamic acid, cinnamic alcohol, sorbic acid, acetic acid, SDS, chitosan, boscalid, 1,10 phenanthroline, epoxiconazole, and quinoxyfen. These compounds inhibited growth and gave a moderate induction of GFP expression. Most of these compounds have been reported to have an increased effect when

the integrity of the cell wall was impaired, suggesting an indirect effect on cell wall biosynthesis (Burt *et al*., 2004, Shimizu *et al*., 1994, Igual *et al*., 1996, Bickle *et al*., 1998, de Groot *et al*., 2001, Rabea *et al*., 2003).

The fourth and final group of compounds behaved similarly as the known cell wall weakening compounds (CFW, caspofungin and tunicamycin). This group includes spiroxamine, fenpropimorph, terbinafine, fludioxonil, and cyprodinil. These compounds inhibited growth and gave a high expression of the reporter construct. The induction of the GFP reporter by the lipid synthesis disturbing compounds spiroxamine, fenpropimorph, and terbinafine, can be explained as an indirect effect on cell wall biosynthesis. Disturbance of the plasma membrane might affect the activity of cell wall biosynthetic enzymes such as chitin, β-1,3-glucan and α-1,3-glucan synthases, thereby affecting the integrity of the cell wall. It was shown in *S. cerevisiae* that the membrane located sensor protein Mid2p (Ketela *et al*., 1999, Green *et al*., 2003) and proteins of the WSC family (Verna *et al*., 1997, Zu *et al*., 2001, Gualtieri *et al*., 2004) are key sensors for cell wall integrity and involved in activation of the cell wall integrity pathway. Through these sensory proteins, the defect in the membrane might trigger the activation of the cell wall integrity pathway.

Kojima *et al*. (2004) reported that the fludioxonil hyperactivates the MAP protein kinase in the osmotic signal transduction (Hog1p). Cross-talk between the cell wall integrity pathway and the osmotic signal transduction pathway, as shown by Alonso-Monge *et al*., 2001, Boorsma *et al*., 2004 or the direct hyperactivation of the MAP protein kinase of the cell wall integrity pathway by the compound could explain the observed induction.

Cyprodinil, which has been described as a compound that interferes with methionine synthesis and the secretion of hydrolytic enzymes (Fritz *et al*., 1997), led to the induction of the GFP reporter. This effect on protein secretion might explain the effect of cyprodinil on the integrity of the cell wall. Cell wall biosynthetic enzymes (chitin synthases, glucan synthases), cell wall crosslinking enzymes (transglucosylases) and cell wall mannoproteins are transported to the cell surface via the secretory pathway. Interfering with an efficient protein secretion might lead to cell wall biosynthetic defect and a subsequent cell wall weakening and activation of the cell wall salvage pathway.

Our results show that the PagsA-(H2B)-GFP reporter strains allow us to monitor the output of the cell wall integrity pathway *in vivo*. This reporter system can be used as a powerful tool to determine the mode of action of unknown antifungal compounds. The PagsA-(H2B)-GFP reporter system is rather specifically activated in response to cell wall related stresses, and not by other forms of stress such as oxidative and high osmolarity stress or in general by growth inhibition. The *agsA* promoter is activated by compounds known to interfere with cell wall directly and, in addition, by compounds that are likely to interfere with

plasmamembrane function (sterol biosynthesis) or the protein secretion machinery, thereby having a more indirect effect on cell wall biosynthesis. Thus the reporter system can give valuable information about the mode of action of a certain antifungal compound.

Another application of the system could be to identify new antifungal compounds in natural extracts. The sensitivity of the reporter system is in most cases, more sensitive in comparison to the growth inhibition determination. Especially for fludioxonil and spiroxamine, induction of the GFP reporter was detected before any growth inhibition was observed, indicating that the GFP-based reporter system has additional value in comparison to growth inhibition assays.

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Summary

Summary

Fungi are a very successful species and are distributed worldwide. However, the presence of fungi is not always desired. Filamentous fungi can grow on living or dead organic material and even inside the host. Current methods to prevent fungal growth are insufficient, causing fatality after fungal infections or loss of crops.

