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Overlapping *Podospora anserina* transcriptional responses to bacterial and fungal non self indicate a multilayered innate immune response

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Provisional

1 **Overlapping *Podospora anserina* transcriptional responses to**
2 **bacterial and fungal non self indicate a multilayered innate**
3 **immune response**

4
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33

Provisional

34 Recognition and response to non self is essential to development and survival of all
35 organisms. It can occur between individuals of the same species or between different
36 organisms. Fungi are established models for conspecific non self recognition in the form of
37 vegetative incompatibility (VI), a genetically controlled process initiating a programmed cell
38 death (PCD) leading to the rejection of a fusion cell between genetically different isolates of
39 the same species. In *Podospora anserina* VI is controlled by members of the *hnwd* gene
40 family encoding for proteins analogous to NOD Like Receptors (NLR) immune receptors in
41 eukaryotes. It was hypothesized that the *hnwd* controlled VI reaction was derived from the
42 fungal innate immune response. Here we analyze the *P. anserina* transcriptional responses to
43 two bacterial species, *Serratia fonticola* to which *P. anserina* survives and *S. marcescens* to
44 which *P. anserina* succumbs, and compare these to the transcriptional response induced under
45 VI conditions. Transcriptional responses to both bacteria largely overlap, however the number
46 of genes regulated and magnitude of regulation is more important when *P. anserina* survives.
47 Transcriptional responses to bacteria also overlap with the VI reaction for both up or down
48 regulated gene sets. Genes up regulated tend to be clustered in the genome, and display
49 limited phylogenetic distribution. In all three responses we observed genes related to
50 autophagy to be up-regulated. Autophagy contributes to the fungal survival in all three
51 conditions. Genes encoding for secondary metabolites and histidine kinase signaling are also
52 up regulated in all three conditions. Transcriptional responses also display differences. Genes
53 involved in response to oxidative stress, or encoding small secreted proteins are essentially
54 expressed in response to bacteria, while genes encoding NLR proteins are expressed during
55 VI. Most functions encoded in response to bacteria favor survival of the fungus while most
56 functions up regulated during VI would lead to cell death. These differences are discussed in
57 the frame of a multilayered response to non self in fungi.

58

59 **Key words:**

60 Bacterial Fungal Interaction, Vegetative Incompatibility, *Podospora anserina*, *Serratia*
61 *marcescens*, *Serratia fonticola*, Transcriptional Response

62

63 **Introduction**

64 All living organisms have developed mechanisms to perceive and respond to non self.
65 Recognition can be conspecific, between individuals of the same species, or heterospecific in
66 the context of host/pathogen or host/symbiont interactions. Fungi display a whole range of
67 beneficial or detrimental heterospecific interactions, with plants, animals, other fungi, bacteria
68 or viruses. In particular, Bacterial Fungal Interactions (BFI) have been extensively studied in
69 different contexts (Frey-Klett *et al.* 2011), including situations where fungi are targeted by
70 pathogenic bacteria (Leveau & Preston 2008). However fungal immune receptors remain
71 largely uncharacterized and the fungal reaction to bacterial pathogens is only beginning to be
72 investigated at the molecular and transcriptional level. Large scale transcriptomic analysis of
73 BFI include pathogenic interactions between *Aspergillus niger* and *Collimonas fungivorans*
74 (Mela *et al.* 2011) or *Rhizoctonia solani* with *Serratia* species (Gkarmiri *et al.* 2015),
75 cooperative interaction between *A. niger* and *Bacillus subtilis* (Benoit *et al.* 2015) or
76 comparative analysis of different behaviors between *Laccaria bicolor* and three different
77 bacterial species (Deveau *et al.* 2015). Fungi also display a con-specific non self recognition
78 mechanism called vegetative incompatibility (VI) which is genetically controlled and leads to

79 the rejection of conspecific non self generated by anastomosis between genetically different
80 isolates of the same species. Co-expression in the same cytoplasm of incompatible alleles of
81 so-called *het* genes initiates the VI reaction that culminates with a programmed cell death
82 (PCD) reaction of the fusion cell, thereby maintaining different isolates separated (Glass *et al.*
83 2000; Saupe 2000). VI has been shown to be selectively advantageous in some circumstances
84 as it can restrict resource plundering (Debets & Griffiths 1998), horizontal propagation of
85 cytoplasmic viruses (Choi *et al.* 2011) or deleterious plasmid (Debets *et al.* 2012). *het* genes
86 have been characterized in three fungal species, *Podospora anserina*, *Neurospora crassa*, and
87 *Cryphonectria parasitica* (Saupe 2000; Choi *et al.* 2011). Although not related in sequences,
88 these genes always display a high degree of allelic polymorphism.

89 STAND proteins are tripartite signal transduction proteins (Leipe *et al.* 2004), and in plants
90 and animals the vast majority of STAND proteins are innate immune receptors, including
91 NOD like receptors in animals and NB-LRR resistance proteins in plants (Rairdan & Moffett
92 2007). Plant innate immune response is a multilayered process. A first line of defense called
93 Pattern Triggers Immunity (PTI) is initiated upon recognition of conserved pathogen
94 molecular markers. Adapted pathogen then develop effectors whose functions are to alter
95 components of the innate immune response, and NB-LRR receptors in turn recognize these
96 effectors to initiate the Effector Triggered Immunity (ETI). Detection of pathogens effectors
97 occurs either by direct interaction with the NB-LRR receptors, but most frequently NB-LRR
98 receptors sense alterations of host proteins as a consequence of the effectors action in a model
99 known as guardian guardee (Jones & Dangl 2006) recently reviewed (Khan *et al.* 2016). This
100 model has also been described for animal NOD like receptors (Ferrand & Ferrero 2013).
101 Fungal genomes do not encode for NB-LRR proteins (Soanes & Talbot 2010; Dyrka *et al.*
102 2014) but instead encode for a great diversity of STAND proteins (Dyrka *et al.* 2014). Some
103 of the protein domains constituting fungal STAND proteins display phylogenetic relationship
104 to domains involved in immunity in plant and mammals, including the central nucleotide
105 binding NACHT domain (Koonin & Aravind 2000), or the N terminal HET domain related to
106 plant and animal TIR domain (Dyrka *et al.* 2014). Interestingly some *het* genes in *P. anserina*
107 and *C. parasitica* encode for proteins of the STAND family (Saupe *et al.* 1995; Choi *et al.*
108 2011). In *P. anserina* *het-d* and *het-e*, members of the *hnwd* gene family encoding STAND
109 proteins (Paoletti *et al.* 2007), form non allelic incompatibility systems with *het-c* encoding a
110 glycolipid transfer protein (Saupe *et al.* 1994). *het-c* and *hnwd* genes are subjected to positive
111 diversifying selection (Chevanne *et al.* 2010; Bastiaans *et al.* 2014), which can be best
112 explained in the context of an evolutionary arms race with pathogens. In addition, by many
113 aspects VI is analogous to hybrid necrosis in plants, a plant auto-immune disease associated
114 with NR-LRR immune receptors (Bomblies *et al.* 2007). These observations lead to the
115 proposition that VI in *P. anserina* was derived from the *P. anserina* innate immune system
116 (Paoletti & Saupe 2009). In this hypothesis HET-c protein would be under the surveillance of
117 HNWD innate immune receptors in the frame of the guardian guardee model (Jones & Dangl
118 2006). Accordingly HET-c protein would be targeted by pathogen effectors and HNWD
119 receptors recognize these alterations. Pathogen driven fast evolution of *hnwd* (Paoletti *et al.*
120 2007) and *het-c* (Bastiaans *et al.* 2014) genes would occasionally generate incompatible
121 combinations of alleles maintained for VI. In a similar fashion *acd11*, an *A. thaliana*
122 homologue of *het-c*, encodes a protein proposed to be under the surveillances of the NB-LRR
123 receptor LAZ5 (Palma *et al.* 2010). This model has several implications, including the fact
124 that HET-c contributes to the defense response against non self, and also that immune
125 response to pathogens and VI reactions induce similar responses. To assess this model we
126 have selected two related bacterial species, *Serratia marcescens* and *S. fonticola*, that trigger a

127 reaction in *P. anserina* WT isolates that is altered in a *Δhet-c* knock out mutant. Briefly *Δhet-*
128 *c* mutants appear more sensitive than wt *P. anserina* to both bacterial species, the full
129 description of the selection process will be presented elsewhere. Here we describe the
130 interactions of WT *P. anserina* to the two *Serratia* species that result either in death or
131 survival of the fungus. We also report the *P. anserina* transcriptional responses to these
132 bacteria as analyzed by a RNA-seq approach. We compare these responses to the STAND
133 triggered VI reaction. While the transcriptional responses to bacteria significantly overlap
134 with the VI reaction in different aspects they also display specificities. These results are
135 discussed in the frame of a multilayered fungal response to heterospecific non self.

136

137 **Material and Methods**

138 **Strains and culture conditions**

139 The WT *P. anserina* strain Cs is almost isogenic to the strain whose genome was sequenced
140 (Espagne *et al.* 2008), except for the *het-s* VI locus. Pa-ATG8-GFP, IDI-GFP and IDI2-GFP
141 as well as confocal microscopy methods were described previously (Dementhon *et al.* 2003;
142 Pinan-Lucarre *et al.* 2003). *S. fonticola* was isolated from compost and identified by PCR
143 amplification and sequencing of 16S ribosomal DNA encoding region. *S. marcescens* DB11
144 strain was a kind gift from D. Ferrandon's lab.

