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Survival in the Presence of Antifungals

GENOME-WIDE EXPRESSION PROFILING OF ASPERGILLUS NIGER IN RESPONSE TO SUBLETHAL CONCENTRATIONS OF CASPOFUNGIN AND FENPROPIMORPH[*S]

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How yeast cells respond to cell wall stress is relatively well understood; however, how filamentous fungi cope with cell wall damage is largely unexplored. Here we report the first transcriptome analysis of *Aspergillus niger* exposed to the antifungal compounds caspofungin, an inhibitor of β-1,3-glucan synthesis, and fenpropimorph, which inhibits ergosterol synthesis. The presence of sublethal drug concentrations allowed *A. niger* to adapt to the stress conditions and to continue growth by the establishment of new polarity axes and formation of new germ tubes. By comparing the expression profile between caspofungin-exposed and nonexposed *A. niger* germlings, we identified a total of 172 responsive genes out of 14,509 open reading frames present on the Affymetrix microarray chips. Among 165 up-regulated genes, mainly genes predicted to function in (i) cell wall assembly and remodeling, (ii) cytoskeletal organization, (iii) signaling, and (iv) oxidative stress response were affected. Fenpropimorph modulated expression of 43 genes, of which 41 showed enhanced expression. Here, genes predicted to function in (i) membrane reconstruction, (ii) lipid signaling, (iii) cell wall remodeling, and (iv) oxidative stress response were identified. Northern analyses of selected genes were used to confirm the microarray analyses. The results further show that expression of the *agsA* gene encoding an α-1,3-glucan synthase is up-regulated by both compounds. Using two P*agsA*-GFP reporter strains of *A. niger* and subjecting them to 16 different antifungal compounds, including caspofungin and fenpropimorph, we could show that *agsA* is specifically activated by compounds interfering directly or indirectly with cell wall biosynthesis.

The fungal cell wall is a dynamic structure that is essential for sustaining cell morphology and for protection against life-threatening environmental conditions. Morphological characteristics during developmental processes in fungi depend upon the temporal regulation and spatial localization of cell wall components and thereby ordered cell wall deposition (1–3). Moreover, cell wall rearrangements that guarantee the structural integrity of the cell wall are of vital importance to withstand environmental stress conditions such as osmotic stress or the presence of antifungal substances compromising cell wall and/or cell membrane integrity (4–6). To prevent cell lysis and to ensure cell survival, fungi have developed mechanisms to sense cell surface stress and to respond to these stresses via a remodeling of the cell wall (see also reviews in Refs. 1, 7, 8).

The composition of fungal cell walls and the mechanisms involved in ensuring cell surface integrity have been studied most intensively in the model yeast *Saccharomyces cerevisiae*. The cell wall of *S. cerevisiae* consists of a moderately branched, flexible β-1,3-glucan network to which to its external face β-1,6-glucan chains are bound which in turn are linked to GPI mannoproteins. At the inner side of the β-1,3-glucan network, chitin chains are attached (reviewed in Ref. 9). Upon cell wall stress, the cell wall becomes reinforced by a massive increase of the chitin content in the lateral wall (9–12) and by increased incorporation of certain cell wall proteins in the cell wall (12–14). At least three signaling pathways, the Pkc1p-Slt2p signaling pathway (also named cell wall integrity (CWI)[3] pathway), the general stress response pathway mediated by Msn2p/Msn4p, and the Ca^{2+}/calcineurin pathway have been shown to be involved in the cell wall compensatory response of *S. cerevisiae* (15). Moreover, genome-wide surveys and large scale phenotypic analyses, aiming at an integrated view of pathways involved in cell wall assembly and integrity of *S. cerevisiae*, have further contributed to the understanding of its cell wall biology. As summarized by Lesage and Bussey (1), five levels of regulation contribute to a controlled cell wall assembly and thereby coordinate cell morphogenesis in yeast as follows: (i) the cell wall synthetic machinery, (ii) surface signaling, (iii) cell cycle regulation, (iv) cell polarization, and (v) the secretory machinery coupled with protein recycling through endocytosis.

In contrast to yeast, information about cell wall biology in filamentous fungi and the mechanisms important for maintain-

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Tables S1 and S2.

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[3] The abbreviations used are: CWI, cell wall integrity; GPI, glycosylphosphatidylinositol; GFP, green fluorescent protein; 8-Br-cAMP, 8-bromo-cAMP; MAP, mitogen-activated protein; ROS, reactive oxygen species; BI, Branching Index; PtdIns(4,5)P_2, phosphatidylinositol 4,5-biphosphate; TOR, target of rapamycin.

ing cell surface integrity is sparse. Although there are indications that architectural principles identified in *S. cerevisiae* may also be valid for filamentous fungi (9, 16), remarkable differences do exist both in the composition of the cell wall as well as the relative amounts of the components. Whereas the presence of β-1,6-glucan in *S. cerevisiae* is undisputed, its presence in filamentous fungi is controversially discussed and, if present, is only in minor amounts (17). The cell wall of filamentous fungi also contains polymers that are not present in the *S. cerevisiae* cell wall such as β-1,4-glucans, α-1,3-glucans, and galactomannans (18, 19). Moreover, the distribution of polymers, such as chitin, varies markedly between yeast and filamentous fungi (20). The compensatory reactions in response to cell wall stress in filamentous fungi were first analyzed in *Aspergillus niger*. It has been shown that the cell wall stress response of *A. niger* involves induced expression of *agsA*, encoding a putative α-glucan synthase (21). In addition, the RlmA transcription factor is, similarly to its *S. cerevisiae* homologue Rlm1p, required for the up-regulation of cell wall stress-induced genes (4). Furthermore, the cell wall stress response of *A. niger* is, like in *S. cerevisiae*, also accompanied by increased chitin deposition, suggesting that part of the remodeling mechanism via the CWI pathway is conserved among fungi (22).

Over the past years, evidence for a close correlation between cell wall assembly and cell morphology in filamentous fungi has been accumulating. Several studies have shown the importance of chitin synthesis in determining hyphal morphology. For example, *Aspergillus nidulans* and *Aspergillus oryzae* strains, in which several chitin synthase genes have been disrupted, are hyperbranched (23, 24). An arrest in polarized growth and the induction of (sub)apical branches have been reported for *A. niger* when treated with the antifungal protein AFP, most recently shown to be an inhibitor of chitin synthase activity in *A. niger* (25). Likewise, inhibition of β-glucan synthesis in *Aspergillus fumigatus* and *A. oryzae* by pneumocandins or by a mutation in β-1,3-glucan synthase gene in *Neurospora crassa* causes considerable changes in morphology, such as swollen germ tubes and highly branched hyphal tips (26, 27). Finally, inhibition of the cross-linking of glycan fibers by the antifungal agent calcofluor white causes an arrest of polarized growth and swelling of hyphal tips in *A. niger* (21). Remarkably, inhibition of polarized growth of filamentous fungi has not only been described as a consequence of direct cell wall perturbations but also for conditions that rather indirectly affect cell wall biosynthesis. For example, interference with the assembly of the cytoskeleton (28), cAMP-dependent protein kinase signaling (29–31), calcium signaling (32), plasma membrane integrity (33), and with the secretory machinery (34) caused apparent morphological changes (see also Ref. 27). However, the underlying molecular mechanisms and the interconnections of the different pathways with cell wall assembly are far from being understood.

The recent sequencing and annotation of the genome of *A. niger* (35) and the availability of the Affymetrix microarray technology for *A. niger* now make it feasible for the first time to study the mechanisms involved in ensuring cell surface integrity and its correlation with polarized growth in this biotechnologically important filamentous fungus. To get first insights into these processes, in this study we screened for antifungal compounds that affected the morphology of *A. niger*. Caspofungin, known as an inhibitor of β-1,3-glucan synthesis in *S. cerevisiae* (36), and fenpropimorph, reported as an inhibitor of *S. cerevisiae* ergosterol biosynthesis (37), were selected, as application of these compounds to *A. niger* resulted in morphological alterations. Both compounds were applied at sublethal concentrations to *A. niger*, and global expression profiling was performed, aimed at the following: (i) identification of cellular responses involved in cell integrity and adaptation to growth-inhibitory conditions, (ii) identification of drug-specific responses and thereby first insights into their mode of action in *A. niger*, (iii) identification of genes whose protein products are important for the establishment and maintenance of polarized growth, and (iv) prevention of secondary drug effects or non-specific responses related to cell death. The experimental setup of the study involved the use of young, unbranched germlings as alteration of their morphology can easily be monitored and quantified by microscopic means and because germlings represent a more homogeneous cell population compared with mycelial hyphae.

