

Transcriptome Analysis of Zones of Colonies of the $\Delta flbA$ Strain of *Aspergillus niger*

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Abstract

Wild-type colonies of *Aspergillus niger* grow and secrete enzymes at their periphery when they are grown on an agar medium. Inactivation of the sporulation gene *flbA* results in colonies that not only secrete proteins at their periphery, but also in central zones. This is accompanied by a more complex secretome, growth throughout the mycelium, and by thinner cell walls. Here, gene expression was studied at the periphery, an intermediate zone, and the centre of wild-type and $\Delta flbA$ colonies using whole genome microarrays. Heterogeneity in gene expression was not reduced in $\Delta flbA$ colonies when compared to wild-type colonies, despite decreased heterogeneity in zonal secretion, sporulation and growth. It was shown that 1152 genes had a fold change difference in expression ≥ 2 , when the averaged expression profiles of the zones of the wild-type were compared with those of $\Delta flbA$ colonies. This gene set contained 13 genes predicted to be involved in reproduction, 12 genes involved in cell wall biosynthesis, modification and degradation, 345 genes encoding secreted proteins and 38 genes encoding transcriptional regulators. These genes may account for the differences between wild-type and $\Delta flbA$ colonies in zonal growth, sporulation and secretion, and the complexity of the secretome and the thickness of the cell wall. The set of differentially expressed genes, in particular, the genes encoding transcriptional regulators, may be instrumental to improve *Aspergillus niger* as a cell factory for the production of enzymes.

Keywords: Fungus; *Aspergillus niger*; Heterogeneity; Sporulation; *flbA*; Regulator of G protein signaling; Transcriptome

Introduction

Aspergillus species are among the most abundant fungi worldwide. They feed on a large variety of organic substrates, in particular, on plant material [1]. To this end, enzymes are secreted that degrade the organic polymers within the substrate into small molecules that can be taken up as nutrients. The capacity of *Aspergillus* species to secrete proteins is enormous. For instance, some strains of *Aspergillus niger* secrete more than 30 grams per liter of glucoamylase [2]. This and the fact that a variety of secreted enzymes of *Aspergilli* are used in the industry or as pharmaceutical proteins makes these fungi important cell factories.

Aspergilli form colonies that consist of hyphae that grow at their tips and that branch subapically [3]. Growth and protein secretion mainly occur at the periphery of the colony when they are grown on an agar medium [4,5]. Hyphae at the periphery of the colony are exposed to unexplored organic material, whereas hyphae in the intermediate and central zones are confronted with a (partly) utilized substrate. The composition of the substrate explains about 50% of the variation in gene expression between different zones of the colonies [6]. The other half of the variation is caused by differentiation processes in the vegetative mycelium.

Recently, it was shown that zones of the colony that have the capacity to sporulate hardly secrete proteins [7]. This suggested that secretion by vegetative hyphae is repressed by the sporulation process. Indeed, a $\Delta flbA$ strain that does not sporulate not only secretes proteins at the colony periphery, but also within its central zones. This is accompanied by a more complex secretome, a reduced width of cell walls, and growth throughout the mycelium [8]. Gene *flbA* encodes an RGS domain protein that stimulates the intrinsic GTPase activity of the $G\alpha$ -subunit FadA [9]. This $G\alpha$ -subunit is part of a heterotrimeric G-protein complex that also consists of the $G\beta$ - and $G\gamma$ -subunits SfaD and GpgA, respectively (Figure 1). The activated $G\alpha$ -subunit and the $G\beta$ - $G\gamma$ dimer both stimulate vegetative growth. FlbA converts the

active $G\alpha$ -subunit-GTP into its inactive GDP bound stage, thereby repressing vegetative growth and promoting asexual development [10-12]. Taken together, these data indicate that FlbA stimulates sporulation and at the same time, represses vegetative growth and secretion by the vegetative mycelium. Moreover, it would promote synthesis of cell wall polymers, resulting in an increased width of the cell wall. The underlying molecular mechanisms are not yet known.

Here, the impact of inactivation of *flbA* on spatial gene expression in the *A. niger* colony was assessed. A total of 1152 genes had changed their expression ≥ 2 fold when RNA profiles of wild-type and $\Delta flbA$ colonies were compared. This set includes genes involved in reproduction and cell wall synthesis, and genes encoding transcriptional regulators and secreted proteins. These genes are of interest to improve *A. niger* as a cell factory.

Materials and Methods

Plasmids, fungal strains and growth conditions

The wild-type *A. niger* strain N402 [13] and its derivative N402 $\Delta flbA$ [8] were used in this study. These strains were grown as sandwiched colonies between porous polycarbonate membranes (0.1 μ m pores, 76 mm diameter; Profiltra, Almere, The Netherlands) placed

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Received March 22, 2013; Accepted October 21, 2013; Published October 23, 2013

Citation: Krijgsheld P, Wösten HAB (2013) Transcriptome Analysis of Zones of Colonies of the $\Delta flbA$ Strain of *Aspergillus niger*. Fungal Genom Biol 3: 109. doi:10.4172/2165-8056.1000109

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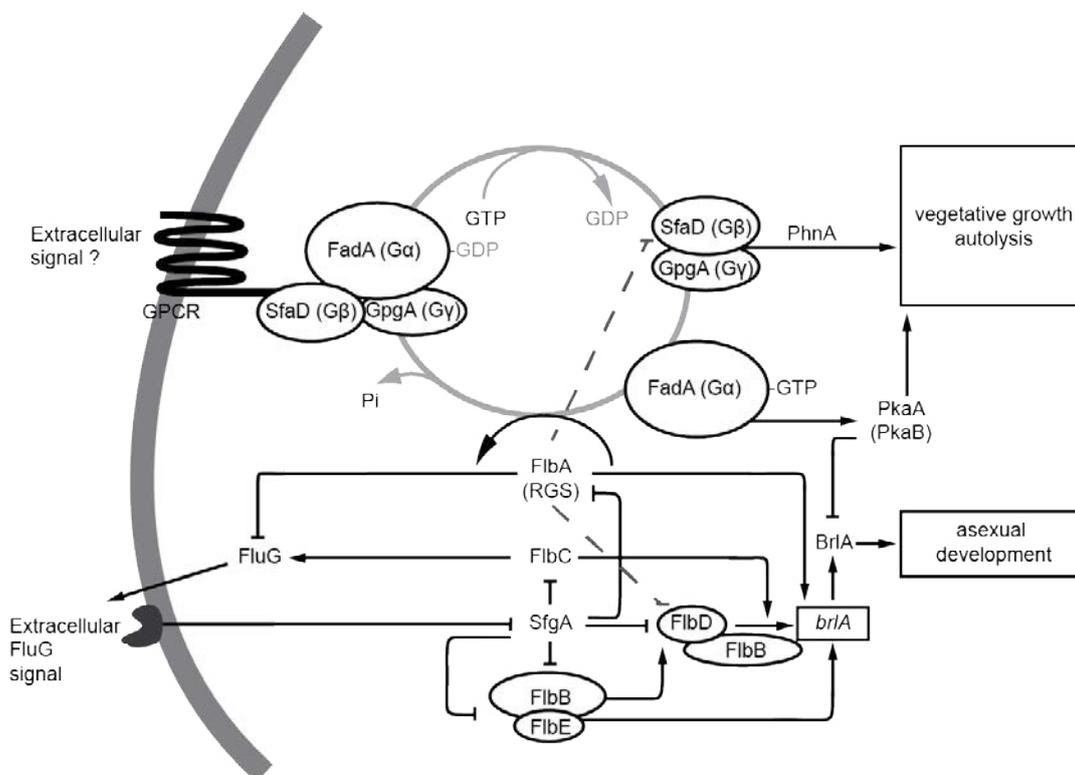


Figure 1: Model of the role of FlbA in asexual sporulation of *Aspergillus*. FlbA is an RGS protein regulating signaling of the $G\alpha$ -subunit FadA. GTP-bound FadA stimulates vegetative growth. FlbA enhances the intrinsic activity of GTP hydrolysis, converting FadA-GTP to the inactive heterotrimeric FadA-SfaD-GpgA GDP-bound G-protein complex. FlbA also inhibits *fluG* and activates *brlA*. The latter may be a direct effect or the result of inactivation of FadA. Stippled lines represent regulation of FlbA in *A. niger* based on the transcriptional analysis described this study. Adapted from [3].

on top of solidified minimal medium with 25 mM xylose as a carbon source [4-6]. Cultures were inoculated with a 1 mm mycelial plug and grown at 30°C.

RNA preparation and analysis

RNA was isolated from a biological duplicate of concentric zones of 7-day-old colonies. Zone 1 represented the most central zone, zone 3 an intermediate zone and zone 5, the peripheral zone [6]. Mycelium of zones was harvested from three colonies, frozen in liquid nitrogen and ground with a TissueLyser (Qiagen, Venlo, The Netherlands) in a 2 ml Eppendorf tube with two metal balls (4.76 in diameter) for 1 min at 25 Hz. The frozen material was taken up in 1 ml TRIzol reagent (Invitrogen, Bleiswijk, The Netherlands) by vortexing. Samples were incubated for 2 min after mixing with 200 μ l chloroform. This was followed by centrifugation at 10000 g for 10 min. RNA was purified using an RNA clean up column (Machery Nagel, Düren, Germany), after addition of 1 volume 70% EtOH to the water phase. After loading the sample on the column, it was centrifuged for 30 sec at 10000 rpm. This was followed by addition of 600 μ l RA3 buffer (provided by the RNA-clean up kit). After 2 min centrifugation at 10000 g, 250 μ l RNA3 was added, followed by another 2 min centrifugation at 10000 g. RNA was eluted after a 10 min incubation in two steps with 40 μ l and 50 μ l RNase free water. The eluted RNA was pooled and 1 μ l was checked for concentration and purity using the nanodrop ND-1000 spectrophotometer (Nanodrop Technology, Wilmington, USA) and the Bioanalyser 2100 (Agilent Technologies, Santa Clara, USA) (Supplemental Figure 1), respectively.

cDNA labeling, microarray hybridization and data analysis

cDNA labeling, microarray hybridization and scanning were performed at ServiceXS (Leiden, The Netherlands), according to Affymetrix protocols. From each RNA sample, 100 ng was used to synthesize biotin-labeled cRNA with the Affymetrix 3' IVT-Express Labeling Kit. Quality of cRNA was checked with the nano-lab-on-a-chip Bioanalyzer 2100 (Agilent Technology). 15 μ g cRNA was fragmented and half of it was used for hybridization on Affymetrix *A. niger* GeneChips [14]. After an automated process of washing and staining, absolute values of expression were calculated from the scanned array using the Affymetrix Command Console v1 software. The Affymetrix probe sets on the chip represent 14,259 annotated ORFs and genetic elements of *A. niger* [14]; (GEO [15] under accession no. GPL6758). The *A. niger* array data of this study have been deposited in the GEO database under accession number GSE44391.MAS5.0 absent/present calls showed that on average 50% and 48% of the genes were expressed in colonies of the wild-type and the $\Delta flbA$ strain, respectively. Genedata Expressionist and Genedata Analyst were used for normalization and statistical analysis of the arrays (Genedata, Basel, Switzerland). The arrays were condensed with the RMA algorithm and normalized on the quantile. Statistical assessment of differential expression was performed with *t*-test ($p \leq 0.01$, using a false discovery rate [BHQ] of ≤ 0.05) or by ANOVA ($p \leq 0.01$, BHQ ≤ 0.05). Differentially expressed genes (change in expression ≥ 2 -fold) were clustered using K-Means clustering. A differentially expressed set of genes obtained by ANOVA was used for a maximal paired contrast analysis to find the zone or strain where the highest mean-difference was observed. The Functional

Catalogue (FunCat, Munich Information Center for Protein Sequence, Munich, Germany) [16] was used for functional classification of genes. Fisher's exact test was used to identify over- and under-represented functional classes in the sets of differentially expressed genes ($p \leq 0.001$, BHQ ≤ 0.05).

cDNA synthesis and quantitative PCR analysis

cDNA was synthesized from biological duplicates of total RNA of zones 1, 3 and 5 of wild type and $\Delta flbA$ colonies using the QuantiTect Reverse Transcription Kit (Qiagen). QPCR was performed on the cDNA using the ABI Prism 7900HT SDS and SYBR Green chemistry (Applied Biosystems, Life Sciences, Foster City, USA). A run without cDNA was used as a negative control for every condition. Cycle threshold (Ct) levels were measured in triplicate for *actin* (An15g00560), *faeA* (An09g00120), *glaA* (An03g06550) and 18S rDNA (An03e03200). Primers were designed according to the recommendations of the PCR master-mix manufacturer (Applied Biosystems, Life Sciences, Foster City, USA), and were described previously [17]. The Ct values of 18S

were used to normalize the qPCR data. For qPCR, each cycle Ct-change in qPCR represents a two-fold change in expression ($2^{-\Delta(\Delta CT)}$, where $\Delta Ct = Ct_{\text{target gene}} - Ct_{18S \text{ gene}}$ and $\Delta(\Delta CT) = \Delta CT_{\text{mutant}} - \Delta CT_{\text{wild type}}$).

Results

Five concentric zones can be distinguished in 7-day-old sandwiched colonies of *A. niger* [4,6]. Zone 1, 3 and 5 represent the most inner zone, an intermediate zone, and the most outer zone of the colony, respectively. Gene expression was assessed in these three zones of xylose-grown colonies of the wild-type and the $\Delta flbA$ strain of *A. niger* using Affymetrix microarrays (Supplemental Table 1). QPCR of *glaA*, *faeA*, and *actin* (Supplemental Table 2) was used to verify micro-array data (Supplemental Table 3). Fold changes of these genes between zone 1 of wild-type and zone 1 of $\Delta flbA$, zone 3 of wild-type and zone 3 of $\Delta flbA$, and zone 5 of wild-type and zone 5 of $\Delta flbA$ as obtained with micro-array analysis and QPCR showed a Pearson's correlation coefficient ≥ 0.97 (Supplemental Table 4).

	wt up intermediate/ center	wt up periphery	$\Delta flbA$ up intermediate/ center	$\Delta flbA$ up periphery
01 Metabolism	O	O	O	O
01.01.10 amino acid degradation (catabolism)	O			O
01.03.19 nucleotide transport				O
01.05.01 C-compound and carbohydrate utilization				O
02 Energy		O		O
02.11.05 accessory proteins of electron transport and membrane-associated energy conservation		O		
03 Cell cycle and dna processing			U	
04 Transcription		U	U	U
04.05.01 mRNA synthesis		U		U
05 Protein synthesis		O	U	
08 Cellular transport and transport mechanisms				O
40 Subcellular localisation				U
99 Unclassified proteins	U	U	U	U

Table 1: Over- (O) and under- (U) representation of functional gene classes in the pool of genes that are ≥ 2 fold up- or down-regulated in the central zone 1 and intermediate zone 3 when compared to the peripheral zone 5 of wild-type and $\Delta flbA$ colonies.

	zone 1 Up	zone 1 Down	zone 3 Up	zone 3 Down	zone 5 Up	zone 5 Down
01 Metabolism	O	O	O	O		O
01.01.07 amino acid transport	O					
01.05.01 C-compound and carbohydrate utilization						O
03 Cell cycle and DNA processing						U
06.13.99 other proteolytic degradation						O
08 Cellular transport and transport mechanisms					O	
99 Unclassified proteins	U	U		U		U

Table 2: Over- (O) and under- (U) representation of functional gene classes in the pool of genes that are ≥ 2 fold up- or down-regulated in the $\Delta flbA$ strain when compared to the wild-type.

	up-regulated in $\Delta flbA$	Down-regulated in $\Delta flbA$
01 Metabolism	O	O
01.01.07 amino acid transport		
01.05.01 C-compound and carbohydrate utilization		O
02 Energy		U
03 Cell cycle and DNA Processing		U
03.03.01 mitotic cell cycle and cell cycle control		U
04 Transcription	U	
05 Protein synthesis		U
08 Cellular transport and transport mechanisms	O	
40 Subcellular localisation		U
99 Unclassified proteins	U	U

Table 3: Over- (O) and under- (U) representation of functional gene classes in the pool of genes that are ≥ 2 fold up- or down-regulated between the mean expression values of zones 1, 3, and 5 of the $\Delta flbA$ strain and those of the wild-type strain.

