Sexual reproduction is controlled by successive transcriptomic waves in *Podospora anserina*

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Abstract

Despite the inherent challenge of finding suitable mating partners, most eukaryotes use sexual reproduction to produce offspring endowed with increase genetic diversity and fitness. The persistence of this mode of reproduction is a key question in evolutionary biology. Fungi offer valuable insights into this question, due to their diversity and short lifecycle. This study focuses on *Podospora anserina*, a pseudohomothallic ascomycete fungus that bypasses self-sterility by maintaining two compatible nuclei in one mycelium. We performed genome-wide gene expression profiling during ten stages of *P. anserina* sexual reproduction and identified five major expression patterns. Our expert annotation approaches identified differentially expressed genes related to secondary metabolite production, fungal vegetative incompatibility, programmed cell death, and epigenetic regulation. In addition, master transcriptional regulators and their target networks were uncovered. This study provides a comprehensive database for future functional genomics experiments and novel pathway characterization during sexual reproduction.

Keywords: transcriptome, time course, sexual differentiation, filamentous fungi

Running head: Transcriptome landscape during fungal sexual development

Introduction

Most eukaryotes rely on sexual reproduction to produce offspring. However, the maintenance of sex remains a fundamental question in evolutionary biology. The amazing diversity of fungi makes them of particular interest for addressing this question. Fungi are found in almost every terrestrial and marine ecosystems (Stajich et al. 2009; Breyer et Baltar 2023). Some play key roles in biogeochemical cycles, while others possess traits that enable them to be pathogenic to plants or animals (Iliev et al. 2024). Understanding the biology of fungi, and in particular why many of them can produce either asexual or meiotic products depending on environmental conditions (Dyer et Kück 2017; Wang et al. 2018), is crucial for fields as diverse as ecology, agronomy and human health.

For fungi, as for all eukaryotes, sexual reproduction has well-documented advantages. In particular, meiosis, the sex-specific division, reduces the accumulation of deleterious mutations and can link beneficial alleles that are subsequently fixed in populations by natural selection (Otto 2009). In harsh environments, sexual reproduction, as opposed to asexual reproduction, can lead to improved fitness of the offspring.

Fungal sexual reproduction relies on a wide range of developmental strategies and mating systems. These include both inbreeding, in which a single individual can self-fertilize, and obligate outbreeding, in which only genetically distinct compatible partners can mate. Some fungi, referred to as pseudohomothallic, have managed to retain the best of each strategie. Pseudohomothallism extends the diversity of reproductive systems evolved in fungi by selecting breeding systems that allow self-sterility to be bypassed by maintaining two populations of compatible nuclei in the same mycelium (Peraza-Reyes et Malagnac 2016). Early forward genetic screens and more recent transcriptomic studies in Sordariomycetes (Wang et al. 2014; Lütkenhaus et al. 2019; Kim et al. 2022) have led to the identification of large sets of genes involved in specialized developmental pathways that control the fertilization and the subsequent developmental stages (Wilson et al. 2019). Some of these species were heterothallic (self-incompatible, self-sterile (Wang et al. 2014; Lütkenhaus et al. 2019)), while others were homothallic (self-compatible, self-fertile (Teichert et al. 2012; Wang et al. 2019)). However, how the development of meiotic tissues within the fruiting body is controlled and which genes are critical in this process remains unknown.

In this study, we propose to approach this question from a different angle using the pseudohomothallic species *Podospora anserina*. This species is used as a model system for the study of the fruiting body development (Peraza-Reyes et Malagnac 2016) but a detailed analysis

79 of the cellular programs allowing its formation from fertilization to ascospore discharge was
80 lacking. In this context, we performed a genome-wide gene expression profiling during the
81 sexual reproduction of *P. anserina* at ten time points representing key developmental steps. To
82 characterize the topology of differential gene expression profiles across sexual reproduction,
83 we analyzed our transcriptomic data using two complementary approaches: *i)* a data-driven
84 approach using k-means clustering and *ii)* a biological expertise-driven approach to mine the
85 gene functions. The data-driven approach revealed five major patterns (waves) of differential
86 expression during sexual development. The expert annotation approach has led to the
87 identification of a number of interesting gene categories, including secondary metabolites and
88 toxins encoding genes (Wang et al. 2022), genes responsible for fungal vegetative
89 incompatibility programmed cell death (Chou, Hsu, et Leu 2022), and gene related to epigenetic
90 effectors that interfere with messenger stability (RNAi/MSUD genes, (Gladyshev 2017)).
91 Finally, we identified several master transcriptional regulators and draw their potential target
92 gene network. Overall, our study has produced a comprehensive database that offers numerous
93 candidates for functional genomics experiments. This database also paves the way for the
94 characterization of new pathways during sexual reproduction.

95 Materials and methods

96 *Podospora anserina* strains and methods

97 *P. anserina* is a filamentous ascomycete whose life cycle and general methods have
98 already been described (Esser, 1974; Rizet and Engelmann, 1949; Zickler et al., 1995). All
99 strains used in this study were derived from the *S* strain. Growth and crosses of *S mat+* and *S*
100 *mat-* strains were carried out on a minimal synthetic medium (M2 medium) at 27°C with
101 constant light.

102 Cytology

103 Sexual cells were either fixed in 7.4% paraformaldehyde and processed for microscopy
104 as previously described (Thompson-Coffe and Zickler, 1994) or directly observed in M2
105 medium. When required, nuclei were stained with DAPI (4',6-diamidino-2-phenylindole, 1
106 mg/mL; Boehringer Ingelheim). Observations were performed with a Zeiss Axioplan
107 microscope, and images captured with a CDD Princeton camera system.

108 Time course procedure and RNA isolation

109 To facilitate biological materials collecting, *S* strains from both mating types were
110 grown on cheesecloth (Sefar Nitex 03-48/31 Heiden) or cellophane sheet (cat#1650193,
111 BioRad laboratories, Hercules) previously placed on Petri dishes containing M2 medium. *S*
112 *mat*- strain has been used as male gamete donor and *S mat*+ as female. The *S mat*+ strains were
113 grown on M2 medium plus cheesecloth except the T-24h (on cellophane sheet). For each ten
114 points of the time course, the number of Petri dishes has been adjusted (five were used for T-
115 24h, T0h, T6h, T18h, T24h, four for T30h, two for T42h, one for T54h and T96h) to collect 20
116 to 100 mg of fertilization competent mycelium or/and perithecia. One to five Petri dishes *per*
117 time course experiment were thus inoculated simultaneously with a growing *S mat*- strain and
118 spermatia were collected by washing with 1.5 ml of H_2O six days later. The collected spermatia
119 (around 10^4 spermatia per ml) were pooled and then used to fertilize a corresponding number
120 of Petri dishes of 4-day-old *S mat*+ strain. The moment just prior the fertilization process was
121 considered as the T0 (0 hours). The biological material was then collected 6, 12, 18, 24, 30, 42,
122 54 and 96 hours after fertilization and an additional time point 24 hours prior fertilization was
123 taken. Three biological replicates issued from three independent time course have been
124 collected for all time points and two for T42. The RNAs pool used as a reference was assembled
125 by combining equal amount of RNAs from various growth and crosses conditions and has
126 already been described (Bidard *et al*., 2010).

127 The biological material collected by scraping gently either cellophane or cheesecloth
128 with a cover glass, was dried out with filter paper, weighted, frozen in liquid nitrogen and stored
129 at –80°C. Total RNAs of *P. anserina* were extracted using RNeasy Plant Mini Kit (Qiagen),
130 including, a grinding process using a Mikro-Dismembrator (Sartorius) and a DNase treatment.
131 The quality and quantity of the total RNAs was determined by using a Nanodrop
132 spectrophotometer (Nanodrop Technologies) and the Bioanalyzer 2100 system (Agilent
133 Technologies) as previously described (Imbeaud et al., 2005 (Bidard et al. 2010; Bidard et al.
134 2011)).

135 **Gene expression array-based hybridization**

136 Transcriptome microarray experimental procedure, *i.e.* targets preparation,
137 hybridization and washing, was done following the two-color microarray-based gene
138 expression analysis instructions (version 5.0, February 2007) as described by the manufacturer
139 (Agilent Technologies). One-µg aliquots of total RNA were labelled using the Low RNA input
140 fluorescent linear amplification (LRILAK) PLUS kit (Agilent Technologies); internal standards
141 came from the Two-color RNA spike kit (Agilent technologies). The labelling efficiency and

142 the product integrity were checked as described by (Graudens et al., 2006). The reference cRNA
143 was labelled with Cy5 and sample probes with Cy3. Then, Cy3- and Cy5-labeled targets were
144 mixed and incubated on an Agilent microarray slide for 17 hours at 65°C, in a rotating oven (6
145 rpm for a 1x44K and 10 rpm for a 4x44K array format), using an Agilent *in situ* hybridization
146 kit. The slides were washed and then any traces of water were removed by centrifugation at 800
147 rpm for 1 min (Bidard et al. 2010; Bidard et al. 2011).

148 **Data acquisition and analysis**

149 Microarrays were scanned using an Agilent dual laser DNA microarray scanner (Agilent
150 Technologies), model G2567AA, with 5-µm resolution. The resulting 16-bit image files were
151 analyzed in the *Feature Extraction* processing system (FE, v9.5.3). Spot and background
152 intensities were extracted and normalized with the Feature Extraction software using the GE2-
153 v4_95_Feb07 default protocol (Local background subtracted and LOWESS normalization).
154 Preliminary array quality was assessed using Agilent control features as well as spike-in
155 controls (Agilent 2-Color Spike-in Kit for RNA experiment). Subsequent flagging was done
156 according to the GenePix Pro software (Molecular Devices Syunnyvale, CA, USA)
157 nomenclature, including four levels of flags (good (100), bad (-100), not found (-50), moderate
158 (0)) and raw data from the FE software ((Bidard et al. 2010; Bidard et al. 2011)).

159 The statistical analysis was conducted using the R environment and the MAANOVA
160 (MicroArray ANalysis Of Variance) package (version 1.14.0) (Wu et al., 2003). It allows fitting
161 an ANOVA model as proposed by Kerr et al (2001) and provides a way to consider multiple
162 sources of variation in the microarray experiment. Technical biases were then corrected by
163 including specific terms in the ANOVA model.

164 The differential analysis was performed on the whole of the replicated probes by excluding
165 control spots. A F-test based on the James-Stein approach using a weighted combination of
166 global and gene-specific variance estimates was used (Cui et al., 2003). P-values were
167 computed by permutation analysis.