EXPERIMENTAL PROCEDURES

Strains, Growth Conditions, and Antifungal Compounds—The *A. niger* strains N402 (wild type, laboratory collection), RD6.47 (38), and JvD1.1 (this study) were used. The strains were grown at 37 °C (unless otherwise stated) in minimal medium (39) or complete medium (CM), consisting of minimal medium (MM) supplemented with 1% yeast extract and 0.5% casamino acids. Fermentation medium (FM) was composed of 0.75% glucose, 0.45% NH_4Cl, 0.15% KH_2PO_4, 0.05% KCl, 0.05% $MgSO_4$, 0.1% salt solution (39), and 0.003% yeast extract. The pH of FM was adjusted to pH 3. Caspofungin was purchased from Merck (Cancidas®) and fenpropimorph from Sigma, respectively. AFP was isolated and purified from *Aspergillus giganteus* cultures as described in Ref. 40. All other antifungal compounds were made available by BASF.

Screening for Morphological Changes Induced by Antifungal Compounds—5×10^5 conidia of strain N402 were inoculated in Petri dishes containing 5 ml of liquid MM supplemented with 0.003% yeast extract. Prior to inoculation, two coverslips were placed onto the bottom of the Petri dishes. Spores were allowed to germinate for 5 h at 37 °C, and small germ tubes became visible in more than 90% of the spores. Compounds were added at various concentrations, whereas the negative control was supplemented with the same volume of H_2O. The following range of concentrations was tested: caspofungin, 1 ng/ml to 12.5 μg/ml; AFP, 0.2–0.9 μg/ml; fenpropimorph, 0.5–60 μg/ml; myriocin, 30–200 μg/ml; 8-Br-cAMP, 1–10 mM; caffeine, 1–10 mM. After further cultivation for 1 h at 37 °C, germlings that were adherent to the coverslips were analyzed by microscopy (see below). From at least 50 germlings per sample, the morphology was characterized as being either unbranched (germlings with a single germ tube) or branched (germlings with apical and/or subapical branches). A "Branching Index (BI)" was calculated that was defined as follows: $BI = (\Sigma \text{ branched germlings}) \times (\Sigma \text{ branched} + \text{unbranched germlings})^{-1}$.

Construction of GFP Reporter Strains—The reporter strain containing the P*agsA*-H2B-GFP-T*trpC* reporter construct

(RD6.47) has been described previously (38). The reporter strain with a cytoplasmically expressed GFP under control of the *agsA* promoter (P*agsA*-GFP-T*trpC*) was constructed as follows. 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* (21), 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 pJD1was cloned into a HindIII opened pTAS5 vector to give pTAS5-P*agsA*-GFP-T*trpC*. The pTAS5 vector consists of the binary vector pSDM14 (41) with the hygromycin expression cassette from pAN7.1 (42) 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 Ref. 43 and digested with PstI or SstII to determine the copy number. Strain JvD1.1, containing multiple copies (≥2) integrated in the genome (data not shown), was selected as a reporter strain.

Screening of Antifungal Compounds in Glass Bottom Microtiter Plates—The PagsA-GFP (JvD1.1) and PagsA-H2B-GFP (RD6.47) reporter strains were used to screen antifungal compounds for their ability to induce the cell wall integrity of *A. niger*. Conidia (2×10^4) from the reporter strains were inoculated in each well of 96-well optical glass bottom microtiter plates (Nunc art) in 100 μl of 2× CM and grown for 6 h at 37 °C. After spore germination, 100 μl of a 2-fold dilution series for each antifungal compound was added to individual wells. The effect of each compound was tested for at least seven different concentrations. After adding the antifungal solution, the microtiter plates were incubated for an additional 3 h at 30 °C. After discarding the medium by inverting the microtiter plate, germlings that adhered to the bottom of each well were analyzed by microscopy (see below). As a positive control, strain MA26.1, containing P*gpdA*-H2B-GFP-T*trpC* single copy at the *pyrG* locus was used.[4] Strain N402 was used as a negative control. Acquired images were analyzed for both growth and GFP levels.

Bioreactor Cultivation—Freshly harvested conidia (5×10^9) from strain N402 were used to inoculate 5 liters of FM. Cultivations were performed in a BioFlo3000 bioreactor (New Brunswick Scientific) using an agitation speed of 250 rpm. Temperature (37 °C) and pH (set to 3) were controlled on-line using the program NBS Biocommand. Aeration was performed via the headspace until the dissolved oxygen tension dropped to 40% and was then switched to sparger aeration. After 5 h of cultivation, caspofungin or fenpropimorph (dissolved in 5 ml of distilled H_2O) or 5 ml of distilled H_2O (negative control) were added. After an additional hour of cultivation, 400 ml of the culture broth were quickly harvested via filtration, and mycelial samples were immediately frozen using liquid nitrogen. In addition, samples were taken for microscopic analysis (see below) and calculation of the BI value.

[4] M. Arentshorst, unpublished strain.

TABLE 1
Primers used in this study

Open reading frame code	Gene	Primer pairs (sequence 5′- to 3′-oriented)
An09g04010	*chsC*	Forward, TGGTGATCATACGCTCTCCAAA
		Reverse, GAACGAACCAAGGAACGCAC
An03g05940	*gfaA*	Forward, TCAGTCTGGTGAAACAGCCG
		Reverse, GGGTAGTGCCTCGTCAACAAGA
An12g10200		Forward, GGCAGCTATGGCAACTACCAAG
		Reverse, CTCGTCACGCTTGTACGCCT
An18g03740	*mkkA*	Forward, CCTCGATGTGGATGATTTGGAT
		Reverse, TCTCGGACCATCGAATACCG
An01g03350		Forward, GCGCCTGGAGCAGTTCTACAT
		Reverse, GGCGTATCCGAAGAAGAGCAT
An01g07000		Forward, CCATCACCATTGGCCTACCTT
		Reverse, CCAGAGAAGATCGCCAAAGGA
An03g06410		Forward, TGACTTTCCTGGAGAAGGCG
		Reverse, CTTCGCCGGTAAAGTAGCCC
An01g14200		Forward, GAGGTCGAGTCTCACAACGATG
		Reverse, GGTGGCATGACCAACCGATA

RNA Extraction, Expression Profiling, and Northern Analysis—Total RNA was isolated from homogenized mycelial samples using TRIzol reagent (Invitrogen). RNA quality control, labeling, microarray hybridization, and scanning were performed at ServiceXS (Leiden, The Netherlands). Briefly, RNA quality was verified using Agilent Bioanalyzer "Lab on Chip" system (Agilent Technologies, Palo Alto, CA). Processing, labeling, and hybridization of cRNA to *A. niger* Affymetrix GeneChips were performed according to Affymetrix protocols for "Eukaryotic Target Preparation" and "Eukaryotic Target Hybridization." For washing and staining, the protocol "Antibody Amplification for Eukaryotic Targets" was followed. Hybridized probe array slides were scanned with a G2500A Gene Array Scanner (Agilent Technologies) at a 3-μm resolution and a wavelength of 570 nm. Affymetrix Microarray Suite software MAS5.0 was used to calculate signals and *p* values and to set the absolute call flag of the algorithm, which indicates the reliability of the data points according to P (present), M (marginal), and A (absent). Microarray analyses for each condition (control, caspofungin-treated germlings, and fenpropimorph-treated germlings) were performed on cells obtained from two independent bioreactor cultivations (biological duplicate). The complete set of transcriptional raw data is available as supplemental Table S1. Expression data were analyzed using the program GeneSpring 7.3. (Agilent Technologies). For normalization, default settings were used (50th percentile per chip, median per gene). Genes were defined as differentially expressed if their expression levels varied at least 1.5-fold in the caspofungin- (or fenpropimorph)-treated samples compared with the control and if the difference was statistically significant (Student's *t* test, *p* value cutoff of 0.05).

Northern analyses using each 5 μg of RNA from the six conditions were performed as described earlier (4). RNA samples were balanced according to their content of the 18 S mRNA (data not shown). PCR amplicons obtained by using different primer pairs as listed in Table 1 were labeled by random primer labeling using ^{32}P-labeled dATP (Amersham Biosciences) and used as probes for Northern analysis. Hybridizations were carried out according to the manufacturer's instructions (Amersham Biosciences).