145 For confrontation assays 6 spots of 10 μl of a bacterial suspension from a fresh pre culture
146 (OD600 nm=1) were laid on top of a corn meal agar plate, and 6 plugs of fungal mycelium
147 were laid about 1 cm from the bacterial spots. The cultures were left to grow at 26°C in the
148 dark. For transfer assays fungal cultures were set up on cellophane stripes on corn meal agar,
149 and bacterial cultures were set up independently as 3 rows of 10 μl spots (OD600 nm=1) on
150 the same medium at 26°C in the dark. After 48 h incubation, cellophane stripes were
151 transferred onto bacteria seeded plates between rows of bacterial spots.

152

153 **RNA extraction**

154 After 2 or 6 h incubation, the mycelium were scraped off the cellophane and freeze dried for
155 48 h. RNA was extracted with Qiagen RNA plant minikit and DNase treated. As control
156 RNA was extracted from fungal mycelium transferred to bacteria free plates. For the VI
157 condition a self-incompatible thermosensitive strain was grown on corn meal agar and treated
158 as described (Bidard *et al.* 2013). All experiments were duplicated.

159

160 **RNA-seq and differential expression analysis**

161 cDNA library construction and sequencing, mapping to *P. anserina* annotated genes and
162 differential expression analysis were performed by Beckman Coulter Genomics using the
163 Trinity pipeline. We selected genes with a log₂FC>2 and p<0.01 for further analysis.

164

165 **Sequence analysis**

166 Gene Ontology assignation (<https://www.blast2go.com/>), Pfam annotation
167 (<http://pfam.xfam.org/>) and promoter sequence analysis (<http://genie.dartmouth.edu/scope/>)
168 were performed with default cut off values. Two tails Fisher's or Chi2 statistical tests were
169 conducted when required. Orthologous genes were identified as reciprocal best hits

170

171 **Versatility of *P. anserina* genes**

172 Versatility of *P. anserina* genes assignment depends on the number of Blastp hit results
173 against NCBI nr database obtained at the genus level by their translation products. For each *P.*
174 *anserina* protein we counted the number of Blastp hit (cut off: p value $<10^{-5}$). Genes whose
175 products result in a single hit (itself) are categorized as orphans, while genes having an
176 orthologue in *S. cerevisiae* are considered as part of the fungal core genome. The remaining
177 genes are arranged in versatility bins numbered one to 10 of about 670 genes each, depending
178 on the increasing number of hits, so that genes in the lower numbered categories produced the
179 fewer hits. Genes were then assigned a versatility index, set to 0 for orphans, 11 for genes
180 belonging to the fungal core genome, and the bin number for the remaining genes. A sliding
181 window analysis of the distribution of gene versatility along chromosomes was performed
182 with a window size of 100 genes. Regions of high versatility level correspond to contiguous
183 genes with versatility value below average versatility minus standard deviation for the whole
184 chromosome.

185 **Confocal imaging and cell death quantification**

186 Light and fluorescent observations were performed on a Leica DRMXA confocal
187 microscope. Cell death was measured as described previously after staining with the Evans
188 blue vital dye (Pinan-Lucarre *et al.* 2005), basically comparing the length of dead to living
189 fungal hyphae.

190 **ROS detection**

191 The fungus was soaked in a solution of nitrotetrazolium blue chloride (NBT, 250 μg / ml) for
192 20 min, washed extensively with water before observing under the microscope. NBT forms a
193 blueish precipitate in presence of ROS.

194

195 **Results and discussion**

196 **Phenotypic characterization of *P. anserina* response to *Serratia* species.**

197 In a confrontation assay against *S. marcescens* or *S. fonticola*, *P. anserina* grew normally
198 away from the bacterial colony, but growth towards the bacterial colony soon stopped before
199 contact was made. Where the edge of the fungal colony appears almost linear in confrontation
200 to *S. fonticola*, it appears altered in confrontation to *S. marcescens* (Figure 1A). After 2-3 days
201 growth resumed in the confrontation to *S. fonticola* and the fungal colony eventually covered
202 the bacterial colony. In the confrontation to *S. marcescens* growth did not resume. Fungal
203 growth arrest in confrontation with *Serratia* species has already been reported (Li *et al.* 2015).
204 Fungal cell morphology was altered in confrontation to both bacteria, with an intense
205 vacuolization, apical cell swelling, and occasional cell death (figure 1B-E). These phenotypes
206 appeared more pronounced in confrontation to *S. marcescens*. We also observed the induction
207 of autophagy as indicated by the vacuolar localization of Pa-GFP-ATG8, along with the

208 expression of IDI1-GFP and IDI2-GFP, two small secreted proteins induced during VI and
209 believed to act as defensins (figure 1F-K) (Bourges *et al.* 1998; Dementhon *et al.* 2003).
210 During VI IDI1-GFP and IDI2-GFP are localized to the membrane, while in response to
211 bacteria they appear vacuolar, which could be a consequence of bacterial toxins altering the *P.*
212 *anserina* secretory pathway (Guichard *et al.* 2014). These phenotypes decrease in intensity as
213 observations are made further away from the colony edge, and are not observed in fungal cells
214 growing opposite from the bacterial colony (figure 1L-M).

215 We also developed a transfer assay where *P. anserina* mycelium is grown on cellophane
216 stripes on a bacteria free medium before transfer onto a bacteria seeded medium (figure 1N).
217 This transfer assay results in induction of a response in the entire mycelium as revealed by
218 monitoring induction of IDI1-GFP, IDI2-GFP and autophagy. This assay also allows for the
219 timing of the response. We estimated *P. anserina* cell death level over time (Pinan-Lucarre *et*
220 *al.* 2005). Transfer to *S. fonticola* seeded plates resulted in a low (<10%) and transient level of
221 cell death while transfer to *S. marcescens* seeded plates lead rapidly to a considerable level of
222 cell death (>40%) (figure 1O). Taken together these results indicated that *P. anserina* was
223 resistant to *S. fonticola* but sensitive to *S. marcescens* in our conditions.

224

225 **High numbers of genes up and down regulated**

226 Using a RNA-seq approach we analyzed *P. anserina* transcriptional response 2 h and 6 h after
227 transfer to *S. marcescens* or *S. fonticola* seeded plates compared to a control response after
228 transfer to bacteria free plates. We also sequenced pooled RNA extracted 1 h and 3 h after
229 induction of VI, conditions largely covering the VI transcriptional response as previously
230 described (Bidard *et al.* 2013). Number of genes up and down regulated by at least a factor 2,
231 and maximum fold changes are reported in Table 1. Number of up regulated genes range from
232 1091 to 1913 (10% to 18% of the genome), while number of genes down regulated genes vary
233 from 781 to 1923 (7% to 18% of the genome). We pooled expression data obtained at different
234 times of exposure to bacteria to generate the set of unique genes regulated in presence of *S.*
235 *fonticola* (VsSf) or *S. marcescens* (VsSm) (table 1). The number of genes up or down
236 regulated are similar in response to bacteria or during VI.

237

238 **Responses to bacteria overlap**

239 We first compared *P. anserina* transcriptional responses to both bacteria. As indicated in
240 figure 2, both responses largely overlap (Fisher test, $p=0$), and the response to *S. marcescens*
241 is almost entirely included in the response to *S. fonticola*. Only 12% and 9.5 % of the genes
242 up or down regulated in the VsSm set were not in the VsSf set, while 21% and 22% of the
243 VsSf set were not in the VsSm set. This observation remains true when looking at regulated
244 genes after 2h or 6h of exposure to the bacteria, or when looking at expressed genes with
245 different LogFc levels. 390 and 211 are up regulated specifically in response to *S. fonticola* or
246 *S. marcescens*, while 420 and 153 genes are specifically down regulated in response to these
247 two bacteria respectively. When focusing on genes regulated in both conditions we observed a
248 good correlation in the response to both bacteria (figure 2), meaning that globally in response
249 to both bacteria gene expression regulation follows a similar pattern. However, we observed
250 that generally the magnitude of the differential expression, whether for up or down regulation,
251 is more important in response to *S. fonticola* than in response to *S. marcescens* (figure 2). In

252 other words globally genes are more up or down regulated in response to *S. fonticola* than to
253 *S. marcescens*. Again, this remains true for different times of exposure or when considering
254 different logF_c level.

255 Overall, the response to *S. fonticola* includes more genes with a greater level of differential
256 expression. Whether the difference comes from a better ability of *P. anserina* to detect and
257 respond to *S. fonticola* than to *S. marcescens*, or from a better ability of *S. marcescens* to
258 subdue *P. anserina* response than *S. fonticola* remains to be investigated.

259

260 **Transcriptional responses to bacteria overlap with vegetative incompatibility**

261 We next observed that transcriptional response to bacteria also significantly overlap with the
262 VI reaction (Chi², p=0) (figure 3). The overlap is significantly more important for genes
263 down regulated than for genes up regulated (Chi², p=0). As the fold change increases, the
264 overlaps between responses to bacteria and VI decrease. Only 6 and 15 genes are common to
265 the 100 most up or down regulated genes in all three conditions. Transcriptional responses to
266 VI and to bacteria thus share a common signature for genes with a low level of regulation
267 while genes the most regulated in response to bacteria are different from the genes most
268 regulated during VI.