168 All p-values obtained were adjusted by the false discovery rate step-up method of
169 Benjamini and Hochberg to account for multiple testing (Benjamini and Hochberg, 1995).
170 Differentially expressed genes were defined as those whose adjusted p-value was statistically
171 significant at a level of $p<0.05$, with a fold change ≥ 2 or ≤ -2 and a Amean ≥ 6 or a fold change
172 ≥ 4 or ≤ -4 and a Amean ≥ 4.5

173 **Classification**

174 Genes significantly differentially expressed in at least one of the comparisons and presenting a
175 maximum value minus a minimal value >2.5 after a median center transformation were
176 classified (1798 genes). To this list, the mating-type transcription factor SMR1, SMR2 and
177 FMR1 were added. Clusters analysis was computed from expression intensity measurements
178 with the correlation coefficient as similarity measure. A hierarchical clustering (UPGMA
179 method) and a K-means clustering to 15 classes were computed independently using Spotfire
180 DecisionSite software package (Spotfire, Somerville, Mass.) with correlation coefficient as
181 similarity measure on the 1798 regulated genes. Intersection of clusters obtained by the two
182 clustering method results in 13 robust clusters with 1197 CDS classified. All microarray data
183 is MIAME compliant. The raw data has been deposited in the MIAME compliant Gene
184 Expression Omnibus database (Edgar et al. 2002) and is accessible through the GEO Series
185 accession number GSE21659
186 https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE93094.

187 **Ortholog analysis**

188 Orthologous genes were searched between *P. anserina* and the other species *N. crassa*,
189 *S. macrospora*, *C. globosom* and *T. reesei*. For this purpose, protein sequences were
190 downloaded from Uniprot (https://www.uniprot.org/) under the accession numbers:
191 UP000001805_367110 (*N. crassa*), UP000001881_771870 (*S. macrospora*),
192 UP000001056_306901 (*C. globosum*) and UP000008984_431241 (*T. reesei*). The OrthoFinder
193 algorithm (Emms et Kelly 2019) was used with default parameters to obtain "orthogroups"
194 (Table S3), *i.e.* groups of genes that are good candidates to share a common ancestor between
195 the compared species.

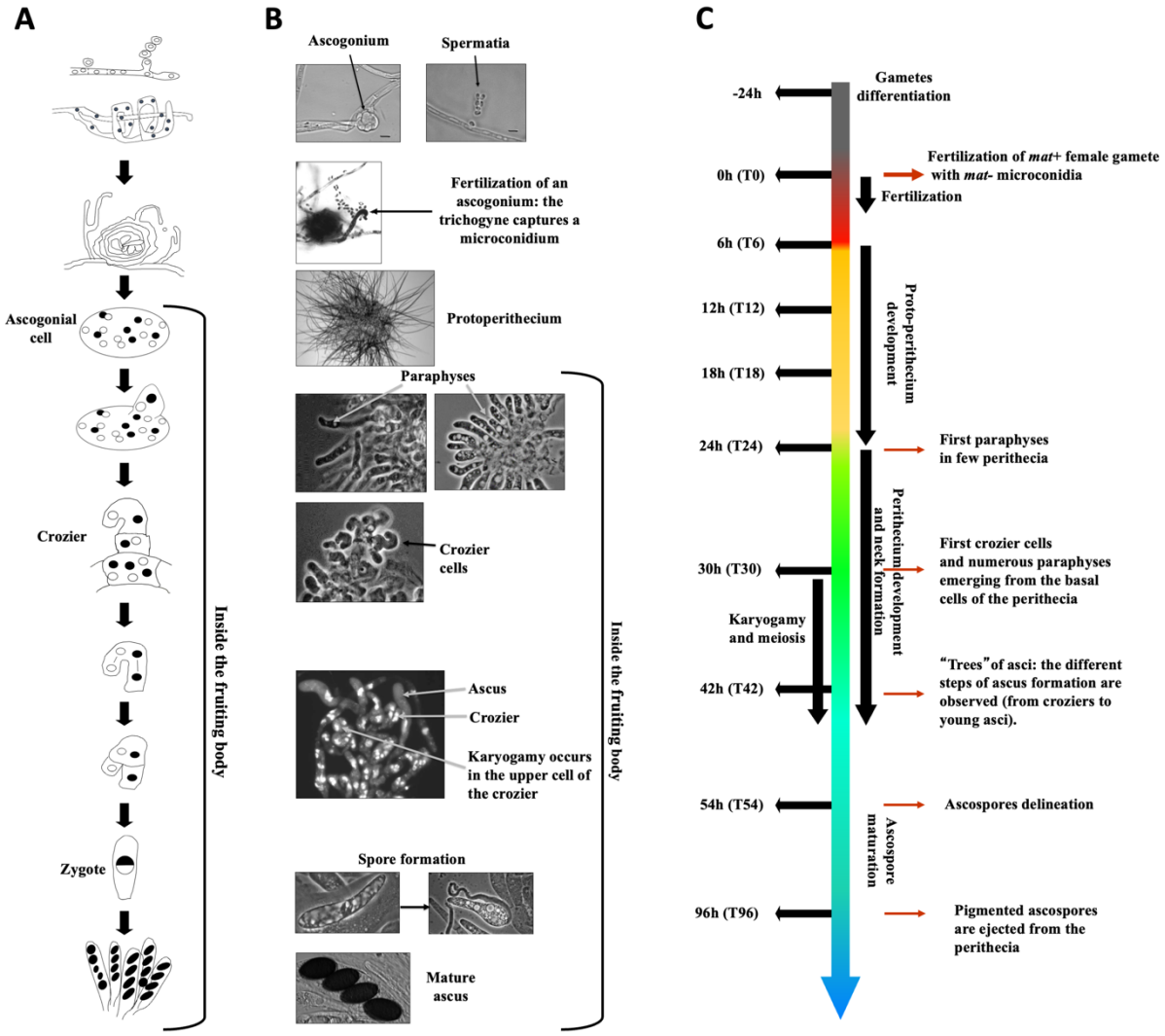
196 **Transcription factors network**

197 To search among all the transcriptional regulators for those that could be involved in
198 setting up the observed transcriptional waves, we listed the candidate genes that encode
199 transcription factors (TFs). This list includes 92 genes (table S7). Gene expression profiles for
200 each of the TF genes were then retrieved from the microarray datasets and used to search for
201 significant correlations with the expression profiles of all genes previously classified in
202 transcriptional waves I, II, III, IV or V. We then considered the situations, in which the
203 calculated correlation between the expression profiles of a TF gene and a gene classified in a
204 transcriptional wave was above a predefined threshold (here 0.95). Our assumption is that such
205 a high correlation value may reveal a potential link of transcriptional control, between the TF
206 and another gene (referred to as "target gene").

207 # Results

208 ## High-resolution kinetics of transcription fluctuations during sexual reproduction

209 To ensure an accurate, high-density representation of transcriptional variation, we
210 collected RNA samples at ten key time points during the sexual reproductive cycle of *P.*
211 *anserina* (Fig. 1).



212

213 **Figure 1 - *P. anserina* developmental time course from fertilization to ascospore**
214 **maturation. A.** Schematic representation of the fertilization and the main steps of
215 zygotic development. **A.** Pictures of the main steps of sexual development. Sexual
216 reproduction began with the production of male (spermatium) and female
217 (ascogonium) gametes. A pheromone/receptor signaling system allows the female
218 gametes to attract specialized hyphae (trichogynes) that recognize and fuse with male
219 gametes of compatible mating type. This event initiates the multi-step process of
220 sexual development that begins with the formation of fructifications and ends with
221 the discharge of ascospores. The nucleus of the male gamete enters the trichogyne,

222 migrates along this specialized hypha, reaches the ascogonium and undergoes
223 several mitotic divisions. Fertilization (T0) triggers the development of
224 protoperithecia (T6 to T24), which recruit multiple layers of protective maternal
225 hyphae to grow into perithecia (T24 to T45). The developing fructifications shelter
226 the fertilized ascogonial cells (T24 to T30), which contain multiple haploid parental
227 nuclei of either *mat*+ or *mat*- genotype. Differentiation of paraphyses begins (T24)
228 just before three-celled hook-shaped structures called croziers emerge from the
229 ascogonial cells (T30 to T42). Croziers are dikaryotic because they contain two
230 haploid nuclei derived from both parents. Karyogamy (T30 to T44) occurs in the
231 upper cells of croziers. The resulting diploid zygote immediately undergoes meiosis
232 (T30 to T44), producing four haploid nuclei, which then undergo mitosis prior to
233 ascospore delineation (T54 to T96). Ascospores are formed around two non-sister
234 nuclei within the developing asci. In rare cases, two ascospores form around a single
235 haploid nucleus, resulting in five-spored asci. **C.** Time line of the sexual cycle of *P.*
236 *anserina* with the steps from which RNAs have been extracted on the left.
237

238 *P. anserina* is a pseudohomothallic fungus, namely natural isolates are self-fertile
239 because they contain nuclei of each mating-type. Self-fertilization does not start at a specific
240 time point and therefore natural isolates cannot be used for a time course analysis of sexual
241 development. However, the genetic basis of the *P. anserina* mating system is heterothallism
242 with two mating-type idiomorphs called *mat*+ and *mat*- (Turgeon et Debuchy 2007). Typical
243 ascospores contain *mat*+ and *mat*- nuclei, but a few asci contain monokaryotic ascospores
244 which produce self-sterile individuals. Those latter individuals were used in the experiments
245 presented here. Fertilization was performed by spreading *mat*- male gametes on a *mat*+ strain.
246 This experimental design allows the time course to start at a well determined time point for
247 synchronization purpose. Under this experimental condition almost all fertilization events
248 occurred within the first six hours (Fig. S1A and S1B). However, despite this experimental
249 design, RNA samples were extracted from biologically non-homogeneous tissues because *P.*
250 *anserina* fructifications are composed of different cell types (Fig. 1A and 1B).
251 We measured the expression of 10,507 nuclear genes at ten time points which are
252 designated as Tm24 for mycelium 24 h before fertilization and T0, T6, T12, T18, T24, T30,
253 T42, T54 and T96 for time 0 to 96 h postfertilization, respectively (Fig. 1C, Table S1). We
254 performed a Principal Component Analysis (PCA) on the complete dataset. More than 75% of
255 the total variance was captured by only three principal components (PCs, Fig. 2A and 2B, Table
256 S2).