Microscopy—Pictures of *A. niger* germlings were captured using an Axioplan 2 (Zeiss) equipped with a DKC-5000 digital

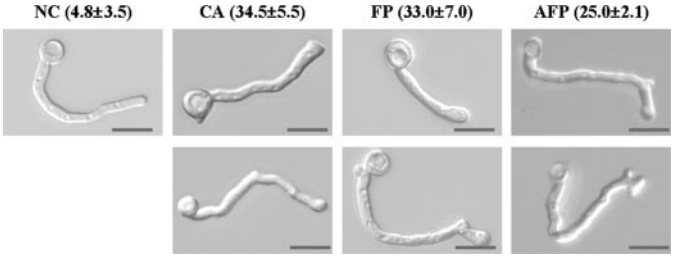

FIGURE 1. **The effect of selected antifungal drugs on the morphology of *A. niger*.** Caspofungin (*CA*, 5 ng/ml), fenpropimorph (*FP*, 5 μg/ml), and AFP (0.9 μg/ml) were added to 1×10^5/ml spores grown for 5 h in MM at 37 °C. After 1 h of further incubation, germlings ($n = 50–100$) were microscopically analyzed. BI is given in *parentheses*. *NC,* negative control. *Bar,* 10 μm.

camera (Sony). Both light (using DIC settings) and fluorescence images (using GFP settings) were obtained with a ×40 objective. For GFP images, a fixed exposure of 2 s was used. Images were processed using Adobe Photoshop 6.0 (Adobe Systems Inc.).

RESULTS

Screening for Morphology-affecting Compounds—To select suitable compounds that affect the morphology of *A. niger*, we have screened various substances proposed to interfere with fungal cell wall synthesis (caspofungin, AFP), ergosterol synthesis (fenpropimorph), sphingolipid synthesis (myriocin), and cAMP-dependent protein kinase signaling (8-Br-cAMP and caffeine). These compounds were applied in different concentrations to 5×10^5 *A. niger* germlings grown on coverslips in 5 ml of minimal medium, and their effect on germ tube elongation and branching was microscopically followed. Morphological alterations were only observed in response to caspofungin, AFP, and fenpropimorph (Fig. 1), whereas no significant effect was observed when germlings were treated with a series of concentrations of myriocin, 8-Br-cAMP, or caffeine (data not shown). In Fig. 1, the morphological changes provoked by caspofungin, AFP, and fenpropimorph are depicted. Tip swelling and an increase in (sub)apical branching were observed after treatment of the germlings with a minimal concentration of 5 ng/ml, 5 μg/ml, and 0.9 μg/ml for caspofungin, fenpropimorph, and AFP, respectively. To judge and quantify the effect on morphology, a BI was determined that gives the percentage of germlings displaying (sub)apical branches ($n > 50$). As shown in Fig. 1, the strongest effects on branching were exerted by caspofungin and fenpropimorph, where the BI value was about 7-fold higher compared with the negative control. Application of higher drug concentrations did not significantly increase the BI but resulted in the presence of dead germlings (data not shown).

Morphological Responses to Caspofungin and Fenpropimorph—Caspofungin and fenpropimorph were selected for further analysis as their effect on morphology was more prominent compared with AFP. The screening assay described above was repeated in large scale using a cultivation of *A. niger* spores in a bioreactor (working volume of 5 liters). Using such an experimental design, we wanted to ensure controlled and equal growth conditions between treated and nontreated germlings and thereby reliable expression data. An increased starting inoculum (1×10^6 spores/ml) and a slightly different minimal medium (FM) were used. During bioreactor runs, the dissolved oxygen tension was followed and used as an indication for equal growth behavior between the different experiments (data not shown). After 5 h of total cultivation, caspofungin or fenpropimorph were added, and the cultivations were continued for an additional hour, after which samples were taken for determination of the BI value and for transcriptomic analysis. Using this experimental setup, we observed that a 10-fold increased concentration of both caspofungin and fenpropimorph was necessary to significantly affect the morphology of *A. niger* germlings when compared with the screening experiment described above (Table 2 and data not shown). On the one hand this can be explained by the higher spore titer used for bioreactor inoculation and on the other hand by different cultivation conditions used in both experiments. In addition to inducing the formation of (sub)apical branches, both caspofungin and fenpropimorph were observed to induce the establishment of new polarity axes that started from the spore and thus resulted in the formation of new germ tubes. In particular, the amount of spores displaying three or four germ tubes, which are usually rarely observed in *A. niger*, was significantly increased by both antifungals (Fig. 2). This observation indicated that the germlings may counteract the disturbance of existing polarity growth sites by the formation of new polarity sites.

TABLE 2

The effect of caspofungin (50 ng/ml) and fenpropimorph (50 μg/ml) on branching efficiency of *A. niger* germlings

More than 200 germlings were counted for each bioreactor run in order to determine the BI for a single experiment. For every duplicate experiment, a mean BI was calculated. —, negative control; CA, caspofungin; FP, fenpropimorph.

Bioreactor run	Compound	BI	Mean BI
		%	%
1	—	14.7	13.7 ± 1.0
2		12.7	
3	CA	43.4	43.7 ± 0.7
4		44.1	
5	FP	33.2	30.5 ± 2.7
6		27.8	

Global Gene Expression Responses to Caspofungin and Fenpropimorph—Affymetrix microarray chips representing 14,509 open reading frames of *A. niger* were hybridized with RNA samples from each of two biological replicates of caspofungin-, fenpropimorph-, and nontreated samples, respectively, as described under "Experimental Procedures." Following normalization to account for deviations in hybridization intensity, genes showing at least 1.5-fold change (p value cutoff of 0.05) in expression level were considered to be differentially expressed.

A total of 172 genes were differentially expressed upon exposure to caspofungin, 165 of which showed increased expression and 7 genes decreased expression. In comparison, a total of 43 genes was found to be responsive to treatment with fenpropimorph, 41 of these were up-regulated and 2 were down-regulated (supplemental Table S2). The modulated genes were functionally classified according to FunCat (44) as shown in Table 3. The category with the largest number of known genes that are modulated by both antifungals is the category involved in metabolism.

Gene Expression Responses to Caspofungin—Caspofungin has been shown to be a potent inhibitor of β-1,3-glucan synthe-

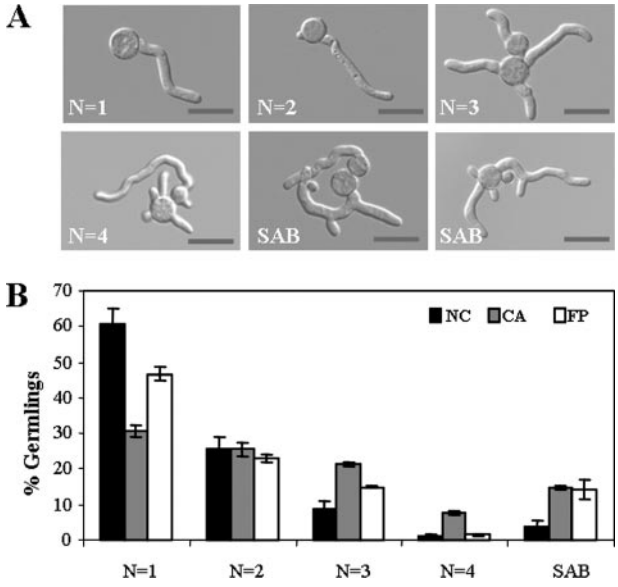

FIGURE 2. **Caspofungin and fenpropimorph provoke the establishment of new polarity axes.** *A,* microscopic images of germlings that were harvested from the bioreactor cultivations (see "Experimental Procedures"). Images were taken using DIC settings; *Bar* 10 μm. *B,* at least 200 germlings per single fermentation run were analyzed for the number of germ tubes present and for the presence of subapical branches. Means ± S.D. were calculated from each two independent fermentation runs. *CA,* cells treated with caspofungin; *FP,* cells treated with fenpropimorph; *NC,* negative control, cells treated neither with caspofungin nor with fenpropimorph. *A* and *B, N,* number of germ tubes per germling; *SAB,* germlings with subapical branches.

TABLE 3

Functional categories induced by caspofungin (CA) and fenpropimorph (FP)

An annotated list of all genes, including fold change, *p* value, and classification, can be found in supplemental Table S2.