269

270 **Genes up regulated in response to non self tend to be lineage specific.**

271 Wapinski and co-authors introduced the term versatility to describe the frequency at which
272 genes can be gained or lost during the course of evolution, and they found that genes encoding
273 for adaptive functions are more versatile than genes encoding for essential functions
274 (Wapinski *et al.* 2007). In previous studies of VI (Hutchison *et al.* 2009; Bidard *et al.* 2013),
275 showed that up regulated gene sets included an excess of orphan genes. We analyzed
276 versatility of genes regulated in response to non self. We approached versatility of *P. anserina*
277 annotated cds by counting the number of blastp hits (cut off e value < 1^{e-5}) of their predicted
278 products at the genus level in fungal genomes. We defined the fungal core genome as the
279 genes having an orthologue in the distantly related ascomycete *S. cerevisiae* (3297 genes
280 described in (Bidard *et al.* 2013)), orphans as having a blastp hit only in *P. anserina* (640
281 genes) and ranked the remaining genes (6698 sequences) in ten versatility bins of
282 approximately 670 sequences according to an increasing number of hits, so that smallest
283 numbered bins contain sequences resulting in the less blastp hits (all bin compositions are
284 presented in additional file 1). Genes belonging to the versatile bins or orphan categories are
285 significantly more up than down regulated (1493 up / 995 down (p=6.2^{e-28}) for VsSf, 1386 up /
286 790 down (p=0) for VsSm, 1597 up / 932 down (p=0) for VI). In contrast the core genome is
287 more down than up regulated (354 up / 886 down (p=0) for VsSf, 282 up / 825 down (p=0)
288 for VsSm, 317 up / 991 down (p=0) for VI). We then analyzed the distribution of
289 differentially expressed genes in these categories for the three responses we analyze (figure
290 4A-C). In all three responses genes belonging to orphan or versatile categories are more up
291 than down regulated. However repartition of differentially expressed genes in versatility bins
292 differ between VI and response to bacteria as exemplified in the comparison between VsSf
293 and VI gene sets (figure 4D-E). Genes up regulated during VI are more represented in the
294 most versatile bins, while genes up regulated in response to the bacteria are more represented
295 in the less versatile bins. Genes expressed in both conditions are equally represented in all

296 bins. Inversely, genes down regulated in response to bacteria are more represented in the most
297 versatile bins. The situation is identical when comparing the reaction to *S. marcescens* and VI
298 (not shown).

299

300 **Versatile genes are clustered**

301 We next analyzed distribution of versatile and conserved genes on chromosomes along with
302 their expression. Genes were assigned a versatility value (0 for orphans, 11 for core genome
303 genes, and the versatility bin numbers for the rest) and we performed a sliding window
304 analysis of the versatility level of genes along the chromosomes. As expected versatile genes
305 are not evenly distributed (Fedorova *et al.* 2008; Klosterman *et al.* 2011) and we identified a
306 total of 19 regions of high density of versatile genes distributed between all chromosomes, 8
307 of which appear to correspond to telomeric / subtelomeric regions (figure 4F). We also
308 identified chromosomal regions with a high density of genes up regulated in response to non
309 self. Thirteen out of 19 (lettered a to i) highly versatile regions coincide with regions of high
310 up-regulation density in at least one condition. Note that inversely regions of highly
311 conserved genes are essentially down regulated (additional file 2). There is thus a clear
312 correlation between versatility gene distribution and expression landscape in response to non
313 self. We also identified regions of high expression level specific either to the response to
314 bacteria or to VI (figure 4F). Clustering of versatile genes could facilitate their coordinated
315 epigenetic transcriptional regulation, as proposed for the regulation of effector encoding genes
316 in the plant pathogen *Leptosphaeria maculans* (Soyer *et al.* 2014)

317 Overall, the core genome is essentially down regulated while less conserved genes are up
318 regulated. This is particularly true for the VI reaction that includes expression of an additional
319 set of highly versatile genes. Upregulated versatile genes tend to cluster in specific regions of
320 the genome. Expression of versatile genes in response to non self suggest an adaptive nature
321 of the responses to non self (Wapinski *et al.* 2007).

322

323 **Functional annotation of differentially expressed genes**

324 We analyzed Gene Ontology (GO) terms and Pfam-A protein domains associated with
325 differentially expressed genes. As expected, annotation level is correlated with the versatility
326 of the genes (Additional file 3), and thus the proportion of annotated up regulated genes is
327 inferior to that of down regulated genes for both GO terms and Pfam-A domains (additional
328 file 3).

329 We first analyzed the 579 and 995 genes up or down regulated in all three conditions (figure
330 5). As observed above, GO terms and Pfam-A annotations of genes up regulated in common
331 are significantly less extensive than annotation of genes down regulated (Fisher test, $p < 10^{-4}$ in
332 both cases). The most represented GO terms and Pfam-A domains associated with these gene
333 sets, representing the common basis for response to non self, are presented figure 5. Down
334 regulated GO terms or Pfam-A annotations are essentially related to growth, development and
335 protein synthesis. Up regulated GO terms or Pfam-A annotations are related to metabolic and
336 catabolic process, secondary metabolism and autophagy (see below). The common basis of
337 response to non self includes a growth arrest accompanied with reduction in protein synthesis,

338 and induction of autophagy, a process associated with response to stress (Kroemer *et al.* 2010)
339 and immune responses (Zhou *et al.* 2014; Shibutani *et al.* 2015).

340 We next identified and compared Biological Process and Molecular Function GO terms
341 significantly enriched compared to their genomic representation (Fisher test, $p < 0.001$) in up
342 or down regulated gene sets (figure 6). We found few GO terms enriched with up regulated
343 gene sets (from 8 to 18) and a single term, autophagy, is enriched in all three conditions (see
344 below). Terms enriched in both VsSf and VsSm sets are concerned with lipid, carbohydrates
345 and Reactive Oxygen Species (ROS) metabolic processes.

346 In contrast, we found many GO terms associated with down regulated gene sets (102 to 138),
347 with 73 terms associated with all down regulated gene sets (figure 6). They are essentially
348 related to developmental processes such as ribosome assembly, RNA processing and protein
349 synthesis and illustrate the transcriptional signature of the growth arrest observed in the three
350 conditions. Note in addition that the 24 terms common to the VsSf and VsSm sets are
351 essentially related to DNA metabolism.

352 Table 2 presents 25 Pfam-A domains significantly enriched or depleted in either of the
353 conditions analyzed. The only Pfam-A domains enriched in all three up regulated gene sets
354 correspond to proteins related to secondary metabolism generally involved in interspecific
355 communication (see below). Domains enriched in presence of both bacteria include Heat
356 Shock Proteins, transcription repressors of the NmrA family suggesting nitrogen repression
357 (Andrianopoulos *et al.* 1998), and transmembrane ion transport. Note that all proteins
358 displaying the Pfam-A domain PF01699 (Na-Ca-exchange) are predicted to be vacuolar
359 which associated to the up-regulation of genes Pa_3_6420 and Pa_7_8320 encoding a
360 regulator of V-ATPase and vacuolar ATP-synthase respectively, may indicate that regulating
361 vacuolar pH is important in response to bacteria. Indeed *S. marcescens* is known to increase
362 vacuolar pH when invading macrophage cells in culture (Fedrigo *et al.* 2011). Interestingly
363 two domains related to transport of small solutes through membranes are specifically enriched
364 in presence of *S. fonticola*. The enrichment of MFS transport often associated with drug
365 extrusion could explain why *P. anserina* can survive to the presence of *S. fonticola*. Finally,
366 the HET, LysM and NACHT domains associated with incompatibility can be linked to NLR
367 signaling related to defense functions (see below). Domains associated with down regulated
368 genes in presence of bacteria are essentially associated with transport through nuclear
369 membrane and RNA. Domains associated with incompatibility are also related to RNA
370 binding and GTPase activity. Finally, note that WD40 repeat containing domains are highly
371 represented in the three down regulated gene sets, while ankyrin repeat containing proteins
372 are tightly regulated during VI but not in presence of bacteria. Four of the ankyrin containing
373 proteins appear to be STAND proteins (see below), the remaining mostly correspond to
374 proteins lacking a predicted function but usually these repeat domain proteins are involved in
375 protein interactions with scaffolding functions (Javadi & Itzhaki 2013).

376

377 **Secreted proteins**

378 Secreted proteins are involved in interspecies communication in situation of host pathogen or
379 host symbiont interactions. We analyzed the expression of the 801 genes encoding predicted
380 secreted proteins from *P. anserina*, and found no bias for genes encoding CAZyme, proteases
381 or lipases, three of the main classifications for secreted proteins (Pellegrin *et al.* 2015).
382 However genes encoding Small Secreted Proteins (SSP, protein of less than 250aa) are

383 overrepresented in the up regulated gene set in response to bacteria, but not during the VI
384 reaction for genes with a fold change over 4 (Table 3). SSPs in the context of host pathogen or
385 host symbiont interactions act on the host cell to modify its behavior, and are thus considered
386 fungal effectors. It appears that they can also be involved in the fungal reaction to
387 heterospecific non self, possibly acting directly on the bacterial cells. In that respect it is
388 relevant to note that within the expressed SSP encoding genes are *idi1*, *idi2* and *idi3*
389 previously identified in the context of VI. IDI2 protein is homologous to the victoriocin
390 displaying an antifungal activity in the species *Chochliobolus victoriae* (de Sa et al. 2010).