The cell wall of a fungus is an intriguing component. It protects the cell from the harsh environment and determines the shape of the cell. Hence the cell wall is an essential component to the cell and provides an attractive target for antifungals. Additionally, the cell wall contains components only found in fungi, and the target is a desirable target as it is exposed on the outside of the cell.

Currently, little is known about the cell wall of filamentous fungi. In order to design new or improved antifungal compounds, a better understanding of the fungal cell wall and of its adaptation to various conditions is required.

In this thesis, we have used *Aspergillus niger* as a model filamentous fungus to study the biosynthesis of the fungal cell wall. The cell wall is a highly dynamic structure and able to adapt to various changes, either developmental (e.g. mating, growth, budding, branching and sporulation), environmental (e.g. heat, pH, osmolarity, chemical compounds), or genetic (e.g. mutations in cell-wall related genes). In chapter 1 an overview is presented of the current state of knowledge about the fungal cell wall. The architecture, biosynthesis and the remodeling are discussed in this chapter.

The response of our model fungus *A. niger* to chemical induced cell wall stress is described in chapter 2. The fluorescent brightener Calcofluor White (CFW) was used to induce cell wall stress. We show that *A. niger*, like *Saccharomyces cerevisiae*, responds to cell wall stress by an increase of chitin deposition in the cell walls. This increase in chitin, a structural cell wall polymer, was accompanied by an increased transcription level of *gfaA*. It was also shown that this mechanism is not only limited to *A. niger* but is also observed in other filamentous fungi like the plant pathogenic fungus *Fusarium oxysporum* and the food spoilage fungus *Penicillium chrysogenum*. It is further shown that *gfaA* is an essential gene and the deletion strain can be rescued by addition of glucosamine.

In chapter 3, a family of five 1,3-α-D-glucan synthase encoding genes is described. The expression of these genes during various types of cell wall stress was monitored and it was found that the expression of *agsA* and *agsE* was induced. The induction of an 1,3-α-D-glucan synthase encoding gene after cell wall stress was also observed in *P. chrysogenum*. The deletion of *agsA* led to an increased sensitivity towards CFW. While in chapter 2 and 3 changes in expression levels of genes encoding proteins involved in cell wall biosynthesis are

described, the mechanism behind the induction of cell wall stress responsive genes is described in chapter 4.

A promoter deletion study combined with an *in silico* analysis indicated that the induction of *agsA* in response to cell wall stress is dependent on a putative Rlm1p binding site in its promoter. Therefore a gene, named *rlmA* encoding for a MADS-box transcription factor was isolated from *A. niger* after database searches. The role of this gene in the induction of *agsA* and *gfaA* after CFW stress was investigated. A deletion of the *rlmA* gene was constructed and this resulted in an increased sensitivity towards cell wall disturbing compounds.

In *S. cerevisiae* an important part of the response towards cell wall threatening conditions is the up-regulation of GPI-anchored cell wall proteins. In chapter 5 the isolation and characterisation of an HF-extractable cell wall protein from *A. niger*, named CwpA, is described. It was shown by simple fractionation experiments that the protein was mainly present in the cell wall fraction. Deletion of *cwpA* resulted in an increased sensitivity towards CFW suggesting a structural role for CwpA.

Chapter 6 describes a novel method for the identification of cell wall mutants. The mutants are first selected based on their compensatory reaction (induction of *agsA*) and subsequenly subjected to various secondary screens, to confirm an altered cell wall integrity. Four out of 240 mutants with induced *agsA* expression levels, named *miaA-D*, were selected for complementation. All four mutants were complemented by cosmids. Further subcloning experiments are underway to identify the mutated genes.

