Transcriptional changes in the transition from vegetative cells to asexual development in the model fungus *Aspergillus nidulans*.

Running Head: Transcriptional analysis of *A. nidulans* early asexual development.

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ABSTRACT.

Morphogenesis encompasses programmed changes in gene expression that lead to the development of specialized cell types. In the model fungus *Aspergillus nidulans*, asexual development involves the formation of characteristic cell types, collectively known as the conidiophore. With the aim of determining the transcriptional changes that occur upon induction of asexual development, we have applied massive mRNA-sequencing to compare the expression pattern of 19 hour-old submerged vegetative cells (hyphae) with that of similar hyphae after exposure to the air for 5 hours. We found that the expression of 2,222 (20,3 %) of the predicted 10,943 *A. nidulans* transcripts was significantly modified after air exposure, 2,035 down- and 187 up-regulated. The activation during this transition of genes that belong specifically to the asexual developmental pathway was confirmed. Another remarkable quantitative change occurred in the expression of genes involved in carbon or nitrogen primary metabolism. Genes participating in polar growth or sexual development were repressed, as those belonging to the HogA stress-response MAP Kinase pathway. We also identified significant expression changes in several genes purportedly involved in redox balance, transmembrane transport, secondary metabolite production or transcriptional regulation, mainly binuclear-zinc cluster transcription factors. Genes coding for these four activities were usually grouped in metabolic clusters, which may bring regulatory implications in the induction of asexual development. These results provide a blueprint for further stage-specific gene expression studies during conidiophore development.

INTRODUCTION.

Public availability of hundreds of fungal genome sequences as well as the advent of high-throughput proteomic and transcriptomic methods have allowed for the acquisition of genome-scale data and the characterization of transcripts and proteins which have no designated function (26, 43).

In the genus *Aspergillus*, which includes model organisms as well as industrially and medically important species, proteomic studies have mainly focused on 2D-PAGE coupled to MS-MS, gaining valuable information into the composition of the proteome under different growth and stress conditions (32). The main transcriptomic approach to the development, stress response or secondary metabolite production of *Aspergilli* has involved microarray analyses (see, for example, references (48, 51, 57, 59)). RNA-sequencing (RNA-seq; (39)) technology allows a deeper and more reproducible analysis of gene expression and regulation with a lower background than microarray analysis (39, 53, 65). It has been successfully used to elucidate transcriptomes of microbes and higher eukaryotes (see references within (64)). Thus, this powerful technique arises as an efficient tool for transcriptomic analyses in the genus *Aspergillus*, as shown by Wang and coworkers in 2010 (64), who published the first RNAseq-based transcriptomic study in this *genus*. In these last two years, the RNAseq-based studies involving *Aspergillus* species analyzed the temperature effect on secondary metabolite synthesis or biofilm formation in *A. fumigatus* or *A. flavus*, respectively (24, 64, 64, 70).

Aspergillus nidulans is the reference organism in the study of fungal asexual development, also known as conidiation or conidiophore development (2, 17, 19, 41). The required morphological changes during conidiophore formation (Figure 1; (38)) are induced by environmental signals and arise from non-specialized cells called vegetative hyphae. Conidiophore development starts with the formation of the foot-cell, which has a thick cell wall. Then a branch emerges from the foot-cell and elongates through apical extension in a negative geotropism way forming the stalk. This is followed by a swelling process of the stalk tip to form the vesicle. Then, a massive multipolar budding process at the dome of the vesicle generates a layer of approximately sixty primary sterigmata or metulae, followed by their respective apical

82 budding to generate 120 secondary sterigmata or phialides. The vesicle, metulae,
83 phialides and conidia are separated by septa, the production of which is regulated by
84 conidiophore non-specific and specific bud-site markers (see for example, (27, 54)).
85 Finally, each phialide produces, via basipetal cell divisions, long chains of more than
86 100 asexual propagules called conidia (Figure 1).

87 The morphological changes described above are programmed at the genetic
88 level. For practical purposes, initial works divided the process into two genetic stages
89 (2). Stage one included factors involved in the perception of environmental changes,
90 transduction of these signals and the launching of the initial morphogenetic
91 transformations leading to vesicle formation. These are the Upstream Developmental
92 Activators (UDA; (17)). Loss-of-function mutations in these genes yield a "*fluffy*"
93 aconidial phenotype that is manifested as cotton-like masses of vegetative cells and
94 the absence of cell differentiation (67). From the genetic point of view, the *fluffy*
95 phenotype of UDA mutants is associated with the inability to induce the second
96 stage, characterized by the control exerted by the C2H2-type transcription factor (TF)
97 *brlA*, the first conidiation-specific TF (1). BrlA and downstream factors of the Central
98 Developmental Pathway of Conidiation (CDP), as AbaA and WetA (6, 37), regulate
99 the spatio-temporal morphological transformations leading to spore formation.

100 In this simplified genetic model, the handover of control between UDA and
101 CDP pathways is not so clear-cut. The requirement of additional proteins than those
102 actually known in early asexual transformations is, in addition, highly feasible. With
103 the aim of identifying the genes and determining the cellular processes significantly
104 altered during this transition, we used in this work a RNA-seq approach to compare
105 the transcriptomes of submerged 19 hours-old vegetative hyphae (non-specialized
106 cells) and 5 hours-old air-exposed samples (in which asexual structures started to
107 develop). We chose this stage of asexual development because previous works
108 showed that this is the time point in which a maximum expression of UDA coding
109 genes coincide with an remarkable induction of *brlA* and the CDP pathway (17).
110 Below are the most remarkable results and conclusions that in our opinion can be
111 extracted.

112

113

MATERIALS AND METHODS.

Fungal strains and culture conditions.

As a reference *A. nidulans* strain we used MAD2666 (kindly provided by Dr. Ane Markina-Iñarrairaegui), an isogenic strain of TN02A3 (40) where the *pyrG89* mutation was eliminated by gene replacement using a wt fragment of *pyrG* gene. Vegetative cell samples were obtained by culturing 10^6 spores/ml in liquid minimal medium (MMA) with the appropriate supplements (46) for 19 hours. Filtered mycelia were processed for RNA extraction.