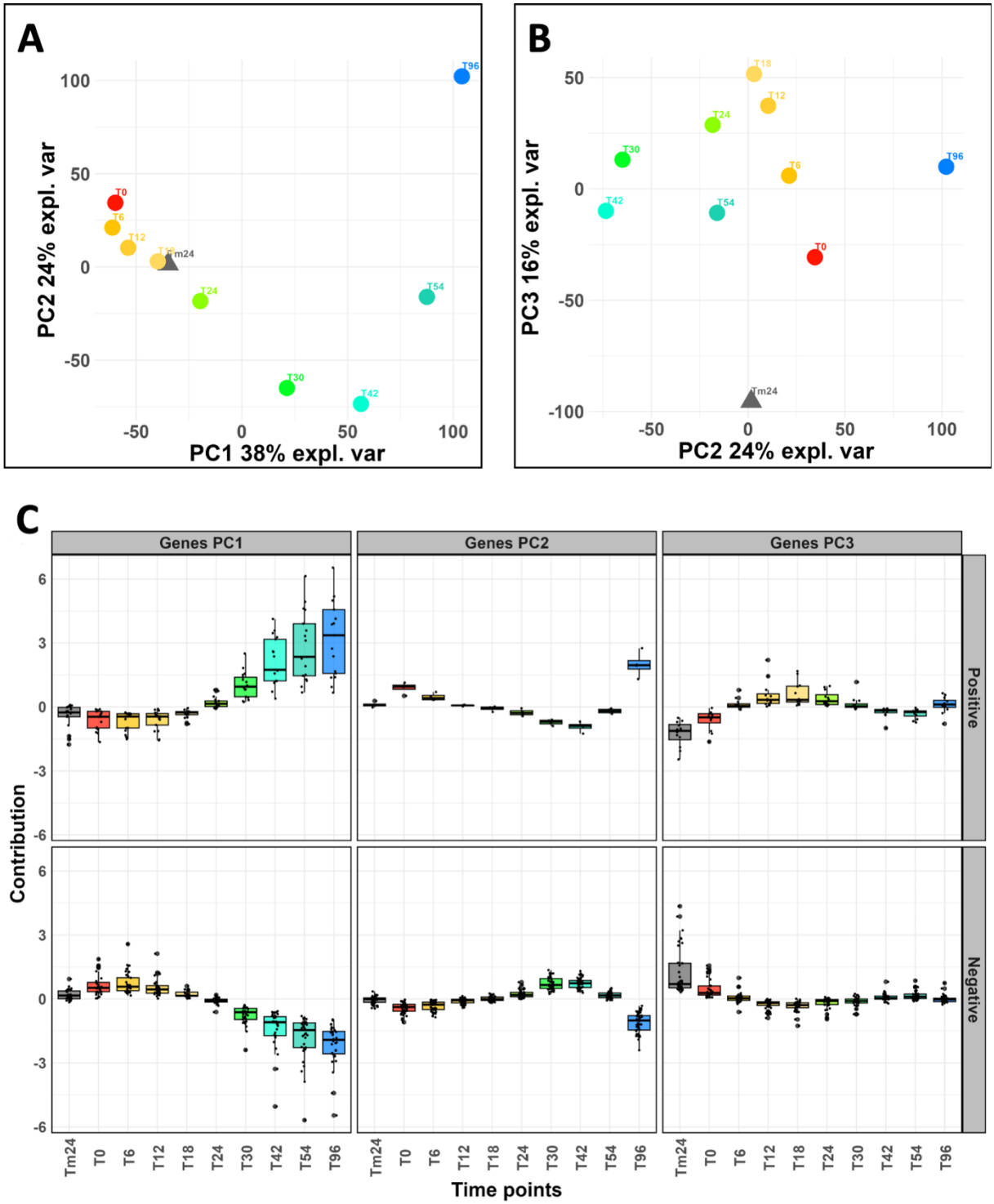


Figure 2 - PCA analysis reveals a strong temporal effect on gene expression variations relevant to the key developmental steps in *P. anserina*

Principal Component Analysis (PCA) on the complete dataset (*i.e.* all genes and all time points). **A.** Axis 1 and 2 of the PCA explain 38% + 24 % = 62% of the variance. **B.** Axis 2 and 3 of the PCA explain 24% + 16 % = 40 % of the variance. Altogether, these first three axes of the PCA explain 78 % of the variance. **C.** Gene expression profiles for the 50 genes, which contribute the most to the definition of the first

265 (PCA1), second (PCA2) and third (PCA3) principal components, as shown in A and
266 B. Detailed lists of these genes are given in Table S2. They were separated according
267 to the type of regulation they experience during sexual development (positive or
268 negative). Tm24, T0, T6, T12, T18, T24, T30, T42, T54 and T96 : time points of the
269 kinetic were the biological samples were collected, described in Fig. 1.
270

271 With the exception of Tm24, we observed that all samples were distributed along the PC1 axis
272 (38%) according to the sexual development time line (Fig. 2A). On PC2 axis, we found T30
273 and T42 samples clustered apart from the others (24%). Interestingly, this period corresponds
274 to the formation of the dikaryotic cells, which are the progenitors of fungal zygotic lineage (Fig.
275 1). The peripheral position of the Tm24 sample (Fig. 2B) which appeared on the PC3 axis
276 (16%), indicated that the gene expression profiles derived from vegetative mycelium (Tm24)
277 were clearly different from those of all samples collected after fertilization. Overall, this
278 unsupervised exploratory data analysis validated both the relevance of our experimental design
279 and the robustness of our differential analysis (see below). The expression profiles for the 50
280 genes whose expression measurements contributed the most to the calculation of the principal
281 components 1, 2 and 3 are shown Figure 2C (and detailed Table S2). The data indicated that
282 the greatest fold changes, both positive and negative, occurred after the formation of dikaryotic
283 cells (>T30). This was also the case for axis 2, but only after the formation of the ascospores
284 (>T54). We observed that the positive fold change values on axis 1 exhibited a higher dispersion
285 after T42.

286 **Topology of global expression variation reveals five major transcriptional waves during**
287 **sexual development**

288 Using k-means clustering, we defined 13 representative expression patterns (Fig. S2),
289 encompassing 1,186 genes. Further data outlined five successive transcriptional waves (Fig. 3
290 and Table S1). Notably, they all shared a common pivot point (Fig. S3), corresponding to the
291 mid-course of the kinetic (T30-T42). This observation indicated a pause in the regulation of
292 transcriptional activity at the time of dikaryon formation. The 1,186 genes were not evenly
293 distributed across the five waves (Fig. 3 and Table S1), resulting in two periods of intense and
294 equal transcriptional fluctuation (waves II and IV) interspersed with three periods of moderate
295 transcriptional fluctuation (waves I, III, and V).

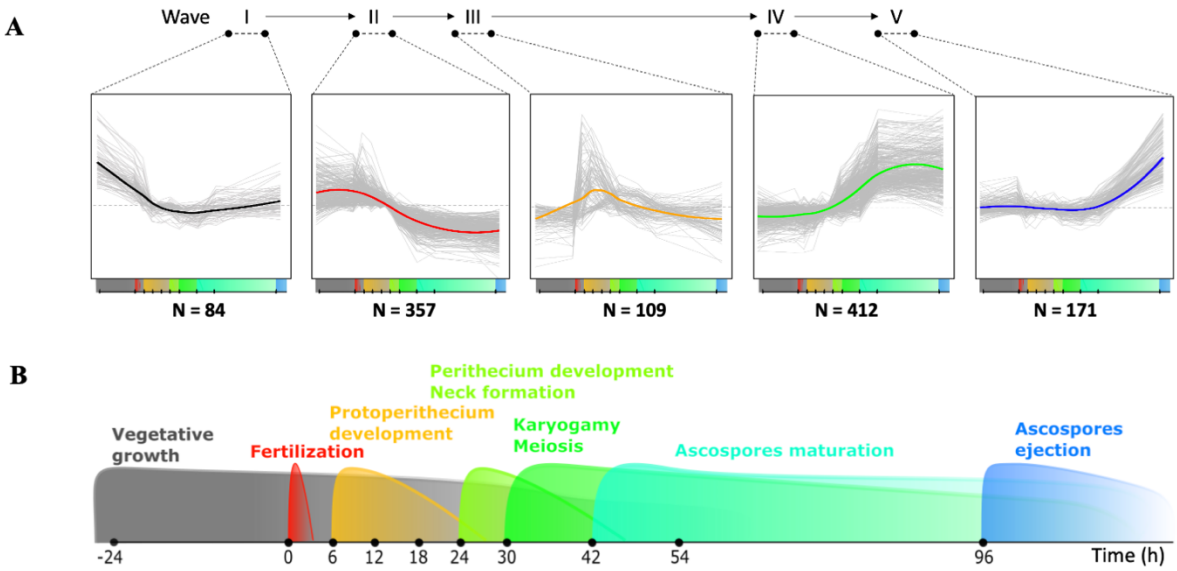


Figure 3 - Temporal transcriptional waves over the course of sexual development

A. Expression profiles of the five major successive transcriptional waves (numbered in Roman figures) that take place during *P. anserina* sexual reproduction (Bottom timeline, see panel B for zoom). Number of DE genes present in each wave is indicated below the corresponding graph. Waves II (early up/late down pattern, 31.51%) and IV (early down/late up pattern, 36.37%) accounted for 67.87% of them (33.98% and 37.42%, respectively), while wave I (early down pattern, 7.41%), wave III (early-mid up pattern, 9.62%) and wave V (late up pattern, 15.02%) contained only 32.13% of them, respectively.

