Mol Gen Genomics (2005) 273: 33–42
DOI 10.1007/s00438-004-1082-9

ORIGINAL PAPER

R. te Biesebeke · A. Levin · C. Sagt · J. Bartels
T. Goosen · A. Ram · C. van den Hondel · P. Punt

Identification of growth phenotype-related genes in *Aspergillus oryzae* by heterologous macroarray and suppression subtractive hybridization

Received: 11 May 2004 / Accepted: 14 October 2004 / Published online: 28 January 2005
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Abstract *Aspergillus oryzae* requires polarized growth for colonization of solid substrates, and this growth phenotype differs from that seen in liquid medium. Various experimental approaches were used to identify genes that are differentially expressed when *A. oryzae* is grown on wheat kernels and in a wheat-based liquid medium. Hybridization of *A. oryzae* RNAs to a macroarray bearing cDNAs isolated from a library representing at least 16% of the total number of *A. niger* genes identified 14 differentially expressed cDNA clones, showing that heterologous macroarray analysis with an *A. niger* cDNA library can be used to identify regulated gene transcripts in the related species *A. oryzae*. Moreover, Northern analysis with a selection of eight probes for *A. niger* genes encoding proteins involved in morphological development and cell wall biosynthesis identified five more differentially expressed genes. A suppression subtractive hybridization procedure revealed another 12 differentially expressed genes. The results presented show that, of the 29 identified genes which are expressed at higher levels during growth on wheat kernels, six encode proteins that are functionally related to polarized growth, four encode products known to be involved in morphogenesis, three code for proteins related to cell wall composition, and nine of the cDNA clones encode novel proteins. These findings pinpoint genes associated with the changes in cellular morphogenesis seen in *A. oryzae* grown on wheat kernels as opposed to wheat-based liquid medium.

Keywords *Aspergillus* · Macroarray hybridization · Polarized growth · Morphology · Heterologous hybridization

Communicated by C. P. Hollenberg

R. te Biesebeke · A. Levin · C. Sagt · J. Bartels
T. Goosen · C. van den Hondel · P. Punt (✉)
TNO Quality of Life, Utrechtseweg 48, 3700 AJ, Zeist,
The Netherlands
E-mail: p,punt@voeding.tno.nl
Tel.: +31-306-944925
Fax: +31-306-944466

R. te Biesebeke · A. Levin · P. Punt
Wageningen Centre for Food Sciences,
P.O. Box 557, 6700 AN Wageningen, The Netherlands

C. Sagt · T. Goosen · A. Ram · C. van den Hondel
Institute of Biology, Leiden University,
Wassenaarseweg 64, 2333 AL Leiden, The Netherlands

Present address: A. Levin
Faculty of Biology, University of Utrecht,
Padualaan 8, 3534 CH Utrecht, The Netherlands

Present address: C. Sagt
DSM Food Specialities, P.O. Box 1, 2600 MA Delft,
The Netherlands

Present address: J. Bartels
Dyadic Netherlands, Utrechtseweg 48,
3700 AJ Zeist,
The Netherlands

Introduction

Aspergillus oryzae has a fast rate of tip growth (>60 μm/h; Müller et al. 2002) and a high productive capacity with respect to polarized secretion of enzymes (Machida 2002). Owing to these properties *A. oryzae* can easily colonize solid substrates by the extension of existing hyphae and the formation of new branches on pre-existing hyphae (Trinci 1974; Prosner 1994; Dynesen and Nielsen 2003). Nutrients that are essential for growth are liberated from the substrate during invasion by the penetrating hyphae. Oxygen is taken up from the air mainly by the aerial hyphae (Rahardjo et al. 2001). In contrast to the case during growth in liquid media, enzymes are trapped in the solid substrate and nutrients are only liberated in close proximity to growing penetrative hyphae. Due to these differences in the availability of nutrients for growth, the growth phenotype of *A. oryzae* on solid substrates clearly differs from that in liquid medium. Comparisons of the transcript profiles

expressed under these different cultivation conditions will allow the identification of genes associated with these differences in the morphology of growing *A. oryzae* cells.

The growth phenotype is the result of a number of cellular processes, including polarized growth, cell differentiation, signal transduction, cell wall synthesis, polarized secretion and maintenance of cell shape, that are controlled by finely tuned molecular mechanisms that involve many gene products (Seiler and Plamann 2003). Previous studies of *A. oryzae* have identified genes for proteins involved in substrate degradation, metabolism, transport and cell wall synthesis associated with surface growth (Akao et al. 2002; te Biesebeke et al. 2004). Since little is known about the microbial growth phenotype during solid-state fermentation (te Biesebeke et al. 2002; Holker et al. 2004), we were interested in identifying genes associated with the growth of *A. oryzae* on a solid substrate. We have used a number of different experimental approaches, including macroarray hybridization, to identify genes expressed differentially in *A. oryzae* grown on wheat kernels and in a wheat-based liquid medium. One of these approaches involved the use of a cDNA library from *A. niger* to perform a heterologous macroarray analysis, since no *A. oryzae* cDNA library was available.

