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ORIGINAL PAPER

FluG affects secretion in colonies of *Aspergillus niger*

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Abstract Colonies of *Aspergillus niger* are characterized by zonal heterogeneity in growth, sporulation, gene expression and secretion. For instance, the glucoamylase gene *glaA* is more highly expressed at the periphery of colonies when compared to the center. As a consequence, its encoded protein GlaA is mainly secreted at the outer part of the colony. Here, multiple copies of *amyR* were introduced in *A. niger*. Most transformants over-expressing this regulatory gene of amylolytic genes still displayed heterogeneous *glaA* expression and GlaA secretion. However, heterogeneity was abolished in transformant UU-A001.13 by expressing *glaA* and secreting GlaA throughout the mycelium. Sequencing the genome of UU-A001.13 revealed that transformation had been accompanied by deletion of part of the *fluG* gene and disrupting its 3′ end by integration of a transformation vector. Inactivation of *fluG* in the wild-type background of *A. niger* also resulted in breakdown of starch under the whole colony. Asexual development of the Δ*fluG* strain was not affected, unlike what was previously shown in *Aspergillus nidulans*. Genes encoding proteins with a signal sequence for secretion, including part of the amylolytic genes, were more often downregulated in the central zone of maltose-grown Δ*fluG* colonies and upregulated in the intermediate part and periphery when compared to the wild-type. Together, these data indicate that FluG of *A. niger* is a repressor of secretion.

Keywords Fungus · Conidiogenesis · Asexual development · Aspergillus fluG · Secretion · Heterogeneity

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Introduction

Aspergillus niger is a common saprotrophic fungus that grows on a wide variety of carbon sources and in a wide range of temperature, pH, and water activity (Krijgsheld et al. 2013a). It is used for the commercial production of organic acids (e.g. citric acid) and enzymes (e.g. glucoamylase) (Andersen et al. 2011). Its role as a cell factory is based on its long history of safe use (Schuster et al. 2002) and enormous secretion capacity (Conesa et al. 2001; Punt et al. 2002).

A. niger forms a mycelium, also known as colony, that consists of a network of interconnected hyphae. Zones in colonies of *A. niger* are heterogeneous with respect to growth (Wösten et al. 1991), RNA composition (de Bekker et al. 2011; Levin et al. 2007a), secretion (Levin et al. 2007b; Wösten et al. 1991; Krijgsheld et al. 2012) and sporulation (Krijgsheld et al. 2013b). For instance, secretion of GlaA (Wösten et al. 1991) and expression of its encoding gene *glaA* (Levin et al. 2007a) mainly take part at the periphery of the colony. AmyR regulates expression of *glaA* and other genes encoding enzymes that are active on glucose- and galactose-containing oligo- and polysaccharides (Petersen et al. 1999; vanKuyk et al. 2011).

Formation of conidiophores mainly takes place in the sub-peripheral part of the colony. This zone does not secrete proteins (Krijgsheld et al. 2013b), suggesting that asexual reproduction inhibits secretion. Indeed, inactivation of the sporulation gene *flbA* results in a strain that secretes proteins throughout the colony (Krijgsheld et al. 2013b). In addition, the non-sporulating strain grows in all zones of the mycelium. Notably, zonal heterogeneity in gene expression is not reduced in Δ*flbA* colonies (Krijgsheld and Wösten 2013).

The *fluG* gene is believed to be at the start of the asexual sporulation program in aspergilli. Overexpression of *fluG* in vegetative hyphae of *Aspergillus nidulans* is sufficient to cause sporulation under conditions that normally suppress conidia formation (Lee and Adams 1996). A Δ*fluG* strain of *A. nidulans* does form aerial hyphae but conidiophores are not being formed in excess of nutrients (Lee and Adams 1994). During nutrient starvation, however, conidiophore development is not affected (Lee and Adams 1996). From these data it was concluded that FluG is involved in a developmental program of sporulation but not in a stress-related sporulation pathway. Experimental evidence indicates that FluG of *Aspergillus oryzae* has a similar role as that of *A. nidulans* (Ogawa et al. 2010). In contrast, a Δ*fluG* strain of *A. fumigatus* still sporulates in air-exposed cultures (Mah and Yu 2006).

Formation of conidiophores in a Δ*fluG* colony of *A. nidulans* is rescued by growing a wild-type colony next to it, even when the colonies are physically separated by a dialysis membrane with a size exclusion of 6–8 kDa (Lee and Adams 1994). This led to the hypothesis that FluG is involved in the production of a low-molecular weight extracellular signaling molecule that is involved in the formation of conidiophores. This signaling molecule would activate an unknown receptor. As a result, the suppressor protein SfgA would be inhibited (Seo et al. 2006), enabling expression of the *flb* genes such as *flbA*. FlbA activates the central regulatory gene of asexual development *brlA* by inactivating the Gα subunit FadA.

Here, multiple copies of *amyR* were introduced in *A. niger*. Zonal heterogeneity in expression of *glaA* and secretion of GlaA were not affected in transformants except for strain UU-A001.13. In this strain, starch was degraded throughout the medium underlying the colony. Genomic sequencing revealed that *fluG* had been inactivated in UU-A001.13 as a consequence of the transformation. Inactivation of this gene in a wild-type background also resulted in starch degradation throughout the medium underlying the mycelium. However, it did not affect conidiophore and conidia formation. These data indicate that FluG represses secretion in the central part of the colony under certain environmental conditions and this may link FluG to asexual development in *A. niger*.

Materials and methods

Growth condition and strains

Strains of *A. niger* used in this study (Table 1) were grown at 30 °C under constant white light (Osram Lumilux L36w/840, Osram, Munich, Germany) of 700 lux. Sporulation assays were performed in the dark and at 1,200 lux light. *A. niger* was grown in minimal medium (MM; see Wang et al. 2014) with 25 mM maltose or 50 mM xylose as carbon source. Alternatively, *A. niger* was grown at 250 rpm in

Table 1 Strains used in this study

Strain	Genotype	Reference
N402	*cspA1* derivative of ATCC 9029	(Bos et al. 1988)
NW249	*cspA1*, ΔargB, *pyrA6, nicA1, leuA1*	(Jalving et al., 2000)
UU-049.1	*cspA1*, *pyrA6, nicA1, leuA1*, Δ*argB*::pIM2101[argB +]	(Battaglia et al. 2011)
UU-A001.13	*cspA1*, *pyrA6, nicA1, leuA1*, Δ*argB*::pIM2101[argB +], pJG01	this study
UU-A001.24	*cspA1*, *pyrA6, nicA1, leuA1*, Δ*argB*::pIM2101[argB +], pJG01	(vanKuyk et al. 2011)
N402Δ*fluG*	*cspA1*, Δ*fluG,* hyg^+	this study
N402Δ*fluG fluG*	*cspA1*, *amdS*$^+$, hyg^+	this study

250 ml Erlenmeyer flasks in 100 ml complete medium (CM; MM with 2 g l^{-1} trypton, 1 g l^{-1} casamino acids, 1 g l^{-1} yeast extract, 0.5 g l^{-1} yeast ribonucleic acids, and 55 mM glucose). If necessary, the medium was supplemented with 1.2 mM arginine, 1.5 mM leucine, 0.8 mM uridine, and/or 8 µM nicotinamide.