Annotation	Gene name*	Description	wild-type zone 1	wild-type zone 3	wild-type zone 5	$\Delta flbA$ zone 1	$\Delta flbA$ zone 3	$\Delta flbA$ zone 5	Regulation	sigP
Top 50 down-regulated genes in the $\Delta flbA$ strain compared to wildtype										
An05g01730	<i>uncharacterized</i>	hypothetical protein	11442 ± 3316	14891 ± 315	4511 ± 2021	42 ± 7	37 ± 0	27 ± 1	Down	y
An09g00840	<i>uncharacterized</i>	weak similarity to antigenic cell wall galactomannoprotein MP1 - <i>Aspergillus fumigatus</i>	3153 ± 2599	10725 ± 146	3462 ± 103	29 ± 11	35 ± 22	14 ± 2	Down	y
An18g05480	<i>uncharacterized</i>	strong similarity to alcohol oxidase AOX1 - <i>Pichia pastoris</i>	7168 ± 839	7322 ± 2676	3105 ± 1353	46 ± 2	44 ± 3	42 ± 5	Down	n
An16g05920	<i>uncharacterized</i>	weak similarity to surface recognition protein PTH11 - <i>Magnaporthe grisea</i>	4282 ± 1762	6936 ± 1040	5405 ± 665	48 ± 1	47 ± 11	44 ± 1	Down	n
An07g06460	<i>uncharacterized</i>	similarity to C-7 hydroxycephem methyltransferase coupling protein of patent WO9529253-A1 - <i>Streptomyces lactamdurans</i>	1807 ± 56	3006 ± 541	4779 ± 1295	29 ± 5	24 ± 5	28 ± 0	Down	n
An16g05930	<i>uncharacterized</i>	strong similarity to predicted protein An08g06890 - <i>Aspergillus niger</i>	5034 ± 1765	7101 ± 440	6203 ± 1646	63 ± 18	58 ± 11	58 ± 9	Down	n
An01g06890	<i>uncharacterized</i>	similarity to hypothetical peptide synthetase pesA - <i>Metarhizium anisopliae</i>	1497 ± 1410	2785 ± 1060	5197 ± 1630	88 ± 10	29 ± 2	18 ± 0	Down	n
An16g05910	<i>uncharacterized</i>	similarity to cholesterol 7alpha-hydroxylase CYP7 - <i>Sus scrofa</i>	902 ± 636	1646 ± 590	2001 ± 725	17 ± 3	25 ± 5	18 ± 2	Down	y
An03g00690	<i>uncharacterized</i>	hypothetical protein	1261 ± 1329	2284 ± 1815	2321 ± 1481	21 ± 7	28 ± 1	26 ± 2	Down	n
An08g02330	<i>uncharacterized</i>	strong similarity to multidrug resistance protein MLP-2 - <i>Rattus norvegicus</i>	2009 ± 37	2116 ± 179	1500 ± 529	45 ± 2	29 ± 3	22 ± 4	Down	n
An07g06480	<i>uncharacterized</i>	similarity to cytochrome 4F8 cyp4F8 - <i>Homo sapiens</i>	420 ± 130	586 ± 213	753 ± 142	9 ± 1	10 ± 2	9 ± 1	Down	y
An05g01710	<i>uncharacterized</i>	strong similarity to hypothetical protein An16g07720 - <i>Aspergillus niger</i>	1738 ± 1486	2310 ± 357	387 ± 170	19 ± 2	18 ± 2	19 ± 3	Down	n
An01g00530	<i>pepB</i>	proteinase aspergillopepsin II - <i>Aspergillus niger</i>	682 ± 649	3677 ± 2802	6268 ± 3005	44 ± 5	35 ± 1	34 ± 0	Down	y
An01g06860	<i>uncharacterized</i>	strong similarity to hypothetical Fum9p protein - <i>Gibberella moniliformis</i>	1135 ± 1295	2702 ± 926	5766 ± 2392	67 ± 10	36 ± 3	31 ± 0	Down	n
An01g06870	<i>uncharacterized</i>	strong similarity to hypothetical protein Fum8p - <i>Gibberella moniliformis</i>	581 ± 599	1632 ± 396	3427 ± 1234	50 ± 2	26 ± 1	21 ± 6	Down	n
An02g08300	<i>uncharacterized</i>	strong similarity to the hypothetical protein encoded by An11g06450 - <i>Aspergillus niger</i>	842 ± 71	710 ± 230	2085 ± 860	30 ± 1	21 ± 2	24 ± 8	Down	n
An15g07700	<i>protD</i>	strong similarity to aspergillopepsin II precursor (acid proteinase A) - <i>Aspergillus niger</i>	649 ± 452	5044 ± 2370	9630 ± 101	75 ± 5	76 ± 5	65 ± 2	Down	y
An09g00670	<i>gelD</i>	strong similarity to beta (1-3) glucanosyltransferase Gel3p - <i>Aspergillus fumigatus</i>	1596 ± 565	2244 ± 607	2063 ± 821	45 ± 1	39 ± 6	59 ± 10	Down	y
An16g06570	<i>uncharacterized</i>	hypothetical protein	7069 ± 619	3576 ± 3514	1162 ± 1292	62 ± 4	52 ± 8	62 ± 4	Down	y
An02g00090	<i>uncharacterized</i>	strong similarity to prolidase - <i>Aureobacterium esteraromaticum</i>	6755 ± 2149	4420 ± 2149	707 ± 467	134 ± 13	58 ± 3	36 ± 11	Down	n
An05g02450	<i>uncharacterized</i>	similarity to halogenase bhaA from patent DE19926770-A1 - <i>Amycolatopsis mediterranei</i>	663 ± 62	1035 ± 59	469 ± 33	25 ± 6	18 ± 2	18 ± 1	Down	y
An07g08950	<i>eglC</i>	endoglucanase B eglB - <i>Aspergillus niger</i>	2511 ± 570	11775 ± 532	7793 ± 1370	52 ± 29	366 ± 244	422 ± 77	Down	y
An08g02300	<i>uncharacterized</i>	weak similarity to enniatin synthetase - <i>Fusarium scirp</i> [truncated ORF]	1123 ± 198	1542 ± 488	1754 ± 1024	79 ± 8	53 ± 1	22 ± 5	Down	n
An01g06900	<i>sm to amyR- A. ory</i>	weak similarity to transcription activator amyR - <i>Aspergillus oryzae</i>	758 ± 96	1056 ± 406	654 ± 233	34 ± 8	25 ± 1	21 ± 1	Down	n

An12g01320	<i>ppoD</i>	strong similarity to linoleate diol synthase - <i>Gaeumannomyces graminis</i>	1123 ± 612	1043 ± 345	132 ± 72	18 ± 2	20 ± 3	14 ± 2	Down	y
An01g06880	<i>uncharacterized</i>	similarity to dihydroflavonol 4-reductase BAA12723.1 - <i>Rosa</i> hybrid cultivar	625 ± 534	1769 ± 746	3403 ± 1745	110 ± 3	36 ± 3	27 ± 6	Down	n
An02g08330	<i>uncharacterized</i>	strong similarity to sequence 253 from Patent WO0100804 - <i>Corynebacterium glutamicum</i>	627 ± 234	624 ± 177	877 ± 309	27 ± 2	25 ± 1	24 ± 6	Down	y
An01g06840	<i>uncharacterized</i>	strong similarity to acid-CoA ligase Fat2p - <i>Saccharomyces cerevisiae</i>	419 ± 441	1295 ± 731	3234 ± 1795	52 ± 0	34 ± 1	29 ± 4	Down	n
An03g06670	<i>uncharacterized</i>	weak similarity to myosin-like protein MLP1 - <i>Saccharomyces cerevisiae</i>	5998 ± 1898	6281 ± 1029	1761 ± 437	171 ± 1	171 ± 7	118 ± 9	Down	n
An01g06830	<i>uncharacterized</i>	similarity to ketosphinganine reductase Tsc10p - <i>Saccharomyces cerevisiae</i>	418 ± 405	1031 ± 265	2067 ± 700	46 ± 1	33 ± 6	24 ± 3	Down	n
An01g06850	<i>uncharacterized</i>	similarity to 4-hydroxybutyrate dehydrogenase - <i>Alcaligenes eutrophus</i>	967 ± 1081	1917 ± 437	4224 ± 1181	112 ± 1	54 ± 2	44 ± 4	Down	n
An03g00640	<i>uncharacterized</i>	similarity to neutral amino acid permease mtr - <i>Neurospora crassa</i> [truncated ORF]	2950 ± 1037	2645 ± 295	2479 ± 1469	149 ± 28	122 ± 9	57 ± 2	Down	n
An01g11670	<i>eglA</i>	strong similarity to endo-beta-1,4-glucanase A <i>eglA</i> - <i>Emericella nidulans</i>	638 ± 408	2367 ± 12	589 ± 232	21 ± 2	42 ± 24	58 ± 1	Down	y
An07g08940	<i>uncharacterized</i>	similarity to acetyl-esterase I of patent WO9502689-A - <i>Aspergillus aculeatus</i>	242 ± 13	1268 ± 407	914 ± 553	20 ± 1	31 ± 7	26 ± 0	Down	y
An11g07020	<i>uncharacterized</i>	strong similarity to the hypothetical protein encoded by An07g00200 - <i>Aspergillus niger</i>	3237 ± 1203	2067 ± 1234	1620 ± 142	125 ± 37	97 ± 1	54 ± 11	Down	n
An07g09330	<i>cbhA</i>	cellulose 1,4-beta-cellobiosidase <i>cbhA</i> from patent WO9906574-A1 - <i>Aspergillus niger</i>	1157 ± 1121	7883 ± 2929	3034 ± 1406	67 ± 16	130 ± 62	170 ± 76	Down	y
An09g06200	<i>uncharacterized</i>	strong similarity to PTH11 transmembrane protein - <i>Magnaporthe grisea</i> strain 4091-5-8	5976 ± 1928	5351 ± 2683	3632 ± 1622	442 ± 58	211 ± 45	82 ± 12	Down	y
An04g09990	<i>uncharacterized</i>	strong similarity to 2,5-dichloro-2,5-cyclohexadiene-1,4-diol dehydrogenase <i>linC</i> - <i>Sphingomonas paucimobilis</i>	749 ± 308	422 ± 138	106 ± 4	36 ± 6	34 ± 6	34 ± 5	Down	y
An04g03840	<i>uncharacterized</i>	similarity to microtubule binding protein D-CLIP-190 - <i>Drosophila melanogaster</i>	365 ± 35	373 ± 52	257 ± 17	16 ± 0	14 ± 0	12 ± 3	Down	n
An05g01720	<i>uncharacterized</i>	strong similarity to hypothetical protein An06g00950 - <i>Aspergillus niger</i>	1552 ± 1286	2068 ± 211	368 ± 231	47 ± 7	42 ± 7	41 ± 1	Down	n
An09g02990	<i>uncharacterized</i>	similarity to probable Sua5 protein APE2397 - <i>Aeropyrum pernix</i>	618 ± 224	781 ± 58	344 ± 112	25 ± 6	26 ± 0	24 ± 2	Down	n
An04g06750	<i>uncharacterized</i>	similarity to hypothetical transmembrane protein - <i>Candida albicans</i>	491 ± 201	386 ± 153	489 ± 169	25 ± 4	20 ± 2	19 ± 2	Down	n
An07g06490	<i>uncharacterized</i>	strong similarity to insulin-degrading enzyme IDE - <i>Rattus norvegicus</i>	213 ± 37	361 ± 124	474 ± 112	17 ± 0	17 ± 0	16 ± 2	Down	n
An12g05390	<i>uncharacterized</i>	weak similarity to integral membrane protein PTH11 - <i>Magnaporthe grisea</i>	2079 ± 127	1355 ± 14	84 ± 32	38 ± 0	31 ± 2	30 ± 3	Down	y
An16g02910	<i>uncharacterized</i>	strong similarity to hypothetical protein CC0533 - <i>Caulobacter crescentus</i>	865 ± 51	1033 ± 82	613 ± 238	52 ± 1	40 ± 8	42 ± 10	Down	y
An08g11680	<i>uncharacterized</i>	strong similarity to 2,5-dichloro-2,5-cyclohexadiene-1,4-diol dehydrogenase <i>linC</i> - <i>Pseudomonas paucimobilis</i>	320 ± 4	214 ± 41	125 ± 4	56 ± 0	68 ± 11	61 ± 1	Down	y