Materials and methods

Strains and media

Aspergillus oryzae ATCC16868 was used in this study. Solid-state fermentation (SSF) was performed using Ritmo wheat kernels (ACM, Meppel, The Netherlands) pretreated as described by Hoogschagen et al. (2001). A 50-g sample of pretreated wheat kernels was inoculated with 2 ml of a conidial suspension (5×10^7 conidia/ml, giving a starting density of 1.0×10^6 conidia/g of substrate) in a sterile 1-l bottle, which was placed on a roller apparatus (Wheaton Modular Roller; Wheaton Science Products, Millville, N.J.) and rotated at maximum speed for 6 h at room temperature. The inoculated wheat kernels were then transferred to sterile petri dishes and incubated for 2, 2.5 or 3 days at 30°C and 98% humidity in a climate incubator (VEA-Instruments, Houten, The Netherlands). Wheat-based Liquid Medium (WLM; 2%) was prepared as described by te Biesebeke et al. (2004). Submerged fermentations (smF) were performed using 2%WLM inoculated with 1×10^5 conidia/ml medium and incubated for 24, 30 and 35 h at 30°C on a rotary shaker (250 rpm).

Purification of total RNA

Total RNA from *A. oryzae* grown on wheat kernels and in 2%WLM was isolated as described by te Biesebeke et al. (2004).

Synthesis of ^{32}P labeled first-strand cDNA

First-strand cDNAs (probes) were synthesized from isolated total RNA with Superscript II H^- Reverse Transcriptase (Invitrogen, Carlsbad, Calif.) according to a modification of the manufacturer's protocol. An aliquot (25–30 µg) of total RNA was mixed with 1 µl of $(dT)_{15}$ mix (containing $(dT)_{15}$, $(dT)_{15}$ A, $(dT)_{15}$ G and $(dT)_{15}$ C, each at 1 mg/ml) in 11 µl of distilled water. The mixture was heated to 70°C for 10 min and cooled on ice. On ice, 3 µl of (lowC)dNTP mix (containing 2.5 mM dATP, 2.5 mM dGTP, 2.5 mM dTTP and 16.6 µM dCTP) and 6 µl of 5× first-strand buffer was added. The reaction mixture was incubated for 2 h at 43°C after the addition of 5 µl of [α ^{32}P]dCTP (~50 μCi) and 2 µl of Superscript II enzyme. Then RNase was added (to a final concentration of 10 mg/ml), and incubation continued for 15 min at 37°C. Probes were purified by passage over Sephadex G50 columns (Amersham Biosciences, Little Chalfont, Bucks., UK), denatured for 2 min at 95°C and used for hybridization.

DNA macroarray

The library of *Aspergillus niger* cDNAs used here was constructed in the yeast/ *Escherichia coli* shuttle vector pEMBLyex4 (Goldman et al. 1992) as described by Veldhuisen et al. (1997). Transformed *E. coli* DH5α (Sambrook et al. 1989) colonies containing the *A. niger* cDNAs (Veldhuisen et al. 1997) were picked at random from selective L-medium plates and distributed into twelve 384-well plates containing L-medium and ampicillin (100 µg/ml) and grown overnight at 30°C. In all, 4608 cDNA clones were inoculated onto a Hybond-N+ membrane with a 96-pin tool containing array spotting robot from Engineering Services Inc. (Toronto, Ont., Canada). The membrane was placed on an OMNI-TRAY (Nunc, Wiesbaden, Germany) containing 50 ml of selective L-medium agar and incubated overnight at 30°C. The resulting colonies were lysed by alkaline lysis using a modification of the method of Clak et al. (1999). Membranes were placed on a sheet of Whatman 3MM paper moistened with 0.5 M NaOH/1.5 M NaCl for 4 min at room temperature, followed by a 4-min incubation over a steaming (85°C) water bath. The membranes were neutralized for 4 min with 1 M TRIS-HCl (pH 7.5)/1.5 M NaCl at room temperature, and transferred to a buffer containing 50 mM TRIS-HCl (pH 7.5), 50 mM EDTA, 100 mM NaCl, 1% Sarkosyl and 250 µl/ml Proteinase K, and incubated for 20 min at 37°C. The cDNAs released from the cells were covalently bound to the membrane by exposure to a UV Crosslinker (Stratagene, La Jolla, Calif.). After hybridization with ^{32}P labeled first-strand cDNA at 60°C in phosphate-buffered hybridization solution (20% SDS, 200 mM NaCl, 5% BSA, pH 7), the membranes were washed twice at 65°C with 2×SSC and 0.5% SDS (Sambrook et al. 1989) for 30 min, and twice at 65°C

with 0.2×SSC and 0.5% SDS for 1 h. The signals on the membranes were visualized after exposure to a phosphoimager screen for 5, 10, 15, 20, 25 and 30 min using the Cyclone (Packard Instruments Division, Applied Biosystems, Foster City, Calif.) storage phosphoimager system and the ImaGene 4.2 software from BioDiscovery (Marina del Rey, Calif.). The membranes that showed equal signal intensities for *A. niger gpdA* cDNAs at 50% of the maximum spot intensity as quantified with the ImaGene 4.2 software were compared. *A. niger* cDNAs that showed differential hybridization intensities (>50% difference) with probes synthesized from total RNA isolated from *A. oryzae* grown on wheat kernels and in 2%WLM were selected for further analysis.