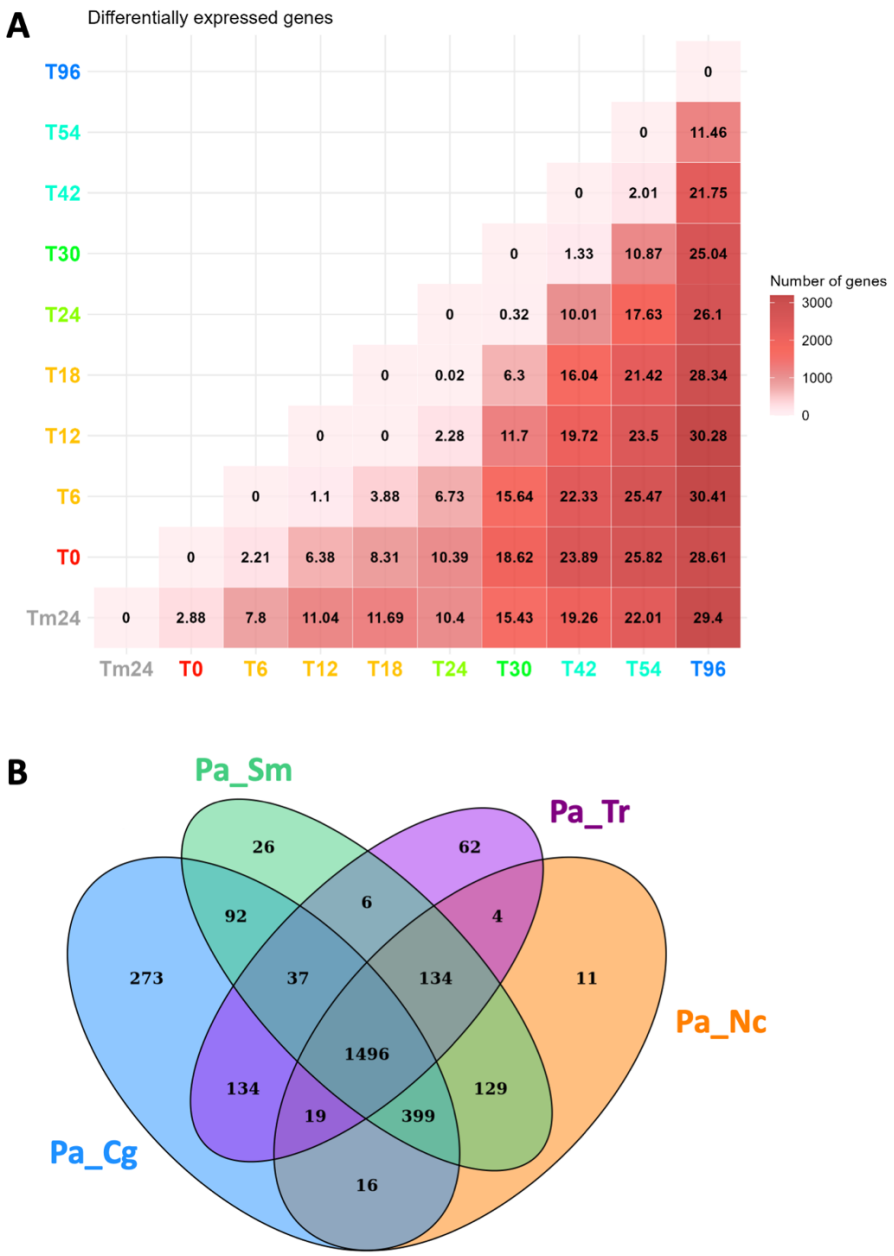
B. Major developmental events according to the sexual reproduction timeline described in Figure 1.

The topology of the transcriptional waves therefore suggests that the genes of waves II and III are down-regulated after a peak of expression during protoperithecium development, while the genes of waves IV and V are up-regulated after perithecium development. These patterns are in accordance with the results obtained in *Neurospora crassa*, during fruiting body development (Wang et al. 2014).

Evolutive conservation and functional annotation of differentially expressed genes during sexual reproduction

Across the entire kinetic, we found 3,466 genes that were differentially expressed (DE, see Methods). They represent 33% of the total gene set for which gene expression measurements were monitored (Table S1, Fig. 4A), including 1,133 of the genes clustered in

the five transcriptional waves (more than 95% of the clustered genes, Table S1 and Fig. 3). Overall, this analysis revealed that in *P. anserina*, one-third of the complete gene set is transcriptionally regulated during sexual reproduction. Of this, one-third follows one of the five transcriptional waves disclosed by the clustering analysis. To test for evolutive conservation of these DE genes, we built group of orthologs, using four species of filamentous fungi, *i.e.* the homothallic species *Chaetomium globosum* and *Sordaria macrospora* and the heterothallic species *N. crassa* and *Trichoderma reesei*. A total of 2,957 DE genes (more than 85%) were identified as belonging to an ortholog group shared with at least one of the four species (Table S3). A core set of 1,496 DE genes is conserved in all four fungi (Fig. 4, Table S3).



330 **Figure 4 - Distribution of differentially expressed genes along the kinetics of**
331 **sexual reproduction and their evolutive conservation**
332 **A.** Heatmap of pairwise comparisons of DE genes across the complete kinetics. For
333 each pairwise comparison, the number of DE genes is expressed as a percentage
334 relative to the total number of DE genes (3,466) and also as a color intensity given
335 by the color key bar (right).
336 **B.** Venn diagram showing the conserved orthologs between the indicated fungal
337 species. Cg: *C. globosum* (homothallic), Sm: *S. macrospora* (homothallic), Nc: *N.*
338 *crassa* (heterothallic) and Tr: *T. reesei* (heterothallic).
339
340 A pairwise comparison of the DE gene set of *P. anserina* with each gene set of the four
341 fungi, showed that the number of conserved DE genes is consistent with fungal evolutionary
342 history (*i.e.* closely related fungi share more DE genes than distantly related fungi). The
343 remaining 506 genes (14.78%) were classified as orphan genes, with 247 (21.84%) belonging
344 to the five transcriptional waves. Notably, the set of DE genes, including that of the five waves,
345 were found to be enriched with orphan genes (p-values: 2.55×10^{-9} and 4.60×10^{-20} respectively).
346 We then search for functional annotation of the proteins encoded by the DE genes. We
347 found that 1,279 (36.95%) of the 3,466 putative proteins encoded by the DE genes lacked Pfam
348 annotation (Table S1, (Mistry et al. 2021)), which is significantly more (p-value: 8.55×10^{-9})
349 than in the complete list of *P. anserina*'s proteins. The lack of annotated conserved domains
350 was further enriched (p-value: 1.48×10^{-13}) in wave-embedded DE genes, as 515 out of these
351 1,133 putative proteins (45.44%) lacked Pfam annotation. We also performed Gene Ontology
352 (GO, (Ashburner et al. 2000; The Gene Ontology Consortium et al. 2023)) analysis on the DE
353 genes (Table S4, Fig. 5A).

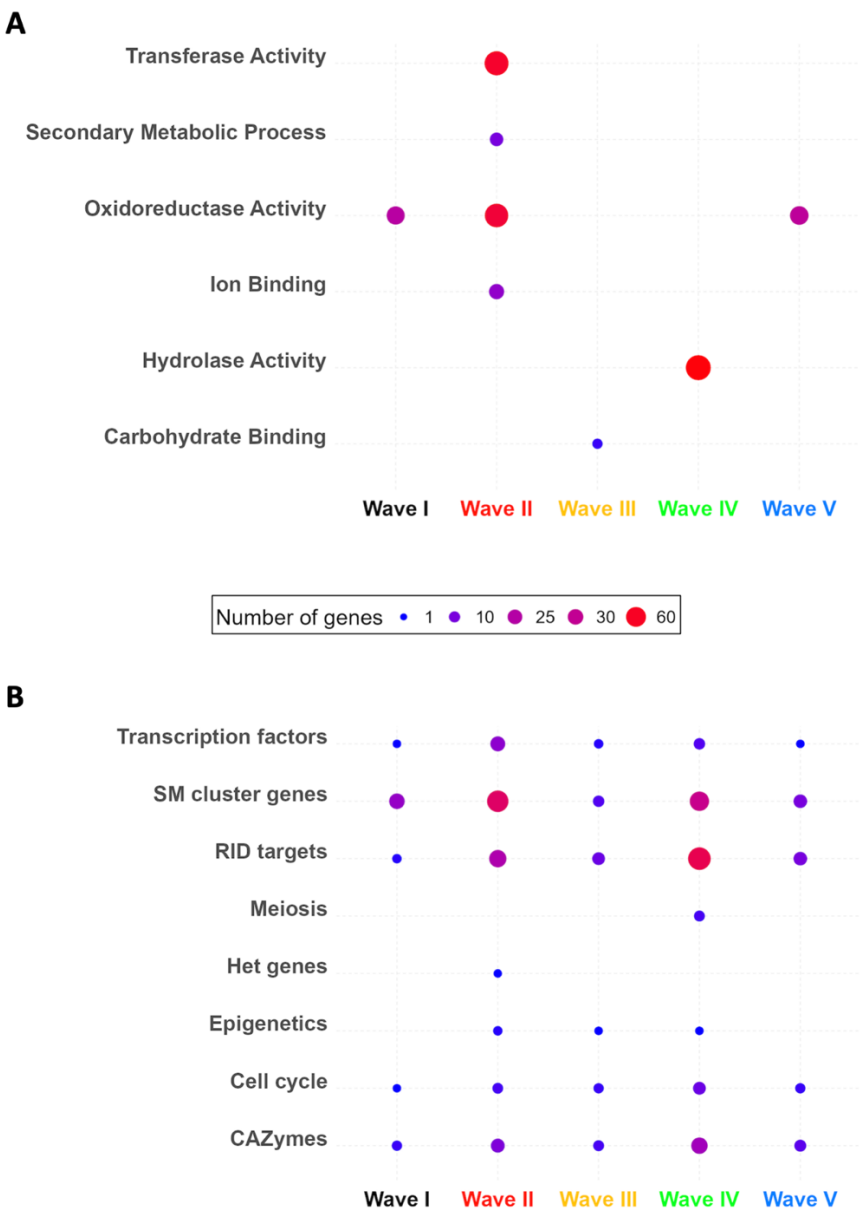


Figure 5 - GO term and expert annotation categories enriched in the transcriptional waves.

A. Descriptions of GO terms significantly enriched (p-value ≤ 0.05) in at least one wave of the transcriptional waves. **B.** Enrichments of genes of interest in at least one wave of the transcriptional waves. TF: transcription factors, SM: secondary metabolites, HET: fungal specific vegetative incompatibility HET domain. Points are scaled and colored according to the number of genes in each category for each wave. No score is shown for zero genes.

In general, the enriched GO terms and Pfam domains (Table S4 and S5) described intense catalytic (GO:0003824) and metabolic (GO:0008152) activities, associated with binding (GO:0005488, PF00187). Accordingly, we observed enrichments in genes involved in

367 redox catalytic activities (GO:0003824, PF00067, PF03055, PF01565, PF00106, PF01494,
368 PF00199, PF08240) and transporter activities (GO:0005215, Pfam PF07690.19). We also
369 uncovered enrichments in DE genes involved in cellular differentiation programs more
370 specifically related to sexual reproduction. They either deal with cellular anatomical entity
371 (GO:0110165), localization (GO:0051179) or encode zinc-binding domain transcription factors
372 (PF13695), necrosis inducing factor (PF14856) and TUDOR domain containing protein
373 (PF11160). However, if the repertoire of GO terms (Table S4) and Pfam domains (Table S1
374 and S5) generated by this study should provide some clues to select candidate genes for *in vivo*
375 functional characterization, our analysis essentially showed that more than one third of the DE
376 genes have no associated function yet.

377 **Searching for gene of interest, the expertise-driven approach**

378 We then performed a biological expertise-driven approach to mine the functions of the
379 full set of DE genes (Table S6, Fig. 5B).

380 **Developmental processes at work**

381 Fertilization of *P. anserina* requires exposure to blue light, but the molecular basis of
382 this signal transduction is still unknown. In *N. crassa*, the blue light signal is transduced by the
383 photoreceptors WC-1 and WC-2 (Froehlich et al. 2002; He et al. 2002). In this study, we found
384 five blue light responsive genes that were differentially expressed (Table S6A, Fig. S4A-B).
385 Consistent with a role in fertilization three of them (including *Pa_ 5_6130/WC-1*) were
386 expressed before meiosis, while the other two were expressed after meiosis, suggesting that
387 blue light may also play a role during ascospore formation.

