INTRODUCTION

Germination of an Aspergillus conidium leads to the formation of hyphae that grow by apical extension and branch sub-apically. As a result, a vegetative mycelium is formed. This interconnected hyphal network forms aerial hyphae and conidiophores (for reviews see Adams et al. 1998, Krijgsheld et al. 2013). Growth of such aerial structures depends on the translocation of nutrients and water from the vegetative mycelium (Jennings 1984, 1987, Wösten & Wessels 2006). Translocation in the higher fungi i.e. the ascomycetes and the basidiomycetes is possible because of the presence of porous septa that separate compartments within and between hyphae. In fact, the diameter of the pores may even allow passage of organelles (Moore & McAlear 1962, Lew 2005). The cytoplasm within the vegetative mycelium is thus considered to be continuous. Yet, it has been shown that the vegetative mycelium is highly heterogeneous with respect to growth, protein secretion and RNA composition (Wösten et al. 1991, Mouka et al. 1993, Vinck et al. 2005, 2011, Masai et al. 2006, Levin et al. 2007a, b, Kasuga & Glass 2008, de Bekker et al. 2011a, b, Krijgsheld et al. 2012). Even neighbouring hyphae can have a distinct RNA profile (Vinck et al. 2005, 2011, de Bekker et al. 2011b). Recent studies have shown that this can be explained, at least partially, by closure of septa by Woronin bodies (Bleichrodt 2012).

In this study, intra- and inter-compartmental streaming of GFP was studied. The results show that GFP can stream from the vegetative mycelium to the aerial structures but its encoding RNA does not seem to do so. Absence of RNA streaming explains the distinct RNA profiles observed in the vegetative mycelium and the aerial structures (i.e. aerial hyphae and conidiophores).

MATERIALS AND METHODS

Strains and growth conditions

Strains of A. niger (Table 1) were grown at 30 °C in the light on minimal medium [0.6 % NaNO₃, 0.15 % KH₂PO₄, 0.05 % KCl, 0.05 % MgSO₄·7H₂O, 0.2 mL l⁻¹ Vishniac (per liter: 10 g EDTA, 4.4 g ZnSO₄·7H₂O, 1.01 g MnCl₂, 0.32 g CoCl₂, 0.315 g CuSO₄·5H₂O, 0.22 g (NH₄)₂MoO₄·4H₂O, 1.47 g CaCl₂, and 1.0 g FeSO₄; Vishniac & Santer 1957), pH 6.0] containing 25 mM xylose or maltose. In the case of standing cultures, 1.5 % agar was added to the medium. Cultures were inoculated with 10⁴ spores taken up in 2 μL 0.8 % NaCl containing 0.005 % v/v Tween-80.

Plasmids for nuclear run on experiments

Genes were amplified by PCR using A. niger N402 chromosomal DNA as template, and Phusion® High-Fidelity DNA polymerase (Finnzymes; www.finnzymes.com). In this study, all primers were designed according to the A. niger CBS 513.88 genome sequence (http://www.ncbi.nlm.nih.gov/). Primers RB1 and RB2 were used to amplify gpdA (An16g01830), RB3 and RB4 for mpdA (An02g05830),
Table 1. Strains used in this study.

| Strain  | Construct | Parental strain | Description of strain or plasmid |
|---------|-----------|-----------------|----------------------------------|
| N402    | pAN52     | NRRL3           | Short conidiophore mutant (cspA1; Bos et al. 1988). |
| AB4.1   | pAN52     | N402            | pyrG mutant (van Hartingsveldt et al. 1987) |
| N593    | pAN52     | N402            | pyrG mutant (Goossen et al. 1987) |
| ARIglaAsGFP | pAN52-10S65TGFPh/3 (a) | AB4.1 | Plasmid containing sGFP under regulation of the glaA promoter of A. niger (Siedenberg et al. 1999). |
| ARIgpdAAsGFP | pGPDGFPh/3 (b) | AB4.1 | As (a) but with the gpdA promoter of A. nidulans (Lagopodi et al. 2002). |
| ARIglaA-H2B-EGFP | pMA25 (c) | AB4.1 | Derivative of pAH2BG (Maruyama et al. 2001) containing a fusion of histone H2B to EGFP under the regulation of the glaA promoter of A. niger (This work). |
| ARIgpdA-H2B-EGFP | pMA26 | AB4.1 | As (g) but with the gpdA promoter of A. nidulans (This work). |
| UIRlmdA-H2B-EGFP | pRI459 | NW249 | As (c) but with the mtdA promoter of A. niger (Aguilar-Osorio et al. 2010). |
| RBIRgpdAPA-GFP | pRB014 | AB4.1 | As (b) but containing PA-GFP instead of GFP (This work). |
| RBIRgpdAGPD-PA-GFP | pRB013 | AB4.1 | As (b) but containing the gene encoding the fusion protein of glyceraldehyde-3-phosphate dehydrogenase and PA-GFP (GPD-PA-GFP) under regulation of the gpdA promoter of A. niger (This work). |

RB5 and RB6 for agy1 (also known as olvA; An14g05350), RB7 and RB8 for flavohemoprotein (flaA; An14g02460), RB9 and RB10 for adhA (An17g01530), RB11 and RB12 for FAD binding oxidoreductase (oxIA; An18g00510), RB13 and RB14 for glaA (An03g06550) and RB15 and RB16 for 18S rDNA (Table 2). The fragments were cloned in the SmaI site of pUC19. This resulted in plasmids pRB001, pRB002, pRB003, pRB004, pRB005, pRB006, pRB007 and pRB008, respectively.

Construction of vectors containing a gene encoding nuclear targeted EGFP.

Fusion PCRs were performed with Phusion® High-Fidelity DNA polymerase to construct vector pMA25 and pMA26 (Finnzymes). For the construction of pMA25, the A. niger glaA promoter was amplified with primers AV1 and AV2 (Table 2) using pANS2-7 (Dr. P. Punt, unpublished vector) as template DNA. The H2B:EGFP sequence was amplified from pAH2BG (Maruyama et al. 2001) using primers AV3 and AV4 (Table 2). The fusion PCR of both PCR products was performed with primers AV1 and AV2 using pAN52-7 (Dr. P. Punt, unpublished vector) as template DNA. The resulting 2.1 kb product encompassing the glaA promoter and the H2B:EGFP sequence with BamHI linkers at both ends. The product was cloned in the BamHI site of pAN52-7 resulting in pMA25.

To construct pMA26, the gpdA promoter was amplified using primers AV5 and AV6 (Table 2) with pAN56-1 (Punt et al. 1990) as a template. This fragment was fused to the H2B:EGFP fragment (see above) in a fusion PCR using primers AV5 and AV4. The resulting 1.7 kb product with a Sall and a BamHI linker at the 5‘ and 3‘ end, respectively, was cloned in pANS2-1Not (Dr P. Punt, unpublished vector) using the Sall/BamHI restriction sites.

Construction of vectors containing PA-GFP

The ORF of PA-GFP was amplified with Phusion® High-Fidelity DNA polymerase (Finnzymes) using primers RB17 and RB18 (Table 2) and plasmid pRSETA-WEGFP (Patterson & Lippincott-Schwartz 2002) as a template. The promoter and ORF of gpdA were amplified with primers RB19 and RB20 (Table 2) using N402 genomic DNA. Both fragments were inserted in the SmaI site of pUC19, resulting in pRB011 and pRB009, respectively. The cloned fragment of pRB009 was cut out with NotI/NcoI. To this end, pRB009 was first partially digested with NcoI, since it contains an internal NcoI site. The fragment was ligated in pGPDGFp (Lagopodi et al. 2002) that had been digested with NotI and NcoI. This resulted in pRB010. The ORF of PA-GFP was cut out of pRB011 with NcoI/HindIII and ligated in the respective sites of pRB010 and pGPDGFp, generating pRB013 and pRB014, respectively. These plasmids express the genes encoding PA-GFP-GPD and PA-GFP under the control of the A. niger gpdA and the A. nidulans gpdA promoters, respectively.