391

392 **Autophagy contributes to survival against bacterial non self**

393 Autophagy, the only GO term associated with all three responses, is generally associated to
394 stress responses (Kroemer et al. 2010), and has also been described as essential to immune
395 response in plants and animals (Zhou et al. 2014; Benoit et al. 2015; Shibutani et al. 2015).
396 Genes encoding components of the *S. cerevisiae* autophagy machinery were recently
397 reviewed (Feng et al. 2014). Eighteen have orthologues in *P. anserina*, 12, 10 and 12 of
398 which are up regulated in the VsSf, VsSm or VI gene sets while none is down regulated
399 (additional file 4). The role of autophagy as a cell death or survival mechanism seems to
400 depend on the context and model (Dickman & Fluhr 2013). Autophagy has been extensively
401 studied in *P. anserina*, in particular in the context of VI where it exerts a pro-survival
402 function, and was hypothesized to restrict spread of death signals from the heterokaryotic cell
403 (Pinan-Lucarre et al. 2005; Pinan-Lucarre et al. 2007). We transferred three *P. anserina*
404 autophagy mutants, $\Delta Pa-ATG1$, $\Delta Pa-ATG8$ (Pinan-Lucarre et al. 2005), and $\Delta pspA$ (Paoletti
405 et al. 2001) involved at different stages of the process onto *S. fonticola* or *S. marcescens*
406 seeded plates. In presence of both bacteria, autophagy mutant cell death rapidly reaches high
407 levels not attained by WT strain (60% to 80%) (figure 7). This is particularly clear in transfer
408 to *S. fonticola* seeded plates, while in response to *S. marcescens* level of WT cell death is
409 initially slightly inferior to that of the autophagy mutant strains. These results indicate that as
410 for VI, autophagy exerts a pro-survival function in response to bacteria. This pro-survival
411 function of autophagy is the clearest in response to *S. fonticola*, but seems attenuated in
412 response to *S. marcescens*. Interestingly *S. marcescens* is known to be able to alter the pH of
413 autophagic like vesicles in mammalian cells (Fedrigo et al. 2011) and one could speculate that
414 such action renders autophagy less efficient in *P. anserina* as well.

415

416 **Reactive Oxygen Species**

417 ROS are often produced in the context of host/pathogen interactions either as a defense
418 mechanism by the host or as a debilitating factor by the pathogen (Gessler et al. 2007). We
419 have compiled *P. anserina* genes encoding ROS producing enzymes or acting as antioxidant
420 agents (additional file 5). It is clear that in reaction to bacteria, expression of antioxidant
421 components is stimulated, which is not the case during the VI reaction, while expression of
422 the main ROS producing enzymes (PaNOX1-3) is not stimulated. Using a probe reactive to
423 ROS we indeed observed their accumulation in dead or dying cells in presence of bacteria
424 (figure 8). These observations suggest that *P. anserina* is confronted to an oxidative stress
425 generated by the presence of the bacteria. It was already observed that *P. anserina*
426 accumulated peroxides in response to certain fungal species but not during VI (Silar 2005).

427

428 **Secondary metabolite**

429 Secondary metabolites constitute central elements of the chemical arsenal for inter organismal
430 communications, and fungal secondary metabolite production has been shown to be
431 stimulated by bacteria in fungi (Brakhage 2013). Thirty five secondary metabolite gene
432 clusters comprising 470 genes have been identified in *P. anserina* (Bills *et al.* 2013). In
433 agreement with the Pfam-A protein domain analysis, they are over represented in the three up
434 regulated gene sets (Fisher's test, $p < 0.005$, Additional file 6), with a total of 126, 122 and 142
435 genes in the VsSf, VsSm and VI up regulated gene sets. We also observed that 33, 31 and 34
436 clusters having at least one gene up regulated, and 22, 22 and 21 have at least three genes up
437 regulated in the VsSf, VsSm or VI gene sets (additional file 6). Only 52 genes (including 8
438 out of 17 expressed polyketide synthase encoding genes) are up regulated in all three
439 conditions suggesting that the final secondary metabolites produced in VI or in response to
440 bacteria likely differ.

441

442 **Signaling**

443 In *P. anserina* we identified 77 such NLR encoding genes (Dyrka *et al.* 2014). They are not
444 transcriptionally regulated in presence of bacteria, but they are overrepresented (Fisher test,
445 $p < 0.001$) in the up regulated genes during the VI reaction (table 4) as already reported (Bidard
446 *et al.* 2013). In *P. anserina* NLR proteins, 7 N-terminal domains with a known annotation
447 were identified, three of which (HET, HeLo, HeLo-like) known or suspected to induce cell
448 death in *P. anserina* (Paoletti & Clave 2007; Daskalov *et al.* 2012; Daskalov *et al.* 2015).
449 Two N terminal domains are related to lipase, one (sesB) altering growth in *Nectria*
450 *haematococca* (Graziani *et al.* 2004), the other (Patatin) controlling PCD and defense in
451 plants (Kumar & Kirti 2012; Kim *et al.* 2014). All these domains are also found in non-NLR
452 proteins (Daskalov *et al.* 2012). As these effector domains are not all Pfam-A annotated, we
453 used an in-house annotation pipeline to identify them in all *P. anserina* cds, and examined
454 expression of the corresponding genes (table 4). For each domain they are expressed in higher
455 numbers during VI than in response to bacteria. Taken altogether they appear over-
456 represented in VI gene set ($p < 0.001$). This remains true when the two most represented HET
457 and HeLo like domains are subtracted from the analysis ($p < 0.05$). Overall VI conditions result
458 in expression of NLR N-terminal or stand-alone effector domains known to induce cell death
459 in *P. anserina*. Note that HeLo and HeLo-like domains act as pore forming toxins through
460 insertion in biological membranes (Mathur *et al.* 2012; Seuring *et al.* 2012), and could thus
461 also be active on membranes of pathogens in addition to *P. anserina*. Indeed HeLo domain is
462 known to induce cell death when expressed in yeast cells (Taneja *et al.* 2007). The same could
463 be true for the other N terminal effector domains.

464 Histidine kinase (HK) function as sensors for external or internal stimuli and are able to
465 activate response pathways either directly, or in two component system via phosphotransfer
466 protein (HPT) (Schaller *et al.* 2011). HK encoding genes are over represented in up regulated
467 gene sets as described in details in additional file 7

468

469

470 **Transcription factors and promoters**

471 *P. anserina* genome encodes for 216 putative transcription factors. TF encoding genes are
472 under-represented in the down regulated gene sets for all three conditions (2.1 folds, $p=1.5^{e-3}$
473 for VsSf, 2.4 folds $p=7.2^{e-4}$ for VsSm, 2.7 folds $p=9.1^{e-5}$ for VI). Of the 30 down regulated TF
474 genes, 12 have orthologues with known functions that are essentially related to development.
475 Of the 84 TF up regulated, only 13 have orthologues with known functions, indicating that TF
476 associated with response to non self remain largely uncharacterized (additional file 8). This
477 figure also presents the overlap between up regulated TF genes between all three conditions.
478 Within the TF genes up regulated in all three conditions is *Atf1*, a general stress responsive
479 transcription factor (Lawrence *et al.* 2007). One of the two TF over-expressed in both VsSm
480 and VI conditions, the two conditions resulting in *P. anserina* cell death, is *idi-4* whose
481 overexpression initiates a cell death reaction comparable to the VI reaction (Dementhon &
482 Saupe 2005). However, deletion of *idi4* does not prevent cell death during VI or in presence
483 of bacteria (not shown). A single TF expressed in presence of both bacteria has an orthologue
484 with a known function in secondary metabolism control. Interestingly, TF genes are
485 significantly over-represented only in the VsSf up regulated gene set ($p=2^{e-4}$), the only
486 condition where *P. anserina* survives, suggesting that the ability to stimulate the expression of
487 a large number of pathways may contribute to the resistance to bacteria. They include genes
488 with orthologues encoding TF involved in response to stresses, regulating expression of
489 secondary metabolite synthesis, and orthologues of *N. crassa* WC-1 and WC-2 (WC-1 and
490 WC-2 are also up regulated in presence of *S. marcescens* but below the FC>2 threshold)
491 involved in response to blue light and regulation of the circadian clock ((Chen *et al.* 2010),
492 see below).

493 We analyzed 500bp promoter sequences using Scope (Carlson *et al.* 2007) to identify putative
494 cis regulating sequences and found some that are significantly enriched or depleted in the up
495 or down regulated gene sets (additional file 8). Interestingly, the binding site ATGANTCAT
496 identified as the IDI4 target during VI (Dementhon & Saupe 2005) is specifically enriched in
497 the genes up regulated in presence of bacteria. We found a single GO term (autophagy)
498 associated with putative cis regulating sequence kACGTCAb in the up regulated gene set,
499 while all sequences enriched in down regulated gene sets are associated with GO terms,
500 essentially related to translation and ribosomal proteins.

501

502 **Comparison to other responses**

503 Fungal transcriptional response in BFI conditions have been investigated in a limited number
504 of cases. Numbers of fungal genes up or down regulated in response to bacteria vary greatly
505 depending on the nature of the interaction and the experimental set ups. For instance, few
506 genes are regulated in *A. niger* confronted to *C. fungivorans* (Mela *et al.* 2011) or in *L. bicolor*
507 confronted to *P. fluorescens* (Deveau *et al.* 2015), while high numbers of regulated genes are
508 reached in *A. niger* confronted to *Bacillus* (Benoit *et al.* 2014) or in *R. solani* confronted to
509 *Serratia proteamaculans* or *S. plymuthica* (Gkarmiri *et al.* 2015).