Spot-blot analysis

The cDNA clones selected from the macroarray analysis were isolated with the Miniprep plasmid isolation kit (Qiagen, Hilden, Germany), and a 2-µl aliquot of each plasmid preparation was spotted onto Hybond-N+ membranes and covalently bound to the membranes by irradiation with the UV-cross-linker (Stratagene). The membranes were subsequently used for hybridization experiments (under the same conditions as used for the DNA macroarray) with ^{32}P labeled first-strand cDNA. The blots were exposed to X-OMAT AR films (Kodak, Rochester, N.Y.) for 2–48 h. The developed films were scanned with the Hewlett Packard 6200C Scanjet at 600 dpi. Films showing the *gpdA* signals equivalent to 50% of the maximum spot intensity, as quantified with Gene Tools from Syngene (Synoptics, Cambridge, UK), were used for the analysis of differential transcription. The signal intensity of the *gpdA* spot was set at 100 and the signal intensities of the other spots on the same blot were normalized with respect to the *gpdA* signal. Furthermore, ratios of spot intensities of cDNAs were determined. The ratio smF for a given cDNA was obtained by dividing the signal intensity obtained with a probe from *A. oryzae* grown in 2%WLM by that observed after hybridization with a probe originating from *A. oryzae* grown on wheat kernels. The ratio SSF is the converse ratio.

Construction of a subtraction cDNA library

cDNA was synthesized from total RNA with the SMART PCR cDNA synthesis Kit (Clontech, Palo Alto, Calif.) according to the supplier's protocol. Starting from two populations of total RNA obtained from *A. oryzae*, a cDNA subtraction library was constructed using the PCR-select cDNA subtraction kit (Clontech), which is based on the suppression subtractive hybridization method of Diatchenko et al. (1996), according to the supplier's protocol. For the forward subtraction protocol cDNA produced from total RNA isolated from *A. oryzae* grown for 3 days on wheat kernels was used as the tester together with driver cDNA produced from total RNA from *A. oryzae* grown for 30 h in 2%WLM. The forward subtraction protocol is designed to enrich for cDNAs derived from transcripts that are expressed at higher levels in *A. oryzae* grown on wheat kernels. The cDNAs were cloned in pGemT-easy (Promega, Madison, Wis.) and used to transform *E. coli* DH5α (Sambrook et al. 1989). Individual *A. oryzae* cDNA clones (380) were randomly picked from selective L-medium agar plates, and four individual *A. niger gpdA* cDNA clones were taken from the ordered *A. niger* cDNA library. These clones were transferred to a 384-well plate containing selective L-medium and grown overnight at 37°C. The 384 cDNA clones were then inoculated manually onto a Hybond-N+ membrane, incubated overnight at 30°C, and lysed as described above. Hybridization with ^{32}P labeled first-strand cDNA and washing of the membranes was performed as for the macroarray membranes, except that both steps were carried out at 68°C. The signal intensities of the spots were quantified and compared after equalizing the signal intensities of the *A. niger gpdA* cDNAs on the different membranes as described earlier. In all, 79 of the cDNAs showed detectable hybridization with ^{32}P-labeled probes. cDNAs that showed three-fold higher hybridization with probes originating from *A. oryzae* grown on wheat kernels compared to probes originating from *A. oryzae* grown in 2%WLM were selected for further analysis.

PCR and DNA sequencing

PCR was used to amplify the cDNA inserts from the clones. PCR was performed with the primers MBL1589 (5′-GGATCAATTCGAGCTCGGTA-3′) and MBL1588 (5′-ATTGGATCATAAGCTTGC-3′) for the *A. niger* cDNA clones, and the primers M13F (5′-GTAAAACGACGGCCAG-3′) and M13R (5′-CAGGAAACAGCTATGAC-3′) for the *A. oryzae* cDNA clones, using 40 cycles of 30 s at 94°C, 1 min at 45°C and 30 s at 72°C. Probes for the cDNA clones were purified from 1% agarose gels after electrophoresis, using Qiaquick DNAeasy columns (Qiagen). Sequencing was performed with primer MBL1589 or primer MBL1588 to obtain the *A. niger* cDNA sequences or with primer M13F or primer M13R to obtain the *A. oryzae* cDNA sequences. The Cycle Sequencing kit from Amersham Pharmacia was used according to the manufacturer's protocol, and sequences were determined using the ABI Prism 310 Genetic Analyzer from Applied Biosystems (Perkin-Elmer Division). The sequences obtained were used for Blast searches in the databases at NCBI (http://www.ncbi.nlm.nih.gov), the *A. oryzae* EST site (http://www.nrib.go.jp/ken/EST/db/blast.html), the Broad Institute (http://www.broad.mit.edu/annotation/fungi/fgi) or the *Saccharomyces* Genome Database (http://www.yeastgenome.org/). Database Accession Nos. were obtained after submission of the sequences to the

EMBL-EBI (http://www.ebi.ac.uk/Submissions/) or NCBI database.

Northern analysis

Total RNA (10 μg) was fractionated by electrophoresis on formaldehyde-agarose (1%) gels, and transferred to Hybond-N+ membranes (Amersham Bioscience). The fractionated RNA was hybridized with at 60°C (in the hybridization and wash buffers used for the macroarray analysis) with [α ^{32}P]dCTP (~50 μCi)-labeled probes, synthesized using the Random Prime labeling kit (Amersham Pharmacia). Blots were exposed to X-OMAT AR film and the exposed films were scanned as described above. The signal intensities were quantified with Gene Tools from Syngene (Synoptics) after adjustment to the *gpdA* signal intensity and the ratio smF and ratio SSF were determined as described above.