An01g06930	uncharacterized	strong similarity to polyketide synthase FUM5 - <i>Gibberella moniliformis</i>	714 ± 704	1442 ± 246	2506 ± 866	110 ± 1	60 ± 2	46 ± 1	Down	n
An14g03130	uncharacterized	hypothetical protein	1109 ± 560	752 ± 164	262 ± 94	49 ± 11	29 ± 4	25 ± 2	Down	y
An08g03760	uncharacterized	similarity to hypothetical protein Rv3472 - <i>Mycobacterium tuberculosis</i>	191 ± 86	197 ± 37	843 ± 343	16 ± 1	16 ± 1	23 ± 5	Down	n
An08g05230	uncharacterized	strong similarity to putative endoglucanase IV - <i>Trichoderma reesei</i>	1152 ± 1281	2411 ± 2729	648 ± 116	55 ± 5	58 ± 11	46 ± 5	Down	y
Top 50 up-regulated genes in the $\Delta flbA$ strain compared to wildtype										
An08g08490	uncharacterized	similarity to the calcium-independent phospholipase A2 2 - <i>Homo sapiens</i>	143 ± 8	118 ± 7	85 ± 3	728 ± 0	847 ± 91	775 ± 76	Up	n
An07g06240	uncharacterized	strong similarity to ferrioxamine B permease sit1 - <i>Saccharomyces cerevisiae</i>	88 ± 24	87 ± 8	94 ± 8	323 ± 28	397 ± 32	1915 ± 383	Up	n
An03g03620	uncharacterized	strong similarity to multidrug resistance protein atrD - <i>Aspergillus nidulans</i>	18 ± 1	22 ± 2	26 ± 10	83 ± 5	110 ± 1	378 ± 123	Up	y
An08g10830	uncharacterized	strong similarity to geranylgeranyl pyrophosphate synthase ggpps - <i>Gibberella fujikuroi</i>	65 ± 5	58 ± 0	68 ± 18	412 ± 9	388 ± 31	555 ± 30	Up	n
An01g12200	uncharacterized	similarity to hypothetical protein F10B6.29 - <i>Arabidopsis thaliana</i>	112 ± 53	68 ± 23	94 ± 12	741 ± 26	726 ± 82	429 ± 36	Up	n
An09g00270	aglC	alpha-galactosidase C aglC - <i>Aspergillus niger</i> [truncated ORF]	89 ± 18	85 ± 22	760 ± 428	879 ± 73	1158 ± 119	1805 ± 251	Up	nc
An07g04900	uncharacterized	strong similarity to mRNA sequence of cDNA clone 2589 - <i>Aspergillus niger</i>	1065 ± 70	929 ± 155	364 ± 239	5638 ± 245	5796 ± 687	3483 ± 369	Up	y
An11g09170	uncharacterized	similarity to the secreted aspartic proteinase SAP8 - <i>Candida albicans</i>	48 ± 5	55 ± 11	42 ± 1	316 ± 18	441 ± 6	290 ± 41	Up	y
An14g01840	uncharacterized	similarity to hypothetical temperature-shock induced protein TIR3 - <i>Saccharomyces cerevisiae</i>	227 ± 122	258 ± 66	426 ± 36	1679 ± 739	2311 ± 995	2553 ± 877	Up	y
An11g02600	uncharacterized	strong similarity to PT2/PHT4 Phosphate transporter - <i>Arabidopsis thaliana</i>	50 ± 15	60 ± 10	104 ± 14	483 ± 47	653 ± 132	394 ± 127	Up	n
An16g07040	sm to btgE -A. nid	similarity to beta-1,3-glucanosyltransferase BGT1 - <i>Aspergillus fumigatus</i> [truncated ORF]	502 ± 224	676 ± 353	2375 ± 144	5982 ± 202	6554 ± 1346	7358 ± 380	Up	y
An17g00120	uncharacterized	strong similarity to major facilitator superfamily transporter protein mfs1 - <i>Botrytis cinerea</i>	30 ± 6	26 ± 0	24 ± 2	237 ± 3	221 ± 13	140 ± 13	Up	n
An16g08360	uncharacterized	weak similarity to cytochrome c1 of ubiquinol-cytochrome-c reductase - <i>Paracoccus denitrificans</i> [truncated ORF]	64 ± 10	75 ± 12	158 ± 35	610 ± 68	591 ± 130	852 ± 12	Up	n
An11g01810	uncharacterized	weak similarity to probable membrane protein YBR005w - <i>Saccharomyces cerevisiae</i>	55 ± 6	62 ± 5	96 ± 20	438 ± 9	541 ± 62	564 ± 34	Up	n
An02g07930	ppoC	strong similarity to linoleate diol synthase precursor - <i>Gaeumannomyces graminis</i>	180 ± 34	126 ± 52	38 ± 2	983 ± 279	747 ± 19	464 ± 32	Up	n
An09g01240	uncharacterized	strong similarity to phospholipase B - <i>Penicillium notatum</i>	78 ± 7	63 ± 9	104 ± 3	499 ± 142	727 ± 111	627 ± 178	Up	y
An01g06280	uncharacterized	strong similarity to IgE-binding protein - <i>Aspergillus fumigatus</i>	267 ± 123	341 ± 167	136 ± 4	2010 ± 28	2634 ± 280	1002 ± 145	Up	y
An13g01250	uncharacterized	strong similarity to the yeast siderophore-iron transporter for enterobactin Enb1 - <i>Saccharomyces cerevisiae</i> .	37 ± 2	35 ± 3	36 ± 3	183 ± 64	331 ± 79	415 ± 58	Up	n

An14g07130	uncharacterized	strong similarity to neutral amino acid permease mtr - <i>Neurospora crassa</i>	95 ± 8	70 ± 4	93 ± 8	481 ± 32	602 ± 135	1245 ± 123	Up	n
An11g04810	aox1	alternative oxidase Aox1 - <i>Aspergillus niger</i>	43 ± 8	54 ± 32	47 ± 20	279 ± 254	470 ± 353	890 ± 194	Up	n
An15g03940	uncharacterized	strong similarity to monosaccharide transporter Mst-1 - <i>Amanita muscaria</i>	244 ± 17	202 ± 47	2589 ± 838	3196 ± 21	3689 ± 943	8423 ± 210	Up	y
An13g00510	uncharacterized	strong similarity to hexokinase 1 hxk1 - <i>Schizosaccharomyces pombe</i>	199 ± 43	202 ± 28	67 ± 10	3335 ± 329	2920 ± 299	223 ± 64	Up	n
An18g01290	uncharacterized	strong similarity to predicted protein An13g01340 - <i>Aspergillus niger</i>	26 ± 2	24 ± 2	38 ± 10	233 ± 53	218 ± 65	405 ± 13	Up	y
An01g07000	uncharacterized	strong similarity to C-14 sterol reductase ERG24 - <i>Saccharomyces cerevisiae</i>	89 ± 14	77 ± 2	270 ± 166	498 ± 24	1009 ± 531	3138 ± 50	Up	y
An10g00680	uncharacterized	strong similarity to H+-ATPase V0 domain 17 KD subunit, vacuolar, CUP5 - <i>Saccharomyces cerevisiae</i>	443 ± 342	220 ± 116	119 ± 50	2783 ± 380	2761 ± 26	1102 ± 42	Up	y
An16g09040	uncharacterized	strong similarity to N-acetylglucosamine-6-phosphate deacetylase CaNAG2 - <i>Candida albicans</i>	629 ± 51	516 ± 71	81 ± 7	6612 ± 950	6653 ± 1009	637 ± 136	Up	n
An01g00390	uncharacterized	hypothetical protein	45 ± 2	47 ± 2	319 ± 99	542 ± 59	685 ± 35	1891 ± 74	Up	n
An16g01850	uncharacterized	similarity to blastomyces yeast phase-specific protein 1 bys1 - <i>Ajellomyces dermatitidis</i>	2984 ± 2759	1197 ± 780	166 ± 54	12215 ± 313	12035 ± 1137	3132 ± 648	Up	y
An16g01880	Lipanl	strong similarity to lysophospholipase - <i>Aspergillus foetidus</i>	648 ± 245	795 ± 248	454 ± 202	10644 ± 663	10585 ± 2214	2676 ± 160	Up	y
An14g02940	uncharacterized	strong similarity to L-sorbose dehydrogenase, FAD dependent - <i>Gluconobacter oxydans</i>	212 ± 101	153 ± 71	139 ± 92	2065 ± 372	1565 ± 349	1690 ± 279	Up	n
An07g03570	uncharacterized	strong similarity to sorbitol utilization protein sou2 - <i>Candida albicans</i>	83 ± 7	85 ± 17	1534 ± 228	1757 ± 42	1613 ± 167	6032 ± 312	Up	n
An18g03360	uncharacterized	similarity to the proteophosphoglycan ppg1 - <i>Leishmania major</i>	32 ± 19	46 ± 26	27 ± 5	473 ± 19	503 ± 4	242 ± 2	Up	y
An14g06980	uncharacterized	strong similarity to delta-12 fatty acid desaturase - <i>Mortierella alpina</i>	33 ± 3	36 ± 2	301 ± 112	465 ± 89	750 ± 251	1939 ± 15	Up	n
An05g00790	uncharacterized	strong similarity to nodulin GmNOD53b - <i>Glycine max</i> [truncated ORF]	25 ± 15	20 ± 4	12 ± 1	213 ± 57	341 ± 30	161 ± 9	Up	n
An03g06660	uncharacterized	strong similarity to peptide transporter ptr2 - <i>Arabidopsis thaliana</i>	2166 ± 1123	1709 ± 1392	80 ± 15	12416 ± 598	10787 ± 865	3842 ± 47	Up	n
An18g01320	uncharacterized	strong similarity to extracellular protease precursor BAR1 - <i>Saccharomyces cerevisiae</i>	148 ± 76	129 ± 56	328 ± 31	2146 ± 67	2897 ± 61	2501 ± 161	Up	y
An05g00800	uncharacterized	similarity to nodulin GmNOD53b - <i>Glycine max</i> [truncated ORF]	47 ± 21	31 ± 11	22 ± 1	395 ± 130	667 ± 69	333 ± 6	Up	n
An12g09870	uncharacterized	hypothetical protein	50 ± 0	57 ± 1	56 ± 0	571 ± 232	432 ± 82	2224 ± 250	Up	n
An11g00100	uncharacterized	strong similarity to triacylglycerol lipase LIP5 - <i>Candida rugosa</i>	71 ± 4	64 ± 3	59 ± 8	800 ± 192	866 ± 397	2265 ± 77	Up	y
An01g01630	uncharacterized	strong similarity to hypothetical protein An09g00510 - <i>Aspergillus niger</i>	69 ± 71	36 ± 26	96 ± 40	1151 ± 99	830 ± 115	802 ± 17	Up	y
An01g07730	uncharacterized	weak similarity to TcSL-2 protein precursor - <i>Toxocara cani</i>	118 ± 74	64 ± 3	63 ± 5	1697 ± 86	1478 ± 312	1257 ± 12	Up	y
An15g03550	uncharacterized	weak similarity to protopectinase patent WO9806832-A1 - <i>Bacillus subtilis</i>	57 ± 44	57 ± 45	20 ± 0	954 ± 34	1066 ± 21	330 ± 22	Up	y

An16g06950	uncharacterized	strong similarity to carboxylic acid transport protein JEN1 - <i>Saccharomyces cerevisiae</i>	31 ± 8	26 ± 0	23 ± 5	837 ± 53	725 ± 9	357 ± 20	Up	n
An03g01770	uncharacterized	strong similarity to the EST an_3645 - <i>Aspergillus niger</i>	758 ± 657	336 ± 176	55 ± 12	10029 ± 207	8451 ± 0	1933 ± 247	Up	y
An03g05360	uncharacterized	strong similarity to neutral amino acid permease mtr - <i>Neurospora crassa</i>	39 ± 0	36 ± 2	31 ± 2	1673 ± 148	1362 ± 88	771 ± 2	Up	y
An14g04210	uncharacterized	similarity to hypothetical protein An07g05660 - <i>Aspergillus niger</i>	30 ± 0	35 ± 7	31 ± 0	931 ± 174	1386 ± 104	2511 ± 5	Up	y
An03g06220	sm to <i>geld</i> - <i>A. nid</i>	strong similarity to beta (1-3) glucanosyltransferase GEL3 - <i>Aspergillus fumigatus</i>	42 ± 3	48 ± 11	58 ± 3	1406 ± 256	1874 ± 54	5043 ± 547	Up	y
An02g08560	uncharacterized	similarity to probable dioxygenase SCOEDB - <i>Streptomyces coelicolor</i>	22 ± 3	23 ± 3	19 ± 0	797 ± 22	1048 ± 83	1682 ± 135	Up	y
An02g08130	uncharacterized	similarity to hypothetical protein ZSCG18.24 - <i>Streptomyces coelicolor</i>	46 ± 3	48 ± 9	41 ± 5	2045 ± 64	2532 ± 185	3306 ± 224	Up	y
An09g05050	uncharacterized	questionable ORF	25 ± 1	24 ± 4	22 ± 3	3522 ± 1024	2872 ± 506	8559 ± 471	Up	y

Table 4: Hybridization values in colony zones 1, 3 and 5 of top 50 up- and down-regulated genes of the $\Delta flbA$ strain when compared to wild-type. Gene name or its closest homologue. A. nid: *A. nidulans*; A. ory: *A. oryzae*.

Principal component analysis (PCA) showed that the transcriptomes of the wild-type zones and those of the $\Delta flbA$ strain cluster in the first component of the analysis. This component accounts for 54% of the variation in the datasets (Figure 2). The second component of the analysis accounted for 27% of the variation and separates the transcriptomes from zones 1 and 3 of the wild-type and the $\Delta flbA$ strain from zone 5 of these strains (Figure 2). Expression in the central zone 1 and the intermediate zone 3 of the wild-type correlated to a high extent (Pearson's $r^2 \geq 0.98$), when compared to the duplicates of these zones ($r^2 \geq 0.97$ and ≥ 0.98 , respectively). Wild-type zone 5 was more distinct from zone 3 and 1 ($r^2=0.91$ and 0.93). The expression profile of zone 5 was also most different within the $\Delta flbA$ colony (Figure 2). The fact that zone 5 has the most distinct expression profile was also illustrated by the number of genes, with a fold change in expression ≥ 2 between the zones of wild-type and $\Delta flbA$ colonies (Figure 3). The number of differentially expressed genes between zone 1 and zone 3 of the wild-type colony was 5, between zone 3 and zone 5, 119, and between zone 1 and zone 5, 325. These numbers were 0, 115 and 595 for the $\Delta flbA$ strain, respectively (Figure 3). Of these differentially expressed genes, only 138 were found in both the wild-type and the $\Delta flbA$ strain.

In the next analysis, the number of genes was determined that are differentially expressed when zones of the wild-type and the $\Delta flbA$ strain were compared (Figure 3). The number of genes differentially expressed in zone 1 of the wild-type and the $\Delta flbA$ strain was 233. Of these, 104 and 129 were up- and down-regulated in the $\Delta flbA$ strain, respectively. A total of 235 genes were found to be differentially expressed in zone 3. Of these, 89 were up-regulated in $\Delta flbA$, whereas 149 were up-regulated in the wild-type. In zone 5, 297 genes were differentially expressed, of which 126 and 153 were up- and down-regulated in the $\Delta flbA$ strain, respectively.

Expression analysis of functional gene classes

Fisher's exact test was used to determine whether functional gene classes [16] were over- or underrepresented in the set of genes that are differentially expressed in zones 1 and 3, compared to zone 5 of the wild-type and the $\Delta flbA$ strain (Table 1). The functional classes metabolism and amino acid degradation were overrepresented in the up-regulated genes of zones 1 and 3 of the wild-type colony (Table 1), whereas the

functional classes metabolism, energy, accessory proteins of electron transport and membrane associated energy conservation and protein synthesis were overrepresented in the peripheral wild-type zone 5 (Table 1). The functional classes transcription, mRNA synthesis and unclassified proteins were under-represented in this cluster of genes, while only the latter functional group was underrepresented in zone 1 and 3 of the wild-type. In the up-regulated genes in the central and intermediate zone of the $\Delta flbA$ strain, the functional class metabolism was over-represented, whereas cell cycle and DNA processing, transcription, protein synthesis and unclassified proteins were under-represented (Table 1). Metabolism, amino acid degradation, nucleotide transport, C-compound and carbohydrate utilization, energy and cellular transport and transport mechanisms were over-represented in the up-regulated genes in the peripheral zone of $\Delta flbA$ colonies (Table 1). On the other hand, transcription, mRNA synthesis, subcellular localization and unclassified proteins were under-represented.

Funcat analysis was performed on the differentially expressed genes between the zones of the wild-type and the $\Delta flbA$ colonies (Table 2). Up-regulated genes in zone 1 of $\Delta flbA$ compared to wild-type zone 1 were overrepresented in metabolism and amino acid transport, whereas unclassified proteins were underrepresented. Metabolism and unclassified proteins were also over- and underrepresented, respectively, in the down-regulated genes in zone 1 of $\Delta flbA$ compared to wild-type zone 1. The same was observed in the down-regulated genes in zone 3, whereas only the gene class metabolism was overrepresented in the up-regulated genes of this zone. Cellular transport and transport mechanisms were overrepresented in the up-regulated genes of the peripheral zone 5 of $\Delta flbA$ colonies. In contrast, the down-regulated genes in this zone showed an over-representation of genes of the functional classes metabolism, C-compound and carbohydrate utilization and other proteolytic degradation, whereas cell cycle and DNA processing and unclassified proteins were underrepresented (Table 2).