RNA isolation

Aspergillus niger was grown as a sandwiched culture (Wösten et al. 1991) in a 0.25 mm layer of 0.6 % agarose between two porous polycarbonate membranes (diameter 76 mm, pore size 0.1 μm; Profiltra; www.profiltra.nl) that had been placed on solid minimal medium containing 25 mm maltose. After 6 d of growth, the top membrane of the sandwich was replaced by a membrane with pores of 10 μm (Profiltra), allowing formation of aerial hyphae and conidiophores. After 24 h, vegetative mycelium and aerial structures of the 7 d old cultures were harvested from 3 and 5 sandwiched colonies, respectively. The aerial structures were scraped from the top membrane of the sandwiched culture with a razor blade. From other colonies, vegetative mycelium was harvested by flipping over the top membrane and scraping it off with a razor blade. In the case of the aerial structures, colonies were first submerged in RNA later ICE (Ambion; www.ambion.com). Vegetative mycelium and aerial structures were frozen in liquid nitrogen and homogenised in a TissueLyser II (Qiagen; www.qiagen.com; setting 2 min, 30 Hz) using stainless steel 10 mL buckets. RNA of the vegetative mycelium was isolated using RNeasy spin columns (Qiagen). RNA of the aerial structures was extracted using a modified protocol of the MasterPure Yeast RNA Purification Kit (Epicentre Biotechnologies; www.epibio.com) (see also van Leeuwen et al. 2013). To this end, homogenised material was taken up in 1.8 mL T&C lysis buffer and vortexed vigorously. 525 μL MPC Protein Precipitation Reagent was added and the sample was incubated on ice for 5 min. After centrifugation at 4 °C for 10 min at 14,000 rpm in an Eppendorf microcentrifuge, the supernatant was transferred to a new tube and 1 mL isopropanol...
Table 2. Primers used in this study.

| Primer | Primer Sequence |
|--------|----------------|
| RB1    | GCCGCCTGCTCCACAGAAAGGAG |
| RB2    | CATTGGGAGGATCCTCAGCTGG |
| RB3    | GCGCCGCTGCTCGTTTCCCG |
| RB4    | CATTGGCTGCTCCCGCGTTCCTG |
| RB5    | GCCTGTTAAATAGCTAGCTACCGAACATGT |
| RB6    | TATGGCCGCGCCCTGTCGAGGCTCCTTG |
| RB7    | GGCCGGTAAATACGCCAAGCCACATGCCATCAAGAAG |
| RB8    | TTAAGGCCCAGGGGCGAGGCAACAGCAGAGTGCCAAAC |
| RB9    | GCCTGTTAAATAGCGGTGTACGTTACATA |
| RB10   | TATGGCCGCGCCCTGTCGAGGCTCCTG |
| RB11   | GCATTAAATACGCAAGCCACACAGCAAGAAG |
| RB12   | TTAAGGCCCAGGGGCAATCCTAGCCTCCTCGGGAG |
| RB13   | CCAGCATCATTACACCTAG |
| RB14   | TGCACACCACTACAT |
| RB15   | CCTCCGGCTTAAATTTAGCT |
| RB16   | CTCCTAAATGACGCCGGTTTG |
| RB17   | CATGTTGGACAGAAGGGGAGG |
| RB18   | AACGTCTTACGTACGGGACACTCAG |
| RB19   | GCGGCGCCCAGCCACAACAGCAGATG |
| RB20   | CATTGGGGGCGCAACATCTTG |
| AV1    | CGGGATCCGCTCCACCA |
| AV2    | CGGCAGTGGGGCACCATCAG |
| AV3    | ATGCCTCCTGCGGAG |
| AV4    | CGGATGCTTCATCTGACGCTCAG |
| AV5    | AAGCTGGCCATGCGACCAT |
| AV6    | CGCAACGCTTGGGAGCCATGAGTCTGCTCAAG |

was added. After centrifugation (see above for the conditions), the RNA was resuspended in DNAse incubated and incubated at 37 °C for 15 min. This was followed by adding 400 μL T & C lysis solution. After vortexing, 400 μL of MPC Protein Precipitation Reagent was added and samples were placed on ice for 5 min. After centrifugation, 800 μL isopropanol was added to the supernatant, immediately followed by centrifugation. The RNA pellet was washed twice with 70 % ethanol and resuspended in 100 μL TE buffer. After addition of 350 μL RLT buffer (RNEasy kit, Qiagen) and 250 μL ethanol, samples were purified using RNEasy spin columns according to the instructions of the manufacturer.

Nuclear run-on transcription assay

Vegetative mycelium and aerial structures were isolated from 7 d old colonies as described above and frozen in liquid nitrogen. The material was homogenised in a TissueLyser II (setting 2 min, 30 Hz) using stainless steel 10 mL buckets and resuspended in ice cold HB 0.5 buffer (10 mM PIPES pH 6.9, 5 mM CaCl₂, 5 mM MgSO₄, 0.5 M sucrose, complete protease inhibitor (Roche; www.roche.com), 0.1 % 2-mercapto-ethanol). From now on all steps were performed at 4 °C. Mycelial fragments were removed from the homogenate by centrifugation for 10 min at 160 g in a swing-out rotor (Harrier; www.mseuk.co.uk) followed by filtering the supernatant over glass wool twice. The filtrate was centrifuged 20 min at 5900 g. The pellet was resuspended in 2 mL HB 2.1 buffer (10 mM PIPES pH 6.9, 5 mM CaCl₂, 5 mM MgSO₄, 2.1 M sucrose, complete protease inhibitor (Roche), 0.1 % 2-mercapto-ethanol) and centrifuged for 20 min at 5900 g to pellet mycelial fragments. The supernatant was transferred to a new tube and brought to a volume of 2 mL with HB 2.1 buffer. Samples were centrifuged for 1 h at 128000 g in 1 mL tubes in a TLA100.1 rotor (Beckman Coulter; www.beckmancoulter.com). The nuclei in the pellet were taken up in 200 μL nuclei resuspension buffer (50 mM Tris-HCl pH 8.3, 40 % glycerol, 5 mM MgCl₂, 0.1 mM EDTA), divided in 100 μL portions, and stored at -80 °C. Nuclei were stained with DAPI for quantification using a haemocytometer. About 2.5 x 10⁷ and 7 x 10⁶ nuclei were isolated from the vegetative mycelium and the aerial structures, respectively, from one 7 d old colony.

Nuclei (4.5 x 10⁷ in 100 μL nuclei resuspension buffer) were thawed on ice and mixed with 67 μL 3x reaction buffer (15 mM Tris-HCl pH 8.0, 0.45 M KCl, 7.5 mM MgCl₂, and 0.75 mM of each of the nucleotides, except dUTP). In total 27 μL DEPC treated demi water and 6.25 μL α-3²P-UTP (100 μCi, 6000 Ci/mmol, PerkinElmer; www.perkinelmer.com) were added. After mixing carefully by pipetting up and down, the mixture was incubated for 30 min at 30 °C. The nuclear DNA was degraded by incubating with 5 μL RNAse free DNAsel (1U µL) for 10 min at rt. Nuclei were lysed by adding 1/9th volume of 10 % SDS and 4 M NaCl, after which 1 mL of TRizol® was added. After incubation for 5 min at rt, 210 μL chloroform was added. The RNA was centrifuged at 10 000 g for 10 min after 3 min incubation at rt. The aqueous phase was transferred to a new tube and centrifuged again. 250 μL of 2-propanol was added to the aqueous phase. After mixing well, the RNA was pelleted at 10 000 g for 10 min. The RNA was washed with 70 % ethanol and centrifuged for 5 min. Pellets were taken up in 150 μL RNAse free water and the RNA was dissolved by incubation for 15 min at 65 °C.

The RNA was hybridised to plasmid DNA containing selected genes of A. niger. To this end, plasmid DNA was isolated from E. coli cultures using a NucleoBond® PC 100 kit (Macherey-Nagel; www.mn-net.com). For each plasmid, 5 μg of DNA was taken up in 180 μL of water. 80 μL 4 M NaOH was added, after which the mixture was incubated for 15 min at rt. This was followed by adding 800 μL of ice cold 2 M NH₄Ac. Dot-blot equipment was incubated for 1 h in 3.5 % H₂O₂ and rinsed with RNAse free water. The dot-blot apparatus was loaded with two Whatmann papers and an Amersham Hybond™-N+ nitrocellulose membrane that had been washed with RNAse free water and 2x SSC. The dot-blot apparatus was put under vacuum, using a standard vacuum pump and wells were washed with 200 μL 2x SSC. This was followed by washing with 200 μL 1 M NH₄Ac. 800 μL of each DNA sample (i.e. 3.8 μg) was spotted. Wells were washed with 200 μL 1 M NH₄Ac, after which the nitrocellulose membrane was air dried. DNA was cross-linked to the membrane by a 30 s exposure to UV-light resulting in a total dose of 0.28 J. DNA was stained with 0.04 % methylene blue in 0.5 M NaAc buffer pH 5.2 to confirm equal loading, after which the membrane was de-stained with RNAse free water. The nitrocellulose membrane containing plasmid DNA was pre-hybridised in 20 mL hybridisation buffer (50 % formamide, 6x SSC, 2x Denhardt’s [0.04 % Ficoll, 0.04 % polyvinylpyrolidone, and 0.04 % bovine serum albumin], 0.1 % SDS, 10 % dextrane sulphate) for 2 h at 42 °C. Radioactively labeled RNA, resulting from the run-on transcription, was added to the hybridisation buffer after incubating the RNA for 2 min at 100 °C and 5 min on ice. After hybridisation for 16 h at 42 °C, the membrane was washed once with 6x SSC and 0.2 % SDS (5 min at rt), twice with 2x SSC and 0.2 % SDS (20 min at 65 °C), and twice with 0.2x SSC and 0.2 % SDS (20 min at 65 °C). The blots were exposed to X-OMAT film at -80 °C using intensifying screens.
Micro-array analysis