Probes for *A. niger* genes

Fragments of the genes for α-1,3-glucan synthases (*agsA, agsB*) and β-1,3-glucan synthase (*fksA*) were isolated by PCR using degenerate primers that were based on conserved amino acid sequences (R. A. Damveld and A. F. J. Ram, unpublished). Fragments of putative MAP kinase genes were isolated by PCR using degenerate primers based on conserved amino acid sequences of the HOG family of MAP kinases (*hogA* and *hogB*) (M. Arentshorst and A. F. J. Ram, unpublished). Fragments of two genes for Rho-related small GTPases were isolated using degenerate primers based on conserved amino acid sequences (*racA*, Punt et al. 2001; *cftA*, A. F. J. Ram, unpublished). A gene encoding a putative GPI-anchored cell wall mannoprotein was identified in a collection of *A. niger* ESTs deposited in GenBank (Acc. No. BE759683, Tsang and Storm, unpublished). Primers based on this sequence were used to clone a fragment of this gene (*cwpA*, R. A. Damveld and A. F. J. Ram, unpublished). All DNA fragments were amplified from *A. niger* genomic DNA, with the exception of *cftA* and *cwpA*, which were isolated from the *A. niger* cDNA library (Veldhuisen et al. 1997). All the DNA fragments were cloned in pGemT easy (Promega) and sequenced to confirm their identity.

Results

Macroarray analysis

To determine the complexity of an ordered collection of 4608 *E. coli* DH5α clones containing *A. niger* cDNAs (Veldhuisen et al. 1997), 50 of the clones were randomly chosen and their inserts were sequenced. Analysis of the sequencing data revealed that 29 cDNA sequences appeared only once among the 50 cDNA clones. Of cDNA sequences that were present more than once, six cDNAs corresponded to *A. niger* ribosomal RNA and two to the *A. niger gpdA* gene. Although these results indicate that the library includes a number of redundant clones, they imply that it contains 2950 (±600, for a confidence interval of 95%) unique cDNAs. More than 14,000 ORFs have been identified during the *Aspergillus niger* Genome Sequencing Project (http://www.aspergillus.man.ac.uk), suggesting that our collection represents 16–25% of the protein-coding genes present in *A. niger*. We considered this amount sufficient to justify its use as a heterologous macroarray for the identification of growth phenotype-specific genes in *A. oryzae*.

The 4608 *A. niger* cDNAs were spotted on several Hybond-N+ membranes. To further evaluate the complexity of the cDNA library, probes for two highly expressed genes, the *A. niger gpdA* and *bipA* genes, were used to screen the cDNAs. Of the 4608 cDNAs screened, 4% (188 cDNAs) hybridized with the *gpdA* probe and 0.3% (13 cDNAs) with the *bipA* probe, confirming the presence of a fraction of redundant clones in the library.

The membranes were then used to identify differentially expressed genes in *A. oryzae* grown for 3 days on wheat kernels and for 30 h in 2%WLM. cDNA robes synthesized from the total RNA of *A. oryzae* grown under these two conditions were hybridized to the *A. niger* library on the membranes. In *A. oryzae* housekeeping genes, like the glyceraldehyde-3-phosphate dehydrogenase encoding gene *gpdA*, are among the most highly expressed genes (Machida 2002). Since the transcript levels of the *gpdA* gene of *A. oryzae* grown on different carbon sources are similar (Nakajima et al. 2000), the intensity of the signals for *A. niger gpdA* clones was used to adjust spot intensities on the different membranes. Furthermore, the *A. oryzae gpdA* cDNA (AB032274) shows 88% identity to the nucleotide sequence of the *A. niger gpdA* gene (X99652), which guarantees hybridization under the chosen conditions. Based on inspection of the membrane images, 46 *A. niger* cDNA clones were selected, of which 34 showed a >50% higher signal after hybridization with cDNA probes from *A. oryzae* grown on wheat kernels than with cDNA from liquid cultures. The other 12 clones showed >50% higher spot intensities after hybridization with cDNA probes from *A. oryzae* grown in 2%WLM than with probes from solid-state cultures.

Spot-blot, Northern and sequence analyses

To determine if the 46 selected *A. niger* cDNAs also hybridized differentially with probes originating from *A. oryzae* grown on wheat kernels and in 2%WLM at different time points during culture, the selected clones and a *gpdA* cDNA clone were spotted on Hybond-N+ membranes. The spot blots were then hybridized with probes synthesized from total RNA isolated from *A. oryzae* grown for 2, 2.5 and 3 days on wheat kernels

or for 24, 30 and 35 h in 2%WLM. Comparison of the signal intensities showed that, of the 46 selected cDNAs, 15 cDNAs showed about equal signal intensities and 17 cDNAs showed differences in signal intensities, but the patterns were inconsistent when the various signal intensities were compared over the time course. Of the remaining 14 cDNAs, 12 showed higher signal intensities with all probes from *A. oryzae* grown on wheat kernels and two cDNAs showed higher signal intensities with probes from cultures grown in 2%WLM. To verify the results obtained for the macroarray and spot-blot analysis, Northern hybridization analysis was performed with total RNA from *A. oryzae* grown on wheat kernels for 3 days or in 2%WLM for 30 h, using the 14 identified cDNAs from *A. niger* as probes. The results presented in Fig. 1A are in agreement with the results obtained with the macroarray and the spot-blot analysis. These 14 cDNAs were sequenced and subjected to BLAST analysis. The results are presented in Table 1. Northern analysis with the probes for the 4,5-dihydroxyphthalate decarboxylase (SSF10)- and the DNAJ domain (SSF12)-encoding genes (Fig. 1A) detected two bands of different sizes indicating that these genes express different mRNA products.