In chapter 7 a GFP-based reporter system is described. The system allows the rapid screening of compounds to see if they trigger the cell wall integrity pathway and thereby induce the P*agsA*(-H2B)-GFP reporter. The method has been evaluated towards various putative antifungal compounds and is a promising tool for the identification of new cell wall related antifungal compounds. In conclusion, this thesis provides evidence for the existence of a cell wall remodeling mechanism in filamentous fungi and in particular *A. niger*. Also, signal transduction pathway components were identified by which cell wall weakening is sensed and transduced into a transcriptional response. Additionally, a cell wall stress reporter system was developed to identify new cell wall related antifungal targets and to identify cell wall related antifungal compounds.

Samenvatting

Samenvatting

Schimmels zijn erg succesvol; deze organismen worden over de gehele wereld aangetroffen. Echter hun aanwezigheid is niet altijd wenselijk. Filamenteuze schimmels kunnen op dood of levend materiaal groeien en zelfs binnenin een organisme. De huidige methoden om de groei van schimmels te voorkomen zijn onvoldoende, met als gevolg sterfte van het geinfecteerde organisme.

De celwand van een schimmel is een intrigerend object. Hij zorgt voor bescherming tegen de vaak schadelijke omgeving en bepaalt de vorm van de cel. De celwand is onmisbaar voor de cel en is daardoor een interessant aangrijpingspunt om de groei van schimmels te voorkomen. Bovendien bestaat de celwand uit componenten die alleen voorkomen in schimmels en deze zijn vaak goed toegankelijk omdat ze aan de buitenkant van de cel zitten.

Momenteel is er niet veel bekend over de celwand van filamenteuze schimmels. Meer kennis is echter wenselijk om de celwand beter te kunnen begrijpen, alsmede de aanpassingen van de celwand ten gevolge van celwandstress. Door deze kennis wordt het mogelijk nieuwe of vebeterde schimmelwerende stoffen te ontwikkelen.

In dit proefschrift wordt *Aspergillus niger* gebruikt als model organisme voor het bestuderen van de celwand van een filamenteuze schimmel. De celwand is een zeer dynamisch onderdeel van de cel. Deze is in staat zich aan te passen aan een verscheidenheid van situaties, zoals onwikkeling (b.v. paring, groei, splijting, vertakking en sporulatie), veranderingen in de omgeving (b.v. hitte, pH, osmolariteit en chemische verbindingen) en mutaties (b.v. mutaties in celwand gerelateerde genen). In hoofdstuk 1 wordt uiteengezet wat de huidige staat van kennis is op het gebied van de celwand. De architectuur, biosynthese en de aanpassingen na stress worden besproken.

De reactie van ons model organisme *A. niger* ten gevolge van chemisch geïnduceerde celwandstress wordt beschreven in hoofdstuk 2. Inductie van de celwandstress gebeurde door middel van het fluorescerende bleekmiddel Calcofuor White (CFW). Er wordt aangetoond dat *A. niger*, net als *Saccharomyces cerevisiae*, op celwandstress reageert door middel van het vermeerderen van de hoeveelheid chitine in de wand. Naast deze toename van chitine, een stucturele celwandpolymeer, werd ook een toename in transcriptie van *gfaA* waargenomen. Tevens werd aangetoond dat deze reactie niet alleen voorkomt in *A. niger* maar ook in andere filamenteuze schimmels zoals de plant pathogeen *Fusarium oxysporum* en de voedselbederf veroorzakende schimmel *Penicilium chrysogenum*. Verder werd aangetoond dat *gfaA* een essentieel gen is en dat een stam waarin dit gen gedeleteerd is gered kan worden door de toevoeging van glucosamine.

In hoofdstuk 3 wordt een familie van vijf genen beschreven; deze genen coderen voor 1,3-α-D-glucaan synthases. Het expressiepatroon van deze genen werd gedurende verschillende soorten van celwandstress bestudeerd. De expressie van *agsA* en *agsE* bleek geïnduceerd te worden. Ook in *P. chrysogenum* werd de inductie van een 1,3-α-D-glucaan synthase na celwandstress waargenomen. De deletie van *agsA* had een verhoogde gevoeligheid voor CFW tot gevolg. In hoofdstuk 2 en 3 worden veranderingen beschreven in expressieniveaus van genen die coderen voor eiwitten die betrokken zijn bij de celwand biosynthese; hoofdstuk 4 beschrijft echter het mechanisme achter deze door celwand stress geinduceerde genen.