Micro-array analysis was performed using biological triplicates hybridised to separate arrays according to Affymetrix protocols (ServiceXS, Leiden, The Netherlands). In brief, RNA concentration was determined by absorbance at 260 nm using the Nanodrop ND-1000 (Thermo Scientific; www.thermo.com). Quality and integrity of the RNA was verified using the RNA 6000 Nano assay on the Agilent 2100 Bioanalyzer (Agilent Technologies; www.agilent.com). Biotin-labeled antisense cRNA was produced from 2 μg of total RNA with the Eukaryotic One-Cycle Target Labeling kit (Affymetrix; www.affymetrix.com). The quality of the cRNA was checked using the Agilent 2100 bioanalyzer. 12.5-20 μg cRNA was used for fragmentation and 10 μg of this was hybridised to Affymetrix A. niger Genome Gene chips. After an automated process of washing and staining, absolute values of RNA levels were calculated from the scanned array using the Affymetrix Command Console v. 1.1 software. The array data has been deposited in NCBI's Gene Expression Omnibus (Edgar et al. 2002) and is accessible through GEO Series accession number GSE32123 (www.ncbi.nlm.nih.gov/geo/). RNA normalisation was done using the MAS5.0 algorithm with a baseline correction of the median. A Fisher’s exact test was used to identify over-represented functional gene classes using the Functional Catalogue FunCat v. 2.0 (Ruepp et al. 2004; www.mips.helmholtz-muenchen.de/projects/functcat).

Transformation

Protoplasting of A. niger was performed according to de Bekker et al. (2009). The protoplasts were transformed (Punt & van den Hondel 1992) by co-transformation with pGW635 that contains pyrA as a selection marker (Goosen et al. 1989). Strains were selected on MMS medium (minimal medium pH 6.0, 0.95 M sucrose and 1.5 % agar; de Bekker et al. 2009) based on pyrA prototrophy.

Monitoring cytosolic and nuclear-targeted GFP

Cultures were grown in glass bottom dishes (MatTek, www.glass-bottom-dishes.com, P35G-1.5-20-C) under water saturated conditions. To this end, glass bottom dishes were filled with 200 μL minimal medium (pre-warmed at 60 °C) containing 1 % agarose and 25 mM maltose. Nicotinamide (1 μg/mL), leucin (200 μg/mL) and arginine (200 μg/mL) were added to the medium in the case of strain UU#PmtdA-H2B-EGFP. On top of the medium, an 18 x 18 mm cover glass was placed. After the medium had solidified, 0.5 μL of spore suspension was placed next to the agarose medium. After three days, hyphae had grown in the agarose medium and conidiophores had formed at the medium/air interface.

Confocal laser scanning microscopy was performed using an inverted Zeiss LSM5 system equipped with a Plan-Neofluar 25x/0.8 Imm corr objective objective lens (Zeiss, www.zeiss.com). GFP was excited with the 488 nm laser line and fluorescence was detected at 505–530 nm bandpass. Bright field images were made using the transmission channel. Laser intensity was kept to a minimum to reduce photobleaching and phototoxic effects. Images were captured as z-series of optical sections. The data sets were displayed as maximum intensity projections (1024 x 1024 pixels) using Zeiss software.
Cytosolic streaming in mycelium and aerial structures of Aspergillus niger

Monitoring PA-GFP

Strains were grown on minimal medium containing 25 mM maltose and 3 % agar. To this end, plates were inoculated in the middle with 2 μL spore suspension. After 24 h of growth, a polycarbonate membrane (pore size 0.1 μm; Procella) was placed on top of the colony and growth was prolonged for another 24 h. For microcopy, the membrane was removed and pieces of the agar medium (10 x 10 mm) with the mycelium on top of it were excised and placed up-side-down on a drop of 100 μL minimal medium with 25 mM maltose. Growth was prolonged for 1 h. PA-GFP was activated in a region of 20 x 30 μm by a 5 s exposure to 405 nm light with 3.75 mM laser power and a pinhole of 3.08 airy units. An inverted Zeiss LSM 5 system was used for imaging in combination with a Plan-Neofluar 25x/0.8 Imm corr objective (Zeiss) with oil immersion. Time lapse movies of PA-GFP fluorescence were made for 2 or 10 min using a 488 nm laser with 4.73 mW power, a pin hole of 3.29 airy units, a pixel dwell time of 3.20 μs and a LP 505 filter. Image resolution was 512 x 512 pixels. Ten hyphae were measured in each experiment.

To determine velocity of PA-GFP streaming, background fluorescence was measured for ten hyphae for each strain. Increase of fluorescence was monitored in time using the ROI tool from the PASCAL software (Zeiss). Rate of streaming was determined using the total distance of streaming and the time point at which fluorescence intensity was twice above the standard deviation of the background fluorescence. Data were statistically analyzed using an independent-samples T-test with a Levene’s test. In all cases, a difference was assumed significant when p < 0.05.

RESULTS

Genes gpdA and glaA are highly expressed in the substrate mycelium, whereas mtdA is expressed in aerial structures

Expression of the glyceraldehyde-3-phosphate dehydrogenase gene gpdA, the glucoamylase gene glaA and the mannitol dehydrogenase gene mtdA was studied in the substrate mycelium and in aerial structures of A. niger using GFP as a reporter. To this end, the GFP gene was fused to the histone gene H2B that contains a nuclear localisation signal (Maruyama et al. 2001). Strain UU#PmtdA-H2B-EGFP that expresses the fusion protein under control of the constitutive gpdA promoter was selected as a representative transformant for further studies. PA-GFP was activated in vegetative hyphae that were in contact with the conidiophore stalk. Streaming of GFP from the vegetative mycelium to conidiophores

Strains AR#PglaA-sGFP and AR#PgpdA-sGFP that express GFP from the glaA and gpdA promoter, respectively, have been described previously (Siedenberg et al. 1999, Lagopodi et al. 2002, Vinck et al. 2005). In this case, both vegetative and aerial hyphae were fluorescent (Fig. 3). In fact, the aerial structures were more fluorescent than the substrate hyphae. These results and the fact that glaA and gpdA are lower expressed in the aerial structures indicate that cytosolic GFP streams from the vegetative mycelium into conidiophores and conidia. Streaming of GFP was further studied using the photo-activatable derivative of GFP (PA-GFP) (Patterson & Lippincott-Schwartz 2002). A construct encompassing the PA-GFP gene under control of the constitutive gpdA promoter of A. nidulans was introduced in A. niger strain AB4.1. Strain RB#PgpdA-PA-GFP was selected as a representative transformant for further studies. PA-GFP was activated in vegetative hyphae that were in contact with the conidiophore stalk. Streaming of GFP from the vegetative hyphae to the conidiophore was observed in approximately 25 % of the cases (Fig. 4; see online Supplemental Movie 1). In contrast, PA-GFP that had been activated at the base of conidiophore stalks did not stream into vegetative hyphae. Similarly, PA-GFP did not stream from conidia and/or the conidiophore vesicle towards the base of the conidiophore stalk (data not shown). Taken together, these data
Fig. 3. Expression of GFP from the gpdA (A, C), and glnA (B, D) promoter. Confocal microscopy images were taken using bright field (A, B) and 488 nm laser light (C, D). Large and small arrow point to a conidiophore and a vegetative hypha, respectively.