Colonies on agar medium were grown as sandwiched cultures in a water saturated box (Wösten et al. 1991). To this end, the fungus was grown in between two perforated polycarbonate (PC) membranes (diameter of 76 mm; pore size 0.1 µm; Osmonics, GE Water Technologies, Trevose, PA) in a 0.2 mm thin layer of 1.25 % agarose. Sandwiched cultures were inoculated in the centre of the PC membrane with 1.5 µl spore suspension (10^5 spores μl^{-1} 0.14 M NaCl, 0.005 % Tween-80). The top membrane was positioned on the agarose layer after 24 h of incubation.

Inactivation of *fluG*

Up- and downstream flanking regions of the coding sequence of *fluG* (An14g03390) were amplified from chromosomal DNA of *A. niger* N402 using Taq polymerase and primer pairs 1 and 2 (Supplementary Table 1). As a result, a NotI and XbaI/XhoI site had been introduced at the 5′ and 3′ ends of the upstream flank, respectively, and a XhoI and KpnI site at the 5′ and 3′ ends of the downstream flank, respectively. The amplified products were blunt end ligated in pJET 1.2 (Fermentas, Thermo scientific, Waltham, USA). The resulting plasmids were digested with NotI and XhoI and XhoI and KpnI, respectively. This was followed by a three point ligation with pBluescriptIISK(+) that had been digested with NotI and KpnI. The XhoI/XbaI fragment of PAN7-1 (Punt et al. 1987) containing the hygromycine resistance cassette was inserted in the pBluescriptIISK(+) vector that contained both flanks of *fluG* and that been digested with the same enzymes. This resulted in the *fluG* deletion construct *BNOfluG*. This construct was digested with NotI and KpnI. The flanking sequences interspersed with the antibiotic resistance cassette were introduced in *A. niger* N402.

Complementation of the Δ*fluG* strain

Up- and downstream flanking regions and the coding sequence of *fluG* were amplified from chromosomal DNA of *A. niger* N402 using Phusion® High-fidelity DNA polymerase (Finnzymes; www.finnzymes.com) and primer pair 3 (Supplementary Table 1). As a result, a NotI and HindIII site had been introduced at the 5′ and 3′ ends of the amplified fragment, respectively. These sites were used to introduce the PCR product into pAN01 that had been cut with the same enzymes. Vector pAN01is a derivative of pAN2-4, which contains the *gpd* promoter and *trpC* terminator (Punt et al. 1990; Mullaney et al. 1985). The *gpd* promoter is replaced by the *glaA* promoter of 822 bp in pAN01. By cutting with NotI and HindIII, the *glaA* promoter was replaced by the flanking regions and the coding sequence of *fluG*. This resulted in the *fluG* complement construct pAN01*fluG*.

Transformation

Transformation of *A. niger* was performed as described (Kusters-van Someren et al. 1991; Meyer et al. 2010). Protoplasts of *A. niger* were generated according to de Bekker et al. (2009). Vector BNO*fluG* was introduced in protoplasts of N402 to inactivate *fluG*. Transformants were selected on MMS agar (MM containing per liter 325 g sucrose and 12 g agar)

supplemented with 200 μg ml^{-1} hygromycine and 50 μg ml^{-1} caffeine. For *amyR* over-expression, plasmids pJG01 and pIM2101 were co-introduced in NW249. Plasmid pJG01 (vanKuyk et al. 2011) contains the *A. niger amyR* gene (An04g06910) as a 4.3 kb NsiI fragment in pGEM11, while pIM2101 contains the *argB* gene (Lenouvel et al. 2002). Transformants were selected on MMS plates in the absence of arginine. Construct pAN01*fluG* was introduced in Δ*fluG* protoplasts together with construct p35R2 that contains the acetamidase gene *amdS* (Kelly and Hynes 1985). Transformants of *A. niger* were selected on MMS medium containing 0.3 g l^{-1} acetamide and 0.85 g l^{-1} CsCl. Complementation was confirmed by PCR analysis using primer pair four (Supplementary Table 1).

Southern blotting

Chromosomal DNA was isolated as described (van Peer et al. 2009) and southern blotting was performed according to (Schuren et al. 1993). DNA was digested with SalI, separated on a 0.8 % agarose gel, and blotted to nitrocellulose. DNA was hybridized at 65 °C with a ^{32}P-labelled 1.2 kb SalI fragment of the *amyR* gene of *A. niger* or a ^{32}P-labelled 800 bp fragment of*18S* rDNA in 0.2 × SSC (150 mM NaCl, 15 mM Na citrate, pH 7.0). Blots were exposed to X-OMAT film (Kodak, New York, USA).

Sequencing of genomic DNA

Genomic DNA of *A. niger* was processed using the Illumina® Paired-end DNA sequencing Sample Prep Kit (Illumina, San Diego, USA). Fragmentation of the DNA, ligation of sequencing adapters, and PCR amplification of the resulting products was performed according to protocols of the manufacturer. Quality and yield of products was measured with Lab-on-a-Chip analysis. Samples were sequenced on the Illumina Genome Analyzer IIx according to manufacturer's protocols using 8 pmol of DNA. The total number of generated 76 bp paired-end reads (480 ± 30 bases) was 52,475,558. The paired end reads were aligned to the hybrid genome that was created by adding the sequences of PIM2101 and PJG01 to the *A. niger* ATCC 1015 sequence (V3;http://genome.jgi-psf.org/pages/blast.jsf?db=Aspni5). 94.15 % of the pair end reads could be mapped to the hybrid genome. Sequences flanking introduced vectors were extracted using IGV (http://www.broadinstitute.org/igv/).

RNA extraction

Mycelium was frozen in liquid nitrogen and homogenized using the TissueLyser II (Qiagen, Venlo, The Netherlands). RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the instructions of the manufacturer and purified using N*ucleoSpin*® RNA II (Macherey-NagelGmBh, Düren, Germany). Concentration and quality of RNA were measured with a Nanodrop ND-1000 (Thermo scientific, Wilmington, DE, USA) and an Agilent 2100 bioanalyzer (Agilent Technology, Palo Alto, CA), respectively.