Expression analysis of specific functional gene classes

The expression profiles of zones 1, 3 and 5 of the wild-type were pooled and compared with the pooled profiles of zones 1, 3 and 5 of the $\Delta flbA$ strain. In other words, for each gene the mean expression

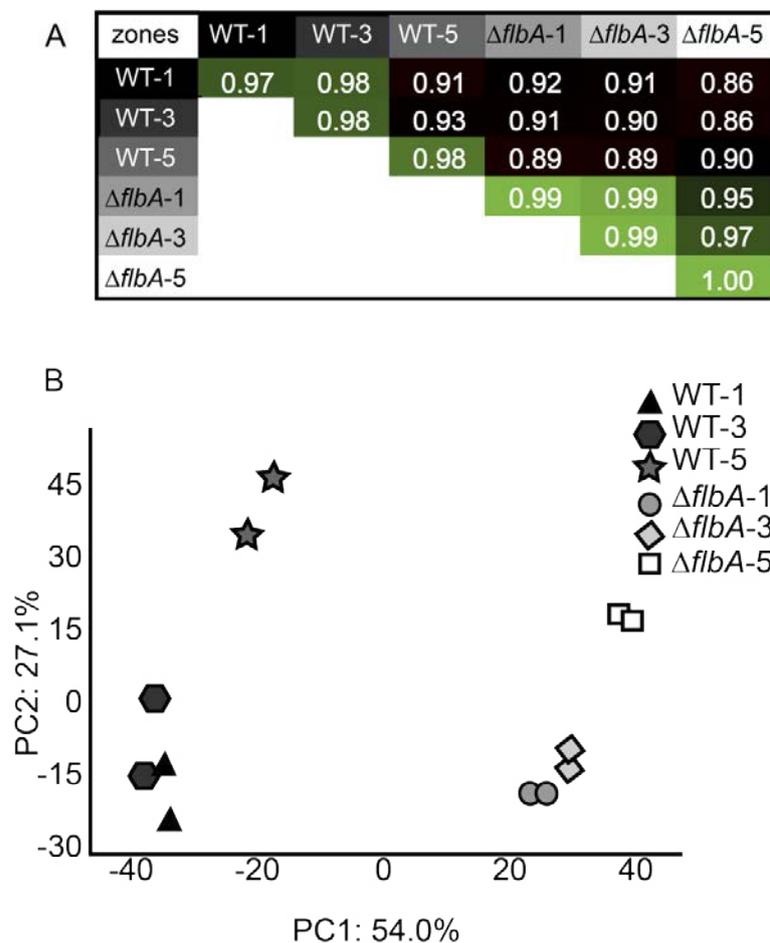


Figure 2: Pearson correlation (r^2)(A) and principal component analysis (B) of the transcriptomes of zones 1, 3 and 5 of wild-type (▲●★) and $\Delta flbA$ (●◆■) colonies.



Figure 3: Differentially expressed genes that show ≥ 2 -fold lower or higher expression between zones of wild-type and/or $\Delta flbA$ colonies.

within the wild-type colony was compared to the mean expression within the $\Delta flbA$ strain. This was done to increase the statistical power of the comparison (comparing 6 instead of 2 arrays in each case). The number of genes up-regulated in $\Delta flbA$ colonies was 520, while 632 genes were down-regulated. Functat analysis showed that the up-regulated genes are overrepresented in the functional categories metabolism and cellular transport and transport mechanisms, whereas transcription and unclassified proteins were underrepresented (Table 3). Down-regulated genes were overrepresented in metabolism and C-compound and carbohydrate utilization, and underrepresented in

energy, cell cycle and DNA processing, mitotic cell cycle and cell cycle control, protein synthesis, subcellular localisation, and unclassified proteins (Table 3).

Twenty-six out of the 50 genes with the highest up-regulation (≥ 2 -fold) in $\Delta flbA$ compared to wild-type are predicted to encode proteins with a signal sequence for secretion (sigP) (Table 4). This number was 22 in the top 50 of genes with the highest down-regulation in $\Delta flbA$ colonies. Among the top 50 up-regulated and down-regulated genes, 47 and 43 genes are not yet characterized, respectively, of which

18 and 23 belong to the family of unclassified proteins. These gene sets also contain genes involved in development and cell wall biosynthesis/remodelling and genes encoding transcriptional regulators. Up- and down-regulated (≥ 2 -fold) genes of these classes [18] were analyzed in more detail and are described below.

Transcriptional changes associated to asexual and sexual

development: Of the 68 genes implicated in asexual and/or sexual reproduction in *A. niger*, 13 are found to be differentially expressed between the wild-type and $\Delta flbA$ strain (Figure 4A, Table 5; Supplemental Table 1). Of these genes, 6 are down-regulated in the $\Delta flbA$ strain and 7 are up-regulated. Of the genes indicated in Figure 1, *flbD* and *sfaD* are ≥ 2 -fold higher expressed in the $\Delta flbA$ strain.

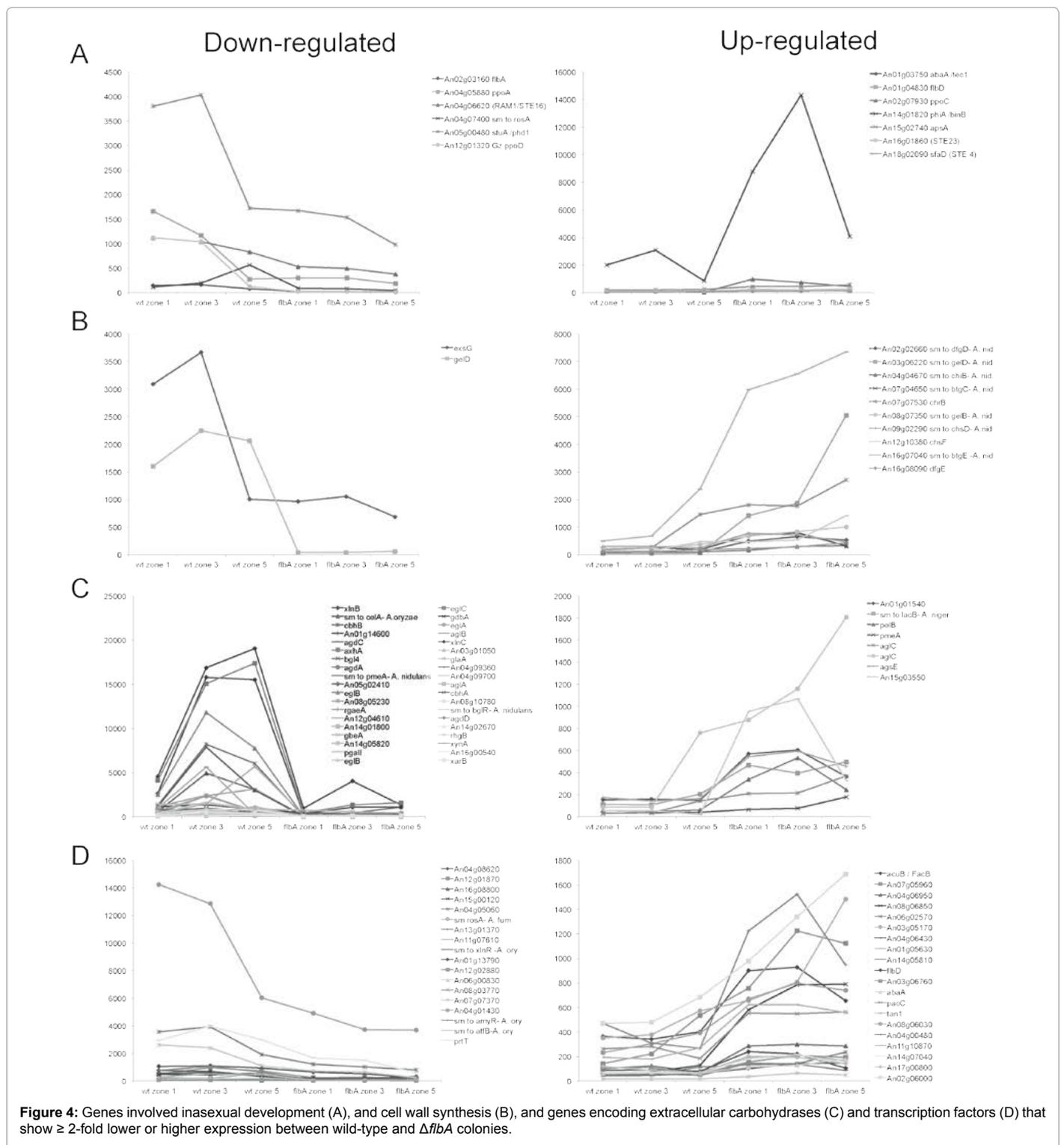


Figure 4: Genes involved in asexual development (A), and cell wall synthesis (B), and genes encoding extracellular carbohydrases (C) and transcription factors (D) that show ≥ 2 -fold lower or higher expression between wild-type and $\Delta flbA$ colonies.

Annotation	Gene Name*	Description	wild-type zone 1	wild-type zone 3	wild-type zone 5	$\Delta flbA$ zone 1	$\Delta flbA$ zone 3	$\Delta flbA$ zone 5	Regulation
An02g03160	<i>flbA</i>	strong similarity to developmental regulator <i>flbA</i> - <i>Emericella nidulans</i>	147 ± 11	166 ± 25	77 ± 24	21 ± 2	24 ± 4	25 ± 3	Down
An04g05880	<i>ppoA</i>	strong similarity to linoleate diol synthase - <i>Gaeumannomyces graminis</i>	1658 ± 245	1167 ± 359	282 ± 22	301 ± 2	298 ± 6	188 ± 11	Down
An04g06620	(<i>RAM1/STE16</i>)	similarity to farnesyl-protein transferase beta chain - Homo sapiens	1125 ± 2	1045 ± 31	831 ± 197	527 ± 31	499 ± 42	378 ± 4	Down
An04g07400	<i>sm rosA</i> - <i>A. fum</i>	strong similarity to C6 zinc finger transcription factor PRO1 - <i>Sordaria macrospora</i> [putative sequencing error]	120 ± 28	195 ± 76	562 ± 77	86 ± 3	83 ± 9	43 ± 6	Down
An05g00480	<i>stuA /phd1</i>	strong similarity to transcription factor involved in differentiation <i>stuA</i> - <i>Aspergillus nidulans</i>	3804 ± 1205	4031 ± 108	1721 ± 59	1672 ± 63	1531 ± 47	978 ± 7	Down
An12g01320	<i>ppoD</i>	strong similarity to linoleate diol synthase - <i>Gaeumannomyces graminis</i>	1123 ± 612	1043 ± 345	132 ± 72	18 ± 2	20 ± 3	14 ± 2	Down
An01g03750	<i>abaA</i>	strong similarity to protein <i>abaA</i> - <i>Aspergillus nidulans</i>	61 ± 9	71 ± 12	67 ± 17	113 ± 28	129 ± 3	182 ± 22	Up
An01g04830	<i>flbD</i>	strong similarity to myb-like DNA binding protein <i>flbD</i> - <i>Aspergillus nidulans</i>	89 ± 18	91 ± 8	81 ± 20	238 ± 35	220 ± 32	107 ± 5	Up
An02g07930	<i>ppoC</i>	strong similarity to linoleate diol synthase precursor - <i>Gaeumannomyces graminis</i>	180 ± 34	126 ± 52	38 ± 2	983 ± 279	747 ± 19	464 ± 32	Up
An14g01820	<i>phiA /binB</i>	strong similarity to hypothetical cell wall protein <i>binB</i> - <i>Aspergillus nidulans</i>	2018 ± 1048	3064 ± 430	843 ± 70	8800 ± 3676	14346 ± 1791	4066 ± 653	Up
An15g02740	<i>apsA</i>	strong similarity to the anucleate primary sterigmata gene <i>apsA</i> - <i>Aspergillus nidulans</i>	199 ± 47	206 ± 53	252 ± 49	453 ± 29	444 ± 68	560 ± 22	Up
An16g01860	(<i>STE23</i>)	strong similarity to protease involved in a-factor processing <i>STE23</i> - <i>Saccharomyces cerevisiae</i>	99 ± 20	95 ± 10	118 ± 8	192 ± 8	215 ± 7	279 ± 28	Up
An18g02090	<i>sfaD (STE 4)</i>	strong similarity to G-protein beta subunit <i>sfaD</i> - <i>Aspergillus nidulans</i>	41 ± 10	43 ± 8	104 ± 4	109 ± 5	113 ± 4	165 ± 13	Up

Table 5: Hybridization values of 13 genes that are implicated in asexual and/or sexual reproduction and that are differentially expressed in wild-type when compared to the $\Delta flbA$ strain of *A. niger*. The table is based on Pel et al. [18], with the addition of *flbE* (An08g07210), *flbB* (An15g03710) and *flbC* (An12g08230). *Gene name or its closest homologue. Sm: Similar; *A. fum*: *A. fumigatus*.

Transcriptional changes associated to cell wall synthesis: A total of 102 genes are predicted to be involved in cell wall synthesis [18]. Of these genes, 10 and 2 were found to be up- and down-regulated in the $\Delta flbA$ strain when compared to the wild-type, respectively (Figure 4B, Table 6, Supplemental Table 1). Among the up-regulated genes are three glucanase genes, two chitin synthase genes, the chitinase gene *chiB*, two glucanase genes, and two glycosylphosphatidylinositol-anchored endo-mannanase genes. One of these glucanase genes, a *gelD* homologue of *A. nidulans* was 48-fold up-regulated in $\Delta flbA$ compared to wild-type. In contrast, the annotated *gelD* gene of *A. niger* was found to be 40-fold down-regulated in $\Delta flbA$. The other down-regulated gene in the $\Delta flbA$ strain is the glucan beta-1,3 exoglucanase gene *exsG*.

Transcriptional changes associated to proteins with a signal sequence for secretion: As mentioned above, wild-type and $\Delta flbA$ were grown on minimal medium supplemented with xylose. This carbon source activates XlnR, a transcriptional regulator that controls 16 genes encoding xylanolytic enzymes [19-25]. Of these, none were higher expressed in the $\Delta flbA$ strain, whereas 6 (*xlnB*, *xlnC*, *axhA*, *aglB*, *eglB*, *cbhA*, *chbB*) were found to be ≥ 2 -fold lower expressed when compared to the wild-type (Figure 4C, Table 7, Supplemental Table 1). In fact, *xlnC*, *xlnB*, and *axhA* are among the highest expressed genes at the periphery of wild-type colonies, while they are hardly expressed in the $\Delta flbA$ strain (Table 4). Thirty-two other carbohydrate genes are

down-regulated in the $\Delta flbA$ strain (e.g. the glucoamylase gene *glaA*), while 8 of these genes are up-regulated in this strain. Among these genes are the α -galactosidase gene *aglC*, the α -glucan synthase *agsE*, the pectin lyase *pelB* and the pectin esterase *pmeA* (Figure 4C, Table 7).

Of the total number of 2612 genes with a predicted signal sequence for secretion, 156 and 189 were up- and down-regulated, respectively (Supplemental Figure 2, Supplemental Table 1 and 5). The up-regulated genes are mainly uncharacterized proteins, but include carbohydrases (see above), a putative cytochrome P450 reductase and two putative proteases. Among the down-regulated are several carbohydrate degrading enzymes (Supplemental Table 1 and 5), a phytase (*phyB*) and six (putative) proteases.