Fig. 4. Intercellular streaming of PA-GFP from vegetative hyphae to the conidiophore stalk. PA-GFP was photo-activated in vegetative hyphae (indicated by the region of the white box). These hyphae included the foot cell hypha from which the conidiophore stalk (large arrow) had formed (A). After 2 min, fluorescence intensity had increased in the conidiophore stalk showing intercellular streaming of PA-GFP from the foot cell to the conidiophore (B). (C) represents bright field image. Small white arrow indicates location of septum in the conidiophore stalk. Bar represents 50 μm.

show that GFP streams from the vegetative mycelium towards the conidiophores but not vice versa.

Streaming of PA-GFP was monitored in vegetative hyphae by confocal microscopy after activation of the reporter at the tip or 100–200 μm from the tip (Fig. 5; see online Supplemental Movies 2 and 3). Streaming of GFP towards the tip (Fig. 5A and B; see online Supplemental Movie 2) or to subapical regions (Fig. 5A, B, D, and E; see online Supplemental Movie 3) occurred at rates that were not significantly different (11.2 ± 0 μm/s and 14.8 ± 7.6 μm/s, respectively). Rate of streaming of GFP to the base of the conidiophore stalk or to the conidiophore vesicles (Fig. 6; see online Supplemental Movies 4 and 5) was similar to that in vegetative hyphae (14.3 ± 0 μm/s).

In the next set of experiments, cytosolic streaming was assessed using A. niger strain RB4PgpdA-GPD-PA-GFP. This strain expresses a GPD-PA-GFP fusion protein under control of the gpdA promoter. Streaming of GPD-PA-GFP was monitored by confocal microscopy after activation of the reporter at the tip or 200 μm from the tip of vegetative hyphae (see online Supplemental Movies 6 and 7). Cytosolic streaming towards the hyphal tip and towards subapical
parts was not significantly different from each other (6.2 ± 1.3 μm/s and 4.1 ± 1.6 μm/s, respectively) but was different from the streaming rate of PA-GFP that was not fused to GPD.

Fig. 5. Streaming of PA-GFP in apical hyphal compartments. PA-GFP was activated 100 μm from the hyphal tip (A–C) or at the hyphal tip (D–F). Fluorescence was monitored directly (A, D) or 2 min (B, E) after activation; (C) and (F) represent bright field images. Arrow indicates distance from the hyphal tip (A–C). Bars represent 50 μm.

Fig. 6. Intracellular streaming of PA-GFP in conidiophores. PA-GFP was photo-activated at the conidiophore base 200 μm from the conidiophore vesicle (A–C) or just under the conidiophore vesicle (D–F). Fluorescence was monitored directly (A, D) or 2 min (B, E) after activation. (C) and (F) represent bright field images. Arrow indicates distance from the conidiophore head. Bars represent 50 μm.

Streaming of GFP into spores formed in the centre and the periphery of the colony

Strain AR#PglA-sGFP expressing GFP from the glaA promoter was grown as a sandwiched culture on solid medium with xylose,
which represses glaA (Fowler et al. 1990). After 5 d, holes were punctured in the upper PC membrane allowing formation of conidiophores both in the centre and the periphery of the colony. Prior to making holes in the PC membrane, the xylose-grown colonies were transferred to medium containing maltose, which induces glaA (Fowler et al. 1990). Spores that had been formed in the centre of transferred colonies were two-fold less fluorescent than those formed at the periphery (Table 3). Differences were less pronounced when cultures had grown continuously on maltose. These results show that the GFP fluorescence of spores depends on the expression of the protein in the underlying mycelium.

**RNA profiles of vegetative hyphae and aerial structures**

Total RNA of vegetative mycelium of 7 d old maltose-grown sandwiched colonies of *A. niger* was isolated using TRIzol®. However, extraction of total RNA from aerial structures was not successful with this commonly used method. Therefore, a novel RNA extraction method was developed, which was based on the MasterPure Yeast RNA Purification Kit (Epicentre Biotechnologies; see Material and Methods). This extraction method yielded high quality total RNA from aerial structures, but not from vegetative mycelium. Therefore, RNA extraction was performed with TRIzol® and the MasterPure Yeast RNA Purification Kit to isolate RNA from vegetative mycelium and aerial structures, respectively (Fig. 7).

Total RNA of biological triplicates was hybridised to separate Affymetrix micro-arrays representing 14259 unique *A. niger* ORFs of strain CBS 513.88 (Pel et al. 2007, Jacobs et al. 2009). A present call was obtained with 5095 and 5939 of the probe sets after hybridisation with RNA from the vegetative mycelium and the aerial structures, respectively. These probe sets represented a total of 6476 genes. Since the arrays were hybridised with RNA that had been extracted with different methods, the RNA levels in vegetative

| Growth condition | Spot | N     | Mean | SD   |
|------------------|------|-------|------|------|
| 5 d 25 mM xylose 8 h 25 mM maltose | cen1  | 101   | 57   | 9.88 |
| 5 d 25 mM xylose 8 h 25 mM maltose | per1  | 127   | 109  | 23.63|
| 5 d 25 mM xylose 8 h 25 mM maltose | cen2  | 49    | 51   | 10.36|
| 5 d 25 mM xylose 8 h 25 mM maltose | per2  | 218   | 98   | 19.18|
| 5 d 25 mM maltose | cen1  | 82    | 73   | 14.90|
| 5 d 25 mM maltose | per1  | 158   | 114  | 25.77|
| 5 d 25 mM maltose | cen2  | 181   | 83   | 21.68|
| 5 d 25 mM maltose | per2  | 118   | 84   | 21.80|

Fig. 7. Bioanalyzer graphs of RNA isolated from vegetative mycelium (upper panel) and aerial structures (lower panel).
Table 4. The top 100 of highest expressed genes in the aerial structures. Also known as olvA, fwnA, ctcB, and brnA, respectively. AU = arbitrary expression units.

| Gene ID          | AU   | Annotation                                                                 |
|------------------|------|-----------------------------------------------------------------------------|
| An08g06730       | 4156 | Weak similarity to hypothetical protein CAD29600.1 - Aspergillus fumigatus   |
| An07g03340       | 4047 | Strong similarity to hydrophobin hYP1 - Aspergillus fumigatus               |
| An08g06960       | 397  | Strong similarity to histone H3 - Aspergillus nidulans                      |
| An03g02400       | 371  | Strong similarity to spore-wall fungal hydrophobin dewA - Aspergillus nidulans |
| An15g07370       | 3312 | Similarity to hypothetical protein encoded by CG4090 - Drosophila melanogaster |
| An16g06570       | 3294 | Hypothetical protein                                                        |
| An11g11310       | 3289 | Strong similarity to histone H2B - Aspergillus nidulans                     |
| An15g07370       | 3217 | Similarity to hypothetical protein encoded by CG4090 - Drosophila melanogaster |
| An14g02140       | 3104 | Weak similarity to Ca-dependent protein kinase CDPK1 - Marchantia polymorpha |
| An16g06520       | 3009 | Hypothetical protein                                                        |
| An18g04840       | 2910 | Strong similarity to translation elongation factor 1 alpha - Podospora anserina [putative sequencing error] |
| An07g00070       | 2887 | Strong similarity to hypothetical protein encoded by An07g00010 - Aspergillus niger |
| An11g11300       | 2806 | Histone H2A httA - Aspergillus niger                                        |
| An04g06500       | 2720 | Strong similarity to rodletless protein rodA - Aspergillus nidulans         |
| An07g00510       | 2687 | Similarity to hypothetical lipoprotein SC4A2.13c - Streptomyces coelicolor   |
| An08g09880       | 2654 | Weak similarity to hydrophobin CoH1 - Coprinus cinereus                     |
| An09g02420       | 2639 | Hypothetical protein                                                        |
| An08g06940       | 2578 | Strong similarity to hypothetical protein CAC28773.2 - Neurospora crassa     |
| An14g05350       | 2578 | Strong similarity to hypothetical yellowish-green 1 ayg1 - Aspergillus fumigatus |
| An11g02720       | 2568 | Similarity to hypothetical protein C50F7.2 - Caenorhabditis elegans         |
| An02g14040       | 2469 | Hypothetical protein                                                        |
| An18g04220       | 2456 | Strong similarity to mitochondrial ADP/ATP carrier anc1p - Schizosaccharomyces pombe |
| An04g07530       | 2365 | Hypothetical protein                                                        |
| An15g02350       | 2302 | Strong similarity to hypothetical precursor of spore coat protein sp96 - Neurospora crassa |
| An12g02680       | 2298 | Weak similarity to hypothetical protein encoded by An02g12900 - Aspergillus niger |
| An08g06940       | 2267 | Strong similarity to histone H4.1 - Aspergillus nidulans                    |
| An04g00710       | 2257 | Hypothetical protein                                                        |
| An15g02250       | 2196 | Hypothetical protein                                                        |
| An18g04220       | 2188 | Strong similarity to hypothetical protein PRO27774.1 - Neurospora crassa     |
| An04g07530       | 2188 | Similarity to nitrogen metabolic repression regulator htmr from patent CN1269419-A - Homo sapiens |
| An09g05730       | 2188 | Strong similarity to hypothetical protein PRO27774.1 - Neurospora crassa     |
| An17g01460       | 2003 | Strong similarity to EST SEQ ID NO:4056 from patent WO200056762-A2 - Aspergillus niger |
| An07g03880       | 1982 | Similarity to hypothetical protein PRO27774.1 - Neurospora crassa           |
| An02g05240       | 1923 | Strong similarity to histone 4 from patent WO9919502-A1 - Homo sapiens      |
| An15g02250       | 1851 | Hypothetical protein                                                        |
| An08g00540       | 1825 | Strong similarity to EST SEQ ID NO:4140 from patent WO200056762-A2 - Aspergillus niger |
| An04g02306       | 1824 | Weak similarity to spore-wall fungal hydrophobin dewA - Aspergillus nidulans |
| An19g00210       | 1820 | Similarity to hemolysin ASP-HS - Aspergillus fumigatus                      |
| An02g05240       | 1753 | Strong similarity to histone 4 from patent WO9919502-A1 - Homo sapiens      |
| An02g11240       | 1723 | Hypothetical protein                                                        |
| An16g07330       | 1718 | Weak similarity to hypothetical extracellular matrix protein AAL47843.1 - Fusarium oxysporum |
| An04g01230       | 1709 | Strong similarity to hypothetical ECM33 homolog SPCC1223.12c - Schizosaccharomyces pombe |
| An07g03340       | 1678 | Strong similarity to antifungal protein precursor pf - Penicillium chrysogenum |
| An03g04530       | 1659 | Similarity to beta-phosphoglucomutase beta-PM - Lactococcus lactis         |
| An04g06510       | 1644 | Strong similarity to polyubiquitin 5 Ubi4 - Saccharomyces cerevisiae         |
| An08g03890       | 1594 | Strong similarity to hypothetical superoxide Cu/Zn dismutase B24P7.320 - Neurospora crassa |
| An01g12450       | 1580 | Strong similarity to hypothetical glucan beta-1,3 exoglucanase exgS - Aspergillus phoenicus |
| An02g14800       | 1560 | Protein disulfide isomerase A pdIA - Aspergillus niger                      |
| An07g08300       | 1554 | Cyclophilin-like peptidyl prolyl cis-trans isomerase cypA - Aspergillus niger |
| An04g08190       | 1535 | Strong similarity to mitochondrial ATP synthase subunit 9 olIC31 - Aspergillus nidulans |
Table 4. (Continued).