388 Orthologs of genes involved in plasmogamy in yeast models (*Pa_3_11320/Prm1* (Curto
389 et al. 2014), *Pa_3_4770/CDC3* (Kurahashi, Imai, et Yamamoto 2002), *Pa_5_9510/Fig1*
390 (Erdman et al. 1998), *Pa_1_1930/KEX2* (Julius et al. 1984), *Pa_4_6420/KAR2* (Rose, Misra,
391 et Vogel 1989), *Pa_7_10810/SEC63* (Young et al. 2001) were found to be up-regulated until
392 T42 and then downregulated (Table S56, Fig. S4C). Development of the zygotic lineage likely
393 involves compartmentalization through active cell wall remodeling, as we found several DE
394 genes encoding different types of cell wall proteins and growth factor receptors (Table S5). One
395 of them was the expansin-like EEL2, involved in cell wall expansion, whose gene *Pa_2_310*
396 was downregulated from karyogamy to ascospore maturation (T30-T96). This expression
397 pattern was similar to that of the C. *globosum* homolog (*CHGG_00523*) but divergent from that
398 of the *N. crassa* homolog (*NCU04603*) (Hutchison et Glass 2010; Wang et al. 2019). Cell-cell
399 interactions may also be tightly regulated, as exemplified by the strict downregulation (T0-T96)
400 of *Pa_1_17480,* which encodes the adhesin protein MAD1 (Wang et St Leger 2007).

401 Since sexual development is rich in cell divisions, we searched for DE cell cycle related
402 genes and found this category slightly enriched (p-value = 2.9×10^{-2}, Table S5B). In addition to
403 the TOR pathway inhibitor *Pa_3_3700/FgFkbp12*, it included homologs of genes required for
404 proper fructification formation (*Pa_1_17170/KIN3*, *Pa_1_23300/cel-2*, *Pa_2_6460/Pah1*,
405 *Pa_2_890/asm-1*), axial budding (*Pa_2_290/axl2*) and genes encoding G-coupled receptors
406 (*Pa_4_5350*, *Pa_1_590*) and ras-like protein (*Pa_1_19030/krev-1*). The expression profile of
407 the *Pa_2_290/axl2* gene, encoding an integral plasma membrane protein, was of particular
408 interest: it was down-regulated when the two haploid parental nuclei actively divide into
409 multinucleated syncytial ascogonial cells (Fig. S4D).
410 As expected, we also found DE genes encoding cytoskeletal elements (Fig. S4E).
411 Interestingly, *Pa_5_5720*, encoding a pyruvate decarboxylase (PDC) was found strongly up-
412 regulated during ascospore maturation (wave V). This observation further enforces the
413 hypothesis that pyruvate decarboxylase is involved as a structural protein (PDC-filaments)
414 associated with the cytoskeleton during the sexual development of filamentous fungi
415 (Thompson-Coffe et al. 1999). Conversely, *Pa_4_7260*, encoding the nucleoprotein Cik1,
416 required for both karyogamy and mitotic spindle organization (Cottingham et al. 1999), was
417 found down-regulated at the same time. Some of the cytoskeleton DE genes showed co-
418 expression with the plasmogamy DE genes (Fig. S4F), making them good candidates to be
419 involved in fusion morphogenesis processes.
420 Three genes essential for ascus development were also found DE (Fig. S4G, Table S5A):
421 *P. anserina*'s orthologs of *Pa_4_9730/asd-1* (wave IV (Nelson, Merino, et Metzenberg 1997)),
422 *Pa_4_9750/asd-3* (Galagan et al. 2003) and *Pa_2_13270/con-6* (wave V (White et Yanofsky
423 1993)). *P. anserina* does not form clonal propagules. However, the orthologs of genes required
424 for conidiation in *N. crassa* and *Aspergillus nidulans* such as *Pa_2_13270/con-6*,
425 *Pa_4_9730/asd-1*, *Pa_4_9750/asd-3, Pa_1_12720/Pyg1*, *Pa_1_30/TmpA* and *Pa_0_1540*
426 were found to be up-regulated after meiosis, suggesting that genes primarily involved in
427 conidiation, *i.e.* asexual reproduction, may have been recruited to participate in ascospore
428 formation. On the contrary, early conidial development-2 *Pa_7_780/ecd-2* was down regulated
429 during the first half of the kinetic (Sun et al. 2012).
430 **Producing energy**
431 The reproductive process is energy consuming and therefore the mobilization of carbon
432 resources is one of the keys to its success. The genome of *P. anserina* contains 276 genes
433 encoding CAZymes ((Espagne et al. 2008) and Table S5D). Among them, 141 were found DE
434 during sexual development, showing an enrichment of this metabolic activity (p-value =

435 $4.36x10^{-5}$). The expression profile of 42 CAZyme genes corresponded to one of the five
436 transcriptional waves, especially waves II and IV. The remaining 99 showed contrasting
437 expression patterns (Fig. S4 H-T), but up-regulation was enriched over down-regulation, as 62
438 of them were up-regulated during the kinetic (Chi^2, p-val < 0.001). We also found that several
439 CAZymes genes had some of the highest positive fold changes measured in this study (Table
440 S1). In addition, three glycolysis enzymes genes (6-phosphofructo-2-kinase *Pa_3_6610*,
441 fructose-bisphosphate aldolase *Pa_0_710*, and phosphoglycerate kinase *Pa_6_10610*) were up-
442 regulated with a similar pattern of overexpression when asci begin to differentiate (Fig. S4U,
443 Table S5E).

444 In *P. anserina*, long-chain fatty acids are shortened in peroxisomes then degraded in
445 mitochondria. The *Pa_6_3500*/*PaPex7* gene, which encodes the peroxisomal-targeting-signal-
446 2 receptor, is found to be up-regulated during ascospore maturation (T54-T96, Table S1 and
447 Fig. S4V). This is in line with the defects in nuclear positioning of the post-meiotic asci
448 observed in *pex7* knock-out mutants, which can result in either abnormal ascospore formation
449 or to ascospores containing an abnormal number of nuclei (Bonnet et al. 2006). The
450 *Pa_1_23790*/*PaFox2* gene (Table S1 and Fig. S4V), which encodes the peroxisomal
451 multifunctional protein 2, a key enzyme in the fatty acid ß-oxidation pathway, was found to be
452 down-regulated during the early stages of sexual development (T12-T54) and up-regulated
453 during ascospore maturation (T54-T96). This expression profile may explain the reduced
454 acetyl-CoA availability within the ascospores that results in the pigmentation defect observed
455 in *PaFox2* knock-out mutants (Boisnard et al. 2009).

456 **Secondary metabolites: major actors of sexual development?**

457 Prokaryotic and eukaryotic micro-organisms produce a wide variety of secondary
458 metabolites (SMs). These molecules are not intended to perform housekeeping functions, but
459 rather are used as adaptive tools to cope with environmental fluctuations or to compete with
460 other living organisms. The *P. anserina* genome contains 35 biosynthetic gene clusters (BGCs)
461 ((Lamacchia et al. 2016), Table S5F, Fig. S4W), containing 474 genes. SM encoding genes
462 were enriched among the complete set of DE genes (p-value = $8.31x10^{-10}$) as 258 of them were
463 found to be DE (33 out of 35 BGCs contained at least two DE genes). These genes were also
464 significantly up-regulated (Chi^2, p-val < 0.001). BGCs N°1, 11, 18, 19, 24, 27 and 34 showed
465 an expression profile topology (Fig. S4W) similar to the general pattern described in *N. crassa*
466 (Wang et al. 2022), which was a coordinated downregulation from fertilization to meiosis
467 followed by a coordinated upregulation during ascospore formation and maturation.

468 We also examined whether co-expression was shared by multiple SM genes within the
469 34 BGCs. This was the case for the sterigmatocystin BGC, where 17 of the 23 genes were
470 differentially expressed during sexual reproduction (Table S5F, cluster 32, Fig. S4X, (Slot et
471 Rokas 2011)), including the transcription factor *PaAflr* (Shen et al. 2019). In addition, we
472 identified four positive regulators of the sterigmatocystin BGC (Table S5F, Fig. S4Y,
473 *Pa_2_7220, Pa_2_7240, Pa_2_7250, Pa_2_7260* (Bhetariya et al. 2011)), that showed the
474 same expression pattern across sexual reproduction. Similarly, 13 of the 20 genes in the
475 terrequinone BGC were differentially expressed (Fig. S4Z).
476 In addition, we identified several isolated genes from different BGCs that showed
477 similar peak expression to each other but no co-expression with other genes from their
478 respective BCG (Fig. S4AA-AB). These expression peaks of genes encoding mostly proteins
479 of unknown function corresponded to two key moments in early development (T6 and T12),
480 just before and during dikaryon formation. Taken together, our analyses further support the key
481 role of SM in sexual reproduction and highlight the role of previously uncharacterized clusters
482 and isolated SM genes.
483 **From regulation of biotic interactions to that of sexual development**
484 Genes encoding STAND proteins (Signal-transducing ATPases with Numerous
485 Domains, (Leipe, Koonin, et Aravind 2004) have been described as involved in fungal biotic
486 interactions with living organisms, either from the same species or from different species but
487 mostly during vegetative growth. The *P. anserina* genome contains 34 genes encoding STAND
488 proteins with a NACHT domain (PF05729), eight of which were found to be down-regulated
489 either throughout the sexual cycle kinetics or prior to perithecium development (Table S5G,
490 Fig. S4AC). This group includes *Pa_3_10930*/*nwd1* (Chevanne et al. 2010), *Pa_7_10370*/*sesB-*
491 *like*, *Pa_2_7940*/*het-d* (Espagne et al. 2002) and *Pa_1_11380* (Bidard, Clavé, et Saupe 2013).
492 Fungal interactions are also under the control of a programmed cell death called vegetative
493 incompatibility that prevents hyphal fusion of two genetically distinct wild-type strains. Most
494 of the effectors of this incompatibility reaction (IR) contain HET domains (PF06985).
495 Strikingly, 59 of the 128 *het* genes annotated in the *P. anserina*'s genome were found to be DE,
496 including *Pa_4_1190*/*hnwd1* and *Pa_7_3460*/*Pin-C* (Chevanne et al. 2010; Kaneko et al. 2006;
497 Bidard, Clavé, et Saupe 2013). The majority of the DE *het* genes were down-regulated either
498 throughout the complete sexual development or from dikaryon formation (Fig. S4AD), but
499 seven of them were up-regulated during some steps of the kinetic, mostly after karyogamy (Fig.
500 S4AE). In addition, three *het* genes showed a peculiar behavior (Fig. S4AF): *Pa_5_2540*
501 expression oscillated during sexual reproduction, *Pa_3_5550* expression peaked during