Northern analysis with probes for genes encoding proteins involved in cell morphology and cell wall biosynthesis

The growth phenotype of *A. oryzae* on wheat kernels differs markedly from that of cultures grown in 2%WLM. To gain more insight into the type of genes that are expressed in each situation we performed a heterologous Northern analysis with a selection of probes prepared from genes previously isolated (Table 2). Probes for genes encoding MAP kinases (*hogA* and *hogB*) and two Rho-related small GTPases (*cftA* and *rac* A) were selected, as their protein products are assumed to be involved in morphological development of *A. oryzae*. Probes for the genes encoding 1,3-*β*-glucan synthase (*fksA*), a cell wall mannoprotein (*cwpA*),and two α-glucan synthases (*agsA* and *agsB*) are involved in the maintenance of cell wall integrity. The labeled probes were hybridized to Northern blots loaded with total RNA from *A. oryzae* grown for 3 days on wheat kernels or for 30 h in 2%WLM. The results are shown in Fig. 1B. Transcripts of the genes *hogA*, *fksA*, *agsA* and *cftA* could only be detected in *A. oryzae* grown on wheat kernels, and the level of the *cwpA* transcript was higher in *A. oryzae* grown on wheat kernels. The transcripts of the *racA*, *hogB* , and *agsB* genes were below the limit of detection (data not shown).

Differentially transcribed *A. oryzae* genes

Northern analysis of total RNA from *A. oryzae* with selected cDNA probes from *A. niger* (Fig. 1) revealed differences in hybridization. It seems probable that these differences reflect differential expression of the corresponding genes in *A. oryzae*. To support this, the nucleotide sequences of the *A. niger* cDNAs were used in a homology search (BLASTN) of an *A. oryzae* EST library (http://www.nrib.go.jp/ken/EST/db/blast.html). The results presented in Tables 1 and 2 show that in about 50% of the cases the *A. niger* cDNAs have sequences similar to an EST sequence from *A. oryzae*. Based on the level of sequence identity between the *A. niger* cDNA and the *A. oryzae* EST, hybridization of these sequences is indeed expected under the conditions used in our screens (Howley et al. 1979).

Suppression Subtraction Hybridization

We also used a different strategy to identify differentially expressed genes associated with the growth phenotype of *A. oryzae*. In this second approach, a cDNA library was constructed that was enriched for cDNAs synthesized from total RNA isolated from *A. oryzae* grown for

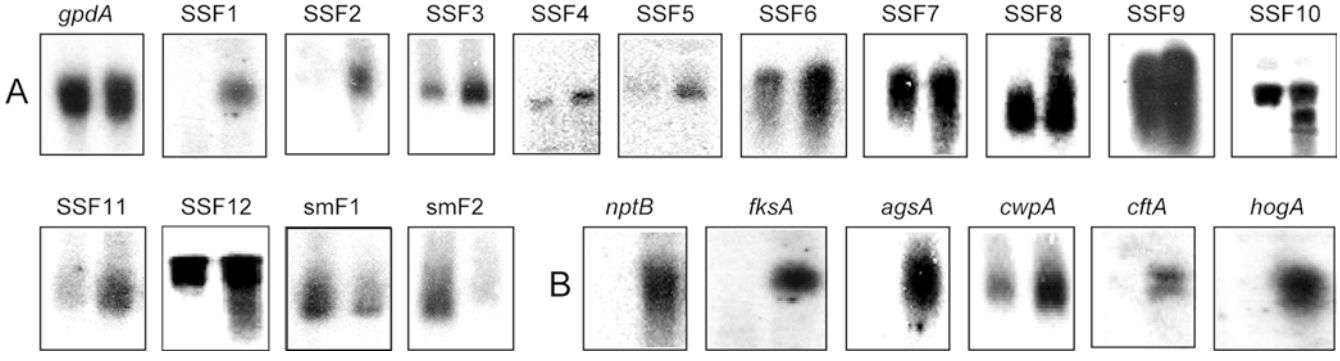

Fig. 1 Differential hybridization of *A. niger* probes with total RNA from *A. oryzae* grown for 3 days on wheat kernels (*right lane* in each panel) or for 30 h in 2%WLM (*left lane* in each panel). **A** Northern analysis with probes for *A. niger* cDNAs (SSF1–12, smF1, smF2). A probe for the *A. niger* glyceraldehyde-3-phosphate dehydrogenase gene was used as a reference. **B** Northern analysis with probes for the *A. niger* genes for beta 1,3-glucan synthase (*fksA*), alpha 1,3-glucan synthase (*agsA*), cell wall mannoprotein (*cwpA*), Cdc42 rho-related small GTPase (*cftA*) and HOG-family MAP kinase (*hogA*). The probe for the *A. oryzae nptB* gene, which was known to be differentially expressed under these conditions (te Biesebeke et al. 2004), was included as a reference

Table 1 Identified *A. niger*cDNAs that were selected in the heterologous macroarray analysis