| Gene ID     | AU  | Annotation                                                                 |
|-------------|-----|-----------------------------------------------------------------------------|
| An14g04180  | 1522| Strong similarity to H+-transporting ATP synthase beta chain - *Neurospora crassa* [truncated ORF] |
| An01g01720  | 1507| Strong similarity to cytoplasmic ribosomal protein of the small subunit Rps31 - *Saccharomyces cerevisiae* |
| An01g03000  | 1499| Strong similarity to 1,3-beta-glucanotransferase g6l1 - *Aspergillus fumigatus* |
| An02g13580  | 1489| Strong similarity to endochitinase from patent EP531218-A - *Aphanocladium album* |
| An04g08190  | 1470| Strong similarity to mitochondrial ATP synthase subunit 9 oliC31 - *Aspergillus nidulans* |
| An02g07470  | 1461| Strong similarity to fructose-bisphosphate aldolase Fba1 - *Saccharomyces cerevisiae* |
| An16g06060  | 1418| Similarity to saframycin Mx1 synthase satA - *Myxococcus xanthus* |
| An02g05330  | 1417| Similarity to hypothetical protein 4MeS - *Metarhizium anisopliae* |
| An12g06380  | 1414| Similarity to mycelial surface antigen Csa1 - *Candida albicans* |
| An03g04860  | 1411| Strong similarity to protein involved in non-classical protein export pathway Nce102 - *Saccharomyces cerevisiae* |
| An16g04940  | 1400| Similarity to cytoplasmic ribosomal protein of the small subunit S12 AS1 - *Podospora anserine* |
| An09g05390  | 1338| Strong similarity to chitinase precursor chit33 - *Trichoderma harzianum* |
| An14g05370  | 1335| Strong similarity to cell surface ferric oxidase precursor Fe3 - *Saccharomyces cerevisiae* |
| An09g01550  | 1323| Similarity to galactose synthase FKS - *Paracoccidioides brasiliensis* |
| An01g00750  | 1317| Hypothetical protein                                                         |
| An04g08980  | 1250| Strong similarity to mannosyl-oligosaccharide 1,2-alpha-mannosidase msdS - *Aspergillus saitoi* |
| An08g07290  | 1262| Aldehyde dehydrogenase stdA - *Aspergillus niger* |
| An06g00180  | 1155| Hypothetical protein                                                         |
| An11g01630  | 1179| Similarity to 6-hydroxy-d-nicotine oxidase 6-HDNO - *Arthrobacter oxidans* |
| An07g00010  | 1176| Similarity to hypothetical protein encoded by An07g00070 - *Aspergillus niger* |
| An08g01960  | 1168| Strong similarity to cytoplasmic ribosomal protein of the large subunit L43a - *Saccharomyces cerevisiae* |
| An11g09500  | 1179| Similarity to cytoplasmic ribosomal protein of the large subunit L8.e P32b - *Saccharomyces cerevisiae* |
| An09g03490  | 1182| Similarity to elongation factor 1 beta EF-1 - *Oryctolagus cuniculus* |
| An01g00750  | 1179| Hypothetical protein                                                         |
| An10g00890  | 1250| Strong similarity to hypothetical protein encoded by An07g00070 - *Aspergillus niger* |
| An18g05000  | 1168| Similarity to hypothetical protein encoded by An07g00070 - *Aspergillus niger* |
| An02g07290  | 1262| Aldehyde dehydrogenase stdA - *Aspergillus niger* |
| An08g06250  | 1097| Strong similarity to cytoplasmic ribosomal protein of the small subunit Rps31 - *Saccharomyces cerevisiae* [putative frameshift] |
| An14g05810  | 1135| Similarity to cytoplasmic ribosomal protein of the small subunit S4.e - *Saccharomyces cerevisiae* |
| An15g03500  | 1124| Weak similarity to hypothetical protein AAP68395.1 - *Saccharomyces cerevisiae* |
| An04g01430  | 1122| Weak similarity to hypothetical protein encoded by B11A5.120 - *Aspergillus niger* |
| An10g05640  | 1115| Similarity to hypothetical mold-specific protein MS8 - *A. niger* |
| An04g02420  | 1085| Strong similarity to ornithine decarboxylase ODC - *Paracoccidioides brasiliensis* [putative frameshift] |
| An11g09500  | 1124| Weak similarity to hypothetical protein AAP68395.1 - *Saccharomyces cerevisiae* |
| An15g02390  | 1135| Similarity to hypothetical membrane protein YDL218w - *Saccharomyces cerevisiae* |
| An05g05810  | 1134| Strong similarity to cytoplasmic ribosomal protein of the small subunit S26 - *Homo sapiens* |
| An07g00020  | 1106| Similarity to hypothetical membrane protein YDL218w - *Saccharomyces cerevisiae* |
| An12g02740  | 1051| Weak similarity to ATP-dependent proteinase Clp from patent WO9743303-A - *Streptococcus pneumoniae* |
| An01g02880  | 1048| Strong similarity to cytoplasmic ubiquitin / ribosomal fusion protein Cep52 - *Saccharomyces cerevisiae* [putative frameshift] |
| An13g02470  | 1040| Hypothetical protein                                                         |
Table 5. The top 100 of highest expressed genes in the vegetative mycelium. AU = arbitrary expression units.