502 protoperithecium development and *Pa_4_1800* expression was down-regulated until ascospore
503 maturation and over-expressed during this process.
504 In the DE gene set, we also found gene families encoding toxins that can induced host
505 cell death or effector-like proteins. All of the six genes encoding the NEP1-like proteins pore-
506 forming toxin NPP1 (necrosis-inducing *Phytophthora* protein, (Ottmann et al. 2009) were
507 down-regulated at least during the second half of the sexual development, i.e. before karyogamy
508 (Fig. S4AG). Six out of the eight genes encoding Hce2 pathogen effectors (Stergiopoulos et al.
509 2013) and one gene annotated as Ptu bug toxin encoding gene (*Pa_6_9450*) were also DE. In
510 contrast to the *NPP1* genes, all of them showed up-regulation during the course of sexual
511 development, five of them belonging to the wave IV pattern of expression (Fig S4AH).
512 **Epigenetic effectors at work in sexual reproduction**
513 In recent years, epigenetic effectors have been identified as key players in fructification
514 morphogenesis (Carlier et al. 2021; Nowrousian 2022). Therefore, we investigated the
515 expression patterns of genes encoding proteins that can either methylate cytosines, write or
516 erase histone post-translational modifications or interfere with messenger RNA synthesis and
517 stability.
518 *PaRid* and *PaDnmt5*, two of the three DNA methyltransferase encoding genes present
519 in the *P. anserina* genome, were found to be DE during this kinetic. *PaRid* expression, which
520 is essential for dikaryon formation (Grognet et al. 2019) peaked at T12, while *PaDnmt5*
521 expression was increasingly up-regulated from T12 to T42 (Fig. S4AI). *Pa_5_2900*, which
522 encodes a RING-type E3 ubiquitin transferase URF1-like, was found to be strongly up-
523 regulated from T42. In mammals, this protein binds both hemi-methylated DNA and tri-
524 methylated lysine 9 of histone H3 (H3K9me3), thereby bridging DNA methylation and
525 chromatin modification (Foster et al. 2018).
526 *Pa_7_10020*/*KAT1* and *Pa_1_7460*/*Hos-2* encodes two chromatin modifiers with
527 antagonistic enzymatic activities, respectively a histone acetyltransferase encoding gene (Tong
528 et al. 2012) and a histone deacetylase (Wang, Kurdistani, et Grunstein 2002) respectively.
529 However, these two genes showed co-expression over the entire kinetic, *i.e.* they were up-
530 regulated from fertilization to karyogamy and then down-regulated (Fig. S4AJ). This pattern
531 was in contrast to that of *Pa_7_5890* encoding the Sin3-associated polypeptide SAP18, which
532 enhances transcriptional repression through histone deacetylation (Cheng et Bishop 2002). Two
533 genes encoding sirtuins NAD(+)-dependent histone deacetylases were also found to be DE:
534 *Pa_4_8570*/*PaSir2* showed a wave III expression pattern that resembled that of
535 *Pa_1_19440*/*PaRid* (Fig. S4AI), while the gene encoding *Pa_4_7390*/*SIRT5* was down-

536 regulated throughout sexual reproduction. Of the 32 genes encoding SET domain proteins,
537 seven were found to be DE (Fig. S4AK), although none has yet been shown to have histone
538 lysine methyltransferase activity. Four genes encoding putative histone demethylase containing
539 JmJC domains were also found DE (Fig. S4AL).

540 Promoter accessibility also depends on nucleosome positioning. The ATP-dependent
541 chromatin remodeling protein SWI/SNF *Pa_5_4310/SMARCA3* (Oh et al. 2013) was one of the
542 most highly up-regulated wave III genes (Fig. S4AM). Its transcription pattern is similar to that
543 of *Pa_1_19440*/*PaRid* (see Fig. S4AI) and *Pa_4_8570*/*PaSir2* (Fig. S4AJ). Transcription
544 requires a large number of proteins assembled into conserved complexes. Two genes
545 (*Pa_1_17860* and *Pa_6_1460*) encoding components of the SAGA complex were found to be
546 up-regulated from T6 to T54 with very similar expression patterns (Fig. S4 AM). However, we
547 also found similarly co-regulated genes although encoding factors with antagonistic effects on
548 transcription, such as the SWD2 subunit of the COMPASS complex (*Pa_7_11070*) and the
549 extra sexcombs subunit of the Polycomb Repressive Complex 2 (*Pa_3_4080*).

550 There is no experimental evidence that post-transcriptional RNAi mechanisms Quelling
551 (Romano et Macino 1992) and MSUD (Shiu et al. 2001) are functional in *P. anserina*. However,
552 genes encoding essential components of Quelling (Fig. S4AN), the RNA-dependant RNA
553 polymerase (RdRP) *Pa_7_4790/Qde1* (Cogoni et Macino 1999) and the dicer *Pa_6_6150/Dcl-*
554 *1* (Yang, Ye, et Liu 2015), were downregulated from T18 and T30, respectively. On the
555 contrary, expression of *Pa_7_9210* encoding the Qde-2 interacting exonuclease QIP was up-
556 regulated from T6 to T30. *N. crassa* Δ*qde-1* quelling mutant strains produced normal-sized
557 perithecia but no ascospore (Wang et al. 2014). Four genes involved in MSUD were also found
558 to be DE (Fig. S4AO). *Pa_2_9830*/*Sad-2* and *Pa_5_11380*/*Sad-5* were up-regulated whereas
559 *Pa_1_19900*/*Sad-1*, encoding the RdRP and *Pa_1_16040*/*Sad-3*, encoding the helicase, were
560 down-regulated. Interestingly, *Pa_6_11690*/*rrp-3*, which encodes an RdRP that could not be
561 clearly assigned to Quelling or MSUD pathways in *N. crassa* (Borkovich et al. 2004), was co-
562 expressed with the Quelling genes *Pa_1_19900*/*Sad-1* and *Pa_1_16040*/*Sad-3* in *P. anserina*.
563 Knockout of the *N. crassa Sad-1* and *Sad-3* resulted in arrest of ascus development in meiotic
564 prophase and production of normal-sized perithecia and young asci without ascospores,
565 respectively (Wang et al. 2014). *Sad2* is essential to *N. crassa* fructification development (Wang
566 et al. 2014). These results suggest that RNAi genes may be silenced during meiosis and
567 sporulation, which may explain the apparent lack of MSUD in *P. anserina* but also that
568 *Pa_2_9830*/*Sad-2* and *Pa_5_11380*/*Sad-5* might have been recruited to play a role during
569 ascospore dormancy.

570 Repeated sequences present in both haploid parental genomes of *P. anserina*'s
571 dikaryotic cells are subjected to cytosine-to-thymine mutations by repeat-induced point
572 mutation (RIP, (Bouhouche et al. 2004)). Modified 5-methylcytosines are known to be hotspots
573 for cytosine-to-thymine transitions, because, unlike unmethylated cytosines, spontaneous
574 deamination of 5-methylcytosines cannot be repaired by uracil excision (Pearl 2000). To
575 counteract this genetic threat, members of the AlkB alpha-ketoglutarate-dependent dioxygenase
576 (PF13532) family oxidize 5-methylcytosines to restore undamaged bases (Bian et al. 2019).
577 Two genes encoding AlkB-like enzymes are present in the *P. anserina* genome. The expression
578 of *Pa_1_12280* was down-regulated throughout the kinetic, while *Pa_1_17260* was up-
579 regulated during two segments of the kinetic: i) from Tm24 to T12, corresponding to the peak
580 of expression of *PaRid* and ii) from T24 to T54, when *PaRid* is no longer expressed (Fig.
581 S4AP). Notably, both genes showed a superimposable flat expression profile at the time of RIP.
582 In filamentous fungi, a conserved post-transcriptional A-to-I mRNA editing process
583 recodes transcription units (Teichert et al. 2017; Blank-Landeshammer et al. 2019), mainly
584 during fructification and ascospore morphogenesis. We identified seven DE genes encoding
585 adenosine deaminase acting on tRNA (ADAT), a class of enzymes which are responsible for
586 A-to-I mRNA editing. *Pa_1_21170*, a wave III gene, showed a peak of expression similar to
587 that of *PaRid*, while *Pa_3_6600* and *Pa_6_7750* were up-regulated from perithecium
588 development to ascospore discharge (Fig. S4AQ).

589 **Co-expression Network: In Search of Potential Transcription Factor Targets During**
590 **Sexual Reproduction**

591 We then focused on the 96 transcription factors (TFs) encoding genes that were found
592 to be DE (Table S6I). The expression profile of each TF gene was used to search for significant
593 correlations with the expression profiles of all genes previously classified as transcriptional
594 waves I, II, III, IV or V. Using this strategy, we build a network of 2002 potential transcriptional
595 links between 76 TFs and 683 target genes (Fig. 6 and Table S7A-C).

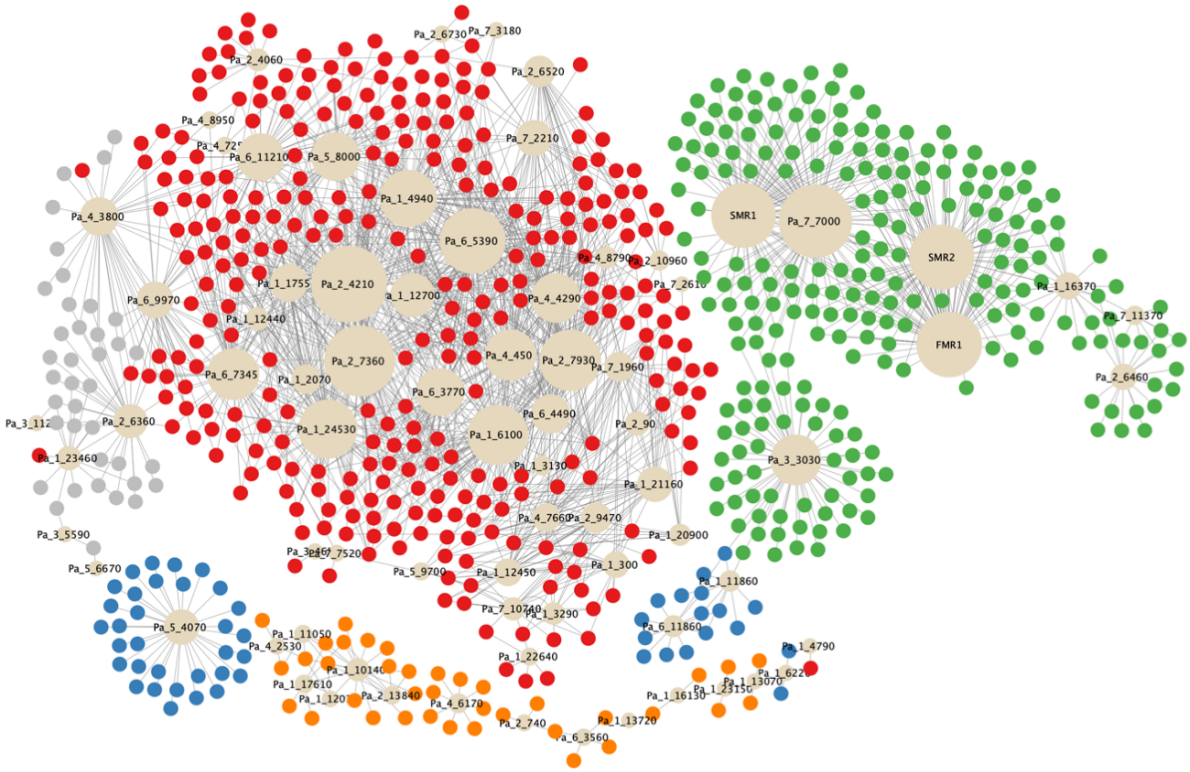


Figure 6 - Co-expression network based on expression profiles of DE genes coding for transcription factors