Induction of asexual development was conducted as described previously (3, 18). Briefly, after 19h culture in liquid MMA as described above, mycelia were filtered using nitrocellulose membranes (0.45 µm; MicronSep; GE Water and Process Technologies). These membranes were placed onto solid minimal medium and mycelia cultured for 5 hours before being collected and processed for RNA extraction. Two biological replicates were processed for each culture condition.

RNA isolation, mRNA library construction, and Illumina sequencing.

Mycelium samples (100 mg [dry weight]) were frozen in liquid nitrogen and total RNA extraction from these samples was performed according to the Invitrogen protocol based on TRIzol reagent using 1 ml of TRIreagent (Fluka) per sample. Isolated total RNA samples were then further purified using the Qiagen RNeasy minikit, following manufacturer's instructions. The concentration and integrity of total RNA was checked using a Nanodrop (Thermo Scientific) and/or a Bioanalyzer 2100 (Agilent Technologies).

mRNA libraries were prepared from *A. nidulans* total RNA samples following Illumina standard protocols (Ilumina, San Diego, USA). Briefly, each total RNA sample (20–50 µg) was treated with DNase and enriched for mRNA using oligo(dT) tags. Samples of poly(A) RNA (0.2–1 µg) were fragmented into smaller pieces (200–500bp; mean for all libraries is approximately 280 bp) and used to synthesize cDNA. The cDNA library construction involved end-repair, A-tailing, adapter ligation, and library amplification followed by cluster generation and sequencing. Sequencing

144 was performed in a pair-end-read, 2x 76-base mode on a GAIIx Sequencer (Illumina,
145 San Diego, USA), and running four samples per lane (multiplexing).

146

147 **Demultiplexing, Mapping, Assembling and Quantifying Sequencing Data.**

148 Sequences were demultiplexed with Demultiplexing software by Brian J. Knaus,
149 freely accessible from his web page (http://brianknaus.com/). They are 75
150 nucleotides in length as the barcodes have been removed or trimmed.

151 Read quality was checked using fastQC program and only reads with quality
152 values higher than Q30 were introduced for mapping. All reads were mapped using
153 Bowtie 2.0.0-beta5 (http://bowtie-bio.sourceforge.net/ index.shtml), using parameters
154 by default. The latest version of the Aspergillus Genome Database
155 (http://www.aspergillusgenome.org/) available provided the annotated genome of
156 *Aspergillus nidulans*, which was used as template for zapping.

157

158 **Differential expression.**

159 The Cuffdiff program (http://cufflinks.cbcb.umd.edu/index.html) was used in
160 order to detect differentially expressed genes between different samples. To
161 associate with predicted genes, we used the fasta file provided by the Aspergillus
162 Genome Database as the reference for the gtf file from previous mapping steps. As
163 input files we used the preformatted mapping files obtained after running TopHat
164 V1.4.1 (http://tophat.cbcb.umd.edu/) program.

165

166 **Data Visualization.**

167 We used an R application called CummeRbund to visualize the results of the
168 RNAseq analysis. This R program converts the different output files from TopHat or
169 Cufflinks into a related database (CuffData.db) in order to obtain customized graphs.
170

171 **Gene ontology analysis.**

172 Gene ontology (GO) terms for each *A. nidulans* gene were obtained from the
173 Aspergillus genome database
174 (http://www.aspgd.org/download/go/gene_association.aspgd.gz) and were related
175 with terms downloaded from OBO
176 (http://www.geneontology.org/ontology/obo_format_1_2/gene_ontology_ext.obo).
177 The Gene Ontology (GO) project provided a standardized set of terms describing the
178 molecular function of genes. We used the topGO package from the Bioconductor
179 project (http://www.bioconductor.org/packages/release/bioc/html/topGO.html) to
180 identify over-represented GO terms from a set of differentially expressed genes.
181 Python (http://www.python.org/) was used to prepare the data, utilizing rpy2
182 (http://rpy.sourceforge.net/rpy2.html) to call R for the statistical analysis.

183

184

185 ## RESULTS AND DISCUSSION.

186 ### Summary of the RNA-seq data set.

187 To identify genes that might be involved in the induction of asexual
188 development and obtain a broad view on the associated cellular processes, total
189 RNA samples from submerged 19 hour-old vegetative hyphae and 5 hour-old air-
190 exposed (asexually induced) hyphae were subjected to high-throughput Illumina
191 sequencing. We obtained an average of 8,566,985 reads of 72bp per sample
192 (34,267,942 reads, for all 4 samples), representing nearly 20 *A. nidulans* genome
193 lengths per sample (~82 genome lengths with all 4 samples).

194 RNA-Seq analysis revealed that almost the whole set of genes encoded by the
195 *A. nidulans* genome is expressed during vegetative or early asexual stages. Of the
196 10,943 transcripts predicted by the Aspergillus Genome Database, 9,763 (89.2%)
197 were expressed in vegetative samples, and 10,059 (91.9%) during the early asexual
198 stage (Supplementary Table S1). 10,192 genes were expressed in one or both
199 conditions, and 751 genes were not expressed in either condition. Of the 10,192
200 expressed genes, 429 were uniquely expressed in vegetative cells and 113 genes at
201 the early asexual stage. Of the remaining 9,650 genes that were found to be
202 expressed in both conditions 2,222 showed a significant differential expression, of
203 which 187 were upregulated (higher transcript levels in asexual than in vegetative
204 samples) and 2,035 were downregulated (Figure 2B and Supplementary Table S2).
205 Table 1 and 2 show the top20 genes with the highest significant increase
206 (upregulated) or decrease (downregulated) in expression levels upon induction of
207 conidiation, respectively. In order to obtain an overview of the process, an envisaged
208 functional analysis of the top20 genes will be presented in the next section together
209 with the rest of significantly regulated transcripts.

210 We also analyzed the distribution of significantly regulated genes along the *A.*
211 *nidulans* chromosomes and confirmed that there was not any obvious genomic
212 region enriched in them (Figure 2C). However, chromosome III contained a
213 significant increase in downregulated genes compared to the rest of the
214 chromosomes, with a ratio (down- vs upregulated genes) of 22.6 in comparison to an
215 average of 10.9. In contrast, chromosome VII contained the highest proportion of
216 upregulated genes, with a down- vs upregulated ratio of 7.7.

Functional analysis of early asexual development.