RNA sequencing and analysis

RNA sequencing of samples were performed in triplo at ServiceXS (Leiden, the Netherlands) using Illumina HiSeq 2000 (Illumina, San Diego, USA), and Illumina protocols. The poly-A containing mRNA was isolated from 10 μg total RNA using poly-T oligo-attached magnetic beads. The mRNA was fragmented using divalent cations and copied into first strand cDNA using reverse transcriptase and random primers. This was followed by second strand cDNA synthesis using DNA polymerase I and RNaseH. Adapters were ligated to the cDNA after end-repair and addition of a single 'A' base at the ends. The cDNA fragments were amplified by PCR to create the final cDNA library. Quality and yield of the fragments were measured with a DNA 1,000 Lab-on-a-Chip (Agilent Technology, Waldbronn, Germany). The size of cDNA fragments was approximately 300 bp, as expected. Fragments were sequenced in paired-end mode resulting in two 100 bp reads with an average insert size of 157 bp (±3.9 bp across samples). For each sample (six conditions, three replicates) an average of 12.1 (±1.7 standard deviation) million fragments was obtained. The minimum fragment number was 10.1 million.Trimmomatic 0.22 (Lohse et al. 2012) was used to remove adapters and leading/trailing bases below quality 3. Reads were cut when a four base stretch had an average quality below 15 and they were removed when they had a length <36 bases. Fragments of which both reads survived (89 %,

±2 %) were mapped to the *A. niger* N402 genome using STAR (Dobin et al. 2013). In total, 45331 splice sites (after quality filtering) were found. This information was used to improve alignments across splice sites, by realigning all reads. An average unique alignment rate of 95.5 % was obtained, while on average 1.82 % of the reads mapped at multiple locations. To perform expression analysis, gene annotations were transferred from the CBS 513.88 genome (http://www.aspergillusgenome.org/SequenceContents.shtml) to the N402 genome using RATT (Otto et al. 2011). Of 14,070 genes, 13,070 genes could be transferred completely and 106 genes partially. Cufflinks 2.1.1(Trapnell et al. 2013) was used to process these annotations with the discovery of new genes/transcripts disabled. Cuffdiff 2.1.1 (Trapnell et al. 2013) was also used to quantify differential expression. Conditions were analyzed simultaneously to make use of expression variability information across multiple conditions.

GO analysis

Significant differentially expressed genes in zones 1, 3, and 5 between the wild type and the Δ*fluG* strain were analyzed by Fetgoat (Nitsche et al. 2011). Fetgoat applies a Fisher's exact test to identify gene ontology terms that are overrepresented/underrepresented in a list of differential expressed genes. Benjamini and Hochberg correction method was used to correct for multiple testing.

Quantification of sporulation and germination

The upper PC membrane was removed from light or dark grown 6 day-old sandwiched colonies and exposure to light or dark was prolonged for 24 h. Spore concentration was measured in duplo (maltose) or triplo (xylose) by dividing the spore number by the surface area of the colony. Germination assays were performed in duplo directly on agar medium and accessed using a MULTIZOOM AZ100 microscope (Nikon Corporation Instruments Company, Japan).

Localization of growth, protein synthesis and protein secretion

Protein synthesis, secretion, and growth were monitored in duplo as described (Wösten et al. 1991, Krijgsheld et al. 2012). Sandwiched colonies were labeled for 4 h with 185 kBq ^{14}C-amino acids (NEC 455E amino acid mixture, L-[^{14}C (U)]—specific activity 1.94G Bq milliatom^{-1}; Perkin Elmer, Waltham MA, USA) to monitor protein synthesis and secretion. A polyvinylidenediluoride (PVDF) membrane (Immobilon-p, Milipore, Bedford, USA) was placed in between the sandwich colony and the agar medium to immobilize the secreted proteins. Growth was visualized by labeling for 10 min with 185 kBq ^{14}C-N-acetyylglucosamine (CFA485 N-acetyl-D-[1-14C] glucosamine, specific activity 1.85-2.29 GBq mmol^{-1}; GE Healthcare, Westborough, MA, USA). Labelled N-acetylglucosamine or amino acids were placed on top of the colony after absorbing it to a piece of rice paper (Schleicher and Schuell, Dassel, Germany) with the size of the colony. Colonies were fixed with 4 % formaldehyde in phosphate buffered saline (PBS) for 1 h at room temperature. Sandwiched colonies and PVDF membranes were washed three times 60 min with 1 % casamino acids (Becton, Dickinson and company, Le-Pont-De-Claix, France) or 0.44 mM N-actyl-glucosamine depending on the label used. After drying over night at room temperature, colonies and PVDF membranes were exposed to Kodak Biomax XAR film (Sigma-Aldrich, Saint Louis, USA).

Localization of starch degrading activity

Starch-degrading activity was detected in triplo by transferring 7-day-old maltose grown sandwiched colonies to agar MM containing 1 % soluble starch. After 4 h, colonies were removed and the medium was sprayed with 5 % (w/v) iodine/10 % (w/v) potassium iodide solution (Lugol) (Wösten et al. 1991).

Scanning electron microscopy

Sandwiched colonies were grown in the absence of an agarose layer between PC membranes. Samples (8 × 5 mm) were cut from the periphery and the center of the colony and prepared as described (Krijgsheld et al. 2013b). A Field Emission Scanning Election Microscope (FEI, Eindhoven, The Netherlands) was used to view the samples using an acceleration voltage of 5 kV and a working distance of about 5 mm.

Statistical analysis

Statistical analysis was performed with IBM Statistics 20 (SPSS statistics; IBM New York, USA) using a p value of 0.05.

Results

Plasmid pJG01 contains the *A. niger amyR* gene under control of his own promoter (vankuyk et al. 2011). This vector was co-introduced with vector pIM2101 into *A. niger* strain NW249 selecting for arginine prototrophy (vanKuyk et al. 2011). Secretion of glucoamylase was monitored by immuno-localization on PVDF membranes that had been placed under sandwiched colonies (Fig. 1). The amount of secreted glucoamylase was higher in most of the transformants (vanKuyk et al. 2011). The majority of the strains (e.g. UU-A001.24) showed a spatial secretion pattern like the control strains N402 and UU-A049.1. These strains secreted glucoamylase mainly at the periphery of the colony. In contrast, transformant UU-A001.13 released glucoamylase throughout the colony. Spatial release and the level of secretion of glucoamylase correlated with the zonal expression of the glucoamylase gene *glaA* (Fig. 1). The control strain and UU-A001.24 mainly expressed *glaA* in the two outer zones of 7-day-old colonies. The latter strain showed a much higher expression than the wild-type and expression was also observed in the colony centre. In contrast, expression of *glaA* was similar throughout the colony in the case of UU-A001.13. Its expression was increased in the central zones when compared to the control.