Functional category	No. of genes responsive to	
	CA	FP
Metabolism	41	26
Energy	3	1
Cell cycle and DNA processing	1	
Transcription	3	
Protein synthesis	11	
Protein fate	17	4
Cellular transport and transport mechanism	5	2
Cellular communication	7	1
Cell rescue, defense, and virulence	3	1
Regulation of/interaction with cellular environment		1
Transport facilitation	4	
Unclassified proteins	70	5

sis in *S. cerevisiae* (36). As β-1,3-glucan is the central cell wall polysaccharide to which other cell wall components of *S. cerevisiae*, such as β-1,6-glucan, chitin, and mannoproteins, are cross-linked (1), the inhibition of β-1,3-glucan synthesis by caspofungin causes cell wall disorganization and cell lysis in *S. cerevisiae*. One of the compensatory responses in yeast described to the presence of caspofungin is the induction of the CWI pathway (69, 106), including induced expression of cell wall protein encoding genes and cell wall remodeling enzymes. In agreement with this, we observed an up-regulation of several *A. niger* genes involved in cell wall assembly and remodeling (Table 4). Genes that were up-regulated included genes coding for proteins involved in UDP-glucose synthesis (An02g07650/PgmB and An12g00820/UgpA), UDP-*N*-acetylglucosamine synthesis (An18g06820/GfaA, An03g05940/GfaB, and An12g07840/GnaA), chitin formation (An09g04010/class III chitin synthase ChsB), α-glucan synthesis (An09g03100/AgtA), β-1,3-glucan remodeling (An03g05290/BgtB, An10g00400/GelA), cross-linkage of chitin to β-1,6-glucan (An07g07530/CrhB, An07g01160/ChrC), GPI anchor processing (An14g03520/DfgC), protein mannosylation (An03g01090/HocA, An18g06500/Sec53, and An16g04330/DpmA), and genes encoding putative cell wall proteins (An12g10200 and An14g01820). Genes involved in signaling cascades ensuring cell wall integrity were also up-regulated such as An16g04200/RhoB (similar to Rho2 GTPase of *Schizosaccharomyces pombe*, regulator of α-1,3-glucan synthesis), An10g00490/Rho-GAP, and An18g03740/MkkA (similar to *S. cerevisiae* MAP kinase kinase 2 involved in CWI signaling).

The presence of sublethal concentrations of caspofungin allowed *A. niger* to adapt to its inhibitory effect in such a way that the fungus survived and continued growth by the establishment of new polarity axes and formation of new germ tubes (Fig. 3). We thus expected, besides identifying genes involved in cell wall maintenance, to also identify genes coding for proteins having a function in cell growth regulation and cell polarity. Indeed, three genes involved in growth control were up-regulated as follows: An17g02350 showing strong similarity to the human Ras-related GTPase Rheb known to be a key component of the TOR signaling pathway (45), An13g00100 (similar to the *S. cerevisiae* cell-cycle checkpoint protein kinase Dun1), and An14g00010 corresponding to the secretion related Rab-GTPase SrgA shown to be important for efficient secretion and maintenance of polarity in *A. niger* (46).

Furthermore, genes for which a function in cytoskeleton organization and maintenance has been established for eukaryotic organisms were up-regulated as follows: (i) An08g06410 and An18g06590 showing homology to actin-binding proteins important for the integrity of cortical actin patches and actin-dependent endocytosis in *S. cerevisiae* and *S. pombe*; (ii) An01g03770 displaying homology to microtubule-based motor proteins; (iii) An16g03000 and An18g03900 with high homology to subunits of the prefoldin complex involved in the folding of tubulin and actin; (iv) An05g00810 with homology to tubulin-specific chaperones; and (v) An01g13120 predicted to be a ADP-ribosylation factor-like 2 of the Ras superfamily of GTPases, which has been shown to be important for tubulin stability and dynamics in human cells (47).

Caspofungin also induced expression of genes predicted to function in lipid metabolism and signaling as follows: An02g01180, coding for diacylglycerol pyrophosphate phosphatase Dpp1 and An02g13220 predicted as lysophospholipase LplB. Moreover, a gene coding for a geranylgeranyltransferase type II (An13g01040) involved in prenylation of proteins and thereby in the membrane targeting and interaction of the modified proteins (48) showed increased expression. Remarkably, a large number of signaling proteins such as GTPases of the subfamilies Rho, Rac, Rab, and Rap require this modification for their cycling between intracellular membrane compartments and hence their activity (48, 49). Increased expression of An13g01040 might thus probably reflect a higher demand for relocalization/recycling of GTP-binding proteins in response to caspofungin treatment.

TABLE 4
Selected caspofungin-responsive genes ordered into different biological processes

Open reading frame code	Gene	Fold change	*p* value	(Predicted) protein function
Cell wall synthesis				
An14g01820		(33.14)[a]	0.020	Cell wall protein related to phiA
An12g10200		(23.03)[a]	0.000	Cell wall protein with internal repeats, hypothetical
An03g05940	*gfaB*	(7.98)[a]	0.051	Glutamine:fructose-6-phosphate amidotransferase
An09g04010	*chsB*	3.08	0.007	Chitin synthase class III
An12g07840	*gnaA*	3.18	0.004	Glucosamine-6-phosphate *N*-acetyltransferase
An10g00400	*gelA*	(3.05)[a]	0.029	β-1,3-Glucanosyltransferase
An07g07530	*crhB*	2.58	0.030	GPI-anchored glucanosyltransferase
An18g06820	*gfaA*	2.32	0.008	Glutamine:fructose-6-phosphate amidotransferase
An18g06500		2.12	0.020	Phosphomannomutase (Sec53)
An09g03100	*agtA*	2.10	0.016	GPI-anchored α-glucanosyltransferase
An07g01160	*crhC*	2.03	0.030	GPI-anchored glucanosyltransferase
An03g01090	*hocA*	(2.03)[a]	0.046	α-1,6-Mannosyltransferase
An01g04650		2.00	0.032	Nucleoside diphosphate-sugar epimerase
An02g07650	*pgmB*	1.95	0.036	Phosphoglucomutase
An16g04330	*dpmA*	1.85	0.032	Mannose phosphodolichol synthase
An03g05290	*bgtB*	1.77	0.040	β-1,3-Glucanosyltransferase
An12g00820	*ugpA*	1.64	0.048	UTP-glucose-1-phosphate uridylyltransferase
An14g03520	*dfgC*	2.40	0.017	Endomannanase
An02g14500		2.21	0.009	GPI-anchored cell wall protein
CWI signaling				
An16g04200	*rhoB*	2.32	0.007	GTPase (Rho2-related)
An10g00490	*rapA*	2.16	0.039	Rho-GAP (ScSag7-related)
An18g03740	*mkkA*	2.03	0.009	MAP kinase kinase
Cell growth and polarity				
An17g02350		3.04	0.007	GTP-binding protein (Rheb-related)
An14g00010	*srgA*	2.51	0.012	GTPase
An13g00100		1.91	0.035	Serine/threonine-protein kinase (Chk2-related)
Cytoskeleton				
An01g03770		5.87	0.001	Dynein light chain
An16g03000		(2.97)[a]	0.037	Subunit of the Gim/prefoldin protein
An01g13120		(2.64)[a]	0.026	ADP-ribosylation factor family protein
An05g00810		2.60	0.016	Tubulin-specific chaperone (Rbl2-related)
An18g03900		2.16	0.027	Prefoldin subunit 2
An08g06410		2.13	0.016	Actin-like protein ARP2
An18g06590		1.89	0.023	ARP2/3 complex subunit 1A
Lipid metabolism				
An08g10110		2.88	0.011	Lipid transfer protein
An02g01180	*dppA*	(2.62)[a]	0.044	Diacylglycerol pyrophosphate phosphatase
An13g01040		1.84	0.043	Rab geranylgeranyltransferase
An02g13220	*lplB*	1.77	0.025	Lysophospholipase

[a] Values given in parentheses as genes have an Absent flag in the control experiment.

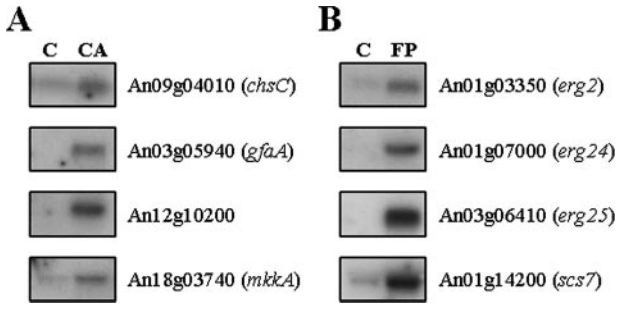

FIGURE 3. **Northern analysis of selected genes that were identified as caspofungin-responsive (*A*) or fenpropimorph-responsive (*B*) genes.** RNA samples extracted for the microarray experiments were used for hybridizations. Each 5 μg of RNA was loaded onto each lane and hybridized with different probes as indicated. Control hybridizations with 18 S RNA and actin probes confirmed equal loading (data not shown). *CA,* cells treated with caspofungin; *FP,* cells treated with fenpropimorph; *C*, control.

Gene Expression Responses to Fenpropimorph—Fenpropimorph belongs to the morpholine fungicides and interferes with ergosterol biosynthesis in *S. cerevisiae* by inhibiting sterol C-14 reductase (*ERG24* gene) and sterol C-8 isomerase (*ERG2* gene) (37). We thus assumed that among the *A. niger* genes up-regulated by fenpropimorph, a considerable number of genes should be connected to lipid metabolism and especially to ergosterol biosynthesis. Indeed, five genes encoding proteins with homology to the *S. cerevisiae* ergosterol pathway were responsive to fenpropimorph treatment (Table 5) as follows: An08g05400 (homologue of Erg10p that catalyzes the first and rate-limiting step in ergosterol biosynthesis), An03g06410 (Erg25p homologue; *p* value 0.06), An01g07000 (Erg24p homologue), An01g03350 (Erg2p homologue), and An15g00150 (Erg3p homologue; *p* value 0.06), indicating that fenpropimorph also targets ergosterol synthesis in *A. niger*.