Een promoter deletie studie samen met een *in silico* analyse toonden aan dat de inductie van *agsA* ten gevolge van celwandstress afhankelijk is van een mogelijke Rlm1p bindingsplaats in de promoter. Het gen, genaamd *rlmA* coderend voor een MADS-box transcriptiefactor, werd daarom na identificatie in een database geisoleerd. De rol van dit gen bij de inductie van *agsA* en *gfaA* na celwandstress werd onderzocht. Van het *rlmA* gen werd een deletie gemaakt; dit resulteerde in een stam die een verhoogde gevoeligheid vertoonde voor stoffen die aangrijpen op de celwand.

In *S. cerevisiae* is het induceren van GPI-verankerde celwandeiwitten een belangrijk onderdeel van de reactie op situaties die de integriteit van de celwand in gevaar brengen. In hoofdstuk 5 wordt de isolatie en karakterisering van een HF-extraheerbaar celwand eiwit uit *A. niger*, CwpA genaamd beschreven. Door middel van eenvoudige fractionatie experimenten wordt aangetoond dat het eiwit voornamelijk in de celwand fractie voorkomt. De deletie van het gen coderend voor CwpA leidde tot een verhoogde gevoeligheid voor CFW, hetgeen een stucturele rol voor CwpA suggereert.

Hoofdstuk 6 beschrijft een nieuwe methode voor de identificatie van celwand mutanten. Allereerst werden de mutanten geselecteerd op de aanwezigheid van een compensatiereactie (de inductie van *agsA*). Vervolgens werden de mutanten onderworpen aan een aantal secundaire screeningsmethoden om een veranderde integriteit van de celwand aan te tonen. Vier van de 240 mutanten met verhoogde agsA expressieniveau's, *miaA-D* genaamd, werden geselecteerd voor complementatie. Alle vier de mutanten werden gecomplementeerd door cosmiden. Subclonering van deze cosmiden wordt momenteel uitgevoerd om de gemuteerde genen te identificeren.

In hoofdstuk 7 wordt een op GFP gebaseerd reportersysteem beschreven. Deze methode maakt het mogenlijk om snel te testen of stoffen in staat zijn de route die de celwandintegriteit waarborgt te activeren en daardoor de P*agsA*(-H2B)-GFP reporter activeren. Verschillende stoffen zijn gebruikt om deze methode te evalueren en het blijkt een veelbelovende methode voor de identificatie van nieuwe celwandgerelateerde

schimmelwerende stoffen. Concluderend levert dit proefschrift bewijs voor het bestaan van een aanpassingsmechanisme van de celwand in filamenteuze schimmels en in het bijzonder *A. niger*. Tevens zijn er componenten geïdentificeerd van de signaleringsroute die een verzwakking van de celwand waarneemt en deze omzet in een transcriptionele reactie. Ook is er een methode ontwikkeld die het mogelijk maakt nieuwe celwandgerelateerde aangrijpingspunten en celwandgerelateerde schimmel werende stoffen te identificeren.

Curiculum vitae

Robbert Damveld was born 29 April 1978 in Vlaardingen (Zuid-Holland, The Netherlands). In 1996, he graduated successfully from High school (VWO, Nieuw-Vennep, The Netherlands). He subscribed to study Biology at the Leiden University (Faculty of Mathematics and Natural Sciences, Leiden, The Netherlands). He fulfilled internships with Prof. Dr. C.A.M.J.J. van den Hondel in the fungal genetics laboratory at Leiden University and with Dr. J.C. Kapteyn in the fungal research group at the University of Amsterdam (SILS, Amsterdam, The Netherlands). After his graduation he started a PhD research with Prof. Dr. C.A.M.J.J. van den Hondel and Dr. A.F.J. Ram at Leiden University. This thesis describes the results obtained during this PhD-project. This work was supported by STW (Technology Foundation).

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