To obtain a comprehensive picture on the pathways and cellular processes switched on/off as the initial stages of asexual development proceeded, we divided the list of 2,222 genes with significant altered expression (Supplementary Table S2) into two groups. On one hand, we studied those genes having a standard name, which meant that they were previously described and had a neatly proposed function (274 genes; 12.3 %). On the other hand, we analyzed those only containing a systematic name and not characterized to date (1948 genes; 87.6 %).

From the 274 genes in the first group, 236 (86.1 %) showed lower expression levels after the induction than in vegetative hyphae (downregulated; log 2FC › 0), while 38 (13.9 %) showed higher expression at the asexual phase (upregulated; log 2FC ‹ 0). This is clearly in agreement with the Volcano graph shown in Figure 2B.

Using the Aspergillus Genome Database (www.aspgd.org) and previously published works, we extracted all the available information on the function, localization, genetic pathway and/or cellular process in which those genes in the first group are described or predicted to participate. This information is available in Supplementary Table S3. Genes were grouped according to their participation in different cellular processes. Genes encoding putative Cytochrome P450s were included in a separated group called “Electron transfer and energy metabolism”. Some genes were included in more than one group, as they have been described to participate in several cellular processes. Figure 3 shows how these cellular processes are represented as well as the proportion of downregulated (green) and upregulated (red) genes. Genes predicted to code for proteins with miscellaneous functions are listed in the group called “Unknown/Other” (Supplementary Table S3). The most represented processes are “Primary carbon and nitrogen metabolism” with 76 genes, 67 down and 9 up-regulated (76; 67 + 9), “Stress response” (37: 34 + 3), “Hyphal morphogenesis” (25: 24 + 1), “Conidiation” (27: 21 + 6), “Cell wall organization and biogenesis” (19: 10 + 9), “Secondary metabolism” (17: 13 + 4), “Nucleic acid assembly, organization and integrity” (18: 18 + 0) and “Sexual development” (15: 14 + 1). Other under-represented processes are “Fatty acid metabolism” (9: 7 + 2), “GTPase, ATPase and channels” (11: 10 + 1), “Nuclear transport” (6: 6 + 0), “7 transmembrane domain and heterotrimeric G protein

249 signalling" (6: 6 + 0), "Cell death" (9: 9 + 0), "Siderophore synthesis and transport" (3:
250 2 + 1) and "Cell cycle regulation" (3: 3 + 0).

251

252 **Induction of asexual development provokes alterations in primary metabolism**
253 **pathways.**

254 The analysis of significantly regulated genes suggests that distinct cellular
255 processes are altered to fulfill the requirements of conidiophore development. For
256 instance, primary metabolism appears to be strongly reoriented. Several genes
257 coding for enzymes acting in glycolysis and gluconeogenesis, TCA cycle, urea cycle
258 or amino acids synthesis pathways are down-regulated and few of them up-
259 regulated. We also identified downregulated transcriptional regulators that play a key
260 role in nitrogen metabolite repression (AreA and MeaB; (68) and (69)) or carbon
261 catabolite repression (CreA; (14)), or are involved in the cross-pathway control of
262 amino acid biosynthesis in response to amino acid starvation (CpcA and JlbA; (29)
263 and (56)). Strong variations in the levels of primary metabolism enzymes have been
264 commonly described under a wide array of growth conditions, including early
265 biochemical studies on fungal asexual development (36, 44). For example,
266 experiments examining calcium-induced conidiation in *Penicillium notatum* (45) show
267 several regulated enzymes like fructose-bisphosphate aldolase, triosephosphate
268 isomerase, pyruvate kinase or glucose-6-phosphate dehydrogenase that also appear
269 in our analysis. This metabolic switch can be attributed to the starvation in nutrients
270 associated to the exposition of hyphae to the aerial environment, the stimulus used in
271 this work to induce conidiophore development (see below).

272

273 **Air emergence represses the HogA MAP Kinase stress-response pathway.**

274 Supplementary Table 3 shows that an important number of significantly up- or
275 downregulated genes are involved in the response to stress situations. A large
276 number of them belong to the HogA-mediated general stress response pathway (see
277 references within (19, 25)). The expression of multiple factors from this pathway is
278 downregulated, starting from those involved in signal perception and transduction, as
279 histidine kinases (TcsA, involved in asexual development (63) and FphA, involved in

280 reception of red light), the phosphotransfer protein YpdA, the response regulators
281 SrrA and SrrC, the MAKK PbsB and the MAPK HogA. TFs that act downstream as
282 NapA and AtfA, which are supposed to activate the expression of proteins involved in
283 the detoxification of stress-causing-agents, such as catalases CatA, CatC or CpeA
284 (30), are also downregulated.

285 Previous works linked elements from this pathway with different stages of
286 asexual development. It was described that the loss of *tcsA,* the homologue of the *S.*
287 *cerevisiae* transmembrane osmosensor Sln1p, did not block initiation of conidiophore
288 development but appeared to prevent the cell divisions preceding conidia formation
289 from phialides (63). Conidiation was also reduced in a Δ*fphA* strain in comparison to
290 wild type (49). The loss of SrrA or SskA activity has been linked to decreased *brlA*
291 levels (61). These previous observations strongly suggested that histidine kinases
292 and components of the phospho-relay system are required to coordinate different
293 stages of conidiophore development and the response to ambient stimuli (19). Our
294 results show that this sensing and signal transduction pathway is mainly required at
295 vegetative phase. However, at the stage of conidiophore formation analyzed in this
296 work, which coincides with the formation of stalks and vesicles, it is inhibited.
297

298 **Proteins involved in vegetative growth are inhibited after asexual induction**
299 **while the composition of the cell wall undergoes strong alterations.**

300 Polar growth-related functions are, in general terms, also inhibited 5 hours
301 after the induction of conidiation, in comparison to vegetative growth. At this stage of
302 asexual development vesicles are forming or already formed and this requires an
303 isotropic mode of growth. In agreement with this morphological observation, we
304 found that of a total of significantly regulated 25 genes that code for proteins involved
305 in different aspects of polar growth (establishment of polarity and germination,
306 endocytosis, polarisome components, proteins required for a proper branching
307 pattern, cytoskeleton proteins, etcetera) all except one are down-regulated
308 (Supplementary Table 3 and Figure 3).

309 Early microscopic observations reported that the wall of the foot-cell was
310 thicker than the cell-wall of vegetative hyphae (2), suggesting that it was a target of
311 important transformations during initiation and progression of conidiophore formation.