| Gene ID   | AU   | Annotation                                                                                                                                 |
|-----------|------|-------------------------------------------------------------------------------------------------------------------------------------------|
| An14g02140 | 2976 | Weak similarity to Ca-dependent protein kinase CDPK1 - Marchantia polymorpha                                                                |
| An18g04840 | 2752 | Strong similarity to translation elongation factor 1 alpha - Podospora anserina [putative sequencing error]                                    |
| An16g01830 | 2616 | Glyceraldehyde-3-phosphate dehydrogenase gpdA - Aspergillus niger                                                                           |
| An03g04530 | 2526 | Similarity to beta-phosphoglucomutase beta-PGM - Lactococcus lactis                                                                    |
| An03g06550 | 2235 | Glucan 1,4-alpha-glucosidase giaA - Aspergillus niger                                                                                  |
| An19g00210 | 2226 | Similarity to hemolysin ASP-HS - Aspergillus fumigatus                                                                                 |
| An14g04710 | 1966 | Aspartic proteinase aspergillopepsin 1 pepA - Aspergillus niger                                                                            |
| An07g08300 | 1924 | Cyclophilin-like peptideyl prolyl cis-trans isomerase cypA - Aspergillus niger                                                             |
| An11g01630 | 1910 | Strong similarity to thiazone biosynthesis protein nmt2p - Schizosaccharomyces pombe                                                          |
| An11g02200 | 1841 | Strong similarity to 4-hydroxyphenylpyruvate dioxygenase tcrP - Coccidioides immitis                                                        |
| An01g12450 | 1835 | Strong similarity to hypothetical glucan beta-1,3 exoglucanase exgS - Aspergillus phoenicis                                               |
| An02g13750 | 1738 | Strong similarity to glutaminase A glaA - Aspergillus oryza                                                                               |
| An07g08640 | 1673 | Strong similarity to mutanase mutA - Penicillium purpureogen                                                                             |
| An09g00840 | 1643 | Similarity to plastic-degradation enzyme within SEQ ID NO:6 from patent WO2004038016-A1 - Aspergillus oryza                                |
| An11g02200 | 1614 | Strong similarity to 4-hydroxyphenylpyruvate dioxygenase tcrP - Coccidioides immitis                                                        |
| An01g12450 | 1571 | Strong similarity to mannosyl-oligosaccharide 1,2-alpha-mannosidase msdS - Aspergillus saltai                                               |
| An02g05620 | 1567 | Weak similarity to hypothetical protein encoded by An07g10060 - Aspergillus niger                                                         |
| An16g01150 | 1566 | Strong similarity to soluble cytoplasmic fumarate reductase YEL047c - Saccharomyces cerevisiae                                              |
| An08g03490 | 1566 | Similarity to elongation factor 1 beta EF-1 - Orzyctolagus cuniculus                                                                       |
| An02g07020 | 1552 | Strong similarity to chitinase 1 precursor cts1 - Coccidioides immitis                                                                       |
| An17g01460 | 1528 | Strong similarity to EST SEQ ID NO:4056 from patent WO200056762-A2 - Aspergillus niger                                                   |
| An14g04090 | 1522 | Similarity to glucose permease Rgl2 - Saccharomyces cerevisiae                                                                         |
| An14g04090 | 1522 | Similarity to phosphopyruvate hydratase ENO1 - Candida albicans                                                                           |
| An14g03080 | 1498 | Similarity to hypothetical membrane protein YDL218w - Saccharomyces cerevisiae                                                             |
| An02g04740 | 1491 | Strong similarity to fructose-bisphosphate aldolase Fba1 - Saccharomyces cerevisiae                                                        |
| An04g06510 | 1466 | Strong similarity to polyubiquitin 5 Ubi4 - Saccharomyces cerevisiae                                                                      |
| An14g04920 | 1349 | Triose-phosphate-isomerase tpiA from patent WO8704464-A - Aspergillus niger                                                               |
| An12g07450 | 1332 | Strong similarity to glucose permease Rgl2 - Saccharomyces cerevisiae                                                                         |
| An18g06250 | 1322 | Strong similarity to hypothetical mold-specific protein MS8 - Ajellomyces capsulatus                                                        |
| An01g03090 | 1299 | Strong similarity to translation initiation factor Eif-5a.2 - Saccharomyces cerevisiae                                                        |
| An04g03290 | 1291 | Strong similarity to long-chain acyl-CoA dehydrogenase - Rattus norvegicus                                                               |
| An11g10490 | 1286 | Strong similarity to ubiquitin conjugating enzyme Ubc4 - Saccharomyces cerevisiae                                                        |
| An04g02420 | 1283 | Strong similarity to ornithine decarboxylase ODC - Paracoccidioides brasiliensis [putative frameshift]                                         |
| An01g03070 | 1281 | Strong similarity to aspergillopepsin aprS - Aspergillus phoenicis                                                                      |
| An11g11180 | 1278 | Strong similarity to hypothetical protein encoded by SPBC1198.08 - Schizosaccharomyces pombe                                              |
| An01g05960 | 1256 | Similarity to cyanovirin-N CV-N - Nostoc ellipsosporum                                                                                   |
| An18g06250 | 1248 | Strong similarity to mitochondrial ADP/ATP carrier anc1p - Schizosaccharomyces pombe                                                      |
| An02g10320 | 1232 | Strong similarity to protein rmt1 - Aspergillus parasiticus                                                                             |
| An08g06960 | 1206 | Strong similarity to histone H3 - Aspergillus nidulans                                                                                |
| An08g07290 | 1186 | Aldohexose dehydrogenase aldA - Aspergillus niger                                                                                          |
| An03g02400 | 1166 | Strong similarity to sporé-wall fungal hydrophobin dewA - Aspergillus nidulans                                                          |
| An07g09990 | 1165 | Strong similarity to heat shock protein 70 hsp70 - Ajellomyces capsulatus [putative frameshift]                                             |
| An01g10720 | 1162 | Strong similarity to cytoplasmic ribosomal protein of the small subunit Rps31 - Saccharomyces cerevisiae                                      |
| An01g08800 | 1154 | Strong similarity to glutamine synthase Gln1 - Saccharomyces cerevisiae                                                                    |
| An02g05700 | 1148 | Strong similarity to translation elongation factor eEF-2 - Cricetulus griseus                                                           |
| An11g02550 | 1136 | Strong similarity to phosphoenolpyruvate carboxykinase KIPck1 - Kluyveromyces lactis                                                   |
| An02g02960 | 1125 | Similarity to acyl-CoA-binding type 2 protein Adbp - Saccharomyces cereviserae                                                          |
| An09g05870 | 1120 | Strong similarity to nucleoside-diphosphate kinase NDK-1 - Neurospora crassa                                                             |
| An17g01530 | 1092 | Alcohol-dehydrogenase adhA from patent WO8704464-A - Aspergillus niger                                                                  |
| An15g00700 | 1076 | Strong similarity to malate dehydrogenase precursor MDH - Mus musculus                                                                 |
| Gene ID      | AU   | Annotation                                                                 |
|-------------|------|-----------------------------------------------------------------------------|
| An04g01430  | 1069 | Weak similarity to hypothetical protein encoded by B11A5.120 - Neurospora crassa |
| An02g05830  | 1058 | Strong similarity to mannitol-1-phosphate 5-dehydrogenase mitD - Streptococcus mutans |
| An02g10550  | 1024 | Strong similarity to endo-alpha,1,5-arabinanase abnA - Aspergillus niger       |
| An02g37650  | 1023 | Strong similarity to phosphoglucomutase pgmB - Aspergillus nidulans           |
| An07g06090  | 1020 | Strong similarity to EST an_3627 - Aspergillus niger                        |
| An03g46660  | 1009 | Strong similarity to peptide transporter PTR2 - Arabidopsis thaliana          |
| An13g02730  | 1003 | Strong similarity to EST an_3461 - Aspergillus niger                        |
| An01g10050  | 986  | Strong similarity to IgE-dependent histamine-releasing factor - Homo sapiens  |
| An07g08710  | 982  | Alpha, alpha-trehalose-phosphate synthase (UDP-forming) 2 (trehalose-6-phosphate UDP-glucose phosphate glucosyltransferase) tpsB - Aspergillus niger |
| An12g07470  | 975  | Weak similarity to cyanovirin-N CV-N - Nostoc ellipsosporum                |
| An16g09070  | 973  | Strong similarity to glucosamine-6-phosphate deaminase from patent WO9835047-A1 - Escherichia coli |
| An07g03770  | 967  | Strong similarity to Cu,Zn superoxide dismutase sodC - Aspergillus fumigatus |
| An19g05930  | 960  | Strong similarity to hypothetical protein encoded by An08g06890 - Aspergillus niger |
| An07g30330  | 958  | Strong similarity to EST SEQ ID NO:4127 from patent WO200056762-A2 - Aspergillus niger |
| An08g06570  | 916  | Strong similarity to transketolase Tkt1 - Saccharomyces cerevisiae            |
| An07g33880  | 912  | Serine proteinase pepC - Aspergillus niger                                  |
| An08g03690  | 904  | Strong similarity to ADP-ribosylation factor arf1 - Ajellomyces capsulatus   |
| An01g11660  | 896  | 1,4-beta-D-glucan cellobiohydrolase B precursor cbhb - Aspergillus niger     |
| An16g01880  | 881  | Strong similarity to lysophospholipase - Aspergillus foetidus                |
| An02g05240  | 878  | Strong similarity to histone 4 from patent WO9919502-A1 - Homo sapiens       |
| An07g32850  | 878  | Strong similarity to transaldolase Tal1 - Saccharomyces cerevisiae           |
| An01g03480  | 864  | Strong similarity to sorbitol dehydrogenase gubB - Bacillus subtilis        |
| An07g10020  | 863  | Strong similarity to microtubule-associated protein Aut7 - Saccharomyces cerevisiae |
| An01g04140  | 849  | Similarity to EST an_2919 - Aspergillus niger                              |
| An02g05240  | 848  | Strong similarity to histone 4 from patent WO9919502-A1 - Homo sapiens       |
| An02g14590  | 847  | Strong similarity to glutamate dehydrogenase Gdh2 - Saccharomyces cerevisiae |
| An15g00410  | 840  | Strong similarity to acetate-inducible gene aciA - Aspergillus nidulans      |
| An17g23340  | 845  | Strong similarity to cytosolic serine--tRNA ligase Ses1 - Saccharomyces cerevisiae |
| An12g10350  | 836  | Strong similarity to hypothetical protein encoded by An15g07090 - Aspergillus niger |
| An01g02500  | 833  | Strong similarity to thioredoxin - Aspergillus nidulans                      |
| An16g04940  | 831  | Strong similarity to cytoplasmic ribosomal protein of the small subunit S12 AS1 - Podospora anserina |
| An04g01230  | 824  | Strong similarity to hypothetical ECM33 homolog SPCC1223.12c - Schizosaccharomyces pombe |
| An01g04140  | 821  | Similarity to EST an_2919 - Aspergillus niger                              |
| An01g01830  | 820  | Strong similarity to catalase/peroxidase cpeB - Streptomyces reticuli         |
| An04g01750  | 818  | Strong similarity to 5-methyltetrahydropteroctylglutamate--homocysteine S-methyltransferase Met6 - Saccharomyces cerevisiae |
| An11g02570  | 813  | Hypothetical protein [truncated ORF]                                         |
| An07g09990  | 813  | Strong similarity to heat shock protein 70 hsp70 - Ajellomyces capsulatus [putative frameshift] |
| An01g06970  | 811  | Strong similarity to D-arabinose dehydrogenase Aral - Saccharomyces cerevisiae |
| An12g10830  | 805  | Similarity to hypothetical protein EAA74834.1 - Gibberella zeae              |
| An16g07150  | 804  | Strong similarity to soluble cytoplasmic fumarate reductase YEL047c - Saccharomyces cerevisiae |
| An15g00560  | 787  | Strong similarity to actin gamma - Aspergillus nidulans                      |
| An12g10350  | 770  | Strong similarity to hypothetical protein encoded by An15g07090 - Aspergillus niger |
| An04g06920  | 768  | Extracellular alpha-glucosidase agU - Aspergillus niger                      |
| An14g04180  | 760  | Strong similarity to coffilin Cof1 - Saccharomyces cerevisiae                |
| An18g00750  | 759  | Similarity to diagnostic protein #11744 from patent WO200175067-A2 - Homo sapiens |
| An14g02460  | 756  | Strong similarity to flavohemoglobin Fhp - Alcaligenes eutrophus            |
| An04g08980  | 751  | Strong similarity to cytoplasmic ribosomal protein of the large subunit L43a - Saccharomyces cerevisiae |
| An08g04120  | 749  | Similarity to hypothetical mold-specific protein MS8 - Ajellomyces capsulatus |
| An04g05300  | 745  | Strong similarity to fructose-1,6-bisphosphatase fbpA - Aspergillus oryzae   |
Table 6. Over-representation of functional FunCat classes (Ruepp et al. 2004) in the top 100 of highest expressed genes in the vegetative mycelium and in the aerial structures. The analysis was performed using the FunCat main-categories (bold) and the FunCat 3 sub-categories.