Previously, we identified 138 proteins with a signal sequence for secretion in the secretome of $\Delta flbA$ colonies when grown on fresh xylose medium [8]. Of these, 70 were not found in the wild-type secretome (i.e. even after partial degradation of the cell wall by the addition of cycloheximide) [8]. Differential expression was observed in the case of 23 out of 70 encoding genes (Table 8). One of these genes was down-regulated, whereas 22 were up-regulated in the $\Delta flbA$ colonies. Most of these genes are uncharacterized, but encode proteins that are putative carbohydrate degrading enzymes (3), proteases (2), cell wall/ morphogenesis enzymes (2), lipases (2), phospholipases (2), oxidases and superoxide-dismutases (5) and other/unknown

Annotation	Gene Name*	Description	wild-type zone 1	wild-type zone 3	wild-type zone 5	$\Delta flbA$ zone 1	$\Delta flbA$ zone 3	$\Delta flbA$ zone 5	Regulation
An01g12450	<i>exsG</i>	strong similarity to putative glucan beta-1,3 exoglucanase - <i>Trichoderma harzianum</i>	3091 ± 197	3667 ± 1573	1008 ± 250	966 ± 4	1053 ± 14	689 ± 27	Down
An09g00670	<i>gelD</i>	strong similarity to beta (1-3) glucanosyltransferase Gel3p - <i>Aspergillus fumigatus</i>	1596 ± 565	2244 ± 607	2063 ± 821	45 ± 1	39 ± 6	59 ± 10	Down
An02g02660	<i>dfgG</i>	strong similarity to the protein required for filamentous growth, cell polarity, and cellular elongation Dfg5 - <i>Saccharomyces cerevisiae</i>	87 ± 5	110 ± 17	102 ± 21	487 ± 30	648 ± 129	532 ± 98	Up
An03g06220	<i>sm to gelD-A. nid</i>	strong similarity to beta (1-3) glucanosyltransferase GEL3 - <i>Aspergillus fumigatus</i>	42 ± 3	48 ± 11	58 ± 3	1406 ± 256	1874 ± 54	5043 ± 547	Up
An04g04670	<i>sm to chiB-A. nid</i>	strong similarity to chitinase cts1 - <i>Coccidioides immitis</i>	106 ± 36	104 ± 26	111 ± 1	171 ± 65	308 ± 102	331 ± 185	Up
An07g04650	<i>sm to btgC-A. nid</i>	similarity to exo-beta-1,3-glucanase BGL2 - <i>Saccharomyces cerevisiae</i> .	203 ± 14	275 ± 109	184 ± 41	699 ± 119	797 ± 4	319 ± 12	Up
An07g07530	<i>chrB</i>	strong similarity to cell wall protein UTR2 - <i>Saccharomyces cerevisiae</i>	176 ± 39	254 ± 48	1452 ± 40	1812 ± 115	1764 ± 453	2719 ± 364	Up
An08g07350	<i>sm to gelB-A. nid</i>	strong similarity to glycopospholipid-anchored surface glycoprotein GAS1 precursor - <i>Saccharomyces cerevisiae</i>	226 ± 26	257 ± 8	363 ± 114	674 ± 9	839 ± 71	1001 ± 69	Up
An09g02290	<i>sm to chsD-A. nid</i>	strong similarity to chitin synthase chsE - <i>Aspergillus nidulans</i>	308 ± 12	314 ± 57	255 ± 23	779 ± 15	723 ± 100	479 ± 18	Up
An12g10380	<i>chsF</i>	strong similarity to chitin synthase C chsC - <i>Aspergillus fumigatus</i>	118 ± 6	137 ± 13	478 ± 239	468 ± 12	527 ± 142	1407 ± 9	Up
An16g07040	<i>sm to btgE-A. nid</i>	similarity to beta-1,3-glucanosyltransferase BGT1 - <i>Aspergillus fumigatus</i> [truncated ORF]	502 ± 224	676 ± 353	2375 ± 144	5982 ± 202	6554 ± 1346	7358 ± 380	Up
An16g08090	<i>dfgE</i>	strong similarity to hypothetical protein B2J23.120 - <i>Neurospora crassa</i>	114 ± 8	125 ± 5	174 ± 10	229 ± 21	280 ± 11	438 ± 28	Up

Table 6: Hybridization values of 12 genes that are implicated in cell wall biosynthesis and that are differentially expressed in wild-type when compared to the $\Delta flbA$ strain of *A. niger*. The table was adapted from Pel et al. [18]. *Description of gene name or its closest homologue. Sm: Similar; A. nid: *A. nidulans*.

Annotation	Gene Name*	Description	wild-type zone 1	wild-type zone 3	wild-type zone 5	$\Delta flbA$ zone 1	$\Delta flbA$ zone 3	$\Delta flbA$ zone 5	Regulation
An01g00780	<i>xlnB</i>	xylanase xynB of patent WO9713853-A2 - <i>Aspergillus niger</i>	4528 ± 1905	16891 ± 683	19085 ± 0	953 ± 977	4014 ± 2052	1371 ± 235	Down
An01g01870	<i>eglC</i>	strong similarity to hypothetical Avicelase III avIII - <i>Aspergillus aculeatus</i>	77 ± 47	501 ± 267	400 ± 240	22 ± 0	23 ± 1	23 ± 2	Down
An01g03340	<i>sm to celA-A.ory</i>	strong similarity to xyloglucan-specific endo-beta-1,4-glucanase - <i>Aspergillus aculeatus</i>	552 ± 60	4966 ± 831	3065 ± 537	151 ± 48	272 ± 34	191 ± 34	Down
An01g06120	<i>gdbA</i>	strong similarity to 4-alpha-glucanotransferase / amylo-1,6-glucosidase GDB1 - <i>Saccharomyces cerevisiae</i>	1138 ± 28	1366 ± 280	915 ± 411	446 ± 27	385 ± 41	290 ± 12	Down
An01g11660	<i>cbhB</i>	1,4-beta-D-glucan cellobiohydrolase B precursor cbhB of patent WO9906574-A1 - <i>Aspergillus niger</i>	1289 ± 335	8224 ± 845	6037 ± 1558	48 ± 13	430 ± 386	1098 ± 313	Down
An01g11670	<i>eglA</i>	strong similarity to endo-beta-1,4-glucanase A eglA - <i>Emericella nidulans</i>	638 ± 408	2367 ± 12	589 ± 232	21 ± 2	42 ± 24	58 ± 1	Down
An01g14600	<i>uncharacterized</i>	strong similarity to the endo-1,4-beta-Xylanase B XynB, patent WO9414965 - <i>Aspergillus tubingensis</i>	256 ± 239	364 ± 349	195 ± 92	30 ± 1	34 ± 6	37 ± 4	Down
An02g11150	<i>agIB</i>	alpha-galactosidase agIB - <i>Aspergillus niger</i>	390 ± 96	2373 ± 668	3155 ± 569	111 ± 5	160 ± 21	126 ± 14	Down
An02g13240	<i>agdC</i>	strong similarity to alpha-1-6-glucosidase glcA - <i>Aspergillus parasiticus</i>	806 ± 223	877 ± 435	313 ± 147	224 ± 5	144 ± 26	77 ± 7	Down
An03g00940	<i>xlnC</i>	endo-1,4-beta-xylanase C precursor xlnC - <i>Aspergillus niger</i>	2605 ± 1941	15761 ± 4701	15538 ± 1230	337 ± 232	1058 ± 553	1067 ± 324	Down

An03g00960	<i>axhA</i>	1,4-beta-D-arabinoxylan arabinofuranohydrolase <i>axhA</i> - <i>Aspergillus niger</i>	4089 ± 1838	15097 ± 1853	17374 ± 0	456 ± 313	1314 ± 776	1602 ± 429	Down
An03g01050	Uncharacterized	similarity to endo-beta-1,4-glucanase - <i>Bacillus polymyxa</i>	137 ± 32	345 ± 202	1027 ± 608	31 ± 8	48 ± 17	89 ± 12	Down
An03g03740	<i>bgl4</i>	strong similarity to beta-glucosidase <i>bgl4</i> - <i>Humicola grisea</i> var. <i>thermoidea</i>	656 ± 278	958 ± 22	587 ± 133	359 ± 34	367 ± 72	312 ± 13	Down
An03g06550	<i>glaA</i>	glucan 1,4-alpha-glucosidase <i>glaA</i> - <i>Aspergillus niger</i>	370 ± 102	1497 ± 1144	5690 ± 1251	182 ± 15	171 ± 29	166 ± 8	Down
An04g06920	<i>agdA</i>	extracellular alpha-glucosidase <i>aglU</i> - <i>Aspergillus niger</i>	236 ± 29	316 ± 16	651 ± 135	176 ± 8	145 ± 5	176 ± 10	Down
An04g09360	uncharacterized	strong similarity to hypothetical protein CC0812 - <i>Caulobacter crescentus</i>	136 ± 33	153 ± 76	90 ± 13	68 ± 0	64 ± 1	38 ± 8	Down
An04g09690	<i>sm to pmeA- A. nid</i>	strong similarity to pectin methylesterase PME1 - <i>Aspergillus aculeatus</i>	60 ± 19	190 ± 41	174 ± 59	38 ± 3	48 ± 0	47 ± 9	Down
An04g09700	uncharacterized	strong similarity to endo-xylogalacturonan hydrolase <i>xghA</i> - <i>Aspergillus tubingensis</i>	60 ± 41	435 ± 272	143 ± 33	28 ± 4	28 ± 0	39 ± 3	Down
An05g02410	uncharacterized	strong similarity to beta-glucuronidase GUSB - <i>Canis familiaris</i>	287 ± 18	343 ± 48	338 ± 149	201 ± 2	132 ± 4	104 ± 8	Down
An06g00170	<i>aglA</i>	alpha-galactosidase <i>aglA</i> - <i>Aspergillus niger</i>	600 ± 56	678 ± 85	124 ± 23	59 ± 7	32 ± 6	18 ± 3	Down
An07g08950	<i>eglC</i>	endoglucanase B <i>eglB</i> - <i>Aspergillus niger</i>	2511 ± 570	11775 ± 532	7793 ± 1370	52 ± 29	366 ± 244	422 ± 77	Down
An07g09330	<i>cbhA</i>	cellulose 1,4-beta-cellobiosidase <i>cbhA</i> from patent WO9906574-A1- <i>Aspergillus niger</i>	1157 ± 1121	7883 ± 2929	3034 ± 1406	67 ± 16	130 ± 62	170 ± 76	Down
An08g05230	uncharacterized	strong similarity to putative endoglucanase IV - <i>Trichoderma reesei</i>	1152 ± 1281	2411 ± 2729	648 ± 116	55 ± 5	58 ± 11	46 ± 5	Down
An08g10780	uncharacterized	strong similarity to hypothetical protein T16K5.230 of <i>A. thaliana</i>	72 ± 40	140 ± 70	1109 ± 145	30 ± 8	26 ± 2	43 ± 0	Down
An09g02160	<i>rgaeA</i>	rhamnogalacturonan acetyl esterase <i>rgaeA</i> - <i>Aspergillus niger</i>	318 ± 9	829 ± 168	409 ± 176	165 ± 41	149 ± 17	58 ± 2	Down
An11g02100	<i>sm to bglR- A. nid</i>	strong similarity to furostanol glycoside 26-O-beta-glucosidase CSF26G1 - <i>Costus speciosus</i>	360 ± 21	1634 ± 301	808 ± 253	105 ± 38	140 ± 12	114 ± 5	Down
An12g04610	uncharacterized	similarity to endoglucanase IV <i>egl4</i> - <i>Trichoderma reesei</i>	966 ± 1072	5636 ± 3072	243 ± 11	76 ± 9	108 ± 31	58 ± 5	Down
An13g03710	<i>agdD</i>	strong similarity to alpha-glucosidase AGLU - <i>Bacillus</i> sp. SAM1606	103 ± 18	130 ± 57	77 ± 28	44 ± 4	42 ± 4	47 ± 3	Down
An14g01800	<i>aglD</i>	alpha-galactosidase <i>aglD</i> - <i>Aspergillus niger</i>	54 ± 4	109 ± 6	136 ± 18	27 ± 0	33 ± 3	34 ± 2	Down
An14g02670	uncharacterized	strong similarity to endoglucanase IV <i>egl4</i> - <i>Trichoderma reesei</i>	347 ± 273	857 ± 754	737 ± 10	37 ± 3	45 ± 3	37 ± 1	Down
An14g04190	<i>gbeA</i>	strong similarity to 1,4-alpha-glucan branching enzyme <i>glc3</i> - <i>Saccharomyces cerevisiae</i>	1445 ± 19	1453 ± 195	915 ± 221	769 ± 3	603 ± 58	451 ± 20	Down
An14g04200	<i>rhgB</i>	rhamnogalacturonase <i>rhgB</i> - <i>Aspergillus niger</i>	83 ± 57	177 ± 184	105 ± 34	33 ± 7	36 ± 5	34 ± 0	Down
An14g05820	uncharacterized	strong similarity to beta-galactosidase <i>lacA</i> - <i>Aspergillus niger</i>	126 ± 37	290 ± 21	275 ± 6	60 ± 13	62 ± 12	72 ± 12	Down
An15g04550	<i>xynA</i>	strong similarity to xylanase A <i>xynA</i> of patent WO200068396-A2 - <i>Aspergillus niger</i>	55 ± 0	384 ± 304	1176 ± 75	35 ± 4	41 ± 21	34 ± 1	Down
An15g05370	<i>pgall</i>	polygalacturonase <i>pgall</i> of patent EP421919-A - <i>Aspergillus niger</i>	224 ± 219	767 ± 877	327 ± 240	29 ± 3	25 ± 5	28 ± 5	Down
An16g00540	uncharacterized	similarity to putative large secreted protein - <i>Streptomyces coelicolor</i> [truncated ORF]	32 ± 1	89 ± 3	159 ± 14	23 ± 1	25 ± 1	26 ± 6	Down
An16g06800	<i>eglB</i>	strong similarity to endoglucanase <i>eglB</i> - <i>Aspergillus niger</i>	154 ± 25	226 ± 100	423 ± 232	87 ± 5	92 ± 5	77 ± 5	Down
An17g00300	<i>xarB</i>	strong similarity to bifunctional xylosidase-arabinosidase <i>xarB</i> - <i>Thermoanaerobacter ethanolicus</i>	156 ± 10	534 ± 22	396 ± 19	88 ± 6	124 ± 19	144 ± 17	Down

An01g01540	<i>uncharacterized</i>	strong similarity to alpha, alpha-trehalase treA - <i>Aspergillus nidulans</i>	154 ± 10	161 ± 8	149 ± 13	568 ± 30	607 ± 43	366 ± 5	Up
An01g10350	<i>sm to lacB- A. niger</i>	strong similarity to secreted beta-galactosidase lacA - <i>Aspergillus niger</i>	113 ± 5	109 ± 20	206 ± 35	469 ± 36	394 ± 38	499 ± 69	Up
An03g00190	<i>pelB</i>	the pectin lyase pelB - <i>Aspergillus niger</i>	56 ± 4	48 ± 4	61 ± 9	338 ± 67	536 ± 67	248 ± 3	Up
An03g06310	<i>pmeA</i>	pectinesterase pmeA- <i>Aspergillus niger</i>	33 ± 6	36 ± 6	43 ± 5	65 ± 4	75 ± 14	179 ± 41	Up
An09g00260	<i>aglC</i>	alpha-galactosidase C aglC - <i>Aspergillus niger</i> [truncated ORF]	31 ± 6	34 ± 4	143 ± 60	208 ± 5	216 ± 38	370 ± 31	Up
An09g00270	<i>aglC</i>	alpha-galactosidase C aglC - <i>Aspergillus niger</i> [truncated ORF]	89 ± 18	85 ± 22	760 ± 428	879 ± 73	1158 ± 119	1805 ± 251	Up
An09g03070	<i>agsE</i>	strong similarity to alpha-glucan synthase mok1 - <i>Schizosaccharomyces pombe</i>	174 ± 2	142 ± 40	163 ± 50	544 ± 44	598 ± 19	458 ± 41	Up
An15g03550	<i>uncharacterized</i>	weak similarity to protopectinase patent WO9806832-A1 - <i>Bacillus subtilis</i>	57 ± 44	57 ± 45	20 ± 0	954 ± 34	1066 ± 21	330 ± 22	Up

Table 7: Hybridization values of 46 genes that are implicated in carbohydrate degradation and that are differentially expressed in wild-type when compared to the $\Delta flbA$ strain of *A. niger*. The table was adapted from Pel et al. [18]. *Description of gene name or its closest homologue. Sm: Similar; A. nid: *A. nidulans*; A. ory: *A. oryzae*.