Ergosterol is most abundant in the plasma membrane and secretory vesicles of *S. cerevisiae* (50), and its metabolism has been shown to be closely linked with the biosynthesis of sphingo- and phospholipids (51–53). Further support for the link between ergosterol biosynthesis and other lipids and fatty acid biosyntheses was provided by the observation of increased transcript levels of three genes predicted to function in sphingolipid synthesis as follows: An01g10030 displaying homology to the *S. cerevisiae* sphinganine hydroxylase Sur2p as well as An01g14200 and An01g14190 coding for sphingolipid α-hydroxylase Scs7p. Furthermore, a gene encoding an inositol-1-P synthase (An10g00530; homologous to *S. cerevisiae* Ino1p) showed increased expression. Perturbation of ergosterol biosynthesis in *A. niger* was also accompanied by alteration of the cellular fatty acid metabolism. Six genes predicted to function in peroxisomal fatty acid β-oxidation were up-regulated as fol-

TABLE 5
Selected fenpropimorph-responsive genes ordered into different biological processes

Open reading frame code	Gene	Fold change	*p* value	(Predicted) protein function
Cell wall synthesis				
An01g11010	*crhD*	1.51	0.024	GPI-anchored glucanosyltransferase
Cell growth and polarity				
An13g02780		(2.90)[a]	0.008	Aldolase and adducin head domain
An04g02340		2.07	0.047	Kinesin light chain
An14g02370		1.71	0.014	Apyrase, nucleoside diphosphatase (Ynd1-related)
Lipid metabolism				
An03g06410		(24.04)[a]	0.062	C-4 methyl sterol oxidase (Erg25-related)
An17g01150		(16.65)[a]	0.022	Acyl-CoA dehydrogenase
An18g05210		(10.70)[a]	0.002	Peroxisomal dehydratase
An01g07000		10.56	0.002	C-14 sterol reductase (Erg24-related)
An08g05400		(5.41)[a]	0.012	Acetyl-CoA acetyltransferase (Erg10-related)
An08g07520		5.24	0.001	3-Oxoacyl-(acyl-carrier-protein) reductase
An15g01280		(5.22)[a]	0.006	Peroxisomal Δ3,Δ2-enoyl-CoA isomerase
An16g04520		4.13	0.004	3-Oxoacyl-(acyl carrier protein) reductase
An14g04050		(4.04)[a]	0.045	Pyridoxamine-phosphate oxidase activity
An16g05340		3.60	0.001	Enoyl-(acyl carrier protein) reductase
An18g01590		2.97	0.018	Mitochondrial carnitine acetyltransferase
An04g00740		2.92	0.003	Sterol carrier protein
An14g00990		(2.92)[a]	0.005	Peroxisomal multifunctional β-oxidation protein
An01g14200		2.75	0.012	Sphingolipid α-hydroxylase (Scs7-related)
An01g14190		2.37	0.018	Sphingolipid α-hydroxylase (Scs7-related)
An01g03350		2.21	0.038	C-8 sterol isomerase (Erg2-related)
An15g00150		2.01	0.062	C-5 sterol desaturase (Erg3-related)
An01g10030		1.72	0.039	Sphinganine hydroxylase (Sur2-related)
An10g00530		1.62	0.028	Myoinositol-1-phosphate synthase

[a] Values given in parentheses as genes have an Absent flag in the control experiment.

lows: An11g00400 and An17g01150 both coding for an acyl-CoA dehydrogenase mediating the first committed step of β-oxidation of fatty acids, two fatty acid dehydrogenases (An18g05210, An14g00990), one fatty acid isomerase (An15g01280), and a sterol carrier protein 2 (An04g00740), which functions *in vitro* as a chaperone for acyl-CoA oxidase (54) and was recently shown to bind to fatty acids (55). The main product of fatty acid β-oxidation is acetyl-CoA that might serve as precursor for *de novo* synthesis of ergosterol (substrate for Erg10p) and fatty acids (substrate for the fatty acid synthase multiprotein complex), which in turn provides fatty acids for sphingolipid and phospholipids biosynthesis. In agreement, three genes coding for proteins involved in fatty-acid synthase reactions showed increased expression (An08g07520, An16g04520, and An08g07520). Interestingly, a gene coding for hexose transporter (first and rate-limiting step of glycolysis; An15g03940) and citrate synthase (important for the shuttle of acetyl-CoA from mitochondria to the cytosol; An15g01920) were also up-regulated, probably hinting at the possibility that the cytosolic demand for acetyl-CoA necessary for fatty acid biosynthesis is accommodated by using glycolysis as alternative source for acetyl-CoA.

Apart from genes connected to lipid metabolism, genes involved in ensuring cell wall integrity were also up-regulated upon fenpropimorph treatment: An01g1010 (CrhD) displaying strong similarity to the *S. cerevisiae* cell wall protein Crh1, most recently shown to function in the cross-linkage of chitin chains to β-1,6-glucan (56) and An03g05000 predicted as ZIP family zinc transporter with homology to the *S. cerevisiae* Yke4p. This protein is important for balancing zinc levels between the cytosol and the secretory pathway in yeast (57).

In response to fenpropimorph, new polar growth sites were developed by *A. niger* germlings, although to a lesser extent when compared with the caspofungin-treated samples (Fig. 3). This might be reflected by the lower number of up-regulated genes that putatively play a role in cell polarity of *A. niger* as follows: An04g02340 (low homology to kinesin light chain), An14g02370 (apyrase, required for Golgi *N*- and *O*-glycosylation in *S. cerevisiae*), and An13g02780 exhibiting similarity to α-adducin, a crucial assembly factor of the spectrin-actin membrane skeleton in higher eukaryotes (58). However, as ergosterol and sphingolipid metabolism have been shown to be important for protein secretion and for the establishment of cell polarity in yeast and filamentous fungi (33, 59, 60) might suggest that some lipid genes mentioned above could also be involved in polarity control of *A. niger*.

Validation of Transcriptome Data by Northern Analysis—To confirm the changes in gene expression detected by the expression profiling, Northern analyses were performed using the same RNA samples as used in the microarray experiments. For cells treated with caspofungin, four genes predicted to function in cell wall biosynthesis and integrity were selected (An09g04010/*chsC* and An03g05940/*gfaA*, An12g10200/hypothetical cell wall protein, and An18g03740/*mkkA*). In the case of the fenpropimorph-treated samples, four genes coding for proteins putatively involved in lipid biosynthesis were selected (An01g03350/*ERG2* homologue), An01g07000/*ERG24* homologue, An03g06410/*ERG25* homologue, and An01g14200/*SCS7* homologue). As shown in Fig. 3, the results of the Northern hybridizations are in good agreement with the microarray data. Genes that showed high/low levels of induction in the expression profiling also showed signals of strong/moderate induction in the Northern experiment (*e.g.* An03g06410 and An18g03740).

agsA Expression Is Specifically Induced by Compounds Affecting Cell Wall Integrity—The expression profiling in this study revealed that the gene coding for the regulator of α-1,3-glucan synthesis (Rho2-GTPase, An16g04200) and *agtA* (GPI-an-

chored α-glucanotransferase, An09g03100) were up-regulated upon caspofungin treatment. We have shown previously that the *agsA* gene coding for α-1,3-glucan synthase (An04g09890) is strongly induced in response to compounds that interfere with cell wall or cell membrane integrity of *A. niger* such as calcofluor white, SDS, caspofungin, and AFP (21, 25), suggesting that α-1,3-glucan synthesis might be generally involved in securing cell surface integrity. In this study, the *agsA* gene was unexpectedly not found among the significantly up-regulated genes. A closer look at the transcriptomic data revealed, however, that *agsA* was not expressed in the control experiment but strongly expressed when *A. niger* germlings were exposed to both caspofungin and fenpropimorph (p value > 0.05, see supplemental Table S2), implying that regulation of *agsA* gene expression is actually under the control of stress conditions that affect the integrity of the plasma membrane and/or the cell wall.