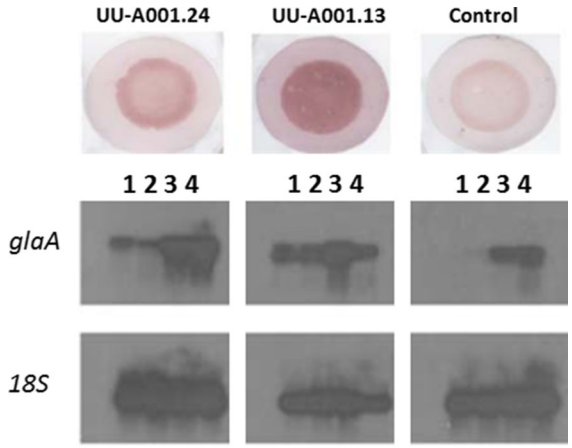

Fig. 1 Immuno-localization of glucoamylase (*upper panels*) and *glaA* mRNA accumulation (*middle panels*) in maltose grown colonies of the *amyR* multicopy strains UU-A001.13 and UU-A001.24 and the control strain UU-A049.1.*18S* rRNA (*lower panels*) was used as a RNA loading control. Zone 4 represents the periphery of the colony; zone 1 the inner center

UU-A001.13 was selected for further analysis because of the changed spatial expression of *glaA* and secretion of its encoding protein. Southern analysis showed the presence of several extra copies of *amyR* in this strain based on the intensity of the hybridization signal (Supplemental Fig. 1). The genome of UU-A001.13 was sequenced with 107-time coverage to localize the insertions of the transforming plasmids. Three insertion points of pIM2101 or pJG01 were identified (Supplemental Fig. 2). It was difficult to discriminate which vector caused the break points due to the high similarity between pIM2101 and pJG01 (their vector backbones pUC19 and pGEM11 share 99 % identity).The insertion in chromosome 1 was identified in the 3′ end of the coding sequence of *fluG* (An14g03390). This was accompanied by a deletion of 850 bp coding sequence upstream of the insertion (Supplemental Fig. 2A). Another insertion site was found in chromosome 4 (Supplemental Fig. 2B). In this case, the integration had occurred in the untranslated region (180 bp from the start codon) of a phosphate/phosphoenolpyruvate translocator gene (An02g08670). An integration in chromosome 6 (Supplemental Fig. 2C) disrupted the 5′ end of the open reading frame of the α-glucosidase gene *agdA* (An04g06920). This was accompanied by a deletion of the coding sequence of the endogenous copy of *amyR* (An04g06910) with its up- and downstream flanking regions.

Phenotypic characterization of Δ*fluG*

It was assessed whether the phenotype of UU-A001.13 could be attributed to inactivation of *fluG*. To this end, *fluG* was inactivated by introducing vector *BNOfluG* in N402 (data not shown). As a control, the coding sequence was reintroduced in the Δ*fluG* strain, resulting in strain Δ*fluG fluG*. Seven-day-old maltose grown sandwiched colonies of N402 (wild type), UU-A001.13, the Δ*fluG* strain, and the Δ*fluG fluG* strain were transferred for 4 h to a starch containing agar plate. Lugol staining demonstrated that the wild-type and the Δ*fluG fluG* strain degraded starch everywhere except for a subperipheral zone and the central zone (note degradation in the subperipheral zone was more

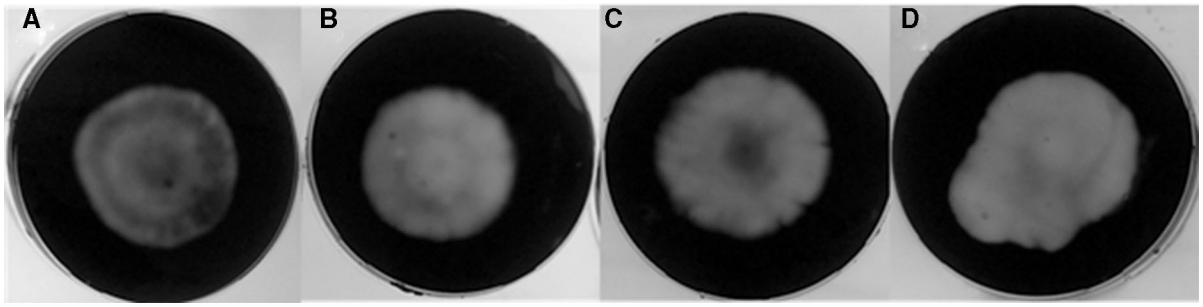

Fig. 2 Lugol staining of starch plates after 4 h incubation of 7-day-old maltose grown colonies of N402 (control) **(a)**, Δ*fluG* **(b)**, Δ*fluG* *fluG* **(c)**, and UU-A001.13 **(d)**

intense in the case of the complemented Δ*fluG* strain) (Fig. 2). On the other hand, the Δ*fluG* strain and strain UU-A001.13 degraded starch throughout the medium underlying the colony. This shows that FluG has an effect on spatial starch degradation.

The role of FluG in asexual sporulation was also assessed. Conidiophore formation was monitored in 6-day-old sandwiched colonies of the wild-type strain, the Δ*fluG* strain, and the complemented Δ*fluG* strain by removing the upper PC membrane for 24 h. Spatial patterns of sporulation (Fig. 3) and spore numbers (Table 2) did not significantly differ between the strains when colonies were grown in the light or in the dark using maltose or xylose as carbon source. All three strains formed less spores on xylose when compared to maltose (Table 2). Scanning electron microscopy revealed that Δ*fluG* colonies form fully developed conidiophores similar to those of wild type (Fig. 4). Moreover, biomass was not significantly different between Δ*fluG* colonies and wild type on either maltose or xylose containing medium (Supplemental Fig. 3).

Growth was localized at the periphery of 7-day-old colonies of the wild-type and the Δ*fluG* strain by labelling with ^{14}C N-acetylglucosamine (Fig. 5). Protein synthesis and secretion, as monitored by labelling with ^{14}C amino acids, was localized throughout the mycelium and at the periphery of the colonies, respectively (Fig. 5). Thus, spatial growth and secretion patterns have not been changed in Δ*fluG* colonies when compared to the wild-type.

RNA profiles in zones of Δ*fluG* colonies

The mycelium of 7-day-old maltose-grown sandwiched colonies of N402 and the Δ*fluG* strain were divided in five concentric zones (Levin et al. 2007a, Levin et al. 2007b). Gene expression was assessed in the central zone 1, the intermediate zone 3, and the outer zone 5 using RNAseq. Principal component analysis (PCA) showed that the transcriptomes from zone 1 and 3 of N402 and Δ*fluG* separated from zone 5 of these strains. This component accounted for 51 % of the variation in the datasets (Supplemental Fig. 4). The second component of the PCA accounted for 14 % of the variation and separated the zones of the wild-type from that of the Δ*fluG* strain. Expression in the central zone 1 and the intermediate zone 3 of the wild-type (N402) correlated to a high extent (Pearson's $r^2 \geq 0.98$, Supplemental Fig. 4), while correlation of these profiles with zone 5 was less ($r^2 = 0.92$ in both cases). A similar result was found in the case of the Δ*fluG* strain with a r^2 of 0.98 and 0.94, respectively. Expression profiles of zones 1 of the wild-type and the Δ*fluG* strain showed a r^2 of 0.97. A similar high correlation was found when zones 3 or zones 5 were compared (r^2 of 0.98 in both cases).

Gene expression comparison and GO analysis

A total of 1,451 genes were differentially expressed in zone 1 of Δ*fluG* colonies (472 up- and 979 down-regulated) when compared to zone 1 of N402 colonies. Similarly, 993 (506 up- and 487 down-regulated) and 1,047 (375 up- and 672 down-regulated) were differentially expressed in zones 3 and 5, respectively. A total of 210 and 620 genes were up- and down-regulated, respectively, in zone 1 when a fold-change ≥ 2 was used as cut-off. These numbers were 340 and 257 and 472 and 187 for zones 3 and 5.