Name	Length (bp)	Acc. No.	Best BLAST hit[a]	Degree of identity[b]	Best BLAST hit among proteins of known function[c]	E-value	SSF[d]	smF[d]	ESTs (*A. oryzae*)[e]	BLASTN
gpdA	349	CAA67966	Glyceraldehyde-3-phosphate dehydrogenase CAA67966 (*A. niger*)	110/110 (100%)	Glyceraldehyde-3-phosphate dehydrogenase CAA67966 (*A. niger*)	0.0	1	1	-	-
SSF1	540	CK769166	Hypothetical protein EAA48840 (*Magnaporthe grisea*)	58/88 (65%)	DNA helicase AAA7040.1 (*Homo sapiens*)	e^{-100}	>5	-	-	-
SSF2	508	CK769167	Hypothetical protein EAA63566 (*A. nidulans*)	31/51 (60%)	Actin cytoskeleton organization and biogenesis PIN3 protein NP595286.1 (*Saccharomyces cerevisiae*)	$4e^{-7}$	>5	-	00576	68/76
SSF3	311	CK769168	Unknown gene	-	-	-	1.7	-	02571	219/225
SSF4	329	CK769169	Unknown gene	-	-	-	2	-	-	-
SSF5	260	CK769170	Unknown gene	-	-	-	2.5	-	-	-
SSF6	233	CK769171	Hypothetical protein EAA62187.1 (*A. nidulans*)	44/46 (95%)	Putative senescence associated protein BAB33421.1 (*Pisum sativum*)	$1e^{-4}$	3	-	02787	271/275
SSF7	333	AJ632136	Hypothetical protein EAA60158.1 (*A. nidulans*)	78/135 (58%)	40S ribosomal protein CAA21965 (*Candida albicans*)	$6e^{-97}$	1.5	-	03131 and 00803	127/158 and 127/158
SSF8	469	AJ632137	Hypothetical protein EAA 56294.1 (*M. grisea*)	29/50 (58%)	Prephenate dehydratase ZP00065118 (*Microbulfer degradans*)	$1e^{-24}$	1.6	-	-	-
SSF9	304	AJ627189	Hypothetical protein EAA66371.1 (*A. nidulans*)	54/72 (75%)	Eukaryotic translation elongation factor 1 BAB64568 (*Carassius auratus*)	$5e^{-36}$	1.5	-	-	-
SSF10	455	AJ632138	4,5-Dihydroxyphthalate decarboxylase BBA03974.1 (*Comamonas testosterone*)	34/102 (53%)	4,5-dihydroxyphthalate decarboxylase BBA03974.1 (*C. testosterone*)	0.0	>5	-	-	-
SSF11	304	AJ632139	orf403 NP 074914 (*Podospora anserina*)	55/92 (59%)	Endonuclease NP 570153.1 (*Hypocrea jecorina*)	$1e^{-88}$	1.5	-	-	-
SSF12	483	AJ632140	Hypothetical protein XP 322593.1 (*Neurospora crassa*)	52/87 (59%)	Tail-anchored ER membrane protein (similar to DnaJ) Hlj1p. NP 013884 (*S. cerevisiae*)	$1e^{-19}$	1.8	-	-	-
smF1	647	CK769172	Unknown gene	-	-	-	-	3	-	-
smF2	522	CK769173	Hypothetical protein EAA65208.1 (*A. nidulans*)	72/87 (83%)	Unknown protein		-	>5	03162 and 00225	181/220 and 145/173

[a]Best result obtained by BLASTX analysis using the cDNA sequence as a query in the nr database at NCBI
[b]Number of (percentage) amino acid sequence identities to the best BLAST hit in the segment compared
[c]In cases where a hypothetical protein emerged as the best BLAST hit, the full-length hypothetical protein was used as the query in a BLASTP search to determine its best hit. The e-value refers to this comparison
[d]SSF is the ratio of the expression level under solid-state growth conditions to that in liquid medium; smF is the converse ratio. These data are based on the results of Northern analysis (see Materials and methods)
[e]Accession No(s). of homologous EST(s) in the *A. oryzae* EST database (http://www.nrib.go.jp/ken/EST/db/blast.html)
[f]Nucleotide sequence identities to EST(s) from *A. oryzae*, based on BLASTN analysis

Table 2 *A. niger* cDNAs used as probes for heterologous Northern analysis of *A. oryzae* RNA

Gene[a]	Length of cDNA (bp)	Acc. No.	Gene ID	SSFb	SmFb	EST (*A. oryzae*) [c]	BLASTN[d]
fksA	365	AY533027	Beta 1,3-glucan synthase	>5	-	01813	185/218
agsA	678	AY530786	Alpha 1,3-glucan synthase	>5	-	06191	40/45
agsB	762	AY530789	Alpha 1,3-glucan synthase	-	-	-	-
cwpA	1155	AY540627	Cell wall mannoprotein	3	-	02990	129/132
cftA	756	AY540628	Rho-related small GTPases	>5	-	01954	278/340
racA	968	AY540629	Rho-related small GTPases	-	-	01170	77/86
hogA	547	AY540624	Hog family of MAP kinases	>5	-	04179	169/196
hogB	509	AY540625	Hog family of MAP kinases	-	-	-	-

[a]All *A. niger* cDNA and DNA fragment sequences were used in a BLASTN search of the *A. oryzae* EST database (http://www.nrib.go.jp/ken/EST/db/blast.html). The probes were chosen on the basis of sequence identity to *A. oryzae* ESTs indicating that cross-hybridization of these sequences can be expected under our experimental conditions (Howley et al. 1979)

[b]The ratios SSF and smF were calculated from the Northern analysis results as described in Materials and methods

[c]Accession No. of *A. oryzae* EST in the *A. oryzae* EST database (see Table 1)

[d]Degree of nucleotide sequence identity between *A. niger* cDNA/DNA and *A. oryzae* EST

3 days on wheat kernels by using the suppression subtractive hybridization method (Diatchenko et al. 1996) with cDNAs synthesized from *A. oryzae* grown in 2%WLM for 30 h. The method combines subtraction and normalization in a single procedure. The *A. oryzae* subtraction cDNA library, consisting of 380 *A. oryzae* cDNA clones, was spotted together with four *A. niger gpdA* cDNA clones onto several Hybond-N+ membranes. A control hybridization with the labeled *A. niger gpdA* probe showed that only the four *A. niger* cDNAs hybridized, suggesting that subtraction of the *A. oryzae gpdA* cDNAs had been successful. Hybridization of the membranes with probes synthesized from total RNA isolated from *A. oryzae* grown for 3 days on wheat kernels or for 30 h in 2%WLM revealed 79 spots. To identify genes that were most relevant for the difference in growth phenotype, 12 spots with an SSF ratio of more than 3 were selected, and the corresponding cDNAs were sequenced. The results are presented in Table 3.