312 Our results show that half of the ten genes significantly regulated are repressed and
313 the other half upregulated. This supports previous observations and suggests that
314 the cell wall of developing stalk and vesicles retains a high compositional dynamism.

315

316 **The sexual-asexual development balance.**

317 A general inhibition of regulators of sexual development was also found in our
318 study. It has been previously described that the deletion of the oxylipin biosynthetic
319 gene *ppoA* increases the conidia/ascospore ratio (58). In this sense, here we found
320 that *ppoA* levels are significantly reduced after conidiation induction, probably to
321 favor asexual development. Light receptors of the velvet complex (7, 12, 49) were
322 also down-regulated, as well as other TFs like NsdC, an activator of sexual
323 development (31) or RosA, a repressor of sexual development under carbon-
324 starvations conditions and submerged culture (62).

325 Among genes included in the group of conidiation genes, there are factors that
326 indirectly regulate asexual development or regulate the balance of asexual cycle and
327 other morphogenetic processes such as sexual reproduction, hyphal growth or
328 development (*veA*, *bemA* or *ppoA*, for example). We also found that the expression
329 of TFs from the CDP pathway of conidiation is differently regulated. While, as
330 expected, *brlA* expression is increased, *abaA* is down-regulated at this time point of
331 asexual development comparing to vegetative growth. Other transcripts coding for
332 factors supposed to act at late stages of asexual development (metulae, phialide and
333 conidia) are down-regulated at early stages (*vosA*, *cetE*, *cetL*, *yA* and *pilA*, for
334 example) while others like *phiA*, required for normal phialide and conidia
335 differentiation, are up-regulated. Recent studies proposed that conidiophore
336 development could be regulated by a discrete number of proteins that modulated
337 their levels and interactions (17). This way, those regulators would fulfill only one of
338 several possible functions during the generation of each cell-type that forms the
339 conidiophore.

340 It is noteworthy that none of the TFs belonging to the UDA pathway was found
341 within this group of significantly altered genes. These TFs are expressed both at
342 vegetative phase and early asexual development (17, 22, 23, 33), and some of them

343 show remarkable differences in expression according to Northern-blot experiments
344 (22, 23, 33). Our RNA-seq results do not correlate with those previously described
345 but support the proposed role of UDA factors at both time points of development
346 studied in this work.

347

348 **Gene Onthology analysis reveals strong alterations in oxido-reduction,**
349 **transcriptional and transmembrane transport processes.**

350 The analysis of the 1948 genes that were significantly altered and only
351 contained a systematic name revealed that the expression of 149 (7.6 %) genes was
352 increased in asexual development, while 1798 (92.3 %) genes were downregulated.
353 Their GO analyses included the prediction of the cellular localization (Cellular
354 component), the function (Biological function) and the cellular process in which they
355 may participate (Biological process). Supplementary Figure S1 shows the statistic
356 distribution of these GO analyses, while Figure 4 only focuses on the most
357 represented Biological functions.

358 Figure 4 indicates that oxidation-reduction reactions are the most represented,
359 including oxidoreductases of different types, each one requiring specific cofactors
360 (Figure 4; Supplementary Figure S1). The second and third groups include those
361 genes with a predicted transmembrane transporter activity or transcriptional
362 regulators. The modifications observed in the expression pattern may be linked to
363 changes in nutrient availability and environmental conditions that occur upon
364 induction of asexual development. The lower availability of nutrients on emergence to
365 the atmosphere likely activates autophagy to sustain the energetic requirements of
366 developmental changes (16, 66). This would involve a re-organization of carbon and
367 nitrogen metabolism (see above; (20, 21, 36, 44, 45)) and a dramatic change in
368 nutrient compartmentalization and transport mechanisms. Furthermore, the highly
369 oxidative air environment might require a higher potential for the detoxification of
370 reactive oxygen species (ROS). The idea that suggests that ROS play important
371 physiological roles was already known (4). It is tempting to suggest that these
372 changes may be controlled by a new genetic or functional relationship of
373 transcriptional regulators.

374 Among under-represented functions, the presence of a number of genes
375 predictably involved in secondary metabolic and biosynthetic pathways is noteworthy.
376 This includes polyketide synthases (PKS) and non-ribosomal Peptide Synthetases
377 (NRPS) significantly regulated during the morphological transition. The
378 corresponding results will be presented and discussed in the following section.

379

380 **Role of Secondary metabolism in asexual development signaling**

381 Secondary metabolite production is tightly linked with development in fungi,
382 both in terms of signaling as well as toxin biosynthesis (10). In our analysis, we found
383 that known genes coding for specific enzymes and TFs acting on known secondary
384 metabolite pathways are differentially regulated (Supplementary Table 3). Besides,
385 the three most represented biological functions in our GO analysis, oxidoreduction,
386 transmembrane transport and transcriptional regulation, are usually involved in the
387 control of secondary metabolite biosynthetic processes, and are grouped in
388 metabolic clusters in fungal genomes (see for example references (5, 13, 24, 35)).

389 It has been described that specific secondary metabolites are required to
390 induce conidiation in fungi (28, 34, 60). One of these metabolites has recently been
391 identified in *A. nidulans* as the meroterpenoid dehydroaustinol (50), but there are
392 additional extracellular and diffusible compounds whose structure has not been
393 elucidated yet (see for example, (18, 55)).

394 Thus, we searched for secondary metabolic enzymes within the list of genes
395 with significantly altered expression when comparing vegetative and early asexual
396 stages. Firstly, we confirmed that genes *An1594*, *An3252* and *An9314*, coding for
397 diterpene synthases (11), and genes *xptA, tdiB*, *An11080*, *An11194* and *An11202*
398 (15), coding for aromatic prenyltransferases, were absent from our list of significantly
399 regulated genes. Secondly, we searched for PKS or NRPS coding genes following
400 the work of Von Döhren, on one hand, and Nielsen and co-workers, on the other
401 hand ((15, 42); Figure 5, Supplementary Table 4). Yellow squares in Figure 5
402 designate significantly altered PKS or NRPS coding genes (Figures 5 and 6; see
403 below). Ten genes belong to this first group, 5 upregulated at the early asexual
404 phase and 5 downregulated. In this group are located the PKS coding genes

405 *An2032*/*pkhA*, related to benzaldehyde derivative biosynthesis, *An6791*, *An8910*,
406 *An9005* and *An12331* (= *An7838*), the NRPS coding genes *An2064*, *An5318*,
407 *An6236*/*sidD*, related to fusarinine type siderophore biosynthesis, and *An9129*, and
408 the hybrid PKS-NRPS coding gene *An8412/apdA*, involved in aspyridone synthesis
409 (5, 9, 15, 42, 47).