510 Recently was reported the transcriptional response of the basidiomycete plant pathogen *R.*
511 *solani* to bacterial species *S. proteamaculans* and *S. plymuthica* used as bio-control agents
512 (Gkarmiri *et al.* 2015). In both of these BFIs fungal growth towards the bacteria stops and the
513 fungal reaction including cell swelling and increased septation appears similar to what we
514 observed in *P. anserina* in response to *S. marcescens* and *S. fonticola*. We thus compared the

515 fungal transcriptional responses. From the 3228 *R. solani* genes found to be up or down
516 regulated in response to *Serratia* species, we found 712 genes with an orthologue in *P.*
517 *anserina* as identified by a reciprocal best hit approach. We then counted the orthologous
518 gene pairs up or down regulated in response to bacteria in both fungal species (table 5).
519 Orthologous gene pairs up regulated by *Serratia* species in both fungi are over represented in
520 the set of up regulated *R. solani* genes. As well, orthologous gene pairs down regulated in
521 response to bacteria in both fungi are over represented in the set of *R. solani* genes down
522 regulated in response to bacteria. Thus *R. solani* and *P. anserina* up and down transcriptional
523 responses to *Serratia* species overlap. However the overlap is significantly more important for
524 the down regulation than for the up regulation as on average the proportion of down regulated
525 orthologous gene pairs represents twice that of up regulated genes. For example 60 out of 220
526 *R. solani* up regulated genes in response to *S. plymuthica* have orthologues in *P. anserina*,
527 while 114 out of 224 *R. solani* genes down regulated in response to the same bacteria have
528 orthologues in *P. anserina*. Pfam-A protein domain annotation and GO term associated with
529 the orthologous gene pairs down regulated in both fungal species are related to growth,
530 development, gene expression and protein synthesis, while up regulated genes are associated
531 with metabolic processes (additional file 8). Note that within the orthologous gene pairs
532 regulated in both fungal species we found the expected number of genes also regulated during
533 VI (table 5). Overall, these results are in accordance with the idea that fungal responses to
534 antagonistic bacteria include a growth arrest associated with the down regulation of genes
535 required for development, DNA, RNA and protein synthesis. It also includes the stimulation
536 of expression of a common set of genes completed with lineage specific genes.

537 In a *P. anserina* colony three distinctive regions of the mycelium can be distinguished
538 corresponding to an outward region where growth occurs, an intermediate region where most of
539 the sexual development takes place, and a central region where growth has stopped. Gene
540 expression in these three zones has been examined and compared to each other (Bidard *et al.*
541 2012). We compared differential gene expression in *P. anserina* in response to bacteria or
542 during incompatibility to gene expression in the different regions of the mycelium obtained by
543 Bidard and colleagues. Genes expressed at a higher level in the growing part of the mycelium
544 (zone 1) are enriched in genes down regulated in presence of bacteria or VI, which was
545 expected as these are essentially related to development and growth. Perhaps more
546 surprisingly, genes expressed to a higher level at the center of the fungal colony (in stationary
547 phase) are significantly enriched in genes up regulated in presence of bacteria or during VI,
548 and depleted in genes down regulated in the same conditions (additional file 10A). These data
549 suggest that when *P. anserina* enters stationary phase, it expresses genes required in reaction
550 to non self, possibly preserving older mycelium from being exploited by invasive
551 microorganisms.

552 *P. anserina* orthologues of *N. crassa* WC-1 and WC-2 are up regulated in response to bacteria.
553 In *N. crassa* transcription factors WC-1 and WC-2 form the White Collar Complex (WCC)
554 required for gene expression in response to blue light and to regulate circadian rhythm (Chen
555 *et al.* 2010). We observed a significant overlap between response to bacteria in *P. anserina*
556 and WCC dependent response to blue light in *N. crassa* as reported in additional file 10B. We
557 also compared *P. anserina* transcriptional responses to VI in *N. crassa* and found a significant
558 but limited overlap between VI conditions as already reported (additional file 10C, (Bidard *et*
559 *al.* 2013)).

560

561 **A multilayered response to non self**

562 In plants and animals, induction of the full innate immune response results from the detection
563 of multiple signals, including detection of conserved pathogen associated molecular patterns
564 (PAMP) through transmembrane receptors, and danger signals detected by cytoplasmic NLRs
565 (Stuart L.M. 2013). In our experimental set up, responses to bacteria initiated without any
566 contact thus detection likely involves diffusible molecules. For instance bacteria are known to
567 produce outer membrane vesicles composed essentially of lipopolysaccharides, a classical
568 PAMP to which *P. anserina* reacts (unpublished). Detection could also occur through danger
569 signals generated by diffusible toxins. In contrast, VI condition is initiated by cytoplasmic
570 NLR thought to mimic detection of a cytoplasmic danger signals (Paoletti & Saupe 2009;
571 Bastiaans *et al.* 2014).

572 From Pfam-A or GO annotations we identified biological functions up or down regulated in
573 response to non self that are summed up in figure 9. All three conditions result in a growth
574 arrest that is reflected by functions encoded by the down regulated genes mostly related to
575 protein synthesis and growth. The conserved up regulated functions common to all three
576 conditions are common in responses to stress (HK signaling, autophagy) and biotic
577 interactions (autophagy and secondary metabolites). Interestingly, all functions specifically up
578 regulated in response to one or both bacteria (SSPs, response to oxidative stress and light
579 response, and response to fungicide through HK signaling, MFS transporters, vacuolar pH
580 regulation) could promote fungal cell survival. For instance, *P. anserina* expresses more
581 transcription factors and MFS transporters and survives better in response to *S. fonticola* than
582 in response to *S. marcescens*, and difference in gene expression and survival may be
583 dependent on the arsenal of bacterial pathogenicity effectors they produce (Guichard *et al.*
584 2014). In contrast, most functions specifically up regulated in the VI reaction seem to promote
585 fungal cell death. The HET domain is an activator of a VI-like cell death (Paoletti & Clave
586 2007), HeLo and HeLo like domains act as pore forming toxins (Daskalov *et al.* 2015).
587 Except for a few NLR controlling VI (Saupe *et al.* 1995; Espagne *et al.* 2002; Choi *et al.*
588 2011) fungal NLR functions have not been characterized but they often display HET or HeLo
589 N-terminal effector domains. LysM domain function has not been defined in fungi, but it is
590 known to bind sugars and promote immune responses in plants (Miya *et al.* 2007; Gimenez-
591 Ibanez *et al.* 2009; Tanaka *et al.* 2013; Akcapinar *et al.* 2015). From these observations, one
592 might propose that like plants and animals, fungi are endowed with a multilayered innate
593 immune system that can be sequentially activated depending on the signals detected.
594 Detection of conserved bacterial PAMPs from long range may initiate a robust and fairly
595 conserved response whose aim is to promote fungal survival while making life of the
596 pathogen difficult. Indeed most fungi will encounter all sorts of bacteria during their life time
597 which will require a global response to keep them at bay. This would be comparable to the
598 plant PTI (Jones & Dangl 2006). Then, adapted bacteria may be able to subdue this first line
599 of defense through the action of specifically evolved effectors. Detection of the resulting
600 danger signals would then initiate the ultimate fungal response by triggering a localized cell
601 death reaction through NLR signaling to restrict further invasion of the entire mycelium in a
602 response similar to the plant Hypersensitive Response (Jones & Dangl 2006). Positive
603 feedback on NLR encoding genes, and standalone N-terminal effector genes, could ensure
604 robustness of the response by initiating multiple death pathways thereby preventing pathogens
605 from developing inhibitors of a single pathway.

606 As for plants and animals responses to pathogenic non self, simply considering the number of
607 genes commonly up or down regulated in response to bacteria or during VI reveal that the

608 different layers of the immune response largely overlap in *P. anserina*. In addition, in all three
609 conditions, versatile genes thought to define adaptive traits (Wapinski *et al.* 2007) are over-
610 represented in up regulated gene sets. This trend is particularly clear in the VI conditions that
611 result in the activation of additional genes in the most versatile categories. A response to non
612 self thus seems to induce mostly adaptive genes, however the detection of conserved PAMPs
613 initiates a more general response than detection of intracellular signals through lineage
614 specific NLR receptors to detect adapted pathogens and require a more specific response.

615

616 **Conflict of Interest:**

617 The authors declare no conflict of interest.

618

619 **Author contribution**

620 ML, WD, AB and MP designed and conducted the experiments, SJS and MP contributed to
621 the writing of the paper

622

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634

635 **Supplementary Material:**

636 The supplementary material can be found online.

637

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842

843 **Titles and legends to figures.**

844 **Figure 1 | Phenotypic characterization of the interaction between *P. anserina* and *S.***
845 ***fonticola* or *S. marcescens*.** (A) Confrontation assay against *S. fonticola* and *S. marcescens*.
846 Fungal growth towards the bacterial colonies stops before contact is made. Fungal colony
847 edge appears linear in confrontation to *S. fonticola* and is altered in confrontation to *S.*
848 *marcescens*. The white line delineates the fungal colony edge. (B) Fungal cell morphology
849 appears altered on the side of the bacterial colony, especially at apices, with cells swelling in
850 confrontation with both bacterial species, here *S. marcescens*. (C) Cell death is observed by
851 Evans Blue staining in confrontation with both bacteria. (D) Cell morphology and (E) Evans
852 Blue staining in absence of bacteria. (F-K) Large vacuoles (F, H, J) and expression of IDI1-
853 GFP (G, I, K) are observed in presences *S. fonticola* (F, G) or *S. marcescens* (H, I), or during
854 the VI reaction (J, K) but not in cells growing away from the bacterial colony (L, M). Scale
855 bars=10 μm . The same induction of expression and localization is observed for Pa-GFP-
856 ATG8 and IDI2-GFP proteins (not shown). (N) In the transfer assay, bacteria and *P. anserina*
857 (on cellophane stripes) are grown for 48h on separate plates, and the fungus is then transferred
858 onto the bacteria seeded plates, setting the initial time point of the reaction. (O) Estimation of
859 the fungal cell death level after transfer to *S. fonticola* or *S. marcescens* seeded plates.