Genes encoding transcription factors (TF) are represented by beige circles whose size represents the number of co-regulated genes. These coregulated genes, which can be considered as potential TF target genes, were classified in transcriptional waves as such: wave I: grey color (61 genes), wave II: red color (1402 genes), wave III: orange color (58 genes), wave IV: green color (426 genes) and wave V: blue color (55 genes).

Some of the prominent nodes of this network were expected TFs (FMR1, SMR1, SMR2, Pa_1_10140/Pro1, see below), confirming its relevance. However, most of the 76 TFs (72%) had no characterized biological function, including those with the broader set of target genes. The distribution of the TFs encoding genes in the five transcriptional waves differed from that of the total set of DE genes (Fig. 6), with the majority belonging to wave II. As with TFs, most of the target genes were of unknown function. Importantly, some TFs were also target genes, e.g. *Pa_1_10140/Pro1*is a target of Pa_1_12070/HOM3Pa_1_11050/HMG4, Pa_1_17610, and Pa_2_13840 (Table S7A).

Among the master regulators of this network, we identified HMG-domain and homeodomain TFs (Espagne et al. 2008; Ait Benkhali et al. 2013). The HMG-domain TFs

616 include the mating-type proteins FMR1 and SMR2, which are both required for the
617 development of the perithecium after fertilization (Zickler et al. 1995; Arnaise et al. 2001).
618 Physical interaction between FMR1 and SMR2 was demonstrated and proposed to be required
619 for their post-fertilization functions (Arnaise et al. 1995; Turgeon et Debuchy 2007; Bidard et
620 al. 2011). Our study shows that FMR1 and SMR2 have a similar expression profile throughout
621 sexual reproduction (Fig. S4AR). Their co-expression networks revealed also an almost
622 identical number of potential target genes (FMR1, n = 79 ; SMR2, n = 78) (Table S7). They
623 share 62 of their 79 potential target genes (Table S7). These observations support the idea that
624 they act together in identical regulatory pathways by forming a heterodimer (Arnaise, Debuchy,
625 and Picard 1997). The third and last *mat-* mating-type gene, *SMR1*, does not belong to the
626 HMG-box family. It encodes a HPG protein (Turgeon et Debuchy 2007), which has been
627 proposed to provide a scaffold for physical interaction of mating-type proteins (Zheng et al.
628 2013). This role is consistent with its presence in wave IV, along with *FMR1* and *SMR2* (Fig.
629 S4AR). Critical genes for the development of ascogenous hyphae are likely to be found among
630 target genes of FMR1, SMR2 and SMR1 (Fig. 6, Table S7A). The only non-mating-type HMG
631 protein present in the network is Pa_1_11050/PaHMG4, related to Nhp6p and Hmo1p from *S.*
632 *cerevisiae* (Ait Benkhali et al. 2013). Its deletion in *P. anserina* resulted in an increased number
633 of male organs. The lack of effect on fructification development is consistent with the down-
634 regulation of *Pa_1_11050*/*PaHMG4* at T42-T96 (Fig. S4AS). Proper shaping of the developing
635 fructifications requires the homeobox protein *Pa_2_6460/Pah1* (Coppin et al. 2012). The
636 expression of the corresponding gene was accordingly up-regulated from T0 to T42 (Fig.
637 S4AS). In contrast, *Pa_3_4650/Pah6* was down-regulated between T0 and T54 (Fig. S4AS),
638 although no fructification defect was associated with its deletion.

639 The $Zn(II)_2Cys_6$ class of regulators is one of the largest families found in fungi
640 (MacPherson, Larochelle, et Turcotte 2006) and consequently they represented almost half of
641 the TFs present in the network (Table S7A). Three of them are of particular interest and have
642 been extensively studied because of their properties. *Pa_1_10140*/*PaPro1* is a regulator of the
643 expression of the mating-type and *IDC* genes, both required for female fertility (Gautier et al.
644 2018). In *S. macrospora*, *Pro1* has been identified as a master regulator of fructification
645 formation (Steffens et al. 2016; Teichert, Pöggeler, et Nowrousian 2020). Its temporal and
646 spatial expression is consistent with the presence of *Pa_1_10140/PaPro1* in wave III (Fig.
647 S4AS). *Pa_7_7000* was characterized in wave IV as a coregulator of FMR1, SMR2 and SMR1
648 target genes (Fig. 6). *Pa_7_7000* is revealed as one of the most prominent nodes in the TF
649 network (Fig. 6 and Fig. S4AT), but has not yet been characterized in *P. anserina*. *Pa_7_7000*

650 has orthologs in the seven species of the *Podospora* complex (Boucher, Nguyen, et Silar 2017)
651 and in some fungi closely related to this species complex (*Cercophora samala*, *Triangularia*
652 *verruculosa* and *Apiosordaria backusii*). However, more distantly related species (*Podospora*
653 *setosa*, *Podospora australis*, *Podospora appendiculata*, *Thermothelomyces thermophilus*,
654 *Neurospora tetrasperma* and *Neurospora discreta*) contain a protein that shares some identity
655 with Pa_7_7000, but they all lack the Zn(II)2Cys6 domain and are therefore unlikely to be
656 functional orthologs. Wave V contains *Pa_6_11860*/*Fluffy* (Bailey et Ebbole 1998) (Fig.
657 S4AT), which is required in *N. crassa* for the formation of conidia. These vegetative spores are
658 absent in *P. anserina*, suggesting that this gene has been recruited for ascospore formation in
659 this fungus, as in others members of the *P. anserina / P. pauciseta / P. comata* species complex
660 (Boucher, Nguyen, et Silar 2017).

661 **Comparison of mating-type target genes at different developmental stages**

662 A transcriptomic analysis of *P. anserina* was performed previously to determine the FMR1 and
663 FPR1 target genes required for fertilization (Bidard et al. 2011). The analysis focused on the
664 comparison of *mat*- and *mat*+ mycelia competent for fertilization, namely the stage preceding
665 the development of the perithecium. It was performed using the same methods as described
666 here. The analysis identified 69 genes upregulated in a *mat*- mycelium and 88 genes upregulated
667 in a *mat*+ mycelium. Among these genes, we search for the DE genes identified in this study.
668 A total of 32 DE genes (46 %) were identified among the 69 genes which were found to be
669 upregulated in the *mat*- mycelium (Table S8) and 45 DE genes (51%) are found among the
670 *mat*+ upregulated genes (Table S9). A statistical analysis indicates that the DE genes are over-
671 represented in both categories, when compared with the 3,466 DE genes in the 10,507 *P.*
672 *anserina*'s gene models. This overrepresentation was higher for the *mat*+ upregulated genes
673 (Chi^2, p-value = 0.0003) than for the *mat*- upregulated genes (Chi^2, p-value = 0.0183). This
674 results is likely due to the use of a *mat*+ maternal strain for the fruiting body development
675 presented here and indicates that half of the genes upregulated in fertilization competent
676 mycelium are also up-regulated in maternal tissues during perithecial development.

677 ## Discussion

678 **Mating-type gene expression**

679 In this study, we analyzed on a genome-wide scale the transcriptional changes associated with
680 sexual development in *P. anserina* over a 10 time points course, from fertilization to ascospore
681 maturation. We found that more than 32% of the *P. anserina*'s genes are differentially

682 expressed during at least one interval covered by this kinetic. Interestingly, more than 36% of
683 the proteins encoded by these genes do not have a Pfam domain, suggesting that a significant
684 proportion of them remain functionally uncharacterized. Overall, the data generated by our
685 study are rich in novel players involved in fungal sexual reproduction and are likely to provide
686 an exploratory framework to analyze the pathways that are required for the differentiation of
687 the 15 different tissues identified in a perithecium (Bistis, Perkins, et Read 2003).
688 Among these 15 tissues, the ascogenous hyphae plays a central role in the sexual cycle,
689 since it is where karyogamy, meiosis and ascospore formation take place. Its formation starts
690 at T24 (Fig. 1) and requires the expression of the four mating-type genes: *FMR1*, *SMR1*, *SMR2*
691 and *FPR1* (Zickler et al. 1995; Arnaise et al. 2001). The ratio of ascogenous tissue to the other
692 tissues of the perithecium is low, potentially masking the zygotic expression profile. This
693 detection limit of our experiments can be assessed by searching the transcripts of *FMR1*, *SMR1*
694 and *SMR2*. These three mating-type genes are specific to the *mat-* nuclei, which come from the
695 male organs and are present only in the ascogonial cell and in the ascogenous hyphae. These
696 three mating-type genes are listed in the DE genes (Table S1), indicating unambiguously that
697 transcripts specific to the ascogenous hyphae are detected in our experiments, in spite of its
698 scarcity. Moreover, upregulations of *FMR1* and *SMR2* transcripts are detected as soon as T24,
699 correlating with the initial development of ascogenous hyphae (Table S1). In addition, 11 genes
700 upregulated in *mat-* fertilization competent mycelium are among the DE genes (Table S8),
701 confirming the detection of transcript from *mat-* male nuclei in the female organ. However, no
702 significant upregulation of the *mat-* mating-type transcripts is detected in the early steps of
703 perithecial development, *i.e.* from T0 to the formation of the ascogonial cell at T18 (Fig. 1).
704 Correspondingly, no wave I DE genes are identified in genes that accumulate transcripts in the
705 *mat-* mycelium (Table S8). The earliest DE genes upregulated in *mat-* competent mycelium
706 belong to wave II (n = 4). Wave IV, which corresponds to upregulation of *mat-* mating-type
707 genes and their correlated genes (Fig. 6), includes 412 genes (Fig. 3), among which six belong
708 to genes upregulated in *mat-* mycelium (Table S8). Overall, these data suggests that critical
709 target genes required for the development from fertilization to the formation of ascogonial cells
710 are not included in the DE genes, because their expression level is below the detection threshold
711 of microarrays. In contrast, the expression of genes required for the subsequent developmental
712 stages (differentiation of ascogenous hyphae, meiosis and ascospore formation) are indeed
713 detected in our experiments.
714 Surprisingly, *FPR1*/*Pa_1_20590*, the *mat+* specific mating type gene, does not belong to the
715 DE genes, although this gene is also required for the formation of ascogenous hyphae (Zickler