Discussion

The growth phenotype of *A. oryzae* during culture on wheat kernels differs from that adopted during growth in 2%WLM, suggesting a difference in cellular morphogenesis. Our results show that, of the genes of known function that were more highly expressed during growth on wheat kernels, about half encode proteins are involved in determining cell morphology or in related processes.

During germination of spores, determination of the site of polarization is one of the first events in hyphal growth. Thereafter various events regulate, determine and maintain polarized cell growth and cell shape. These events include signal transduction involving cAMP and MAP kinase pathways, the re-organization of the actin cytoskeleton, cell wall formation and polarized secretion (see Seiler and Plamann 2003, and references therein). The maintenance of a highly polarized actin cytoskeleton is required to direct polarized growth (Cali et al. 1998; Bretscher 2003; Seiler and Plamann 2003). Interestingly, six of the products identified as showing enhanced expression during growth on wheat kernels in our analyses are related to, or involved in, the organization of the actin cytoskeleton and polarized growth. The identification of the *A. oryzae* homologues (SSF16 and SSF17; Table 3) of the *S. cerevisiae* genes *SAC6* and *TPM2* suggests that the morphological differences between *A. oryzae* cells grown on wheat kernels and those grown in 2%WLM could be a consequence of a difference in actin cytoskeleton organization. Sac6 is also known as fimbrin, a calponin (Ca2+-binding) domain-containing protein involved in bundling actin filaments (Goodman et al. 2003) and Tpm2 is one of the two tropomyosins in *S. cerevisiae* required for the formation and stability of actin cables in vivo (Korman and Tobacman 1999). A change in the organization of the cytoskeleton during solid-state growth was further corroborated by the identification of a homologue of the *S. pombe* gene *mok1+* (*Aspergillus agsA*; Table 2), which codes for an α-glucan synthase involved in morphogenesis (Hochstenbach et al. 1998). This protein requires the actin cytoskeleton for localization of the sites of growth (Katayama et al. 1999). An *A. oryzae* homologue of *PIN3* (SSF2; Table 1) was also recovered. The product of this *S. cerevisiae* gene is suggested to be involved in actin cytoskeleton organization and biogenesis (Goffeau et al. 1997; Madania et al. 1999). In agreement with the macroscopically observed difference in growth phenotype, the differential expression of the *A. nidulans hogA* and *CDC42* homologues (*Aspergillus hogA* and *cftA*; Table 2) suggests a difference in polarized growth between *A. oryzae* grown on wheat kernels and in 2%WLM. The *A. nidulans hogA* gene product is a MAP kinase involved in the maintenance of polar growth (Han and Prade 2002). A *cdc-42* mutant of *Neurospora crassa* was found to perturbed in cell polarization during growth (Seiler and Plamann 2003), which is compatible with the finding that the Rho-

Table 3 Identity of the *Aspergillus oryzae* cDNAs selected from the cDNA subtraction library

Clone name	Length (bp)	Acc. No.	Best BLAST hit[a]	Degree of identity (%) [b]	Best BLAST hit among proteins of known function[c]	E-value	SSF[d]	EST (*A. oryzae*) [e]
SSF13	208	CV066918	Hypothetical protein EAA59495.1 (*Aspergillus nidulans*)	59/61 (96%)	COPI-coated vesicle related protein YJL123C (*Saccharomyces cerevisiae*)	2e-19	> 3	00954
SSF14	370	CV066919	Hypothetical protein EAA53697.1 (*Magnaporthe grisea*)	38/79 (48%)	CsgA protein AAA25391.2 (*Myxococcus xanthus*)	3e-29	> 3	-
SSF15	571	CV066920	Hydrophobin CoH1 CAA71652.1 (*Coprinopsis cinerea*)	44/101 (43%)	Hydrophobin CoH1 CAA71652.1 (*C. cinerea*)	0.0	> 3	02217
SSF16	323	CV066921	Ca2+-binding actin-bundling protein (fimbrin) NP_990678.1 (*Gallus gallus*)	45/78 (57%)	SAC6 fimbrin homolog (*S. cerevisiae*)	2e-113	> 3	02824
SSF17	322	CV066922	Hypothetical protein XP_409823.1 (*A. nidulans*)	82/106 (77%)	TPM2 tropomyosin (*S. cerevisiae*)	3e-25	> 3	02851
SSF18	199	CV066923	Septin B AAB41233.1 (*A. nidulans*)	65/66 (98%)	Septin B AAB41233.1 (*A. nidulans*)	0.0	> 3	07110
SSF19	296	CV066924	Hypothetical protein AN5422.2 (*A. nidulans*)	28/33 (84%)	Putative esterase NP_945813.1 (*Rhodopseudomonas palustris*)	3e-48	> 3	01498
SSF20	416	CV066925	Hypothetical protein EAA54143.1 (*M. grisea*)	89/112 (79%)	Cyclopentanone 1,2-monooxygenase CAD10798 (*Comamonas testosteroni*)	7e-79	> 3	04908
SSF21	280	CV066926	Hypothetical protein XP_383324.1 (*Gibberella zeae*)	40/62 (64%)	Unknown protein	-	> 3	02544
SSF22	300	CV066927	Unknown	-	Unknown protein	-	> 3	-
SSF23	344	CV066928	Hypothetical protein XP_411922.1 (*A. nidulans*)	156/226 (69%)	Unknown protein	-	> 3	03077
SSF24	346	CV066929	Hypothetical protein XP_406323.1 (*A. nidulans*)	44/81 (54%)	Unknown protein	-	> 3	06676