| Functional classes over-represented in the top 100 of highest expressed genes in the vegetative mycelium |
|--------------------------------------------------------------------------------------------------------|
| 01 METABOLISM                                                                                           |
| 01.03.19 nucleotide transport                                                                          |
| 02 ENERGY                                                                                               |
| 02.04.02 elongation                                                                                     |
| 05 PROTEIN SYNTHESIS                                                                                   |
| 05.04.01 mRNA synthesis                                                                                |
| 05.04.02 elongation                                                                                     |
| 05.05.01 Coompond and carbohydrate utilization                                                        |
| 05.06.01 lipid, fatty acid and isoprenoid biosynthesis                                                  |
| 06 PROTEIN FATE (folding, modification, destination)                                                    |
| 06.09.10 assembly of protein complexes                                                                     |
| 06.10.99 other proteolytic degradation                                                                  |
| 11 CELL RESCUE, DEFENSE AND VIRULENCE                                                                   |
| 11.01 stress response                                                                                   |
| 40 SUBCELLULAR LOCALISATION                                                                           |
| 40.01.09 stress response                                                                                |
| 40.04.02 elongation                                                                                     |

mucell and the aerial structures cannot be directly compared. Therefore, we only focused on the top 100 of most highly expressed genes in both fractions (Tables 4, 5). Using a cut-off p-value of 0.05, the Fisher’s exact test showed that the main functional FunCat gene categories metabolism (including C-compound and carbohydrate degradation); energy; protein synthesis; protein fate; cell rescue, defense and virulence and subcellular localisation are over-represented in the top 100 of highest expressed genes in the vegetative mycelium (Table 6). On the other hand the categories energy; protein synthesis and subcellular localisation were over-represented in the top 100 of highest expressed genes in the aerial structures (Table 6). The top 100’s of most highly expressed genes in the vegetative mycelium and the aerial structures shared 34 genes. These genes include histones 3 and 4, several ribosomal proteins, gpdA, a hydrophobin homologous to dewA, and several enzymes (Tables 4, 5). The top 100 of highest expressed genes of the vegetative mycelium and the aerial structures contain 16 and 40 genes, respectively, that encode a secreted protein (based on SignalP v. 4.0). The pools share 7 genes. The 40 genes encoding a secreted protein in the top 100 of the aerial structures include 6 out of the 8 predicted hydrophobin genes (Pel et al. 2007, Jensen et al. 2010). Conidia of A. niger are characterised by a bright spore pigment. Four genes have been described that are involved in the formation of this pigment (Jørgensen et al. 2011). Three of them (i.e. fwnA, olvA and brnA) were among the top 100 of highest expressed genes in the aerial structures (Table 4). Seven carbohydrate degrading enzymes are in the top 100 of highest expressed genes in the vegetative mycelium (Table 5). These are the glucoamylase gene glaA, the α-glucosidase gaiU, glucan beta,1,3 exoglucanase exgS, glutaminase A gtaA, 1,2-alpha-mannosidase msdS, endo-alpha 1,5-arabinanase abnA, and the 1,4-beta-D-glucan cellobiohydrolase B precursor cbhB. The proteases aspergillopepsin pepA and pepC were also found in the top 100 of the vegetative mycelium.

DISCUSSION

Growth of fungal aerial structures depends on the translocation of water and nutrients from the vegetative mycelium (Jennings 1984, 1987, Wösten & Wessels 2006). The presence of porous septa enables this translocation in the case of the higher fungi (i.e. the ascomycetes and the basidiomycetes). The fact that the pores even allow passage of organelles (Moore & McAlear 1962, Lew 2005) suggests that RNA and proteins can stream from the vegetative mycelium to aerial structures. We here show that this is indeed the case for the reporter protein GFP but this seems not to be the case for its encoding RNA. Absence of RNA streaming would explain why vegetative mycelium and aerial structures have distinct RNA profiles.