410 However, the up- or downregulation of these genes may not be associated
411 with a direct increase or decrease in the concentration of the secondary metabolite
412 linked to their activity. Different studies have shown that the transcriptional control of
413 each cluster can be exerted at different levels, through the up- or downregulation of
414 genes coding for specific oxidoreductases, transcriptional regulators or transporters
415 (5, 24, 35). Thus, we decided to study the genomic flanking regions of specific PKS
416 or NRPS coding genes in Figure 5. We included three more PKS or NRPS coding
417 genes to this analysis since, although they were not significantly regulated, defined
418 genomic regions where contiguous genes were significantly regulated (see black
419 squares in Figure 5; (5, 15): *An2035/pkhB*, located in the same metabolic cluster as
420 *An2032/pkhA* (see before), *An3230/pkfA*, involved in orsellinaldehyde derivative
421 synthesis, and the NRPS gene *An11820* (=*An9291*). Based on these criteria, we
422 focused in seven clusters in which at least three genes were significantly regulated,
423 one of them being the PKS or NRPS coding gene or not (Figure 6). The extension of
424 each cluster in Figure 6 was delimited according to previous publications or
425 tentatively determined by us using multiple *in silico* tools as blast, functional, synteny
426 and evolutionary analyses.

427 PKSs *An2032* and *An2035* define a clearly up-regulated cluster. According to
428 our synteny analyses (not shown), this cluster is not conserved in the genus
429 *Aspergillus* but maintains genes, including their position and orientation, comparing
430 to a cluster in *Metharizium robertsii*, an endophytic insect-parasitic fungus that
431 translocate nitrogen directly from insects to plants (8). This suggests that the activity
432 of this cluster is not directly required for the induction of conidiation or could be
433 required at morphological stages that occur exclusively during *A. nidulans*
434 conidiophore development but not in the rest of *Aspergillus* spp. included in the
435 synteny analysis.

436 The cluster defined by the NRPS *An6236*/*sidD* is also upregulated. Most
437 genes from this cluster maintain their position in the genome of *Aspergillus* spp.
438 included in the synteny analysis (not shown), suggesting that products related to
439 fusarinine C and tryacetylfusarinine C from *A. fumigatus* (52) are induced during *A.*
440 *nidulans* conidiation. Siderophore biosynthesis requires L-ornithine as the starting
441 product. Thus, it is tempting to speculate with the conidiation defects caused by
442 mutations in the ornithine transcarbamylase coded by *argB* (2) being related to
443 alterations in the siderophore biosynthetic pathways.

444 The three clusters defined by NRPSs *An2064*, *An5318* and *An11820* are
445 clearly downregulated, suggesting that the unknown metabolites linked to their
446 activity are preferentially required at vegetative phase. Finally, some genes (mainly
447 oxidoreductases and membrane transporters) from clusters defined by the PKSs
448 *An3230/pkfA*, involved in orsellinaldehyde derivative synthesis (5), or *An9005* are
449 upregulated while others are downregulated. This strongly suggests that the
450 availability, concentration and/or final structure of the related secondary
451 intermediates are finely tuned through complex regulatory mechanisms.

452 Overall, the results presented in this section suggest that secondary metabolism is
453 transcriptionally re-oriented during the initial stages of conidiophore development,
454 while cluster analysis reveals the existence of multiple regulatory mechanisms for
455 those metabolic pathways.

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Figure legends.

Figure 1: Morphogenetic transformations leading to conidia production: Time after the induction of conidiophore development is indicated in hours.

Figure 2: Summary of the RNA-seq data set: A) Boxplot (csBoxplot) showing the distribution of the FPKM values. B) Volcano graph showing differentially (in blue) and non-differentially (in red) expressed genes. Values >0 correspond to down-regulated genes while values <0 correspond to up-regulated genes. C) Schematic representation showing the location of significantly up- (in red) and down-regulated (in green) genes in each *Aspergillus nidulans* chromosome.

Figure 3: Cellular processes significantly regulated during the transition from vegetative hyphae (19 hours) to early stages of asexual development (5 hours after induction) in *A. nidulans*. Block size is determined by the number of previously known genes listed in Supplementary Table 3 and participating in each of these processes (Bar = 1 gene). In green are shown down-regulated genes while up-regulated genes are in red. See the complete list of genes in each process and their described or putative function in Supplementary Table 3.

Figure 4: GO functional enrichment analysis of significantly regulated genes between non-inducing and inducing conditions.

Figure 5: Schematic representation of *Aspergillus nidulans* chromosomes showing the location of genes coding for secondary metabolite producer polyketide synthases (PKSs, in blue), non-ribosomal peptide synthetases (NRPS, in orange) and dimethylallyltryptophan prenyltransferases (DMAT, in pink). Those genes significantly regulated during the morphological transition analyzed in this work are in yellow squares. In black squares are those that, being non-significantly regulated, belong to secondary metabolite gene clusters in which at least three genes are significantly regulated.

Figure 6: Expression patterns of those *A. nidulans* secondary metabolism gene clusters in which at least three genes are significantly regulated. The position of PKSs or NRPSs in each cluster is indicated as well as the first and last gene names. Non-significantly regulated genes are in black, those up-regulated in red and those down-regulated in green.

510 **Tables.**

511 **Table 1:** Upregulated top 20 genes.

512 **Table 2:** Downregulated top 20 genes.