GO analysis (biological process, cellular component, and molecular function) was performed on the differentially expressed genes with a fold change ≥ 2.

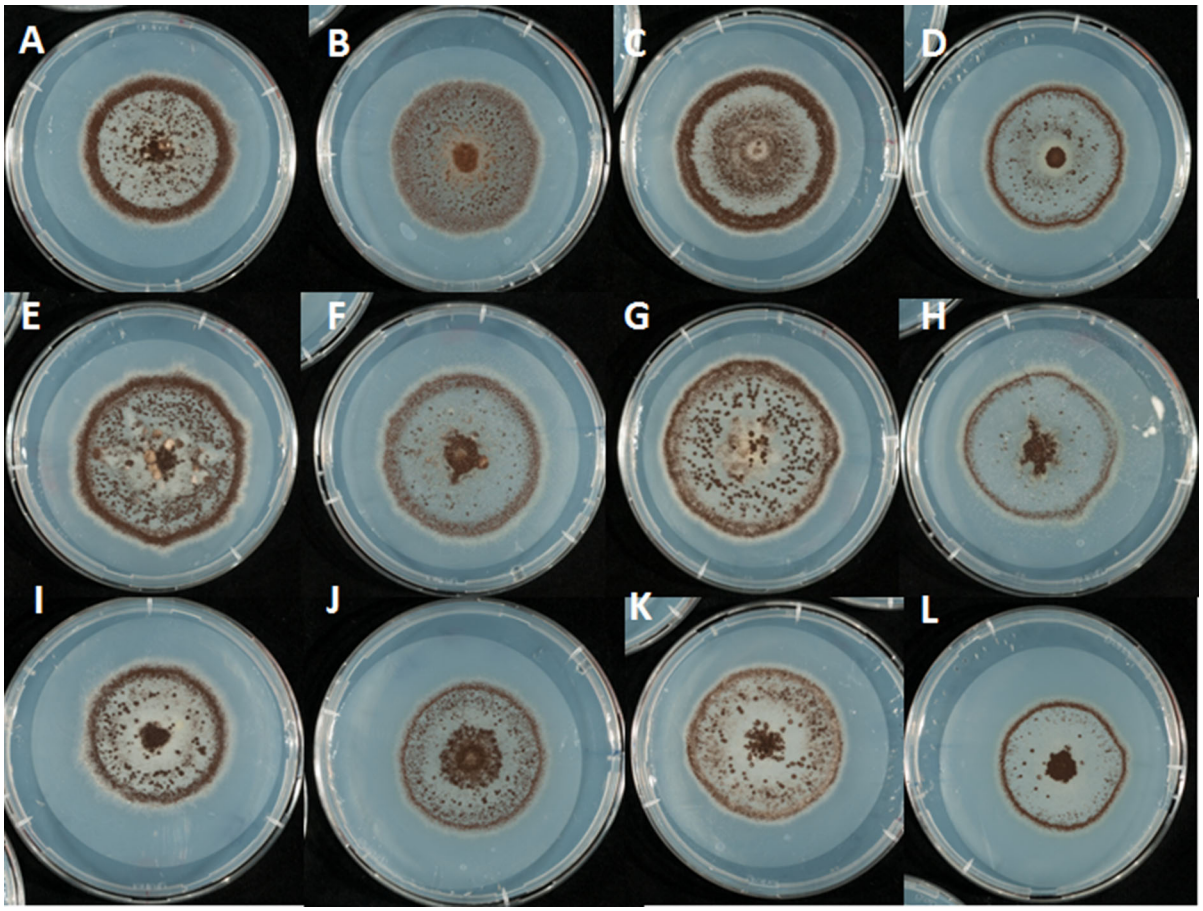

Fig. 3 Sporulation profiles of wild type N402 (*a-d*), the Δ*fluG* strain (*e–h*), and the Δ*fluG* complemented strain (*i-l*) when grown in light (1,200 lux) (*a, b, e, f, i,* and *j*) or dark (*c, d, g, h, k,* and *l*) using maltose (*a, c, e, g, i,* and *k*) or xylose (*b, d, f, h, j,* and *l*) as a carbon source. The upper PC membrane of 6-day-old sandwiched colonies was removed and spores were allowed to form for 24 h

Table 2 Density (spore number cm^{-2}) of spores produced by wild type N402, the Δ*fluG* strain, and the Δ*fluG* complemented (FC) strain under light (1,200 lux) and dark conditions using maltose or xylose as a carbon source. The upper PC membrane of 6-day-old sandwiched colonies was removed and spores were harvested after 24 h. Standard deviation of duplo (maltose) and triplo (xylose) experiments is indicated

	Light			dark		
	N402	Δ*fluG*	FC	N402	Δ*fluG*	FC
Maltose						
Average	$67\ 10^6 \pm 0$	$57\ 10^6 \pm 0{,}3\ 10^6$	$60\ 10^6 \pm 3\ 10^6$	$59\ 10^6 \pm 7\ 10^6$	$62\ 10^6 \pm 1\ 10^6$	$53\ 10^6 \pm 2\ 10^6$
Xylose						
Average	$15\ 10^6 \pm 0.02\ 10^6$	$15\ 10^6 \pm 4\ 10^6$	$22\ 10^6 \pm 1\ 10^6$	$18\ 10^6 \pm 7\ 10^6$	$11\ 10^6 \pm 2\ 10^6$	$30\ 10^6 \pm 5\ 10^6$

A single cellular component GO term (plasma membrane enriched fraction) was overrepresented in the upregulated gene group in zone 1 of the wild-type strain when compared to the respective zone in the Δ*fluG* colony (Supplemental Table 2A). In this gene group ten biological process (Supplemental Table 2B) and six molecular function (Supplemental Table 2C) terms were overrepresented. Of the biological process terms, eight were related to carbohydrate metabolic and catabolic processes, while three out of six

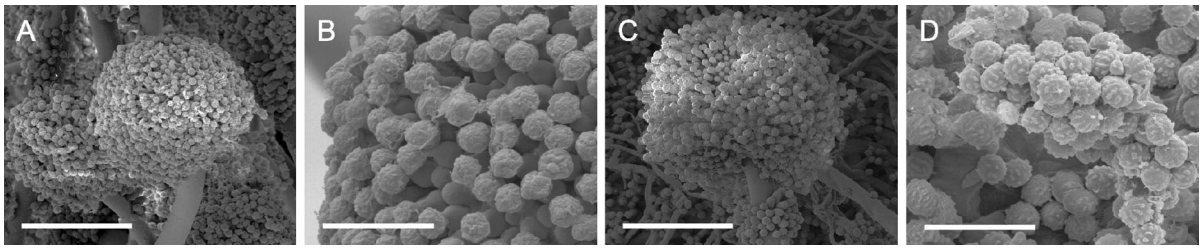

Fig. 4 Scanning electron microscopy of conidiophores formed during a 24 h period after removing the upper PC membrane from 6-day old sandwiched colonies of *A. niger* wild-type N402 (*a, b*) and the Δ*fluG* strain (*c, d*). *Bars* represent 40 μm (*a, c*) and 10 μm (*b, d*)