Annotation	Gene Name*	Description	wild-type zone 1	wild-type zone 3	wild-type zone 5	$\Delta flbA$ zone 1	$\Delta flbA$ zone 3	$\Delta flbA$ zone 5	Regulation
protein products identified in the secretome of $\Delta flbA$ that are predicted to be secreted									
An11g06480	<i>uncharacterized</i>	weak similarity to antigenic protein f86.aa. of patent WO9859071 - <i>Borrelia burgdorferi</i>	165 ± 5	154 ± 2	119 ± 6	43 ± 9	38 ± 1	43 ± 4	Down
An09g00260	<i>aglC</i>	alpha-galactosidase C aglC - <i>Aspergillus niger</i> [truncated ORF]	31 ± 6	34 ± 4	143 ± 60	208 ± 5	216 ± 38	370 ± 31	Up
An03g06310	<i>pmeA</i>	pectinesterase pmeA- <i>Aspergillus niger</i>	33 ± 6	36 ± 6	43 ± 5	65 ± 4	75 ± 14	179 ± 41	Up
An15g03550	<i>uncharacterized</i>	weak similarity to protopectinase patent WO9806832-A1 - <i>Bacillus subtilis</i>	57 ± 44	57 ± 45	20 ± 0	954 ± 34	1066 ± 21	330 ± 22	Up
An11g00100	<i>uncharacterized</i>	strong similarity to triacylglycerol lipase LIP5 - <i>Candida rugosa</i>	71 ± 4	64 ± 3	59 ± 8	800 ± 192	866 ± 397	2265 ± 77	Up
An16g08870	<i>uncharacterized</i>	strong similarity to the triacylglycerol lipase I precursor lipI - <i>Geotrichum candidum</i>	139 ± 16	125 ± 28	84 ± 2	395 ± 28	267 ± 13	181 ± 12	Up
An09g01240	<i>uncharacterized</i>	strong similarity to phospholipase B - <i>Penicillium notatum</i>	78 ± 7	63 ± 9	104 ± 3	499 ± 142	727 ± 111	627 ± 178	Up
An16g01880	<i>lipanI</i>	strong similarity to lysophospholipase - <i>Aspergillus foetidus</i>	648 ± 245	795 ± 248	454 ± 202	10644 ± 663	10585 ± 2214	2676 ± 160	Up
An02g00740	<i>uncharacterized</i>	similarity to 6-Hydroxy-D-nicotine oxidase 6-HDNO - <i>Arthrobacter oxidans</i>	31 ± 1	30 ± 2	35 ± 3	61 ± 5	69 ± 11	130 ± 14	Up
An02g08560	<i>uncharacterized</i>	similarity to probable dioxygenase SCOEDB - <i>Streptomyces coelicolor</i>	22 ± 3	23 ± 3	19 ± 0	797 ± 22	1048 ± 83	1682 ± 135	Up
An03g00460	<i>uncharacterized</i>	strong similarity to the 6-hydroxy-D-nicotine oxidase 6-HDNO - <i>Arthrobacter oxidans</i>	129 ± 69	90 ± 27	102 ± 7	913 ± 19	751 ± 8	198 ± 2	Up
An03g05210	<i>uncharacterized</i>	strong similarity to reticuline oxidase bbe1 - <i>Eschscholzia californica</i>	146 ± 47	180 ± 82	308 ± 23	423 ± 2	453 ± 23	767 ± 34	Up
An06g00720	<i>uncharacterized</i>	similarity to chloroperoxidase CPO - <i>Caldariomyces fumago</i>	59 ± 24	59 ± 16	40 ± 14	109 ± 12	134 ± 32	109 ± 2	Up
An03g06220	<i>sm to geID- A. nid</i>	strong similarity to beta (1-3) glucanosyltransferase GEL3 - <i>Aspergillus fumigatus</i>	42 ± 3	48 ± 11	58 ± 3	1406 ± 256	1874 ± 54	5043 ± 547	Up

An16g07040	<i>sm to btgE -A. nid</i>	similarity to beta-1,3-glucanoglucosyltransferase BGT1 - <i>Aspergillus fumigatus</i> [truncated ORF]	502 ± 224	676 ± 353	2375 ± 144	5982 ± 202	6554 ± 1346	7358 ± 380	Up
An05g02170	<i>uncharacterized</i>	strong similarity to serine-type carboxypeptidase F CPD-II - <i>Aspergillus niger</i>	60 ± 17	57 ± 6	70 ± 11	109 ± 12	121 ± 19	240 ± 17	Up
An18g01320	<i>uncharacterized</i>	strong similarity to extracellular protease precursor BAR1 - <i>Saccharomyces cerevisiae</i>	148 ± 76	129 ± 56	328 ± 31	2146 ± 67	2897 ± 61	2501 ± 161	Up
An01g06280	<i>uncharacterized</i>	strong similarity to IgE-binding protein - <i>Aspergillus fumigatus</i>	267 ± 123	341 ± 167	136 ± 4	2010 ± 28	2634 ± 280	1002 ± 144	Up
An03g00770	<i>uncharacterized</i>	strong similarity to allergic bronchopulmonary aspergillitis allergen rAsp f 4 of patent WO9828624-A1 - <i>Aspergillus fumigatus</i>	93 ± 3	98 ± 34	454 ± 19	473 ± 118	437 ± 60	2858 ± 7	Up
An04g07160	<i>uncharacterized</i>	similarity to hypothetical protein MLD14.3 - <i>Arabidopsis thaliana</i>	202 ± 34	215 ± 74	246 ± 38	548 ± 65	656 ± 82	666 ± 81	Up
An06g00160	<i>uncharacterized</i>	weak similarity to hypothetical cell wall protein binB - <i>Aspergillus nidulans</i>	319 ± 29	330 ± 70	128 ± 21	3226 ± 732	2940 ± 1230	453 ± 184	Up
An07g02730	<i>uncharacterized</i>	strong similarity to SUN family protein Psu1 - <i>Schizosaccharomyces pombe</i>	261 ± 96	317 ± 133	1534 ± 12	2045 ± 210	2424 ± 353	3717 ± 85	Up
An09g03650	<i>uncharacterized</i>	weak similarity to hypothetical protein Ta0309 - <i>Thermoplasma acidophilum</i>	84 ± 40	52 ± 1	133 ± 16	375 ± 55	488 ± 99	438 ± 1	Up
protein products identified in the $\Delta flbA$ secretome that are predicted to be released by non-classical secretion									
An09g02830	<i>uncharacterized</i>	strong similarity to acylaminoacyl-peptidase DPP V - <i>Aspergillus fumigatus</i>	385 ± 95	420 ± 131	622 ± 25	251 ± 45	224 ± 14	116 ± 2	Down
An09g00270	<i>aglC</i>	alpha-galactosidase C <i>aglC</i> - <i>Aspergillus niger</i> [truncated ORF]	89 ± 18	85 ± 22	760 ± 428	879 ± 73	1158 ± 119	1805 ± 251	Up
protein products identified in the $\Delta flbA$ secretome without signal sequence for secretion									
An01g06970	<i>ara1</i>	strong similarity to D-arabinose dehydrogenase ARA1 - <i>Saccharomyces cerevisiae</i>	2370 ± 516	3061 ± 357	2476 ± 456	643 ± 50	627 ± 28	1838 ± 51	Down
An02g11970	<i>uncharacterized</i>	strong similarity to 4-nitrophenylphosphatase pho2 - <i>Schizosaccharomyces pombe</i>	85 ± 12	93 ± 21	123 ± 9	164 ± 6	183 ± 36	416 ± 16	Up
An08g10830	<i>uncharacterized</i>	strong similarity to geranylgeranyl pyrophosphate synthase ggpps - <i>Gibberella fujikuroi</i>	65 ± 5	58 ± 0	68 ± 18	412 ± 9	388 ± 31	555 ± 30	Up
An09g06250	<i>uncharacterized</i>	strong similarity to vacuolar aminopeptidase yscl - <i>Saccharomyces cerevisiae</i>	184 ± 42	187 ± 30	405 ± 70	526 ± 16	555 ± 41	430 ± 39	Up
An12g08610	<i>glkA</i>	glucokinase GlkA - <i>Aspergillus niger</i>	302 ± 29	324 ± 114	306 ± 96	652 ± 16	622 ± 75	660 ± 65	Up
protein products identified in wild-type and $\Delta flbA$ secretome that are predicted to be secreted									
An02g11150	<i>aglB</i>	alpha-galactosidase <i>aglB</i> - <i>Aspergillus niger</i>	390 ± 96	2373 ± 668	3155 ± 569	111 ± 5	160 ± 21	126 ± 14	Down
An01g00780	<i>xlnB</i>	endo-1,4-beta-xylanase B precursor <i>xlnB</i> - <i>Aspergillus niger</i>	4528 ± 1905	16891 ± 683	19085 ± 0	953 ± 977	4014 ± 2052	1371 ± 235	Down
An03g00940	<i>xlnC</i>	endo-1,4-beta-xylanase C precursor <i>xlnC</i> - <i>Aspergillus niger</i>	2605 ± 1941	15761 ± 4701	15538 ± 1230	337 ± 232	1058 ± 553	1067 ± 324	Down
An03g00960	<i>axhA</i>	1,4-beta-D-arabinoxylan arabinofuranohydrolase <i>axhA</i> - <i>Aspergillus niger</i>	4089 ± 1838	15097 ± 1853	17374 ± 0	456 ± 313	1314 ± 776	1602 ± 429	Down

An14g01800	<i>aglD</i>	alpha-galactosidase <i>aglD</i> - <i>Aspergillus niger</i>	54 ± 4	109 ± 6	136 ± 18	27 ± 0	33 ± 3	34 ± 2	Down
An17g00300	<i>xarB</i>	strong similarity to bifunctiona xylosidase-arabinosidase <i>xarB</i> - <i>Thermoanaerobacter ethanolicus</i>	156 ± 10	534 ± 22	396 ± 19	88 ± 6	124 ± 19	144 ± 17	Down
An01g11660	<i>cbhB</i>	1,4-beta-D-glucan cellobiohydrolase B precursor <i>cbhB</i> of patent WO9906574-A1 - <i>Aspergillus niger</i>	1289 ± 335	8224 ± 845	6037 ± 1558	48 ± 13	430 ± 386	1098 ± 313	Down
An07g09330	<i>cbhA</i>	cellulose 1,4-beta-cellobiosidase <i>cbhA</i> from patent WO9906574-A1- <i>Aspergillus niger</i>	1157 ± 1121	7883 ± 2929	3034 ± 1406	67 ± 16	130 ± 62	170 ± 76	Down
An16g06800	<i>eglB</i>	strong similarity to endoglucanase <i>eglB</i> - <i>Aspergillus niger</i>	154 ± 25	226 ± 100	423 ± 232	87 ± 5	92 ± 5	77 ± 5	Down
An08g05230	<i>uncharacterized</i>	strong similarity to putative endoglucanase IV - <i>Trichoderma reesei</i>	1152 ± 1281	2411 ± 2729	648 ± 116	55 ± 5	58 ± 11	46 ± 5	Down
An03g06550	<i>glaA</i>	glucan 1,4-alpha-glucosidase <i>glaA</i> - <i>Aspergillus niger</i>	370 ± 102	1497 ± 1144	5690 ± 1251	182 ± 15	171 ± 29	166 ± 8	Down
An01g12450	<i>exsG</i>	strong similarity to putative glucan beta-1,3 exoglucanase - <i>Trichoderma harzianum</i>	3091 ± 197	3667 ± 1573	1008 ± 250	966 ± 4	1053 ± 14	689 ± 27	Down
An02g09690	<i>uncharacterized</i>	strong similarity to lipase I precursor TFL I - <i>Geotrichum fermentans</i> [putative sequencing error]	652 ± 18	1719 ± 762	529 ± 399	195 ± 22	294 ± 108	356 ± 50	Down
An01g14940	<i>uncharacterized</i>	similarity to nonhemolytic phospholipase C PC-PLC - <i>Burkholderia pseudomallei</i>	299 ± 58	486 ± 126	203 ± 35	122 ± 2	138 ± 34	57 ± 2	Down
An01g01540	<i>uncharacterized</i>	strong similarity to alpha,alpha-trehalase <i>treA</i> - <i>Aspergillus nidulans</i>	154 ± 10	161 ± 8	149 ± 13	568 ± 30	607 ± 43	366 ± 5	Up
An07g07530	<i>crhB</i>	strong similarity to cell wall protein UTR2 - <i>Saccharomyces cerevisiae</i>	176 ± 39	254 ± 48	1452 ± 40	1812 ± 115	1764 ± 453	2719 ± 364	Up
An14g01820	<i>phiA /binB</i>	strong similarity to hypothetical cell wall protein <i>binB</i> - <i>Aspergillus nidulans</i>	2018 ± 1048	3064 ± 430	843 ± 70	8800 ± 3676	14346 ± 1791	4066 ± 653	Up
An08g07350	<i>sm to gelB- A. nid</i>	strong similarity to glycopospholipid-anchored surface glycoprotein GAS1 precursor - <i>Saccharomyces cerevisiae</i>	226 ± 26	257 ± 8	363 ± 114	674 ± 9	839 ± 71	1001 ± 69	Up
An08g00490	<i>uncharacterized</i>	strong similarity to lactonohydrolase - <i>Fusarium oxysporum</i>	160 ± 14	237 ± 17	346 ± 177	484 ± 34	516 ± 99	806 ± 14	Up
An14g02470	<i>uncharacterized</i>	strong similarity to the protein PRO304 of patent WO200104311-A1 - <i>Homo sapiens</i>	326 ± 177	470 ± 61	384 ± 107	2772 ± 181	3044 ± 235	770 ± 49	Up
Protein products identified in wild-type secretome that are predicted to be secreted									
An01g14600	<i>uncharacterized</i>	strong similarity to the endo-1,4-beta-Xylanase B XynB, patent WO9414965 - <i>Aspergillus tubingensis</i>	256 ± 239	364 ± 349	195 ± 92	30 ± 1	34 ± 6	37 ± 4	Down
An06g00170	<i>aglA</i>	alpha-galactosidase <i>aglA</i> - <i>Aspergillus niger</i>	600 ± 56	678 ± 85	124 ± 23	59 ± 7	32 ± 6	18 ± 3	Down
An04g06920	<i>agdA</i>	extracellular alpha-glucosidase <i>aglU</i> - <i>Aspergillus niger</i>	236 ± 29	316 ± 16	651 ± 135	176 ± 8	145 ± 5	176 ± 10	Down
An04g09690	<i>sm to pmeA- A. nid</i>	strong similarity to pectin methyl-esterase PME1 - <i>Aspergillus aculeatus</i>	60 ± 19	190 ± 41	174 ± 59	38 ± 3	48 ± 0	47 ± 9	Down
An07g08940	<i>uncharacterized</i>	similarity to acetyl-esterase I of patent WO9502689-A - <i>Aspergillus aculeatus</i>	242 ± 13	1268 ± 407	914 ± 553	20 ± 1	31 ± 7	26 ± 0	Down