860

861 **Figure 2 | Comparison of the differentially expressed genes in response to bacteria.** (A-
862 B) Venn diagram showing *P. anserina* genes up or down regulated in response to *S. fonticola*
863 or *S. marcescens*. (C-D) Correlation of the fold change level in response to both bacteria. For
864 each gene expressed in both conditions, LogF_c levels in response to both bacteria are
865 reported. Genes are ordered along the X-axis according to increasing LogF_c values (C) in
866 response to *S. marcescens* for genes up regulated and decreasing LogF_c values (D) in
867 response to *S. marcescens* for genes down regulated. (E-F) Magnitude of transcriptional
868 regulation is more important in response to *S. fonticola* than in response to *S. marcescens*.
869 Histograms represent the number of genes that are more up (E) or down (F) regulated in
870 response to *S. fonticola* or *S. marcescens* in the VsSf or VsSm data sets, at each time point, or
871 for different LogF_c values. Fisher tests were conducted to compare number the number of
872 genes with a greater level of regulation in response to both bacteria (**: $p < 0.001$, *: $p < 0.05$).

873 **Figure 3 | Overlap between transcriptional response to bacteria and vegetative**
874 **incompatibility.** (A) Area proportional Venn diagram showing *P. anserina* genes up or down
875 regulated after transfer to *S. fonticola* or *S. marcescens* seeded plates, and during the
876 vegetative incompatibility reaction. Number in brackets represent the total number for each
877 gene set with a FC>2 and $p < 0.01$, values within the graph represent number of genes for each
878 intersect. (B) Histogram representing the percentage of genes up or down regulated by the
879 presence of *S. fonticola* or *S. marcescens* that are also up or down regulated during the VI
880 reaction for different fold change values. In all cases the overlap between response to bacteria
881 and VI response is greater than expected by chance (** $p < 0.001$, * $p < 0.01$).

882 **Figure 4 | Versatility of differentially expressed genes.** (A-C) The histograms represent
883 the number of differentially expressed genes in response to non self for each versatility
884 category. The left scale is relevant for number for orphan or versatile genes, the right scale for
885 core genome genes. Fisher's tests were conducted to compare number of up and down
886 regulated genes in each category (* $p < 0.05$, ** $p < 0.001$). (D-E) Number of genes up or down
887 regulated during VI only, in response to *S. fonticola* only or in both conditions. Fisher's test

888 were conducted to compare the number of genes up or down regulated specifically in either
889 condition (*p<0.05, **p<0.001). (F) Distribution of the versatility level (blue line) or up
890 regulated genes in response to *S. fonticola* (Red line) or during VI (green line) along each *P.*
891 *anserina* chromosomes. Each of the seven *P. anserina* chromosomes was analyzed by a
892 sliding window analysis (window size of 100 genes). For each 100 genes window, versatility
893 is expressed as an average value (left axis), while fraction of up regulated genes is referred to
894 the right axis. The horizontal axis represents the chromosome. Purple bars (lettered a-s)
895 indicate regions of high density of versatile genes; arrowheads indicate regions of high
896 density of expression specific to a given condition (red for VsSf, green for VI).

897

898 **Figure 5 | GO terms and Pfam-A protein domains differentially expressed in all three**
899 **non self conditions.** Only GO terms (left) and Pfam-A protein domains (right) found at least
900 five times in one of the conditions in the up and down regulated gene sets are reported

901

902 **Figure 6 | Enriched GO terms in differentially regulated gene sets in presence of**
903 **bacteria or during vegetative incompatibility for up or down regulated gene sets.**

904

905 **Figure 7 | Autophagy exerts a pro-survival function in response to bacteria.** (A) Light or
906 Evans blue staining of WT or *Δatg1* autophagy mutant 6h after transfer onto a *S. fonticola*
907 seeded plate. (B) Cell death level measure for WT, *Δatg1*, *ΔpspA* and *Δatg8* autophagy
908 mutants after transfer onto *S. fonticola* or *S. marcescens* seeded plates. Cell death was
909 estimated from 10 different pictures of three independent experiments for each time point
910 (two tailed t test, *: p<0.05, **: p<0.001).

911 **Figure 8 | *P. anserina* is submitted to an oxidative stress in response to bacteria:** Upon
912 transfer for 2h or 6h onto *S. fonticola* or *S. marcescens* seeded plates ROS are detected in *P.*
913 *anserina* dead or dying cells that are not detected after transfer to bacteria free plates.

914

915 **Figure 9 | Overview of the biological functions regulated in response to non self.**
916 Biological functions or pathways significantly enriched in up (red) or down (green) regulated
917 gene sets in response to different non self signals are represented. Functions differentially
918 regulated by the same non self signals are grouped in colored boxes, arrows indicating the
919 inducing conditions.

920

921 **Table 1 | *P. anserina* genes differentially expressed in response to non self.** Number of
922 genes up and down regulated after transfer 2h or 6h to *S. fonticola* or *S. marcescens* seeded
923 plates, or during the vegetative incompatibility reaction are reported. VsSf and VsSm genes
924 sets correspond to the total number of genes differentially regulated in presence of *S. fonticola*
925 or *S. marcescens* at least one of the two periods of incubation respectively.

926 **Table 2 | Pfam-A protein domains enriched in at least one differentially expressed gene**
927 **set.** Along with the Pfam-A domain accession numbers and names are indicated the number

928 of annotations in the genome and in the differentially expressed gene sets, the numbers in
929 brackets indicating the enrichment factor (Two tails Fisher's test, $p < 0.001$ in bold, otherwise
930 $p < 0.01$). A brief description of the domain function is also presented.

931

932 **Table 3 | Expression of Small Secreted Protein encoding genes.** For each reaction the
933 number of SSP encoding genes, the enrichment compared the expected number, and the
934 Fisher's two tail test p value are indicated (Number / Enrichment / p value), depending of the
935 fold change (FC) threshold level. Ns = non-significant.

936

937 **Table 4 | NLR and STAND alone effector domains gene expression.**

938 The numbers of NLR and standalone effector domain encoding genes up regulated in response
939 to non self are reported. For responses to bacteria numbers of genes also regulated during VI
940 are indicated in brackets. Fisher tests were conducted to identify genes significantly enriched
941 in comparison to their representation in the genome (*: $p < 0.05$, **: $p < 0.001$).

942 **Table 5 | Responses of *P. anserina* and *R. solani* to *Serratia* species overlap:** For each *R.*
943 *solani* up or down regulated genes sets in response to *S. Plymuthica* or *S. proteamaculans*
944 with orthologues in *P. anserina* (numbers in brackets) we counted genes up or down regulated
945 in response to bacteria in *P. anserina* or during VI, calculated the enrichment compared to the
946 expected number, and the Fisher two tail p value. (Number / Enrichment / p value). We also
947 report for orthologous gene pairs regulated in both fungal species the number of genes
948 regulated during VI (Numbers in italics). The Down / Up columns reports the ratio of the
949 down to up enrichment factors, and the Fisher two tail test p-value.

950 |

951 **Table 1:**

952

	<i>Vs S. fonticola</i>			<i>Vs S. marcescens</i>			<i>Incompatibility</i>
	2h	6h	VsSf	2h	6h	VsSm	1h and 3h
Up	1232	1325	1847	1091	1289	1668	1913
Max log ₂ Fc	10.2	8.8	10.2	9.2	8.5	9.2	11.5
Down	838	1605	1882	781	1458	1615	1923
Max log ₂ Fc	-9.5	-9.9	-9.9	-10.1	-8.4	-10.1	-9.5

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Table 2 :

Annotation	Name	Genome	Up regulated genes			Brief description
			VsSf	VsSm	VI	
PF00153.22	Mito_carr	102	40 (2,24)	-	-	Membrane transport of small solute across mitochondrial and other membranes
PF07690.11	MFS_1	158	51 (1,84)	-	-	Membrane transport of small solutes including drugs
PF01699.19	Na_Ca_ex	16	12 (4,28)	10 (4,16)	-	Ion transport, Vacuolar homeostasis
PF08240.7	ADH_N	36	18 (2,85)	19 (3,51)	-	Alcohol deshydrogenase
PF00107.21	ADH_zinc_N	42	19 (2,58)	20 (3,17)	-	Alcohol deshydrogenase (partially included in ADH-N)
PF00106.20	adh_short	79	28 (2,02)	26 (2,19)	-	Short chain deshydrogenase, reductase
PF07859.8	Abhydrolase_3	20	12 (3,42)	12 (4)	-	Hydrolases including lipases
PF00011.16	HSP20	6	6 (5,7)	6 (6,66)	-	HSP, Chaperonne in response to stress, pathogenesis in ustilago
PF01494.14	FAD_binding_3	38	17 (2,55)	17 (2,98)	-	Electron tranfer
PF00840.15	Glyco_hydro_7	7	5 (4,07)	7 (6,66)	-	Glycoside hydrolase
PF05368.8	NmrA	20	11 (3,14)	10 (3,33)	-	Negative transcriptional regulator, Nitrogen metabolite repression
PF00501.23	AMP-binding	49	21 (2,44)	20 (2,72)	-	AMP-binding in peptide synthases and 4 coumarate CoA ligase
PF13193.1	AMP-binding_C	18	10 (3,1)	10 (3,7)	-	All included in AMP-binding
PF00668.15	Condensation	30	15 (2,85)	14 (3,11)	-	Antibiotic synthesis in peptide synthase
PF00550.20	PP-binding	50	25 (2,85)	21 (2,8)	24 (2,59)	Prosthetic group, associated to PKS (includes 4 condensation)
PF00698.16	Acyl_transf_1	24	12 (2,85)	11 (3,05)	15 (3,37)	Acyl transferase, PKS
PF00109.21	ketoacyl-synt	24	12 (2,85)	10 (2,77)	15 (3,37)	PKS
PF02801.17	Ketoacyl-synt_C	23	11 (2,72)	9 (2,6)	15 (3,52)	PKS
PF14765.1	PS-DH	21	10 (2,71)	-	15 (3,85)	PKS
PF08659.5	KR	14	-	-	11 (4,24)	PKS
PF06985.6	HET	129	-	-	62 (2,59)	Vegetative incompatibility cell death
PF01476.15	LysM	31	-	-	18 (3,13)	Sugar binding, bacterial cell wall degradation
PF05729.7	NACHT	34	-	-	17 (2,69)	NLR oligomerisation domain
PF00400.27	WD40	433	13 (0,17)	17 (0,26)	-	Repeat, protein ligand interaction
PF12796.2	Ank_2	167	7 (0,24)	4 (0,16)	76 (2,46)	Repeat, protein ligand interaction