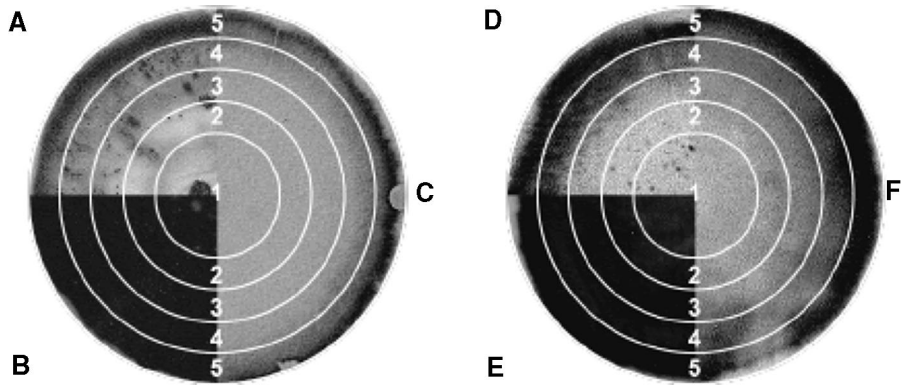

Fig. 5 Growth (*a,d*), protein synthesis (*b, e*), and secretion (*c, f*) in 7-day-old maltose-grown wild-type (*a–c*) and Δ*fluG* (*d–f*) colonies. Growth was monitored by labelling with ^{14}C N-acetylglucosamine, while protein synthesis and secretion were monitored by incorporation of ^{14}C-labeled amino acids. Secreted proteins were immobilized by a PVDF membrane that had been placed underneath the colony

molecular function terms were related to hydrolase activity. No GO terms were overrepresented in the intermediate zone 3 and the peripheral zone of wild type colonies. Only four biological process GO terms (carboxylic acid biosynthetic process, secondary metabolic process, fatty acid biosynthetic process, and organic acid biosynthetic process) were overrepresented in the upregulated gene group of zone 1 of Δ*fluG* colonies (Supplemental Table 3). In contrast, 18 and 16 biological process and molecular function terms, respectively, were overrepresented in the upregulated gene group of zone 3 of Δ*fluG* colonies (Supplemental Table 3). Of these terms, 12 and 4, respectively, were related to carbohydrate catabolic and metabolic processes. Similarly, 27 and 15 biological process and molecular function terms, respectively, were overrepresented in the upregulated gene group of zone 5 of Δ*fluG* colonies (Supplemental Table 3). Of these, 16 and 4 were related to carbohydrate metabolic and catabolic processes.

Expression analysis of selected functional gene classes

Genes involved in sexual and sexual development

72 genes have been implicated in asexual and sexual development in *A. niger* (Pel et al. 2007; Krijgsheld and Wösten 2013). Of these genes, only five genes had a ≥2-fold change in expression in at least one of the zones of Δ*fluG* colonies when compared to the respective zones of the wild-type (Supplemental Table 4). For instance, the *brlA* gene of *A. niger* was downregulated in all three zones of Δ*fluG* colonies, while *flbC* was downregulated in zone 1.

Cell wall biosynthesis genes

102 genes have been implicated in cell wall synthesis (Pel et al. 2007). Of these genes, 11 had a ≥2-fold change in expression in at least one of the zones of

Δ*fluG* colonies when compared to the respective zones of the wild-type (Supplemental Table 5). This set doesn't include chitin synthase or β-glucan synthase genes. The glucanosyltransferase genes *bgt1* (An08g03580) and *gel1* (An01g03090) were down-regulated (fold change ≥ 2) in the central zone 1 and upregulated in zones 3 and 5, respectively, of Δ*fluG* colonies. Moreover, chitinase genes *cfcA* and *chiD* were up- and downregulated in zone 3 of Δ*fluG* colonies, respectively, whereas chitinase gene *cfcI* was upregulated in zone 5 of Δ*fluG* colonies.

Genes encoding proteins with a signal sequence for secretion

The *A. niger* genome contains 2,554 genes that encode proteins with a predicted signal sequence for secretion (Pel et al. 2007). Within this group, 433 genes had a significant ≥2-fold change in expression in at least one zone of the Δ*fluG* colony when compared to wild type (Supplemental Table 6). A total of 60 and 157 genes were ≥2-fold up- and down-regulated, respectively, in the center of the Δ*fluG* colony when compared to the wild-type. Similarly, 135 and 50 genes and 165 and 57 genes were up- and down-regulated in the intermediate zone 3 and the peripheral zone 5 of the Δ*fluG* colony. Thus, genes encoding proteins with a signal sequence were more often downregulated in zone 1 and upregulated in zone 3 and 5 of the Δ*fluG* colony.

AmyR regulated genes

Gene *amyR* and the 16 genes it regulates did not show significant changed expression with a fold change ≥2 when zones 1 and 3 of the wild-type strain were compared (Table 3A). Five genes (*agdB, glaB, bgl, bglA, mstF*) were ≥2-fold upregulated in zone 1 or 3 when compared to zone 5, while two genes (α-galactosidase genes *aglB* and *aglC*) were down regulated when compared to zone 5 (Table 3A). Spatial expression profiles were different in strain Δ*fluG* (Table 3B). 8 out of 16 AmyR regulated genes as well as *amyR* itself were ≥2-fold upregulated in more peripheral zones, while only two genes (*mstF* and *glaB*) were ≥2-fold downregulated at the periphery. The changed spatial expression of the *amyR* regulated genes in Δ*fluG* was also reflected in the ratios of the mRNA levels when the 3 zones of Δ*fluG* were compared with the respective zones of N402 (Table 3C). Down-regulation (≥2-fold) of 6 out of the 16 AmyR regulated genes (including *glaA*) was observed in the central zone of the Δ*fluG* strain when compared to the wild-type. On the other hand, six genes (including acid amylase *aamA*) were up-regulated more than 2-fold at the periphery of colonies of the Δ*fluG* strain. Together, FluG plays a role in zonal expression of AmyR-regulated genes.

Genes involved in carbohydrate degradation

In *A. niger,* 172 genes show carbohydrase activity (Pel et al. 2007). Within this gene group, 131 genes are secreted and 40 genes encode intercellular enzymes (Pel et al. 2007). When compared to wild type, 5 and 22 genes encoding extracellular carbohydrases were significant up- and down-regulated in zone 1 of Δ*fluG* colonies with a fold change ≥2, respectively (Supplemental Table 7). 26 and 1 gene and 32 and 3 genes were significant up- and down-regulated in the intermediate zone 3 and the peripheral zone 5 of the Δ*fluG* colony, respectively.