To further support this conclusion, we used two *A. niger* reporter strains, containing either a cytoplasmically (strain JvD1.1) or nuclear (strain RD6.47) targeted *gfp* gene under the control of the *agsA* promoter. Both strains were exposed to 16 antifungal compounds (including caspofungin and fenpropimorph) that target different cellular processes, and their effect on growth and *agsA* expression was monitored by light and fluorescence microscopy (Table 6). Based on their effects, we have divided the compounds into four groups. The first group of compounds includes calcofluor white, caspofungin, tunicamycin, spiroxamine, fenpropimorph, terbinafine, fludioxonil, and cyprodinil. These compounds inhibited growth and provoked high expression of the GFP reporter. In response to calcofluor white, fungal growth became inhibited, and aberrant hyphal morphology such as tip swelling as well as a clear induction of GFP expression was observed (Fig. 4*A*). Similarly, the induction of *agsA* in response to the presence of caspofungin was also observed in both reporter strains, confirming previous results (21) and the results of the expression profiling in this study (Fig. 4*B*). When the reporter strains were stressed with tunicamycin (inhibitor of protein *N*-glycosylation), swollen hyphae and high GFP expression were visible (Fig. 4*C*). As *N*-glycosylation mutants in *S. cerevisiae* have been shown to have defects in cell wall integrity (15, 61), it is very likely that the addition of tunicamycin to *A. niger* also results in weakening of the cell wall and activation of the cell wall integrity pathway. The induction of the GFP reporter by the lipid synthesis disturbing compounds spiroxamine, fenpropimorph, and terbinafine suggests that disturbance of the plasma membrane integrity negatively affects the integrity of the cell wall and substantiates the expression data with respect to fenpropimorph. Activation of *agsA*::*gfp* expression by fludioxonil (activator of the Hog1 osmotic signal transduction pathway in *S. cerevisiae*) could hint at the existence of a cross-talk between the cell wall integrity pathway and the osmotic signal transduction pathway in *A. niger* as shown recently for *S. cerevisiae* (62, 63). Interestingly, cyprodinil (interferes with methionine synthesis and secretion of hydrolytic enzymes (64)) also leads to an up-regulation of the reporter, suggesting that an efficient secretory pathway is required for proper cell wall biosynthesis. Interfering with protein secretion might lead to cell wall weakening and subsequently to the activation of the cell wall salvage pathway.

The second group of compounds includes chitosan and epoxiconazole. These compounds inhibited growth of *A. niger* and gave a moderate induction of GFP expression. These compounds have been reported to have an effect when the integrity of the cell wall was impaired, suggesting an indirect effect of these compounds on cell wall biosynthesis (65, 66). The third group of compounds consisting of hydrogen peroxide (Fig. 4*D*), pyraclostrobin, benomyl, and cycloheximide showed an inhibition of growth at one or more concentrations used but showed no induction of GFP expression. To our knowledge, none of these compounds were reported so far to affect directly or indirectly cell wall biosynthesis. Into the last group, compounds were sorted that did not have any effect on either growth or GFP expression such as myriocin and nikkomycin. Although nikkomycin was shown to interfere with chitin synthesis in *S. cerevisiae* (67), Li and Rinaldi (68) showed that *A. niger* was not sensitive to nikkomycin concentrations (minimal inhibitory concentration >64 μg/ml). These results are consistent with our finding that nikkomycin had no effect on either growth or GFP expression.

Taken together, the data show that the *agsA* promoter is specifically activated by compounds interfering directly with cell wall biosynthesis or by compounds inhibiting plasma membrane function or the protein secretion machinery, thereby disturbing cell wall biosynthesis more indirectly.

DISCUSSION

Growth and development of fungi as well as their ability to withstand internal turgor pressure and to survive environmental stress conditions depend on maintaining the integrity of their cell surface. As a first step toward a comprehensive understanding of the regulatory network(s) of *A. niger* involved in maintenance of cell surface integrity, we examined in this study the global gene expression profile of *A. niger* in response to treatments with caspofungin and fenpropimorph.

Responsive Genes to Caspofungin—The category with the highest number of genes showing enhanced transcription in response to caspofungin is the group of genes required for cell wall biogenesis and maintenance. About 12% of the up-regulated genes can be classified into this category, suggesting that the primary (or an important) response to caspofungin is to counteract the inhibitory effect of caspofungin on β-1,3-glucan synthesis by transcriptional activation of cell wall reinforcing genes. Caspofungin inhibits β-1,3-glucan synthesis in *S. cerevisiae* and several *Aspergilli* species (7, 36) and has been shown to (mainly) up-regulate genes involved in the synthesis of cell wall components and cell wall strengthening in the yeasts *S. cerevisiae* and *Candida albicans* (69, 70), implying that caspofungin triggers a similar response in *A. niger* as in yeast to reinforce the strength of the cell wall.

One of the signal transduction pathways that becomes activated in *S. cerevisiae* in response to caspofungin is the CWI pathway (69). In brief, the CWI pathway of *S. cerevisiae* consists of the plasma membrane-localized sensor proteins (Wsc1–4p and Mid2p) that mediate the cell wall stress signal through the Rho1-GTPase and the Pkc1p kinase. Pkc1p initiates a phospho-

TABLE 6
The effect of selected compounds tested for antifungal activity

Compound	Proposed target	Reference	Concentration [a]	GFP expression [b] / Growth inhibition [c]						
Calcofluor white	Chitin synthesis	(96)	1.6-102.4	0	0	0	0	1	1	2
				0	1	1	2	2	2	2
Caspofungin	β-1,3-glucan synthesis	(36)	0.4-25.6	0	1	2	2	2	2	2
				0	1	2	2	2	2	2
Tunicamycin	N-glycosylation	(97)	2.6-166.7	0	0	2	2	2	2	2
				1	2	2	2	2	2	2
Spiroxamine	Sterol synthesis	(98)	1.6-104.2	1	2	2	2	2	2	1
				0	1	1	1	2	2	2
Fenpropimorph	Sterol synthesis	(37)	1.6-104.2	0	2	2	2	2	2	2
				1	1	1	1	1	2	2
Terbinafine	Sterol synthesis	(99)	1.6-104.2	2	2	2	2	2	2	2
				1	1	1	1	1	1	1
Fludioxonil	MAP kinase signaling	(100)	1.6-104.2	2	2	2	2	2	2	2
				0	1	1	2	2	2	2
Cyprodinil	Protein secretion	(64)	1.6-104.2	2	2	2	2	2	2	2
				1	1	1	1	1	1	1
Chitosan	Membrane integrity	(66)	5-310	1	1	1	1	1	1	1
				1	1	1	2	2	2	2
Epoxiconazole	Sterol synthesis	(65)	1.6-104.2	0	0	0	1	1	1	1
				0	0	0	0	1	1	1
Hydrogen peroxide	Redox balance	(101)	3.2-202 mM	0	0	0	0	0	0	0
				1	2	2	2	2	2	2
Pyraclostrobin	Respiratory chain	(102)	1.6-104.2	0	0	0	0	0	0	0
				1	1	1	1	1	2	2
Benomyl	Tubulin assembly	(103)	1.6-104.2	0	0	0	0	0	0	0
				0	0	1	1	1	2	2
Cycloheximide	Protein synthesis	(104)	1.6-104.2	0	0	0	0	0	0	0
				1	1	1	1	2	2	2
Myriocin	Sphingolipid synthesis	(105)	1.6-104.2	0	0	0	0	0	0	0
				0	0	0	0	0	0	0
Nikkomycin	Chitin synthesis	(67)	1.6-104.2	0	0	0	0	0	0	0
				0	0	0	0	0	0	0

[a] The concentration range of the antifungal compounds is given in μg/ml. 2-Fold serial dilutions giving seven different concentrations were tested. The value of the highest and lowest concentration is shown.
[b] Schematic representation of the average green fluorescent protein levels in both reporter strains (RD6.47 and JvD1.1) grown in the presence of different compound concentrations. The left column represents the lowest antifungal concentration used. The numbers 0, 1, and 2 represent low/basal, intermediate, and high green fluorescent protein 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. No effect on growth or on green fluorescent protein expression was found when the solvents Me_2SO or ethanol were used (data not shown).

rylation cascade involving the MAP kinases Bck1p, Mkk2p, and Slt2p. Slt2p finally phosphorylates the transcription factor Rlm1p that induces expression of genes involved in cell wall reinforcement (71). All components of the yeast CWI pathway are present in the genome of *A. niger* (35), suggesting that this pathway is not only important for ensuring cell integrity in yeast but also in filamentous fungi. The results of this study provide indications that the CWI pathway becomes