Down regulated genes

PF13634.1	Nucleoporin_FG	12	11 (4,85)	11 (5,28)	-	Nuclear pore
PF00493.18	MCM	6	6 (5,29)	6 (5,76)	-	Replication licensing factors
PF14551.1	MCM_N	6	6 (5,29)	6 (5,76)	-	Comprised in MCM
PF00271.26	Helicase_C	77	33 (2,27)	34 (2,54)	-	Helicase, mostly associated to RNA
PF00270.24	DEAD	47	28 (3,15)	29 (3,55)	22 (2,43)	RNA helicase included in Helicase_C
PF00076.17	RRM_1	83	39 (2,49)	35 (2,42)	30 (1,88)	RNA binding
PF00153.22	Mito_carr	102	-	-	53 (2,71)	Membrane transport of small solute across mitochondrial and other membranes
PF01926.18	MMR_HSR1	12	-	-	11 (4,77)	GTPase
PF06985.6	HET	129	-	-	5 (0,20)	Vegetative incompatibility cell death
PF00400.27	WD40	433	138 (1,69)	137 (1,82)	122 (1,47)	Repeat, protein ligand interaction
PF12796.2	Ank_2	167	10 (0,32)	9 (0,31)	3 (0,09)	Repeat, protein ligand interaction

955

956 **Table 3:**

957

	VsSf	VsSm	VI
FC>2	64 / 1.2 / ns	56 / 1.2 / ns	57 / 1 / ns
FC>4	52 / 1.8 / 5^{e-5}	46 / 1.8 / 9^{e-5}	45 / 1.3 / ns
FC>6	38 / 2.5 / 8^{e-10}	30 / 2.5 / 2^{e-7}	25 / 1.3 / ns
FC>8	20 / 2.5 / 2^{e-6}	17 / 2.8 / 2^{e-6}	11 / 1 / ns

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Provisional

959 **Table 4:**

960

			NLR				Non NLR			
Domain	Function	Cell death	Genome	Up VsSf	Up VsSm	Up VI	Genome	Up VsSf	Up VsSm	Up VI
GOOD-BYE	–	–	10	2 (2)	1 (1)	6*	4	1	0	2
HeLo-like	Pore forming	Suspected	4	1 (1)	0	3*	20	4 (2)	4 (3)	18**
HeLo	Pore forming	Yes	–	–	–	–	7	3 (3)	3 (3)	4
HET	–	Yes	5	0	0	2	124	16 (9)	19 (13)	61**
Patatin	Lipase	Suspected	1	0	0	2	9	5* (2)	5* (1)	3
PNP_UDP	Sugar metabolism	–	1	0	0	2	5	0	0	3
REL-SPO	ppGpp metabolism	–	1	0	0	1	–	–	–	–
SesB	Lipase	Suspected	12	0	0	3	24	4 (2)	4	7
Unk	–		39	2	1	10	–	–	–	–
total			73	5	2	29	193	33	35	98

961

962 **Table 5:**

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<i>R. solani</i> vs <i>S. plymuthica</i> AS13			<i>R. solani</i> vs <i>S. proteamaculans</i> S4		
<i>R. solani</i> up vs AS13 (220)	<i>R. solani</i> down vs AS13 (224)	Down / Up	<i>R. solani</i> up vs S4 (279)	<i>R. solani</i> down vs S4 (352)	Down / Up
Up VsSf: 60 / 1.5 / 0.02 /10	Down VsSf: 114 / 2.9 / 7 ^{e-14} / 79	1.9 / 3.8 ^{e-7}	Up VsSf: 78 / 1.6 / 0.006 / 19	Down VsSf: 178 / 2.9 / 1.6 ^{e-20} 125	1.8 / 1.6 ^{e-8}
Up VsSm: 55 / 1.6 / 0.02 /12	Down VsSm: 107 / 3 / 2.3 ^{e-13} 79	1.9 / 7.3 ^{e-7}	Up VsSm: 68 / 1.6 / 0.01 / 19	Down VsSm: 171 / 3 / 8.8 ^{e-21} 124	2 / 1.1 ^{e-8}
Up VI: 20 / 0.5 / 0.004	Down VI: 87 / 2.1 / 1.1 ^{e-6}	4.2 / 4.8 ^{e-9}	Up VI: 36 / 0.7 / ns	Down VI: 144 / 2.2 / 4.5 ^{e-11}	3.2 / 7.4 ^{e-9}