Transcription factor genes

The genome of *A. niger* contains 623 genes encoding transcription factors (Pel et al. 2007) Of these genes, 56 were differentially expressed with a fold change ≥2 in at least one zone of Δ*fluG* colonies (Supplemental Table 8). 6 and 14 genes showed significant ≥2-fold up- and down- regulation respectively in the center of the Δ*fluG* colony when compared to the wild-type. 13 and 17 genes and 15 and 8 genes were ≥2-fold up- and down- regulated in the intermediate zone 3 and the peripheral zone 5, respectively.

Discussion

The sporulation pathway in *A. niger* has been proposed to be similar, if not identical, to that of *A. nidulans* (Pel et al. 2007). Indeed, BrlA and FlbA have a similar role in asexual reproduction in these fungi (Krijgsheld et al. 2013b). However, the role of VeA is different (Wang et al. 2014). Here it was shown that FluG also has different roles in aspergilli. FluG of *A. nidulans* and *A. oryzae* is involved in initiation of conidiophore formation. In contrast, Δ*fluG* strains of *A. fumigatus* and *A. niger* still form conidiophores. Notably, *A.*

Table 3 Expression of *amyR* and its regulated genes in the outer zone 5, the intermediate zone 3, and the central zone 1 of 7-day-old maltose grown colonies of N402 (A), Δ*fluG* (B), and of N402 and Δ*fluG* (C)

A

Gene id	gene name	Description	log2_fold_change		
			n402 zone 3 / n402 zone 1	n402 zone 5 / n402 zone 1	n402 zone 5 / n402 zone 3
An04g06920	agdA	extracellular α-glucosidase	0,357362	0,63302	0,275658
An01g10930	agdB	Putative α-glucosidase	0,506088	-0,790265	-1,29635
An11g03340	aamA	acid alpha-amylase aamA	0,240682	-0,549849	-0,79053
An03g06550	glaA	glucoamylase glaA	0,166282	0,413587	0,247305
An12g03070	glaB	Putative glucoamylase	-0,421863	-4,04902	-3,62715
An04g06930	amyC	α-amylase	-0,137068	-0,7216	-0,584532
An05g02100	amyA	extracellular α-amylase amyA	ND	ND	ND
An06g00170	aglA	alpha-galactosidase	ND	ND	ND
An06g00290	lacC	β-galactosidase lacC	ND	ND	ND
An14g01770	bgl	strong similarity to β-glucosidase	0,459257	-0,813096	-1,27235
An09g00260	aglC	α-galactosidase C aglC [truncated ORF]	0,698624	1,413	0,714374
An09g00270	aglC	α-galactosidase C aglC [truncated ORF]	0,253318	0,965988	0,71267
An04g02700	agl	hypothetical protein	0,603066	0,643724	0,0406572
An06g02040	bgl	strong similarity to β-glucosidase	0,144934	0,41446	0,269527
An02g11150	aglB	identical to α-galactosidase	-0,288768	0,905355	1,19412
An18g03570	bglA	β-glucosidase	0,349416	-1,29361	-1,64303
An02g00590	mstF	strong similarity to high-affinity glucose transporter	-0,0816058	-2,41745	-2,33585
An04g06910	amyR	transcription regulator of maltose utilization	0,375819	0,0967851	-0,279034

oryzae and *A. niger* are most related of these four aspergilli, while *A. nidulans* is most unrelated (Houbraken et al. 2014). This implies that loss/gain of function of FluG in asexual development has occurred at least two independent times in the evolution of aspergilli. We also showed that asexual development does not depend on light in the case of *A. niger,* while it preferentially takes place in the light in the case of *A. nidulans* (Purschwitz et al. 2008) and is repressed by white and red light in *A. oryzae* (Hatakeyama et al. 2007).

Multiple copies of *amyR* were introduced in *A. niger* to assess whether over-expression of this regulator would impact production levels of glucoamylase and its spatial release in colonies. Most transformants did indeed show higher production of glucoamylase (vanKuyk et al. 2011). This confirms that introduction of multiple copies of *amyR* is an effective strategy to improve production of glucoamylase as was shown in *Aspergillus awamori* (Vinetskii et al. 2010). Presence of multiple copies of *amyR* did not affect the ratio of glucoamylase released at the periphery and within the center of most of the transformants. However, over-expression did increase absolute levels of *glaA* mRNA and release of its encoded protein in these zones of the transformants. Expression of *glaA,* however, was identical in all zones of transformant UU-A001.13 being equivalent to that found at the periphery of wild-type colonies. This loss of heterogeneity in *glaA* expression was accompanied by loss of spatial heterogeneity in release of glucoamylase and starch degrading activity. Clearly, this loss of heterogeneity was not the result of over-expression of *amyR* since colonies of other transformants still showed heterogeneous zonal expression of *glaA* and heterogeneous GlaA release. Therefore, it was assessed whether an integration site of the introduced vectors caused the loss of

Table 3 continued

B

Gene id	gene name	Description	log2_fold_change		
			Δ*fluG* zone 3 / Δ*fluG* zone 1	Δ*fluG* zone 5 / Δ*fluG* zone 1	Δ*fluG* zone 5 / Δ*fluG* zone 3
An04g06920	agdA	extracellular α-glucosidase	2,14493	3,49202	1,34709
An01g10930	agdB	Putative α-glucosidase	1,99175	1,69288	-0,298861
An11g03340	aamA	acid alpha-amylase aamA	3,27008	3,3757	0,105613
An03g06550	glaA	glucoamylase glaA	1,93835	3,13647	1,19812
An12g03070	glaB	Putative glucoamylase	0,262712	-2,04267	-2,30538
An04g06930	amyC	α-amylase	0,618824	0,288562	-0,330263
An05g02100	amyA	extracellular α-amylase amyA	ND	ND	ND
An06g00170	aglA	alpha-galactosidase	ND	ND	ND
An06g00290	lacC	β-galactosidase lacC	ND	ND	ND
An14g01770	Bgl	strong similarity to β-glucosidase	0,54222	-0,39406	-0,93628
An09g00260	aglC	α-galactosidase C aglC [truncated ORF]	2,32574	3,47434	1,1486
An09g00270	aglC	α-galactosidase C aglC [truncated ORF]	2,12952	3,57457	1,44504
An04g02700	Agl	hypothetical protein	0,113626	0,101274	-0,0123518
An06g02040	Bgl	strong similarity to β-glucosidase	-1,17933	-0,445808	0,73352
An02g11150	aglB	identical to α-galactosidase	-0,121398	1,7121	1,83349
An18g03570	bglA	β-glucosidase	0,883157	1,05544	0,172287
An02g00590	mstF	strong similarity to high-affinity glucose transporter	1,16005	-0,200083	-1,36014
An04g06910	amyR	transcription regulator of maltose utilization	1,3399	1,40368	0,0637803

heterogeneity in UU-A001.13. Whole genome sequencing revealed three integration sites affecting four genes. For instance, vector insertion in chromosome 1 was accompanied by a deletion of 0.9 kb coding sequence of *fluG*. Thus, integration of plasmids is accompanied with deletion events near the integration site. This is something to consider when phenotypes of deletion or over-expression strains are being evaluated.