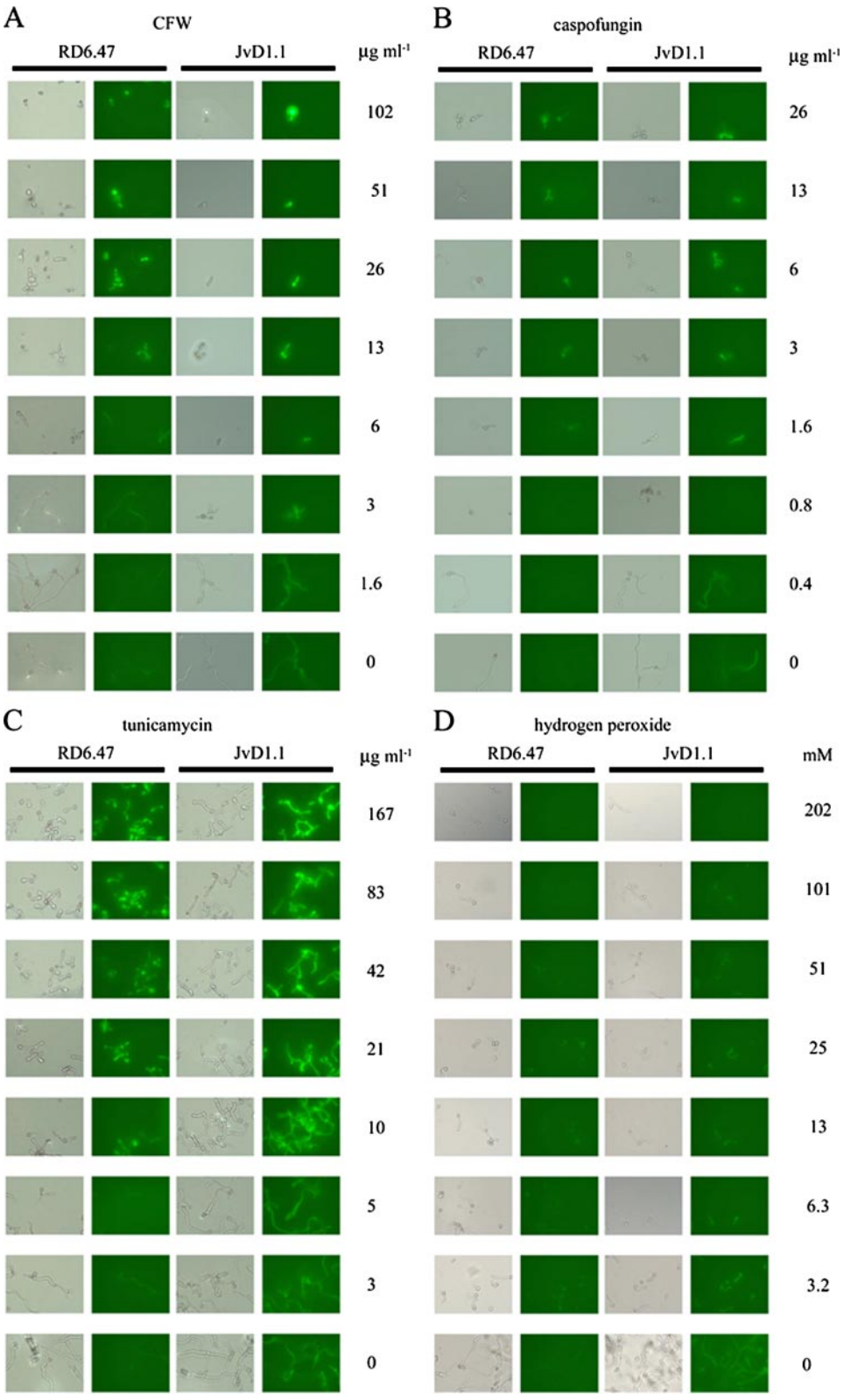

FIGURE 4. **Microtiter plate-based screening of antifungals by microscopic analysis of the morphology and GFP expression.** Strains RD6.47 and JvD1.1 were pre-grown for 6 h and subsequently stressed for 3 h at 30 °C as described under "Experimental Procedures." Different compounds (calcofluor white, caspofungin, tunicamycin, and hydrogen peroxide) were used, *A–D*, respectively. The compounds were 2-fold serial diluted. The final concentration of the compounds is indicated to the *right* of each image.

activated in *A. niger* and is required for adaptation to caspofungin-mediated inhibition of cell wall biogenesis (Fig. 5). First, An02g01180 displaying homology to diacylglycerol pyrophosphate phosphatase is up-regulated. This enzyme generates diacylglycerol that has been shown to be a physiological activator of fungal Pkc1p homologues (72, 73). Second, a homologue to the *S. pombe* Rho2p (An16g04200/RhoB) showed increased expression. In *S. pombe*, the Rho2-GTPase has been shown to stimulate α-1,3-glucan synthesis through activation of the Pkc1p homologue Pck2p (74). Third, enhanced expression of An18g03740/MkkA (homologous to Mkk2p) is further indicative for an involvement of the CWI pathway. Finally, targets of the *A. niger* RlmA transcription factor such as *gfaA* (chitin synthesis) and *agsA* (α-1,3-glucan synthesis (4)) showed enhanced expression.

The motility of the *S. cerevisiae* β-1,3-glucan synthase Fks1p toward the polar growth site is strongly dependent on cortical actin patch movement which itself requires the activity of the Arp2/3 complex (see Ref. 75 and references therein). In this study, two components of the Arp2/3 complex (An08g06410 and An18g06590) showed increased expression upon caspofungin treatment. As other proteins predicted to function in actin and tubulin folding/stability were up-regulated (An16g03000, An18g03900, An05g00810, and An01g13120) suggests that inhibition of β-1,3-glucan synthesis may also affect actin stability in *A. niger*, which is counteracted by the induction of genes encoding for proteins that assist in actin stabilization and cytoskeleton maintenance. The induced expression of this class of genes has not been observed in studies in yeast (*S. cerevisiae* and *C. albicans* (69, 70)) and might therefore be related to the filamentous growth of *A. niger*.

One pathway that has been described to be involved in actin polarization in *S. cerevisiae* is the

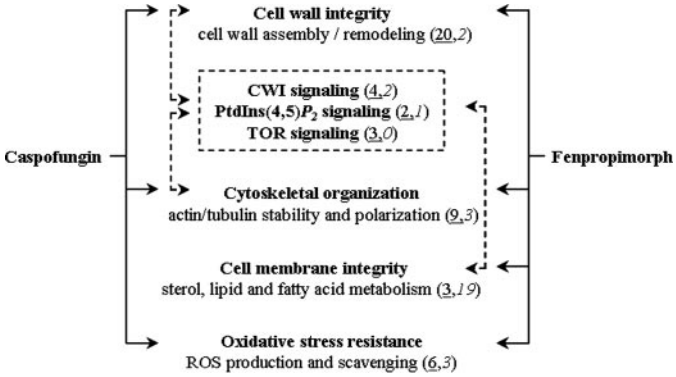

FIGURE 5. **Overview of the major gene responses provoked by caspofungin and fenpropimorph.** The number of responsive genes is given in *parentheses*; and *underlined numbers* refer to caspofungin, and *italic numbers* to fenpropimorph. Signaling processes that counteract the inhibitory effect of both compounds are given as *dashed lines*. For further information, see "Discussion."

TOR signaling pathway. Basically, TOR signaling is conserved from yeast to humans and consists of two signaling branches in *S. cerevisiae* (Tor1p branch and Tor2p branch) that couple nutrient signals to growth-related processes such as protein synthesis, uptake of amino acids, actin organization, and endocytosis (45). In *A. niger*, as in other filamentous fungi and higher eukaryotes, only a single Tor protein is present.[5] The upstream activator of TOR signaling is the GTPase Rheb, and one of the downstream effectors of Tor2p is the Rho1-GTPase of the CWI pathway (45, 60). As in this study, we have observed an up-regulation of a Rheb homologue (An17g02350), which may suggest that Tor activates the Rho-Pkc-MAP kinase cascade in *A. niger* and thereby actin polarization. An additional hint for involvement of the TOR pathway comes from the observation that two putative targets of TOR signaling (An09g03660 and An04g09420 coding for amino acid permeases; supplemental Table S2) showed increased expression, as also observed for *S. cerevisiae* when subjected to caspofungin (69). Surprisingly, a homologue of the Rho1-GTPase activator protein Sac7p showed also increased expression (An10g00490). This GTPase-activating protein has been shown to be important for turning off Rho1p activity (76), which contradicts the conclusion that the *A. niger* Rho1p homologue becomes activated by Tor. However, in plants, it has most recently been shown that the activity of GTPase-activating proteins is necessary to spatially restrict the action of Rho-type GTPases to the tip of pollen tubes and thereby maintains the subapical location of the GTPase and hence polarity of the cell (77). Thus, it might be conceivable that a similar down-regulation of the *A. niger* Rho1p homologue at the flanks of the apex ensures that the active form of the GTPase is only present at the hyphal tip.