[a]Best result obtained by BLASTX analysis using the cDNA sequence as a query in the nr database at NCBI
[b]Number of (percentage) amino acid sequence identities to the best BLAST hit in the segment compared
[c]In cases where a hypothetical protein emerged as the best BLAST hit, the full-length hypothetical protein was used as the query in a BLASTP search to determine its best hit. The e-value refers to this comparison
[d]The SSF ratio was calculated from the results of spotblot analysis as described in Materials and methods
[e]Accession No(s). of homologous EST(s) in the *A. oryzae* EST database (http://www.nrib.go.jp/ken/EST/db/blast.html)

family GTP-binding protein Cdc42 and several actin-binding proteins direct the assembly of actin (Stamnes 2002) and thus control microtubular dynamics.

The actin cytoskeleton also plays an important role in the intracellular distribution of several organelles (e.g. vesicles) and in polarized secretion of proteins (Stamnes 2002; Bretscher 2003). Therefore, the suggested difference in the organization of the actin cytoskeleton during growth of *A. oryzae* on wheat kernels and in 2%WLM could have an impact on the polarized secretion of proteins. In this context it is interesting to note that a homologue of the *S. cerevisiae* ORF *yjl123c* was identified as being differentially expressed in in *A. oryzae* (SSF13, Table 1). The product of ORF *yjl123c* gene has been localized to the COPI-coated vesicles (Huh et al. 2003) and is therefore suggested to be involved in Golgi to endoplasmic reticulum (retrograde) vesicle transport, and possibly in intra-Golgi transport. The correlation between the suggested polarized organization of the actin cytoskeleton and the protein secretion capacity of *A. oryzae* needs to be elucidated further. However, both amylase and protease activities were more than 10 times higher in extracts of *A. oryzae* grown for 2 days on wheat kernels (expressed in U per g per h) than those measured (in U per ml per h) in the growth medium after 24 h growth in 2%WLM (R. te Biesebeke, unpublished results).

In addition to the previously identified *brlA* gene (te Biesebeke et al. 2002), which codes for a zinc-finger transcription factor that induces sporulation (Timberlake 1991), other *A. oryzae* gene homologues encoding proteins suggested to be involved in differentiation and/or morphogenesis were identified as induced under solid-state growth conditions. The *A. nidulans aspB* homologue of *A. oryzae* SSF18 encodes a septin involved in cell division and the organization of branching and conidiation (Westfall and Momany 2002). The *Myxococcus xanthus csgA* homologue of *A. oryzae* SSF14 encodes a C-signal cell surface protein (Hagen and Shimkets 1990) with conserved residues in the NAD(P)+ binding pocket domain and a short-chain alcohol dehydrogenase (SCAD) catalytic domain (Lee et al. 1995). The C-signal protein is involved in the co-ordination of multicellular fruiting body morphogenesis and sporulation in *M. xanthus* (Kruse et al. 2001; Li and Shimkets 1992). The *Coprinopsis cinerea* CoH1 homologue of *Aspergillus oryzae* SSF15 encodes a hydrophobin (Table 3) containing eight conserved cysteine residues that allow formation of four disulphide bridges that are essential for hydrophobin I function (Wosten and de Vocht 2000). Self-assembling hydrophobins are involved in the formation of aerial structures and in the attachment of hyphae to hydrophobic surfaces, while monomeric hydrophobins have been implicated in cell-wall assembly (Wosten 2003).

The probes for the *A. niger agsA, fksA* and *cwpA* genes (Table 2) detected higher levels of transcripts in *A. oryzae* grown for 3 days on wheat kernels. These results may reflect differences in cell wall composition relative to cells grown for 30 h in 2%WLM.

The results presented in Tables 1 and 3 are similar to those of a study by Akao et al. (2000) in showing that about half of the differentially expressed genes are novel genes. The number of novel genes identified in this study reflects the potential of the solid-state growth system for the identification of novel protein functions in *A. oryzae* that might be involved in the control of cellular morphogenesis.

Traditionally, macroarray analysis is performed with cDNA libraries constructed from nucleic acids derived from the investigated species. However, in many cases where such homologous libraries are not available, it would be desirable to utilize heterologous cDNA libraries. We have shown here that genes that are differentially expressed in *A. oryzae* can be identified using an ordered *A. niger* cDNA library. Therefore, the availability of heterologous macroarray analysis for the identification of differentially expressed fungal genes should allow genome-wide expression analysis of fungal species for which cDNA libraries are not available.

Acknowledgements This work was supported by the Wageningen Centre for Food Sciences (WCFS), an alliance of research organizations and food industry partners in the Netherlands set up to carry out strategic fundamental research. The authors thank Dr. Ted van der Lende from Kreatech Biotechnology (Amsterdam, The Netherlands), Drs. Rolf Boesten, Drs. Robert van den Berg, Dr. Roy Montijn and Dr. Frank Schuren from the TNO Nutrition and Food Research Institute (Zeist, the Netherlands) for fruitful discussions

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