Inactivation of *fluG* in a wild-type background showed a starch degrading activity under the colony similar to that of UU-A001.13. However, expression of AmyR regulated genes in Δ*fluG* was not in line with the starch degrading activity. The starch degrading activity predicted that AmyR regulated genes would be up-regulated in the center of Δ*fluG* colonies. However, 6 out of 16 AmyR regulated genes (including *glaA* and *amyR*) were ≥2-fold down-regulated in the center of Δ*fluG* colonies when compared to N402. At the same time, six AmyR regulated genes (including *glaA* and *amyR*) were ≥2-fold up-regulated in the peripheral zone of Δ*fluG* colonies. The discrepancy between amylolytic gene expression in UU-A001.13 and Δ*fluG* may be explained by AmyR levels in the different zones of the colony. Over-expression of *amyR* in UU-A001.13 may result in AmyR levels in the central part of the colony high enough to elevate expression of amylolytic genes like *glaA* in this zone of the colony when compared to wild-type. But how can we explain the discrepancy between expression of the AmyR regulated genes (being high in the periphery of the colony) and the starch degrading activity (being

Table 3 continued

C

Gene id	gene name	Description	log2_fold_change		
			n402 zone 1 / Δ*fluG* zone 1	n402 zone 3 / Δ*fluG* zone 3	n402 zone 5 / Δ*fluG* zone 5
An04g06920	agdA	extracellular α-glucosidase	2,17737	0,389808	-0,681626
An01g10930	agdB	Putative α-glucosidase	1,4731	-0,012554	-1,01005
An11g03340	aamA	acid alpha-amylase aamA	2,49646	-0,532945	-1,42909
An03g06550	glaA	glucoamylase glaA	1,98288	0,210809	-0,740003
An12g03070	glaB	Putative glucoamylase	2,43551	1,75094	0,429164
An04g06930	amyC	α-amylase	0,68023	-0,07566	-0,329929
An05g02100	amyA	extracellular α-amylase amyA	ND	ND	ND
An06g00170	aglA	alpha-galactosidase	ND	ND	ND
An06g00290	lacC	β-galactosidase lacC	ND	ND	ND
An14g01770	Bgl	strong similarity to β-glucosidase	-0,0545	-0,137424	-0,473498
An09g00260	aglC	α-galactosidase C aglC [truncated ORF]	0,08726	-1,53986	-1,97408
An09g00270	aglC	α-galactosidase C aglC [truncated ORF]	1,07795	-0,798254	-1,53063
An04g02700	Agl	hypothetical protein	-0,3673	0,122091	0,1751
An06g02040	Bgl	strong similarity to β-glucosidase	-0,7789	0,545328	0,081335
An02g11150	aglB	identical to α-galactosidase	0,71179	0,544418	-0,0949533
An18g03570	bglA	β-glucosidase	0,66005	0,126312	-1,689
An02g00590	mstF	strong similarity to high-affinity glucose transporter	1,06434	-0,177316	-1,15303
An04g06910	amyR	transcription regulator of maltose utilization	0,767391	-0,19669	-0,539505

The ^{2}log ratio of differences in gene expression is indicated. A number ≥ 1 shows a ≥ 2 fold decrease in the second zone in the comparison; whereas a number ≤ -1 shows a ≥ 2 fold increase in this zone. The green and red shading indicate significant ≥ 2 fold upregulation and downregulation of genes; light green and red shading indicates ≤ 2-fold but significant up and down regulation of genes. An09g00260 and An09g00270 represent truncated ORFs of α-galactosidase C (aglC) and thus represent one gene

increased in the colony center when compared to the wild-type)? A discrepancy between gene expression and secretome activity was also observed in the case of the Δ*flbA* strain of *A. niger*. It was shown that 70 proteins are secreted by the *ΔflbA* strain that are not found in the medium of the wild-type (Krijgsheld et al. 2013b). Only in the case of 22 proteins this could be explained by upregulation of the encoding genes (Krijgsheld and Wösten 2013). This discrepancy was suggested to be due to (post)-translational regulation or by induction of genes after transfer to fresh medium. Gene expression profiling was done with colonies that had not been transferred, while secretome analysis was done after transfer to fresh medium. We used a similar approach in this study. RNA profiling was done without transfer, while starch degrading activity included a transfer to fresh medium. The discrepancy between starch degrading activity and gene expression could also be explained by an increased retention time of secreted proteins in the cell wall. Proteins secreted at the periphery would thus be mainly released in the medium when this part of the colony has become a more central zone. Differential expression of the *amyR* regulated genes is illustrative for other genes encoding proteins with a signal sequence for secretion. Out of 2,554 of these genes, 433 were differentially expressed. A total of 60 and 157 genes were ≥ 2-fold up- and down-regulated, respectively, in the center of the Δ*fluG* colony when compared to the wild-type. This ratio was reversed in

the intermediate and peripheral zones. 135 and 50 genes and 165 and 57 genes were up- and down-regulated in these zones, respectively.

Deletion of *fluG* gene did not cause a change in biomass formation and zonal growth profiles in *A. niger*. In agreement, only few genes predicted to be involved in cell wall synthesis showed differential expression in these two strains. For instance, chitin and glucan synthases showed similar expression in the two strains. Zonal formation of conidiophores was also similar in wild-type and Δ*fluG* colonies of *A. niger*. In addition, morphology of the conidiophores, and the number of spores formed per surface area were similar. This was observed in light- and dark-grown colonies and in colonies grown on maltose or xylose. The impact of *fluG* inactivation on expression of genes involved in asexual development of *Aspergillus* was minimal. Only 5 out of 72 genes were differentially expressed in the Δ*fluG* strain when compared to the wild-type strain. Of these five genes, *brlA* and *flbC* are known to play a central role in asexual development of *A. nidulans*. Gene *brlA* of *A. niger* was downregulated in all three zones of Δ*fluG* colonies, while *flbC* was downregulated in zone 1. The downregulation of *brlA* in the Δ*fluG* strain was expected from expression data of *A. nidulans* (Lee and Adams 1996). However, in *A. niger* this did not result in a phenotypic change in asexual development. Apparently, the reduced *brlA* expression is still sufficient to induce sporulation. The impact of FluG on *flbC* expression was not expected a priori. Expression of this gene in the Δ*fluG* strain of *A. nidulans* was not assessed in the past.

Together, it is concluded that FluG of *A. niger* is not involved in growth and sporulation but does affect zonal expression of genes including genes encoding secreted proteins. A role as a suppressor of secretion under particular environmental conditions would link FluG to asexual development. Experimental evidence indicates that FluG may also have a role as a secretion suppressor in *A. nidulans* (Emri et al. 2005; Szilágyi et al. 2010). A Δ*fluG* strain of this *Aspergillus* shows reduced formation of extracellular β-1,3-glucanase, β-glucosidase, protease and chitinase.

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Conflict of interest The authors declare that they have no conflict of interest.

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