An01g11670	<i>eglA</i>	strong similarity to endo-beta-1,4-glucanase A <i>eglA</i> - <i>Emericella nidulans</i>	638 ± 408	2367 ± 12	589 ± 232	21 ± 2	42 ± 24	58 ± 1	Down
An07g08950	<i>eglC</i>	endoglucanase B <i>eglB</i> - <i>Aspergillus niger</i>	2511 ± 570	11775 ± 532	7793 ± 1370	52 ± 29	366 ± 244	422 ± 77	Down
An01g01870	<i>eglC</i>	strong similarity to hypothetical Avicelase III <i>avIII</i> - <i>Aspergillus aculeatus</i>	77 ± 47	501 ± 267	400 ± 240	22 ± 0	23 ± 1	23 ± 2	Down
An03g01050	<i>uncharacterized</i>	similarity to endo-beta-1,4-glucanase - <i>Bacillus polymyxa</i>	137 ± 32	345 ± 202	1027 ± 608	31 ± 8	48 ± 17	89 ± 12	Down
An08g10780	<i>uncharacterized</i>	strong similarity to hypothetical protein T16K5.230 of <i>A. thaliana</i>	72 ± 40	140 ± 70	1109 ± 145	30 ± 8	26 ± 2	43 ± 0	Down
An14g02670	<i>uncharacterized</i>	strong similarity to endoglucanase IV <i>egl4</i> - <i>Trichoderma reesei</i>	347 ± 273	857 ± 754	737 ± 10	37 ± 3	45 ± 3	37 ± 1	Down
An08g08370	<i>uncharacterized</i>	similarity to the alpha-1,2-mannosidase <i>aman2</i> - <i>Bacillus</i> sp. M-90	1771 ± 843	2086 ± 211	376 ± 19	179 ± 33	78 ± 14	38 ± 4	Down
An16g00540	<i>uncharacterized</i>	similarity to putative large secreted protein - <i>Streptomyces coelicolor</i> [truncated ORF]	32 ± 1	89 ± 3	159 ± 14	23 ± 1	25 ± 1	26 ± 6	Down
An02g02540	<i>uncharacterized</i>	strong similarity to acetyl-esterase I from patent R63066 - <i>Aspergillus aculeatus</i>	296 ± 66	375 ± 174	350 ± 139	83 ± 16	94 ± 1	74 ± 12	Down
An04g09700	<i>uncharacterized</i>	strong similarity to endo-xylogalacturonan hydrolase <i>xghA</i> - <i>Aspergillus tubingensis</i>	60 ± 41	435 ± 272	143 ± 33	28 ± 4	28 ± 0	39 ± 3	Down
An14g04200	<i>rhgB</i>	rhamnogalacturonase <i>rhgB</i> - <i>Aspergillus niger</i>	83 ± 57	177 ± 184	105 ± 34	33 ± 7	36 ± 5	34 ± 0	Down
An15g05370	<i>pgall</i>	polygalacturonase <i>pgall</i> of patent EP421919-A - <i>Aspergillus niger</i>	224 ± 219	767 ± 877	327 ± 240	29 ± 3	25 ± 5	28 ± 5	Down
An07g02360	<i>uncharacterized</i>	similarity to 6-hydroxy-D-nicotine oxidase 6-HDNO - <i>Arthrobacter oxidans</i>	60 ± 24	49 ± 23	104 ± 15	32 ± 3	31 ± 2	25 ± 4	Down
An08g09850	<i>uncharacterized</i>	strong similarity to phosphate-repressible acid phosphatase precursor <i>phoA</i> - <i>Penicillium chrysogenum</i>	424 ± 125	551 ± 364	636 ± 431	245 ± 15	256 ± 8	132 ± 7	Down
An08g11030	<i>phyB</i>	acid phosphatase <i>aph</i> , 3-phytase <i>phyB</i> - <i>Aspergillus niger</i>	436 ± 438	975 ± 1113	973 ± 889	54 ± 4	59 ± 5	44 ± 0	Down
An08g04640	<i>protB</i>	strong similarity to putative lysosomal pepstatin insensitive protease CLN2 - <i>Canis familiaris</i>	98 ± 32	218 ± 127	827 ± 628	62 ± 13	58 ± 5	59 ± 2	Down
An16g09010	<i>protH</i>	strong similarity to carboxypeptidase I protein of patent WO9814599-A1 - <i>Aspergillus oryza</i>	1018 ± 249	1107 ± 340	353 ± 147	312 ± 2	298 ± 3	161 ± 9	Down
An06g00190	<i>uncharacterized</i>	strong similarity to lysosomal pepstatin insensitive protease CLN2 - <i>Homo sapiens</i>	305 ± 23	733 ± 87	1175 ± 28	144 ± 2	114 ± 5	58 ± 5	Down
An12g05960	<i>uncharacterized</i>	strong similarity to dipeptidyl peptidase II DPPII - <i>Rattus norvegicus</i>	105 ± 22	126 ± 40	207 ± 38	81 ± 22	81 ± 4	47 ± 7	Down
An14g02150	<i>uncharacterized</i>	strong similarity to serine-type carboxypeptidase precursor <i>cpdS</i> - <i>Aspergillus phoenicis</i>	49 ± 10	57 ± 12	38 ± 7	23 ± 2	21 ± 1	23 ± 4	Down
An09g00670	<i>geld</i>	strong similarity to beta (1-3) glucanosyltransferase <i>Gel3p</i> - <i>Aspergillus fumigatus</i>	1596 ± 565	2244 ± 607	2063 ± 821	45 ± 1	39 ± 6	59 ± 10	Down
An09g00840	<i>uncharacterized</i>	weak similarity to antigenic cell wall galactomannoprotein MP1 - <i>Aspergillus fumigatus</i>	3153 ± 2599	10725 ± 146	3462 ± 103	29 ± 11	35 ± 22	14 ± 2	Down

An01g15200	uncharacterized	strong similarity to mature penicillin V amidohydrolase PVA of patent US5516679-A - <i>Fusarium oxysporum</i> [truncated ORF]	1551 ± 24	1626 ± 657	351 ± 58	146 ± 9	92 ± 0	108 ± 31	Down
An13g01880	uncharacterized	strong similarity to cephalosporin esterase - <i>Rhodospiridium torulooides</i>	407 ± 104	3000 ± 374	3350 ± 147	96 ± 37	194 ± 69	155 ± 2	Down
An01g00210	uncharacterized	hypothetical protein	105 ± 13	174 ± 37	91 ± 31	36 ± 2	37 ± 0	50 ± 8	Down
An02g11890	uncharacterized	strong similarity to hypothetical protein An14g01330 - <i>Aspergillus niger</i>	61 ± 14	161 ± 33	124 ± 11	24 ± 1	30 ± 1	29 ± 2	Down
An08g04630	uncharacterized	hypothetical protein	361 ± 349	674 ± 706	305 ± 86	51 ± 1	48 ± 13	36 ± 1	Down
An15g02250	uncharacterized	hypothetical protein	1220 ± 1202	1162 ± 905	78 ± 37	47 ± 2	49 ± 6	50 ± 9	Down
An06g01000	uncharacterized	strong similarity to hypothetical protein AN5357.2 - <i>Aspergillus nidulans</i>	201 ± 49	252 ± 68	397 ± 160	601 ± 188	760 ± 179	892 ± 307	Up
An11g00040	uncharacterized	weak similarity to cDNA for 59-kDa readthrough protein RT - Sorghum chlorotic spot virus	780 ± 377	869 ± 597	1222 ± 357	3463 ± 134	3264 ± 0	2983 ± 97	Up
Protein products identified in wild-type without signal sequence for secretion									
An14g01790	uncharacterized	hypothetical protein	93 ± 2	199 ± 15	220 ± 21	37 ± 4	54 ± 8	47 ± 16	Down
An01g00610	uncharacterized	weak similarity to hypothetical protein yukJ - <i>Bacillus subtilis</i>	455 ± 238	550 ± 223	1226 ± 644	47 ± 3	40 ± 6	73 ± 6	Down

Table 8: Hybridization values of 87 differentially regulated genes whose protein products were identified in the secretome of xylose-grown sandwiched colonies of the $\Delta flbA$ [8] strain and/or the wild-type [7] of *A. niger*. *Description of gene name or its closest homologue. Sm: Similar; A. nid: *A. nidulans*.

proteins (6) (Table 8). Furthermore, 2 and 5 genes were up- and down-regulated, respectively, of the genes encoding 30 proteins of the $\Delta flbA$ secretome [8] that were absent in the wild-type secretome [7], and that are predicted to be secreted *via* non-classical secretion (15) (SecP), or have no prediction for secretion (15) [8] (Table 8). The secretome of the cycloheximide treated wild-type strain contained 55 proteins [7] that were absent in the $\Delta flbA$ secretome [8], of which 35 encoding genes were differentially expressed. The two up-regulated genes in the $\Delta flbA$ strain encode uncharacterized proteins. The 33 down-regulated genes encode carbohydrate degrading enzymes (17), phytases (2), proteases (5), oxidase (1) and other/unknown proteins (5) (Table 8). Of the 37 proteins that were identified in both the wild-type and $\Delta flbA$ secretome, 6 and 14 encoding genes were found to be 2-fold higher and lower expressed in the $\Delta flbA$ strain, respectively (Table 8). The up-regulated genes consist of putative cell wall/ morphogenesis enzymes (3), carbohydrate degrading enzymes (1), a protease (1), and a superoxide dismutase (1), while the down-regulated genes encode putative carbohydrate degrading enzymes (11), a cell wall/ morphogenesis enzyme (1), a phospholipase (1) and a lipase (1).

Transcriptional changes associated to transcription factors: Pel et al. [18] described 623 *A. niger* transcription factors, of which 20 and 18 are found to be up- and down-regulated in the $\Delta flbA$ strain, respectively (Figure 4D, Table 9, Supplemental Table 1). Of these genes, 28 encode transcription factors with an unknown function, while 3 are homologous to transcription factor genes of *A. oryzae* and 1 to *A. fumigatus*. These orthologues encode *atfB* (An14g06250), *xlnR* (An11g06290), *amyR* (An01g06900) and *rosA* (An04g07400), respectively. Moreover, the regulator of extracellular protease *prtT* was found to be down-regulated in the $\Delta flbA$ strain. In contrast, *pacC* that is activated upon exposure to alkaline pH and *acuB* that activates acetate metabolism are ≥ 2 -fold higher expressed in the $\Delta flbA$ strain. Similarly, *abaA* and *flbD*, both involved in reproductive development (see above), are upregulated in $\Delta flbA$.

Discussion

Colonies of an *A. niger* strain in which *flbA* is inactivated do not sporulate. Instead, they grow throughout the colony and show secretion in a large part of the mycelium [8]. Moreover, cell walls of the $\Delta flbA$ strain are thinner than those of the wild-type [8]. The molecular mechanisms underlying these phenomena are not known. Here, the impact of inactivation of *flbA* on spatial gene expression in a sandwiched grown *A. niger* colony was assessed with emphasis on genes encoding secreted proteins, genes involved in cell wall synthesis, genes involved in asexual and sexual development and genes encoding transcriptional regulators. The latter genes may control spatial growth and secretion, cell wall synthesis or may have a role in reproduction.

Genes that could account for a sexual pathway have been identified in *A. niger* [18]. However, so far only asexual reproduction has been shown to occur in this fungus. Asexual development involves the formation of conidia forming conidiophores. This process has been studied in most detail in *A. nidulans* [3,26]. Based on its genome, *A. niger* is expected to have similar mechanisms underlying conidiophore development. Indeed, the central regulator of conidiophore formation *brlA*, as well as *flbA* have a similar role in asexual development in *A. niger* and *A. nidulans* [8,27]. Inactivation of *flbA* of *A. niger* does not have a major impact on the expression of genes involved in asexual development, at least in colonies grown as a sandwiched culture as used in this study. Of the 68 genes implicated in reproduction, only 13 were differentially expressed when compared to the wild-type. Genes *flbD* and *sfaD* (Figure 1) were among the 6 genes that were up-regulated ≥ 2 -fold in the $\Delta flbA$ strain. Notably, *flbD* and *sfaD* expression was not increased in the $\Delta flbA$ strains of *A. nidulans* and *A. oryzae* [28,29]. Transcripts of *brlA* were absent in wild type *A. niger* colonies, while some *brlA* expression was observed in the center of $\Delta flbA$ colonies. In contrast, *FlbA* directly or indirectly stimulates expression of *brlA* in *A. nidulans* [9], *A. oryzae* [29] and *A. fumigates* [30]. It can thus be concluded that the sporulation pathway of *A. niger* is similar but

not identical to that of *A. nidulans*, *A. oryzae* and *A. fumigatus*. Our data also indicate that FlbA represses asexual reproduction in zones of *A. niger* colonies that do no longer have the potential to sporulate. It might be that this also occurs in other *Aspergilli*. This should be studied by extracting RNA of zones rather than the whole mycelium, as is routinely done.