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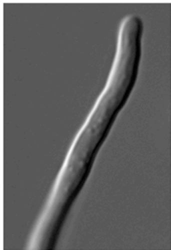
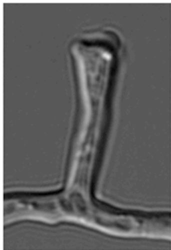
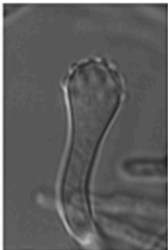
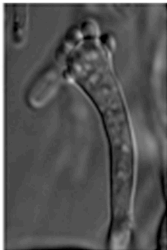
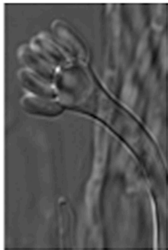
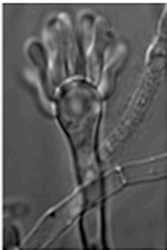
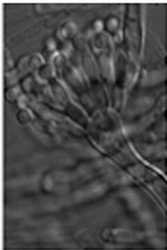
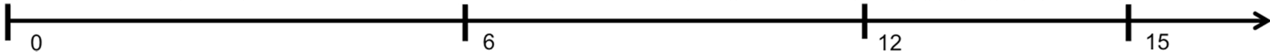
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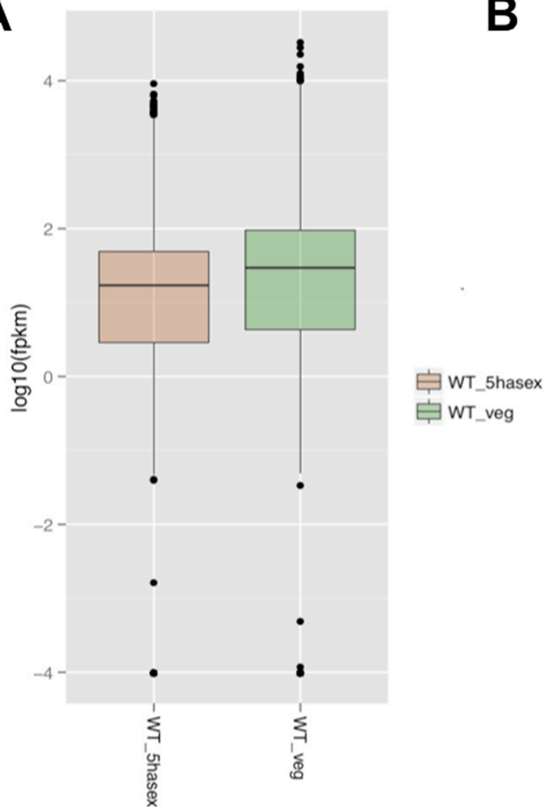
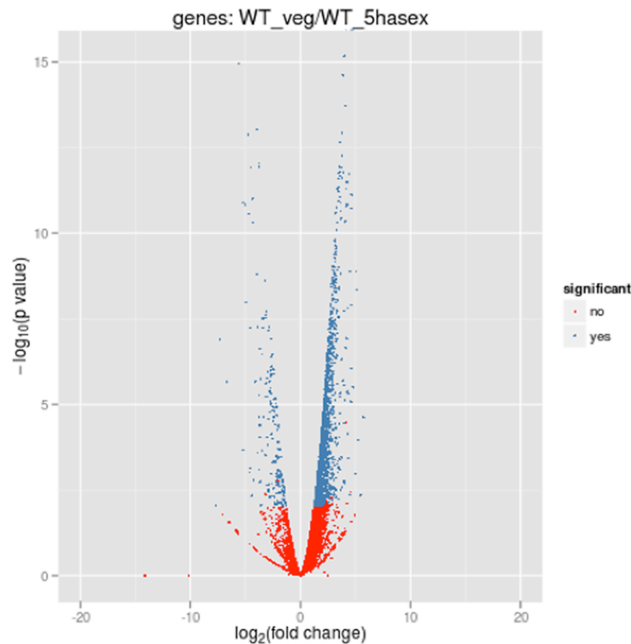
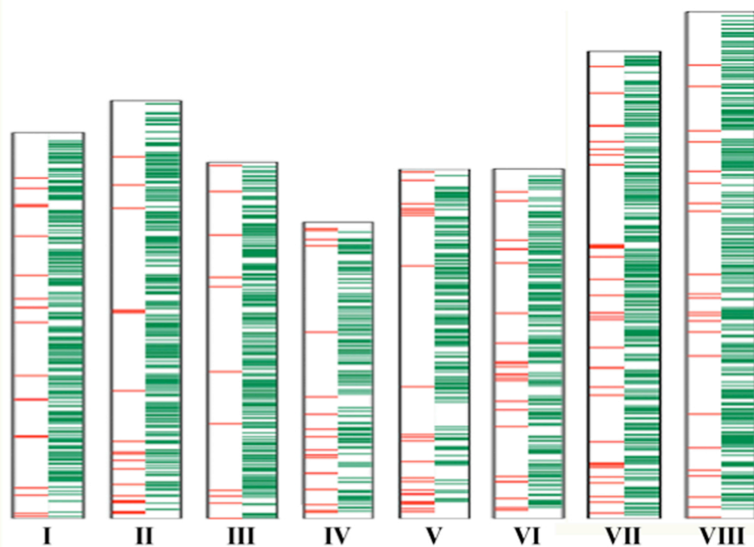
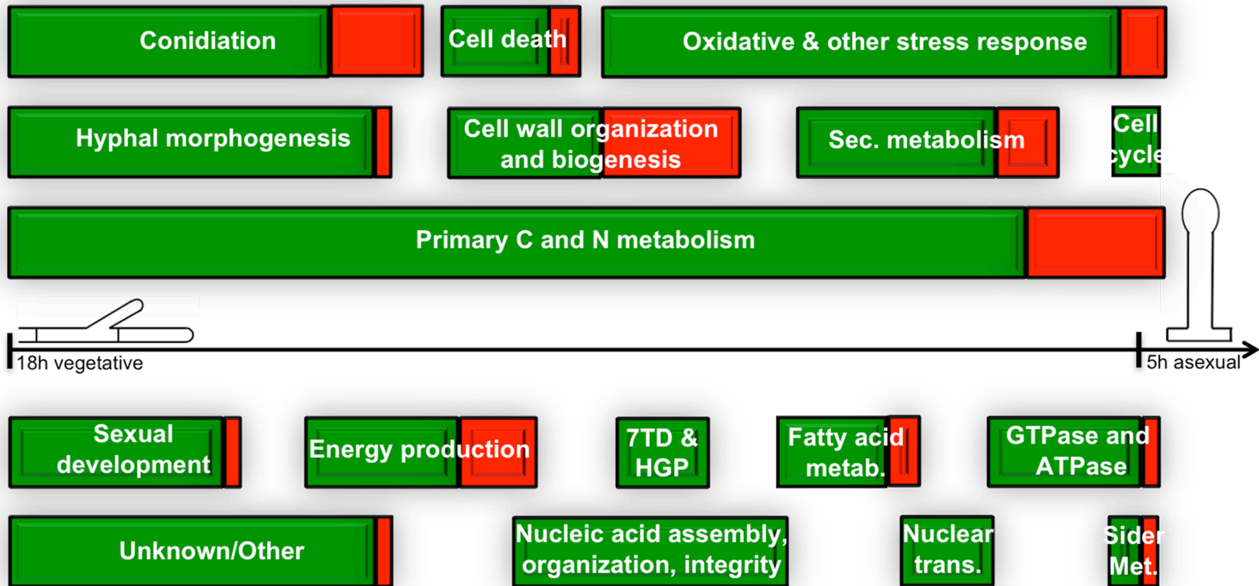
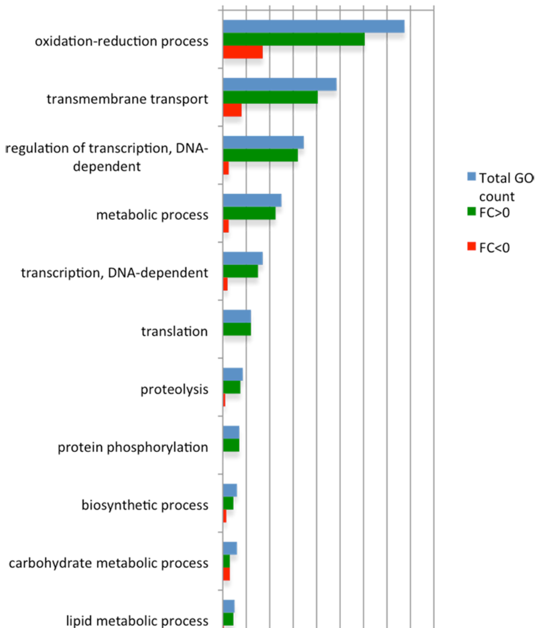