Substrate hyphae, conidiophores and conidia were all fluorescent when cytosolic GFP was expressed from the gpdA or the glaA promoter. In contrast, highly fluorescent nuclei were only observed in substrate hyphae when nuclear targeted GFP was expressed from these promoters. This discrepancy can be explained by assuming that GFP with a nuclear localisation signal is rapidly imported in the nucleus after it has been formed and will thus not stream into the aerial structures. Streaming of cytosolic GFP from the vegetative mycelium to the aerial structures was confirmed by using a photo-activatable version of this reporter called PA-GFP. On the other hand, PA-GFP did not stream from the aerial structures to the vegetative mycelium. Taken together, cytosolic GFP can be used to study streaming in a fungal mycelium but nuclear targeted GFP is the method of use to localise gene expression in a fungal colony. The latter is supported by the fact
that results obtained with a nuclear run-on transcription assay agreed with the localisation of nuclear-targeted GFP resulting from gpda and glaA driven expression.

The rate and direction of streaming of PA-GFP was studied in individual compartments of hyphae of A. niger. It was shown that PA-GFP streams to apical and subapical regions within such compartments (Vinck et al. 2005, Bleichrodt 2012, this study). This finding contrasts results obtained in Neurospora crassa. Oil droplets that had been injected into hyphae only moved to the tip with an average speed of approximately 5 μm/s and a maximum speed of 60 μm/s (Lew 2005). PA-GFP had a rate of streaming in A. niger of approximately 10–15 μm/s. The speed was similar in both directions and in vegetative hyphae and conidiophores. The fact that cytosolic GFP is translocated into aerial reproductive structures suggests that also other cytosolic proteins stream from the substrate mycelium into conidiophores and conidia. Of interest, the streaming rate of PA-GFP was decreased to about 4–6 μm/s when the reporter protein was fused to the glyceraldehyde-3-phosphate dehydrogenase (GPD) protein. Possibly, this is due to the fact that GPD is part of a large protein complex. The yeast GPD homologs Tdh1, Tdh2 and Tdh3 were found to be member of in total 17 unique protein complexes (Gavin et al. 2006). Tdh3 is the core of a complex and interacts with Tdh1 and Tdh2. This complex includes two transmembrane proteins Gpi17 and Plm1 that may well decrease the streaming rate by temporally immobilising the complex at the membrane. These results indicate that streaming of proteins depends on their presence or absence in immobile protein complexes. The studies of Gavin et al. (2006) have indicated that the fast majority of the proteins is in protein complexes but their mobility within a cell has not yet been established. Future studies should reveal which protein species are translocated to aerial structures and what the relative contribution is of this transport when compared to the novo synthesis within the aerial structures. The same question holds for RNA. The fact that gpda- or glaA-driven expression of nuclear targeted GFP only resulted in fluorescent nuclei in vegetative hyphae implies that GFP mRNA is not translocated into the aerial structures, at least not efficiently. The GFP mRNA may have been part of polysomes and these structures may have a relatively low mobility within the mycelium.

Immobility of mRNA in the mycelium would explain differences in RNA composition between zones of the mycelium, between neighboring hyphae or between the vegetative mycelium and aerial structures. In this study, we presented micro-array data of the RNA composition of the vegetative mycelium and the aerial structures of 7 d old maltose-grown colonies of A. niger strain N402. This common lab strain has the cspA1 mutation. This mutation leads to decreased strength and integrity of the spore cell wall in A. fumigatus (Levdansky et al. 2010), but conidiophores are still being formed. We therefore do not expect a major impact on the expression profile when compared to a wild-type strain. We could only isolate RNA from the vegetative mycelium and the aerial structures using different RNA isolation techniques. We cannot exclude that these procedures have an effect on the efficiency of extraction of individual RNA species. Therefore, the array data of the vegetative mycelium and the aerial structures cannot be directly compared. From the 14 259 genes, a total of 6 476 were expressed in the colony. Of these genes, 5 095 and 5 939 were expressed in the vegetative mycelium and the aerial structures, respectively. The higher number of genes that are expressed in the aerial structures may be explained by the different cell types that make up the aerial structures. Recently, it was found that aerial hyphae of the basidiomycete Ustilago maydis have a RNA composition very similar to that of vegetative hyphae. Only 31 genes were differentially expressed (Teertstra et al. 2011). It would be of interest to perform a similar study in A. niger. Possibly, also A. niger aerial hyphae have a RNA profile similar to that of the vegetative mycelium. The conidiophore and conidia are expected to have RNA profiles different from that of the vegetative mycelium because these structures are the result of a developmental program.

The vegetative mycelium feeds the aerial structures. It is therefore not surprising that the functional gene category C-compound & carbohydrate utilisation was over-represented in the top 100 of most highly expressed genes in the vegetative mycelium. Within this top 100, seven genes encode enzymes that are involved in carbohydrate degradation. One of these genes is the glucoamylase gene glaA. Three genes involved in spore pigmentation (i.e. fwnA, olvA, and bnnA) and six out of eight hydrophobin genes were part of the top 100 of most highly expressed genes within the aerial structures. One of these hydrophobin genes is the ortholog of rodA of A. nidulans (Stringer et al. 1991), whereas another is predicted to be the ortholog of hyp1 of A. fumigatus (Parta et al. 1994). RodA and Hyp1 form rodlets at the surface of conidia. RodA has also been shown to coat metulae and phialides.

It had previously been shown that zones within the mycelium of A. niger differ with respect to gene expression (Levin et al. 2007a, de Bekker et al. 2011a, Vinck et al. 2005) and protein secretion (Wösten et al. 1991, Levin et al. 2007b, Krijgheld et al. 2012b). We here showed that translocation of GFP to spores also depends on the zone of the colony. Spores produced at the periphery of induced colonies contained more reporter protein resulting from glaA driven GFP expression than spores formed in the centre. In agreement, the glaA promotor is more active at the periphery than in the centre of colonies (Vinck et al. 2005). These results indicate that spore composition depends on a restricted part of the underlying substrate mycelium. The colony is thus predicted to form spores with a variable composition when nutrients are not evenly distributed in the substrate. So far, we were unable to show differences in germination of spores formed at the colony centre or at the periphery before or after freeze/thawing or freeze-drying (data not shown). However, it cannot be excluded that there are differences in viability under particular conditions. Previously, it has been shown that the age of the culture as well as environmental conditions affect properties (e.g. viability and cytotoxicity) of fungal spores (Hallsworth & Magan 1996, Clijquet & Jackson 1999, Murtoniemi et al. 2003, Clijquet & Jackson 2005). Normally, spores are collected from the whole mycelium. This study indicates that variability in spore properties can be reduced by extracting spores from selected parts of the colony. Defined spore properties are of interest for biocontrol applications (Clijquet & Jackson 1999, Clijquet & Jackson 2005) but may also be of interest for starter cultures of fungal fermentations.

ACKNOWLEDGEMENTS

This work was in part financed by the Netherlands Organisation for Scientific Research (NWO).

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Supplementary movies, online only through CBS website www.cbs.knaw.nl

Supplemental Movie 1: Intercellular streaming of PA-GFP from vegetative hyphae to the conidiophore stalk. PA-GFP was photo-activated in vegetative hyphae of strain R8IBPgpdPA-GFP (indicated by red arrow) and streaming was monitored for 2 min. The activated hyphae included the foot cell hypha from which the conidiophore
stalk (large arrow) had formed. Small white arrow indicates location of septum in the conidiophore stalk.

**Supplemental Movie 2:** Intracellular streaming of PA-GFP in a leading hypha of strain RB#PgpdA-PA-GFP. PA-GFP was activated 200 μm from the tip of the leading hypha and streaming was monitored for 10 min.

**Supplemental Movie 3:** Intracellular streaming of PA-GFP in a leading hypha of strain RB#PgpdA-PA-GFP. PA-GFP was activated at the tip of the leading hypha and streaming was monitored for 10 min.

**Supplemental Movie 4:** Intracellular streaming of PA-GFP in conidiophores of strain RB#PgpdA-PA-GFP. PA-GFP was photo-activated just below the conidiophore vesicle and streaming was monitored for 2 min. White circle indicates conidiophore vesicle.

**Supplemental Movie 5:** Intracellular streaming of PA-GFP in conidiophores of strain RB#PgpdA-PA-GFP. PA-GFP was photo-activated 200 μm from the conidiophore vesicle and streaming was monitored for 2 min.

**Supplemental Movie 6:** Intracellular streaming of PA-GFP in a leading hypha of strain RB#PgpdA-GPD-PA-GFP. PA-GFP was activated 200 μm from the tip of the leading hypha and streaming was monitored for 10 min.

**Supplemental Movie 7:** Intracellular streaming of PA-GFP in a leading hypha of strain RB#PgpdA-GPD-PA-GFP. PA-GFP was activated at the tip of the leading hypha and streaming was monitored for 10 min.