A total of 102 genes are predicted to be involved in cell wall synthesis

[18]. Of these genes, only 10 and 2 were found to be up- and down-regulated in the $\Delta flbA$ strain, respectively. It is tempting to speculate that these genes are involved in the changed spatial distribution of growing hyphae in the colony. In this respect, the up-regulation of two chitin synthase genes is of particular interest. The *chsD* homologue of *A. nidulans* is expressed throughout the *A. niger* colony. This gene is upregulated in all zones of $\Delta flbA$ colonies. The *chsF* chitin synthase gene is even more interesting. This gene is periphery specific in wild-

Annotation	Gene Name*	Description	wild-type zone 1	wild-type zone 3	wild-type zone 5	$\Delta flbA$ zone 1	$\Delta flbA$ zone 3	$\Delta flbA$ zone 5	Regulation
An04g08620	<i>uncharacterized</i>	similarity to heme activator protein HAP1 - <i>Saccharomyces cerevisiae</i>	1079 ± 14	1118 ± 11	942 ± 47	642 ± 42	512 ± 28	191 ± 21	Down
An12g01870	<i>uncharacterized</i>	similarity to positive regulator of the lactose-galactose regulon LAC9 - <i>Kluyveromyces lactis</i>	495 ± 135	1003 ± 137	720 ± 305	254 ± 24	271 ± 9	167 ± 16	Down
An16g08800	<i>uncharacterized</i>	similarity to the zinc finger transcription factor ACE1 - <i>Trichoderma reesei</i>	543 ± 121	642 ± 119	310 ± 34	77 ± 17	78 ± 5	77 ± 1	Down
An15g00120	<i>uncharacterized</i>	similarity to Wilms tumor susceptibility protein WT1 - <i>Homo sapiens</i>	476 ± 307	442 ± 230	580 ± 259	112 ± 11	111 ± 1	65 ± 3	Down
An04g05060	<i>uncharacterized</i>	similarity to XFIN protein - <i>Xenopus laevis</i>	3584 ± 911	3946 ± 117	1943 ± 688	1248 ± 6	1042 ± 73	810 ± 91	Down
An04g07400	<i>sm rosA- A. fum</i>	strong similarity to C6 zinc finger transcription factor PRO1 - <i>Sordaria macrospora</i> [putative sequencing error]	120 ± 28	195 ± 76	562 ± 77	86 ± 3	83 ± 9	43 ± 6	Down
An13g01370	<i>uncharacterized</i>	strong similarity to the hypothetical protein An01g14540 - <i>Aspergillus niger</i>	568 ± 230	603 ± 155	359 ± 47	207 ± 8	157 ± 8	192 ± 26	Down
An11g07610	<i>uncharacterized</i>	strong similarity to the hypothetical protein encoded by An15g04740 - <i>Aspergillus niger</i>	226 ± 129	212 ± 48	104 ± 38	54 ± 1	43 ± 2	33 ± 0	Down
An11g06290	<i>sm to xlnR -A. ory</i>	strong similarity to transcriptional activator xlnR - <i>Aspergillus niger</i>	145 ± 30	141 ± 35	84 ± 17	54 ± 2	40 ± 4	30 ± 1	Down
An01g13790	<i>uncharacterized</i>	weak similarity to homeodomain protein Prep-1 - <i>Homo sapiens</i>	792 ± 126	685 ± 218	368 ± 78	220 ± 10	203 ± 29	317 ± 8	Down
An12g02880	<i>uncharacterized</i>	weak similarity to hypothetical protein SPBC530.05 - <i>Schizosaccharomyces pombe</i>	80 ± 51	80 ± 48	39 ± 1	24 ± 1	27 ± 0	31 ± 0	Down
An06g00830	<i>uncharacterized</i>	weak similarity to hypothetical transcription regulatory protein SPBC530.08 - <i>Schizosaccharomyces pombe</i>	121 ± 26	146 ± 14	221 ± 35	55 ± 6	67 ± 22	49 ± 8	Down
An08g03770	<i>uncharacterized</i>	weak similarity to mucin MUC5AC - <i>Homo sapiens</i>	85 ± 33	77 ± 21	132 ± 63	23 ± 2	19 ± 3	18 ± 2	Down
An07g07370	<i>uncharacterized</i>	weak similarity to PC-MYB2 - <i>Arabidopsis thaliana</i>	2629 ± 9	2410 ± 318	1104 ± 502	724 ± 44	649 ± 44	253 ± 2	Down
An04g01430	<i>uncharacterized</i>	weak similarity to the chitinase ChiA - <i>Emericella nidulans</i>	14247 ± 1226	12839 ± 0	6031 ± 975	4922 ± 660	3743 ± 121	3693 ± 284	Down
An01g06900	<i>sm to amyR- A. ory</i>	weak similarity to transcription activator amyR - <i>Aspergillus oryzae</i>	758 ± 96	1056 ± 406	654 ± 233	34 ± 8	25 ± 1	21 ± 1	Down
An14g06250	<i>sm to atfB-A. ory</i>	weak similarity to transcription factor atf1+ - <i>Schizosaccharomyces pombe</i>	269 ± 19	523 ± 113	434 ± 242	67 ± 0	67 ± 5	89 ± 8	Down
An04g06940	<i>prtT</i>	similarity to hypothetical transcriptional regulator SPAC1399_1 - <i>Schizosaccharomyces pombe</i>	2945 ± 971	3996 ± 1429	2983 ± 738	1702 ± 175	1499 ± 130	608 ± 77	Down

An08g06580	<i>acuB / FacB</i>	DNA binding protein facB - <i>Aspergillus niger</i>	364 ± 29	340 ± 18	400 ± 148	899 ± 47	929 ± 83	656 ± 14	Up
An07g05960	<i>uncharacterized</i>	similarity to finger protein msn2 - <i>Saccharomyces cerevisiae</i>	143 ± 21	220 ± 133	537 ± 288	759 ± 389	1226 ± 285	1125 ± 434	Up
An04g06950	<i>uncharacterized</i>	similarity to homeobox transcription factor hth - <i>Drosophila melanogaster</i>	102 ± 1	124 ± 6	67 ± 16	288 ± 24	302 ± 50	287 ± 54	Up
An08g06850	<i>uncharacterized</i>	similarity to hypothetical C2H2 zinc-finger protein SPBC1105.14 - <i>Schizosaccharomyces pombe</i>	66 ± 3	75 ± 1	129 ± 30	583 ± 676	787 ± 517	791 ± 668	Up
An06g02570	<i>uncharacterized</i>	similarity to nitrogen assimilation regulatory protein nit-4 - <i>Neurospora crassa</i>	56 ± 3	56 ± 10	114 ± 11	133 ± 0	144 ± 7	235 ± 27	Up
An03g05170	<i>uncharacterized</i>	similarity to sterol regulatory element binding protein-1 srebp-1 - <i>Homo sapiens</i>	352 ± 68	378 ± 126	574 ± 9	654 ± 59	806 ± 134	1485 ± 37	Up
An04g06430	<i>uncharacterized</i>	similarity to zinc finger protein BMZF3 of patent WO9962951-A1 - <i>Homo sapiens</i>	43 ± 3	45 ± 6	63 ± 23	103 ± 1	143 ± 1	147 ± 0	Up
An01g05630	<i>uncharacterized</i>	strong similarity to cytoplasmic aspartate--tRNA ligase APS - <i>Saccharomyces cerevisiae</i> [possible sequencing error]	70 ± 1	75 ± 5	63 ± 0	189 ± 5	214 ± 24	164 ± 7	Up
An14g05810	<i>uncharacterized</i>	strong similarity to histone acetyltransferase GCN5 - <i>Saccharomyces cerevisiae</i>	63 ± 15	58 ± 2	46 ± 1	148 ± 17	140 ± 22	85 ± 2	Up
An01g04830	<i>flbD</i>	strong similarity to myb-like DNA binding protein flbD - <i>Aspergillus nidulans</i>	89 ± 18	91 ± 8	81 ± 20	238 ± 35	220 ± 32	107 ± 5	Up
An03g06760	<i>uncharacterized</i>	strong similarity to probable transcription activator SPAC139.03 - <i>Schizosaccharomyces pombe</i>	106 ± 0	112 ± 14	57 ± 13	158 ± 8	209 ± 42	193 ± 22	Up
An01g03750	<i>abaA</i>	strong similarity to protein abaA - <i>Aspergillus nidulans</i>	61 ± 9	71 ± 12	67 ± 17	113 ± 28	129 ± 3	182 ± 22	Up
An02g07890	<i>pacC</i>	transcription factor pacC - <i>Aspergillus niger</i>	263 ± 12	282 ± 35	184 ± 19	552 ± 69	552 ± 118	562 ± 23	Up
An07g09460	<i>tan1</i>	transposase Tan1 of patent WO9808960-A1 - <i>Aspergillus niger</i>	17 ± 4	16 ± 3	20 ± 2	38 ± 3	67 ± 10	51 ± 6	Up
An08g06030	<i>uncharacterized</i>	weak similarity to putative zinc finger transcription factor stzA - <i>Aspergillus nidulans</i>	232 ± 9	305 ± 46	394 ± 61	668 ± 211	801 ± 153	741 ± 116	Up
An04g00480	<i>uncharacterized</i>	weak similarity to the vitellogenin II precursor - <i>Gallus gallus</i>	469 ± 62	309 ± 76	266 ± 36	1222 ± 145	1525 ± 171	947 ± 325	Up
An11g10870	<i>uncharacterized</i>	weak similarity to transcription factor CHA4 - <i>Saccharomyces cerevisiae</i>	193 ± 83	180 ± 27	273 ± 6	626 ± 226	622 ± 163	560 ± 43	Up
An14g07040	<i>uncharacterized</i>	weak similarity to transcriptional activator of lysine pathway genes LYS14 - <i>Saccharomyces cerevisiae</i>	76 ± 5	71 ± 10	70 ± 1	178 ± 10	217 ± 56	141 ± 8	Up
An17g00800	<i>uncharacterized</i>	weak similarity to transcriptional activator prtT of patent WO200020596-A1 - <i>Aspergillus niger</i>	119 ± 48	89 ± 35	69 ± 5	201 ± 38	195 ± 22	142 ± 5	Up
An02g06000	<i>uncharacterized</i>	weak similarity to ZMS1 - <i>Saccharomyces cerevisiae</i>	471 ± 242	481 ± 196	683 ± 362	980 ± 41	1338 ± 83	1688 ± 256	Up

Table 9: Hybridization values of 38 transcription factor genes [18] that are differentially expressed in wild-type when compared to the $\Delta flbA$ strain. Names of transcription factors are indicated that show homology to or that are encoded by *A. niger* genes. Description of gene or its closest homologue. Sm: Similar; A. fum: *A. fumigatus*; A. ory: *A. oryzae*.

type colonies, while it is expressed throughout $\Delta flbA$ colonies. Zonal expression of *chsF* thus correlates with growth in colonies of wild-type and $\Delta flbA$. The differentially expressed genes involved in cell wall synthesis may also impact cell wall thickness in the $\Delta flbA$ strain. This

may involve synthesizing activity, cell wall processing activity (i.e. crosslinking), as well as degradation activity.

The secretome of the wild-type and the $\Delta flbA$ strain was determined

by transferring 7-day-old colonies that had been grown on agar medium to a ring plate containing fresh medium. Proteins were released in the wells of the ring plate that had been formed during the 24 h incubation time. In addition, proteins are released that had been formed during the 7 days of growth on the agar plate and that slowly diffuse through the cell wall [4]. FlbA was shown to have a more complex secretome when compared to the wild-type [8]. Out of 138 secreted proteins of the $\Delta flbA$ strain [8], 101 had not been identified in the secretome of wild-type colonies [8]. Cycloheximide treatment releases proteins into the culture medium that had been trapped in the cell walls of the wild-type strain [8]. Still, 70 secreted proteins were found in the $\Delta flbA$ secretome that were absent in the medium of cycloheximide treated wild-type colonies. Of these proteins, 23 were differentially expressed, of which 22 were up-regulated in the $\Delta flbA$ strain. This shows that part of the differences in the secretome are caused by transcriptional control during the 7 days of growth on the agar medium. Another part of the differences may be explained by induction of genes after transfer to fresh medium and by (post)-translational regulation. Moreover, we can not exclude that part of the proteome is still trapped in or associated with the wild type cell wall after cycloheximide treatment. 55 proteins were identified in the wild-type secretome that were absent in the culture medium of the $\Delta flbA$ strain [8]. Of the genes encoding these proteins, 35 were differentially expressed, of which 33 were down-regulated in the $\Delta flbA$ strain. This shows that transcriptional control during growth on the agar medium has a major impact on genes encoding proteins that are released by the wild-type strain but not by the $\Delta flbA$ strain. Finally, 20 out of 37 genes encoding proteins that were released both in wild-type and the $\Delta flbA$ secretome were differentially expressed. Quantitative proteomics should reveal whether this differential expression also results in quantitative differences in levels of these proteins in the medium. In this study, *A. niger* was grown on xylose. XlnR is the xylanolytic regulator that controls expression of at least 16 genes [19-25]. All encoded proteins were identified in the wild-type secretome after cycloheximide treatment, whereas 15 out of 16 proteins were identified in the $\Delta flbA$ secretome. 6 out of 16 XlnR regulated genes were down-regulated in the $\Delta flbA$ strain. This is not in conflict with the proteomics data per se, since quantitative proteomics was not performed on wild-type versus $\Delta flbA$. Moreover, proteomics was performed on colonies that had been transferred to fresh medium (see above).

Spatial growth and secretion and cell wall synthesising, modifying and degrading activities are most probably regulated by transcriptional regulators. In total, 38 transcriptional regulators were found to be differentially regulated in the $\Delta flbA$ strain. Most of these regulators have not been characterised. However, the list includes 9 genes with a known function or that are homologous to a transcription factor that has been characterized in other *Aspergillus* species. Genes that have homology to *rosA* of *A. fumigatus* and *atfB* of *A. oryzae* (both down-regulated in $\Delta flbA$), and to *flbD* and *abaA* of *A. nidulans* (both up-regulated in $\Delta flbA$) are involved in development (*rosA*, *flbD*, and *abaA*) and stress tolerance of conidia (*attB*) [3,31-33]. Genes homologous to *xlnR* and *amyR* of *A. oryzae*, *prtT* [34] and *acuB* [35,36] (all down-regulated in $\Delta flbA$) are predicted to be involved in degradation of polysaccharides, proteins and acetate. Gene *prtT* has been shown to be the transcriptional activator of protease genes in *A. niger* [34]. Its down-regulation in $\Delta flbA$ colonies is in agreement with the finding that only 6 protease genes were up-regulated in $\Delta flbA$, while 13 were down-regulated. The transcriptional regulatory gene *pacC* [36] was found to be up-regulated at the periphery of $\Delta flbA$ colonies. It is not known how *pacC* expression is exactly regulated in *Aspergillus*. The pH does not seem to regulate its expression or that of the six pH-sensing

pal components [37]. However, PacC protein is abundant in alkaline growth conditions, where it is activated by proteolytical cleavage [37-39]. At alkaline pH, alkaline-associated genes of *A. nidulans* are activated by PacC (*palD*, *prtA*, *xlnA*, *acvA*, *ipnA*, *pacC*, *sidA*, *mirA*, *mirB*) [40], while acid-associated genes (*pacA*, *xlnB*, *abfB*, *gabA*, and *stcU*) are repressed [41]. These genes are not fully annotated in *A. niger*, but homologues of *mirA* (An02g14190), *mirB* (An03g03560) and *sidA* (An05g00220) are up-regulated in $\Delta flbA$, whereas *xlnB*, putative *gabA* (An12g10000) and *xlnA* are down-regulated when compared to wild-type. This might be explained by the overexpression of PacC since the pH of the agar-medium underneath wild-type sandwiched colonies was 3, while it was 6 in the case of the $\Delta flbA$ strain. Genes An11g06290 and An01g06900 are homologous to the xylanolytic and amylolytic regulatory genes *xlnR* and *amyR* of *A. oryzae*, respectively. However, they do not encode the XlnR and AmyR regulators of *A. niger* since these functions have been attributed to An15g05810 [20,24,25] and An04g06910, respectively [42]. The *xlnR* gene of *A. niger* was down-regulated 1.5 fold, which may explain the high number (i.e. 9 out of 16) of xylanolytic genes that are down-regulated compared to wild-type. Apart from the 9 transcriptional regulatory genes with a predicted function, there are 29 of such genes with an unknown role in growth and development of *A. niger* [43]. One or more of these genes may be involved in spatial growth, spatial secretion, cell wall formation and asexual development. As such, they are of interest to improve *A. niger* as a cell factory.

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