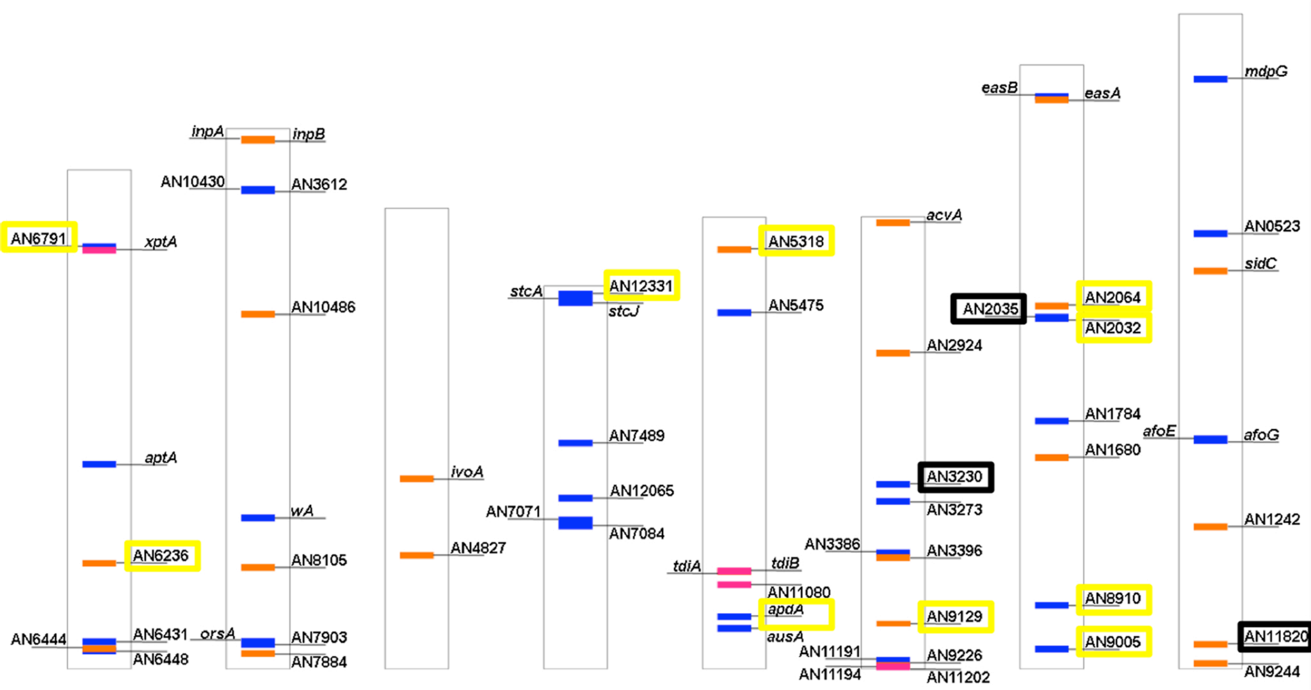




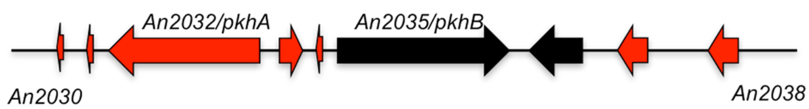
	down	up	ratio down/up
Chr I	262	23	11,4
Chr II	278	22	12,6
Chr III	271	12	22,6
Chr IV	159	19	8,4
Chr V	196	23	8,5
Chr VI	221	26	8,5
Chr VII	300	39	7,7
Chr VIII	348	23	15,1
Total	2035	187	10,9


Conidiation
Cell death
Oxidative & other stress response
Hyphal morphogenesis
Cell wall organization and biogenesis
Sec. metabolism
Cell cycle
Primary C and N metabolism
18h vegetative
5h asexual
Sexual development
Energy production
7TD & HGP
Fatty acid metab.
GTPase and ATPase
Unknown/Other
Nucleic acid assembly, organization, integrity
Nuclear trans.
Sider Met.