716 et al. 1995; Arnaise et al. 2001). It is likely that any changes in expression of this gene in zygotic
717 tissues is hidden by its transcription in the maternal tissues, which are predominant in the
718 fruiting body. We have however found 45 DE genes that are specifically upregulated in *mat+*
719 mycelium competent for fertilization (Table S9). Among these DE genes, 32 are direct or
720 indirect target genes of FPR1 (Bidard et al. 2011). Their detection proved that FPR1 target
721 genes do not escape our method, although FPR1 itself is not present in DE genes. Moreover,
722 detection starts in the early stages of perithecium development as suggested by FPR1 target
723 genes belonging to wave II (n = 9). Later stages are represented by FPR1 target genes belonging
724 to wave IV (n = 3) and V (n = 2). However, our experiments do not allow us to determine
725 unambiguously whether the differential expression of a gene occurs in the maternal or in the
726 zygotic tissues

727 **Secondary metabolite gene are actively regulated during sexual development.**

728 As in *N. crassa* (Wang et al. 2022), *C. globosum* (Wang et al. 2019) and *A. nidulans* (Liu et al.
729 2021), our results further support the active role of SMs in fungal sexual reproduction. The
730 evolutionary conservation of their expression profile, *i.e*. downregulation from fertilization to
731 meiosis followed by a coordinated upregulation during ascospore formation and maturation
732 suggests that SMs protect fungal progeny, *i.e*. ascospores, from animal predation. However, we
733 identified several genes of unknown function that were overexpressed during early phases. It is
734 therefore possible that these metabolites protect newly-formed fructification but also that they
735 are recruited to mediate developmental processes. This is reminiscent of the *Pa_5_2090/pks-6*
736 gene, which encodes the insecticidal polyketide neurosporin A produced at a similar
737 developmental stage by *S. macrospora* (Nowrousian 2009) and *N. crassa* (Zhao et al. 2017) to
738 protect fructifications from arthropod attack. Whether this gene product is also involved in the
739 process of morphogenesis is still an open question (Wang et al. 2022).

740 Our data reveals a relationship between sterigmatocystin synthesis and mating-type
741 genes by correlating expression of *PaAflR/Pa_2_7360*, *HMG5/Pa_1_13940* and
742 *HMG8/Pa_6_4110* (Table 6 and Fig. 6). PaAflR/Pa_2_7360 is a regulator of the
743 sterigmatocystin synthesis cluster (Shen et al. 2019), while HMG5/Pa_1_13940 and
744 HMG8/Pa_6_4110 are HMG-domain TFs acting as hubs of regulation for the mating-type
745 genes and mating-type target genes (Ait Benkhali et al. 2013; Gautier et al. 2018). Our results
746 suggest that PaAflR/Pa_2_7360 controls positively the transcription of the two other genes.
747 Over expression of PaAflR/Pa_2_7360 results in sterility, as does the over-expression of
748 HMG5/Pa_1_13940 (Shen et al. 2019; Ait Benkhali et al. 2013). This result is in agreement

749 with the positive transcriptional control of *HMG5/Pa_1_13940* by PaAflR/Pa_2_7360. To date,
750 *PaAflR/Pa_2_7360* has not been deleted in *P. anserina*, and although its orthologue in *A.*
751 *nidulans* has been knocked out, the effect on the sexual cycle remains poorly understood (Wang
752 et al. 2022).

753 **Het genes operates important but yet unknown roles in sexual reproduction**

754 This study unveiled an interesting regulation of the *het* genes, which are involved in
755 allorecognition and thus control the viability of heterokaryons formed after hyphal fusion
756 during vegetative growth (Daskalov 2023). However, heterokaryotic syncytia are not restricted
757 to vegetative thalli, as fertilization also leads to the formation of such multinucleate cells, and
758 accordingly, several *het* loci have been shown to control hybrid fertility in *P. anserina* (Ament-
759 Velásquez et al. 2020). When we compared the *het* genes expression profiles during sexual
760 reproduction with those assayed during IR (Bidard, Clavé, et Saupe 2013) and bacterial defense
761 response (Lamacchia et al. 2016) (Table S6 G), we found that they fall into two categories and
762 four classes.

763 The first category consists of het genes that were always down-regulated during sexual
764 reproduction but were either up-regulated during IR and/or bacterial confrontation (class 1) or
765 down-regulated during IR and up-regulated during bacterial confrontation (class 4). This
766 category therefore contains *het* genes that play a role only in vegetative growth and/or defense
767 against pathogens, but must otherwise be silenced.

768 The second category consisted of *het* genes that were either up-regulated during IR but
769 also at some time points during sexual reproduction (class 2) or were not DE during IR and
770 bacterial confrontation but during sexual reproduction (class 3). Class 2 genes may be recruited
771 to control cell fusion during morphogenesis of fructifications and/or of early zygotic lineage for
772 wave II genes, or of the developing asci and ascospores for waves IV and V genes. Class 3
773 genes may have been recruited to carry out specific programmed cell death events, not related
774 to hyphal fusion but necessary for sexual morphogenesis. In this context, *Pa_3_5550* is of
775 particular interest since it shows a peak of expression during dikaryon formation. Hce2 proteins
776 also belong to this category, suggesting a specific role during both the pre-zygotic (*Pa_2_4765*)
777 and post-zygotic (*Pa_1_17065*) stages.

778 The *het-6* and *het-13* genes were found to be DE during sexual reproduction in the
779 heterothallic fungus *N. crassa*, whereas no such observation was made in the homothallic
780 fungus *C. globosum* (Wang et al. 2019). In *P. anserina*, both orthologs *Pa_6_6160/het-6* and
781 *Pa_0_150/het-13* were also DE but showed opposite expression patterns compared to *N. crassa*.

782 This suggests that the roles of *het* genes, although central in the sexual developmental network
783 of fungi endowed with two different mating types, are polymorphic.

784 **Rewiring genes in the RNAi pathway?**

785 In *P. anserina* (Grognet et al. 2019), as in *T. reesei* (Li, Chen, et Wang 2018) and *A.*
786 *immersus* (Malagnac et al. 1997), the *rid* (*RIP defective*) ortholog is required for proper sexual
787 reproduction, which is not the case in *N. crassa*. *Pa_1_19440*/*PaRid* showed a short peak of
788 expression at T12 (Fig. S4AI), which is consistent with its requirement for the individualization
789 of the dikaryotic cells leading to meiocytes (Grognet et al. 2019). In contrast, *N. crassa rid* up-
790 regulation started at dikaryon formation and increased continuously throughout the second half
791 of sexual reproduction (Wang et al. 2014). Interestingly, the *N. crassa rid* expression pattern
792 was similar to that of the *P. anserina* DNA methyltransferase encoding gene *PaDnmt5*, which
793 is absent in *N. crassa*. These results were in agreement with our previous data (Grognet et al.
794 2019) and strengthened the body of evidence pointing to a role for DNA methylation during
795 sexual development.

796 Although there is no experimental proof that either somatic or meiotic RNAi pathways
797 (Quelling or MSUD, respectively) works efficiently in *P. anserina*, some of the corresponding
798 genes are found to be DE during sexual reproduction. Globally the expression patterns of *P.*
799 *anserina* genes involved in the RNAi pathways are opposite to those of *N. crassa*, except for
800 *Pa_2_9830/Sad-2* and *Pa_5_11380/Sad-5*. During MSUD, Sad-2 is thought to interact with the
801 RdRP Sad-1 as a scaffold. Yet, *Sad-1* and *Sad-2* showed opposite patterns of expression during
802 *N. crassa* sexual development, which likely discards this hypothesis (Wang et al. 2014).
803 However, *Sad-5*, which role is yet unknown, has an expression profile that is similar to that of
804 *Sad-2*. The evolutive conservation of *Pa_2_9830/Sad-2* and *Pa_5_11380/Sad-5* profiles of
805 expression in both *N. crassa* and *P. anserina*, suggests that they have been recruited together to
806 play a role in sexual reproduction. In the homothallic ascomycete *S. macrospora*, *Dcl1*, *Dcl2*,
807 *Sms2* and *Qde2* are involved in meiocyte formation (Girard et al. 2021), which is not consistent
808 with the expression pattern of the *Pa_6_6150/Dcl-1* but rather with that of *Pa_5_8910/Sms-2*.
809 Altogether, these results suggest a complex role for the RNAi machinery during fungal sexual
810 reproduction.

811 Conclusion

812 Our work reveals 3,466 DE genes during the differentiation of fruiting bodies of *P.*
813 *anserina*. Only 86 of these DE genes have been previously functionally analyzed (Table S10),

leaving a large number of genes for future investigations. Although our analysis does not allow us to determine the DE genes specifically expressed in each tissue of the fruiting body, our method is sensitive enough to detect DE genes in ascogenous hyphae, at a stage where this tissue is represented by few cells in the mass of the fruiting body. The sensitivity of our method is emphasized by the detection of a peak of transcription of *Pa_1_19440*/*PaRid* at a time of the early perithecial development corresponding to its expected function. Among the DE genes, we have identified at least two pathways that may control mating-type genes. The first one involves *Pa_7_7000*, possibly revealing a new regulatory pathway upstream of mating-type genes. The second one is the relationship between *PaAflR*/*Pa_2_7360*, *HMG5*/*Pa_1_13940* and *HMG8*/*Pa_6_4110*. The latter two TFs control mating-type genes, thus revealing an additional putative pathway regulating indirectly these genes. Our analysis reveals also the intriguing expression patterns of the genes from the MSUD and RNAi pathways, although MSUD has not been found in *P. anserina* and RNAi not investigated. Further research should be focused in *P. anserina* on the role of the genes involved in these pathways in other species.

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Conflict of interest disclosure

The authors declare that they comply with the PCI rule of having no financial conflicts of interest in relation to the content of the article.

Data, scripts, code, and supplementary information availability

Data are available online: Go to https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE93094. Enter token whcrwemexfkfhqr into the box.

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