It is interesting to note that the synthesis of two important lipid second messengers, diacylglycerol and phosphatidylcholine, seems to be induced in response to caspofungin (enhanced expression of An02g01180 and An02g13220, respectively). Both lipids are involved in the synthesis of the major phosphoinositide PtdIns(4,5)P_2, shown to be important for the activity and localization of different GTPases involved in actin assembly and membrane trafficking (78, 79). Moreover, PtdIns(4,5)P_2 mediates the plasma membrane localization of Rom2p that activates the Rho1/Rho2 GTPases, which in turn positively affects Pkc1p activity in *S. cerevisiae* (71, 80). Fadri *et al.* (81) have shown that the two PtdIns(4,5)P_2- interacting proteins Slm1p and Slm2p are essential for actin polarization and interact with the Tor2p signaling complex, suggesting a link between PtdIns(4,5)P_2 signaling, the TOR pathway, and organization of the actin cytoskeleton, which might also be the case for *A. niger*.

Responsive Genes to Fenpropimorph—The effect of the morpholine fenpropimorph on *A. niger* or other filamentous fungi so far has not been studied. In yeast, the primary target of morpholines is inhibition of the ergosterol biosynthetic enzymes Erg24p and Erg2p (37). Microarray analyses have shown that the adaptation of *S. cerevisiae* and *C. albicans* to inhibition of ergosterol synthesis involves up-regulation of *ERG24*, *ERG2*, and other *ERG* genes such as *ERG3* and *ERG25* (51, 82). Homologues to all of these genes showed enhanced expression in *A. niger* when treated with fenpropimorph, suggesting that fenpropimorph also targets the ergosterol pathway in filamentous fungi and that the response mechanism to ergosterol biosynthesis inhibition is similar (Fig. 5). Perturbation of ergosterol biogenesis in *A. niger* also affected the expression of genes belonging to related lipid pathways (sphingolipid, phospholipid, and fatty acid metabolism). Overall, the group of up-regulated genes involved in sterol, lipid, and fatty acid metabolism represents the largest category of genes responding to fenpropimorph (44%), suggesting a strong (inter)connection of the different metabolic pathways as also observed in yeast (51–53) and may further point toward a restructuring of the cell membrane as a compensatory response to fenpropimorph.

Lipids are essential components of eukaryotic membranes affecting membrane permeability, fluidity, the activity of membrane-associated proteins, and vesicle targeting and also participate in diverse signal transduction pathways (50, 83). Moreover, sterols and sphingolipids have been observed to form segregated plasma membrane microdomains ("lipid rafts") in organisms from yeast to human (84). The asymmetric distribution of lipid rafts in membranes is thought to provide a platform for signaling proteins such as GPI-anchored proteins and transporters (60) and contribute to polarization events in different yeast such as *S. cerevisiae* and *C. albicans* (85, 86). In *A. nidulans*, it has been reported that inhibition of sphingolipid biosynthesis results in defects of actin polarization and thereby abolishes cell polarity (33, 59). The parallel up-regulation of ergosterol and sphingolipid biosynthesis in *A. niger* could thus point toward a reestablishment of membrane polarization within the adaptation process to fenpropimorph.

In this context, it is interesting to stress the increased expression of An01g10030 (homologue of the *S. cerevisiae* sphinganine hydroxylase Sur2p). The Sur2p product phytosphingosine is thought to stimulate Pkc1p phosphorylation and thereby activation of the CWI pathway in *S. cerevisiae* (60). A further hint for the involvement of the CWI pathway in the adaptive response of *A. niger* to fenpropimorph comes from the observation that a ZIP family zinc transporter (An03g05000 homologous to Yke4p) showed enhanced expression. Yke4p was shown to be strongly up-regulated during cell wall stress via

[5] V. Meyer and A. F. J. Ram, unpublished data.

Pkc1p activity (6, 69). Moreover, expression of the known RlmA target *agsA* was also modulated in response to fenpropimorph treatment, suggesting that cell membrane rearrangements are accompanied by remodeling of the cell wall via the CWI pathway.

Remarkably, An10g00530 (homologous to *S. cerevisiae* inositol-1-P synthase Ino1p) showed also an increased expression. Ino1p catalyzes the conversion of glucose 6-phosphate to myoinositol phosphate, which is the first committed step in the production of all inositol-containing compounds, including inositol phosphates and phosphoinositides (87, 88). Inositol phosphates and phosphoinositides are lipid second messengers necessary for diverse cellular functions and signaling processes in eukaryotes such as transcriptional regulation, mRNA transport, vacuole function, calcium homeostasis, cytoskeletal organization, cell wall biosynthesis, and pseudohyphal growth (89). Moreover, myoinositol phosphate serves as substrate for sphingolipid biosynthesis. The inositol phosphorylceramide synthase catalyzes one of two rate-limiting steps in sphingolipid biosynthesis by transferring myoinositol phosphate to ceramides (60). Cheng *et al.* (59) show that inhibition of inositol phosphorylceramide synthase activity resulted in remodeling of the actin cytoskeleton at the hyphal tips and eventually in the initiation of new branches in *A. nidulans*. Thus, a cellular demand for myoinositol phosphate might further emphasize an important role of sphingolipid metabolism for the adaptation to fenpropimorph.

Common Responses to Both Compounds—As summarized in Fig. 5, a common theme of the response to both caspofungin and fenpropimorph seems to be the induction of the CWI pathway. However, the data deduced from the transcriptional responses point toward varying ways of signal perception and mainly different effector genes. Particularly noteworthy is the *agsA* gene, the expression of which becomes induced not only by both compounds but also by other compounds affecting (directly or indirectly) the integrity of the cell surface (Fig. 3 and Table 5). Therefore, we propose that the *agsA* gene can be considered as marker for the CWI response.

Another related response of *A. niger* to both compounds is increased expression of genes involved in oxidative stress resistance (supplemental Table S2). Caspofungin induced expression of six genes predicted to protect *A. niger* from the toxic effect of oxidative stress (An03g03540/siderophore biosynthesis, An07g03770/Cu-Zn superoxide dismutase, An03g02980/thioredoxin, An04g00150/glutaredoxin, An08g05450, and An08g10600/ABC transporter), whereas fenpropimorph induced expression of three oxidative stress genes (An01g09830/glutathione *S*-transferase, An02g08110/glutathione peroxidase, and An01g12380/ABC transporter). The expression of these genes points toward increased cellular levels of reactive oxygen species (ROS) in response to both compounds. In general, ROS production has been associated with a result of aerobic respiration or with defense mechanisms against pathogen attack (90). However, recent observations mainly gained from studies on plant root hairs (which like filamentous fungi grow in a highly polarized fashion) have shown that the production and localization of ROS are essential for controlling rapid polar growth. Localized ROS production has been shown to be dependent on the activities of Rac-GTPases and NADPH oxidases and is thought to cause nonenzymatic cell wall loosening at the cell tip to allow incorporation of new cell wall building blocks and/or to control calcium influx into the cells (91, 92). Moreover, a dual role has been attributed to the plant GTPase OsRac1. This protein has been reported to act as an inducer of ROS production and as suppressor of ROS scavenging by down-regulating the expression of the metallothionein OsMT2b (93). In this study we also identified a copper-binding metallothionein (An14g00530) down-regulated in response to both caspofungin and fenpropimorph, suggesting that ROS production and scavenging are involved in the morphological response toward these compounds. To the best of our knowledge, an involvement of ROS production in regulation of fungal tip growth has not been reported so far; however, there might be some indications for it. Chen *et al.* (94) show that a dominant activation of the Rho-GTPase Cdc42 in the fungus *Colletotrichum trifolii* results in the production of large amounts of ROS. Moreover, a Cu-Zn superoxide dismutase has been found to be clustered in lipid rafts of *Cryptococcus neoformans* (95). The enhanced expression of ROS-related genes in this study (note that An07g03770 is homologous to Cu-Zn superoxide dismutase) makes it tempting to speculate that a link between ROS production and polar growth of *A. niger* might exist.

CONCLUSIONS

The approach followed in this study allowed the identification of genes not only related to the adaptation of *A. niger* to the antifungal compounds caspofungin and fenpropimorph but also the identification of genes whose functions might be related to the morphogenetic program of *A. niger*. Thus, the genes discovered in this study represent a valuable collection of candidate genes, whose further elucidation will reveal to what extent they actually contribute to the morphogenesis of *A. niger*. The data presented in this work further demonstrate the usefulness of expression profiling to get insights into the mode of action of antifungal compounds and to predict their cellular targets in *A. niger*. Understanding the signaling networks and identifying the responsive target genes make it possible to identify antifungal combinations that might act additively or even synergistically. As such, the prediction of appropriate drug combinations that affect different cellular processes could open new leads in the management of fungal contaminations and infections.

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Survival in the Presence of Antifungals: GENOME-WIDE EXPRESSION PROFILING OF ASPERGILLUS NIGER IN RESPONSE TO SUBLETHAL CONCENTRATIONS OF CASPOFUNGIN AND FENPROPIMORPH

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