0
20
40
60
80
100120140160180
oxidation-reduction process
transmembrane transport
regulation of transcription, DNA-dependent
metabolic process
transcription, DNA-dependent
translation
proteolysis
protein phosphorylation
biosynthetic process
carbohydrate metabolic process
lipid metabolic process
Total GO count
FC>0
FC<0



AN6791
xptA
aptA
AN6236
AN6444
AN6431
AN6448
inpA
inpB
AN10430
AN3612
AN10486
wA
AN8105
orsA
AN7903
AN7884
ivoA
AN4827
stcA
AN12331
stcJ
AN7489
AN12065
AN7071
AN7084
AN5318
AN5475
tdiA
tdiB
AN11080
apdA
ausA
acvA
AN2924
AN3230
AN3273
AN3386
AN3396
AN9129
AN11191
AN11194
AN9226
AN11202
easB
easA
AN2035
AN2064
AN2032
AN1784
AN1680
AN8910
AN9005
mdpG
AN0523
sidC
afoE
afoG
AN1242
AN11820
AN9244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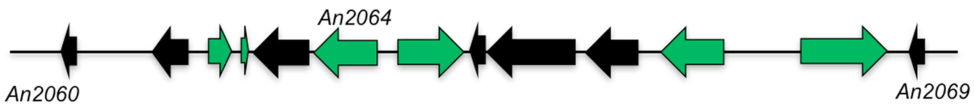
An2035/pkhB
An2030
An2038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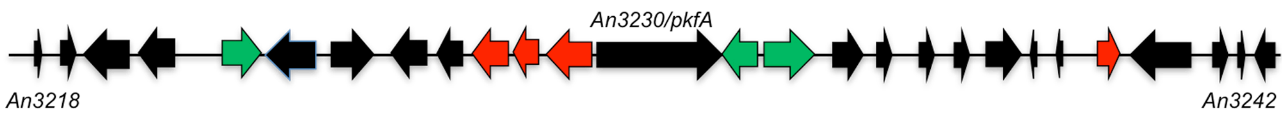
An2069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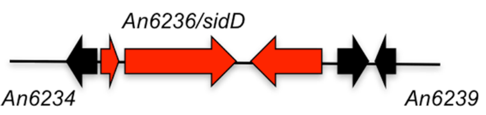
An3218
An3242

An5318
An5312
An5330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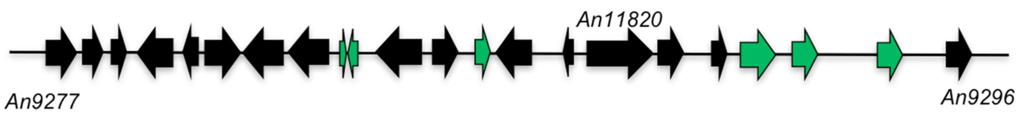
An6234
An6239

An9005
An8997
An9015


An11820
An9277
An9296

Top	gene	value WT asex	value WT veg	log2FC	Description
1	AN3227	24,1786	0,113703	-7,73232	predicted monooxygenase activity
2	AN7521	77,5662	0,482759	-7,32798	Unknown
3	AN3247	20,8309	0,199484	-6,70631	predicted ATP binding, ATPase activity
4	AN4119	404,778	8,4472	-5,58252	Putative major facilitator superfamily protein
5	gelD	341,63	9,37753	-5,18708	Putative 1,3-beta-transglycosidase
6	AN8459	20,258	0,560254	-5,17626	predicted role in transmembrane transport
7	AN6401	929,352	28,7111	-5,01655	Putative hydrophobin
8	rodA	93,0036	3,02418	-4,94267	Hydrophobin; protein involved in conidium development
9	AN6477	110,534	4,10222	-4,75194	predicted role in transmembrane transport
10	ivoB	214,457	8,3293	-4,68635	Conidiophore-specific phenol oxidase
11	AN3336	15,8021	0,632131	-4,64375	Putative enodomannanase
12	AN12331	5,72154	0,241462	-4,56653	Putative polyketide synthase (PKS)-like enzyme
13	AN2841	12,5751	0,540246	-4,54081	predicted role in transmembrane transport
14	AN8308	285,408	12,347	-4,5308	Unknown
15	AN7898	6,75941	0,312821	-4,43349	predicted role in transmembrane transport
16	phiA	188,471	9,37504	-4,32938	Protein required for normal phialide development
17	atrA	37,9445	1,91388	-4,30932	Putative plasma membrane ATP-binding cassette (ABC) transporter
18	AN7891	13,9495	0,732813	-4,25062	Putative beta-1,4-endoglucanase
19	AN5370	21,7855	1,27754	-4,09192	predicted role in transmembrane transport
20	apdA	10,4226	0,670061	-3,95928	Putative hybrid polyketide synthase-nonribosomal peptide synthase (PKS-NRPS)

Top	gene	value WT asex	value WT veg	log2FC	Description
1	AN2808	0,214378	11,3192	5,72247	Unknown
2	AN9006	0,467779	20,7339	5,47002	Unknown
3	AN4392	0,636518	23,7142	5,21941	Unknown
4	AN7200	0,606272	21,4411	5,14427	predicted role in transmembrane transport and integral to membrane localization
5	AN8779	0,189746	6,34872	5,06433	predicted hydrolase activity
6	AN12277	0,138087	4,50668	5,02842	predicted iron ion binding, nucleotide binding, oxidoreductase activity
7	AN8159	0,321225	10,4837	5,02842	predicted DDE1 transposon-related
8	AN4586	35,07	951,717	4,76222	predicted nucleic acid binding, zinc ion binding activity and intracellular localization
9	AN7954	0,78813	21,1459	4,7458	Unknown
10	AN5332	11,8009	294,354	4,64059	predicted nutrient reservoir activity
11	AN11313	5,67334	141,02	4,63556	Unknown
12	AN10039	3,54792	88,0903	4,63394	putative histidine acid phosphatase
13	AN8733	1,43304	35,0303	4,61146	predicted oxidoreductase activity
14	AN7357	29,2723	705,244	4,59052	Unknown
15	AN3341	0,351559	8,42835	4,58341	predicted chromate transmembrane transporter activity
16	AN3175	1,5925	35,8123	4,49109	predicted transferase activity
17	AN1320	0,233046	5,11311	4,45552	predicted serine-type peptidase activity and role in proteolysis
18	AN8621	5,62032	122,55	4,44657	predicted role in transmembrane transport
19	mdpA	1,12835	22,6884	4,32967	secondary metabolite regulatory protein
20	AN5505	0,32594	6,49	4,31554	Unknown