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Provisional

Figure 01.JPEG

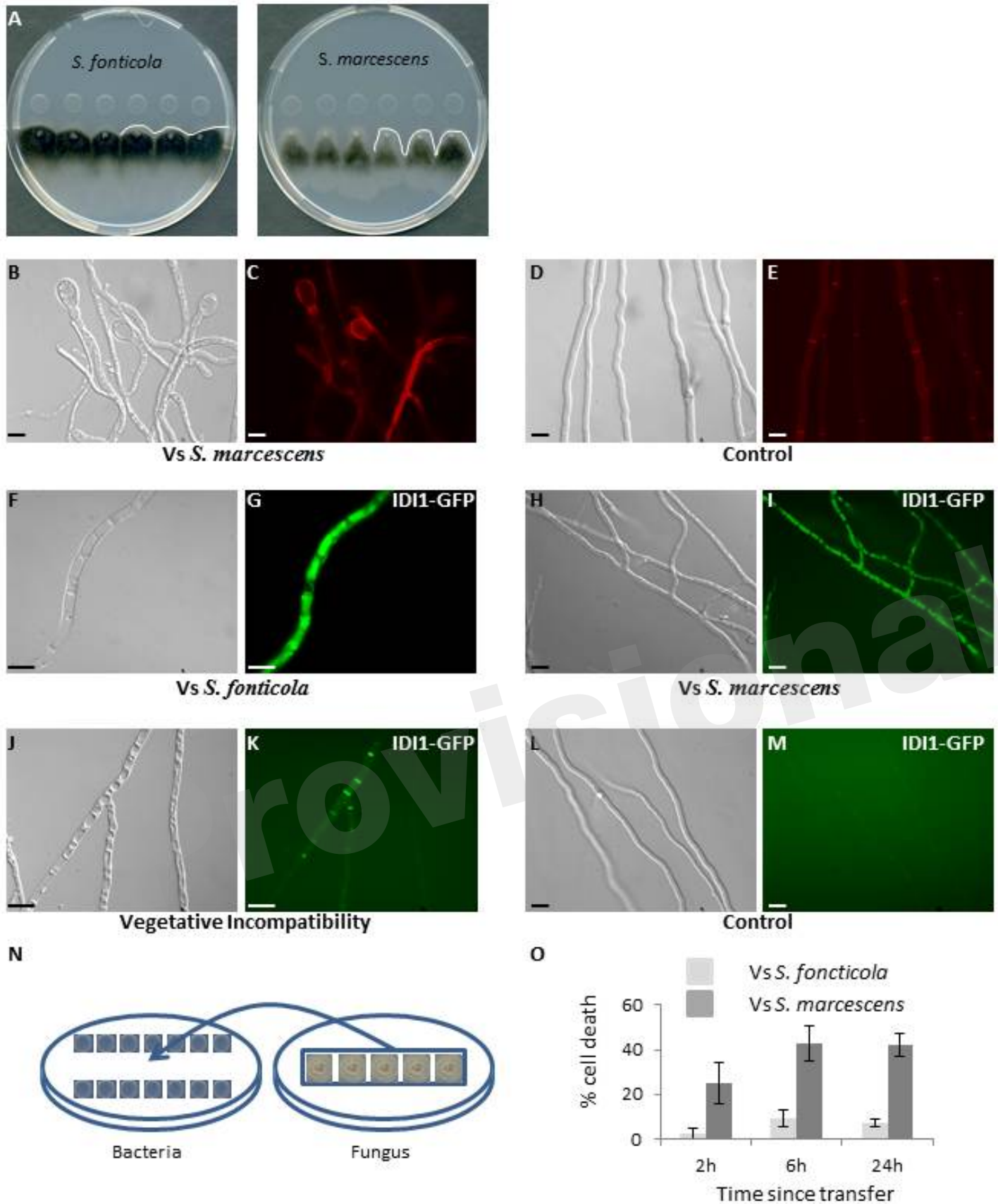


Figure 02.JPEG

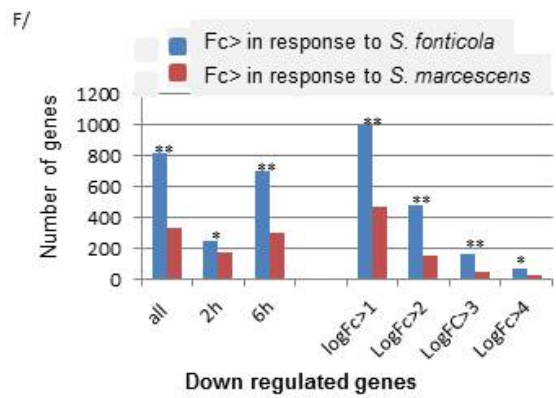
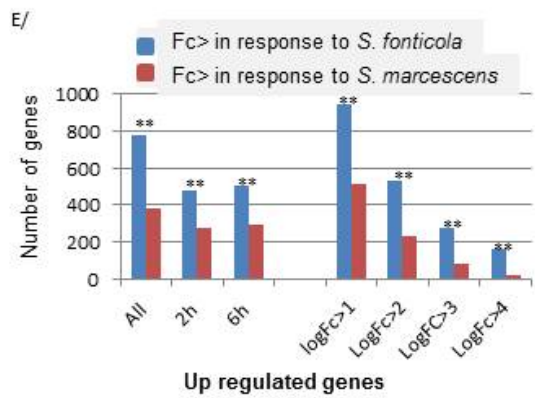
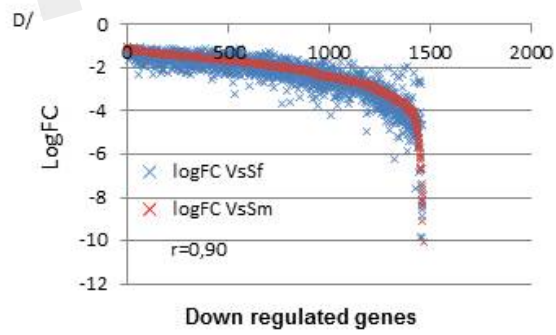
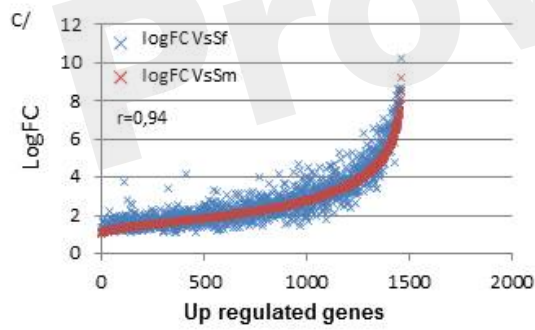
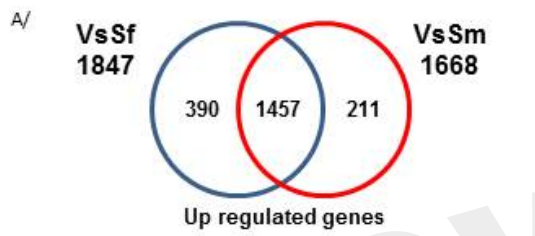


Figure 03.JPEG

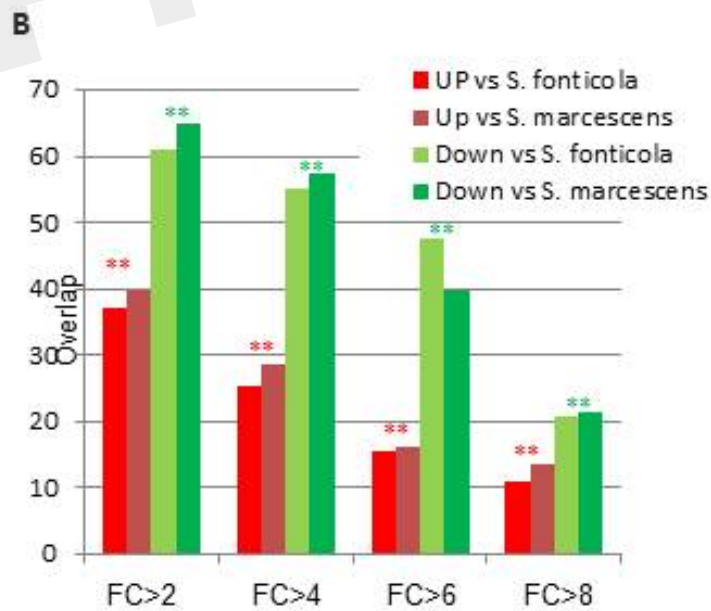
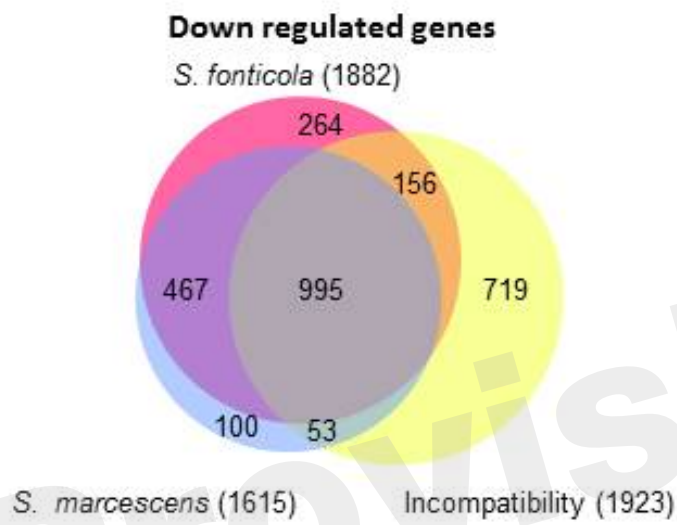
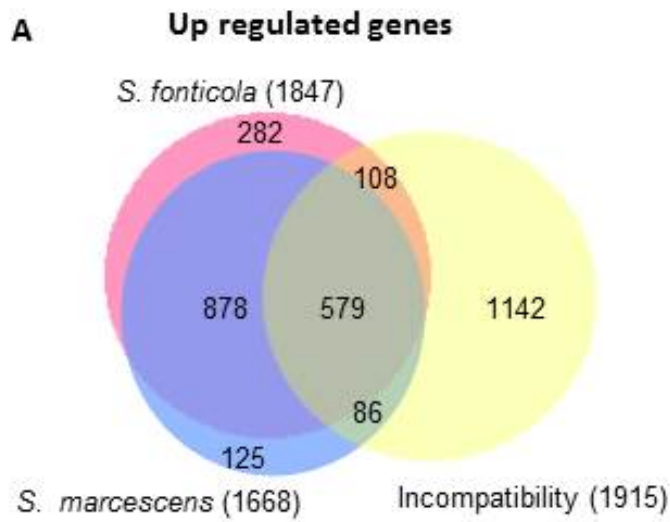
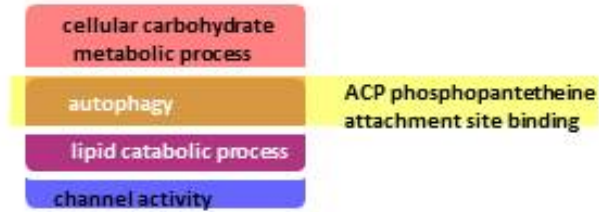


Figure 06.JPEG

Up regulated

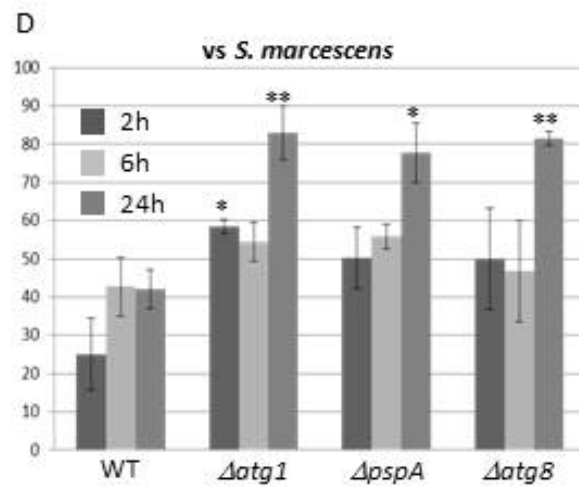
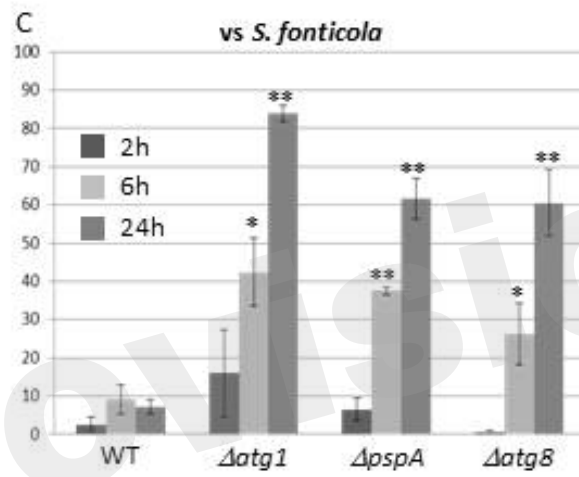
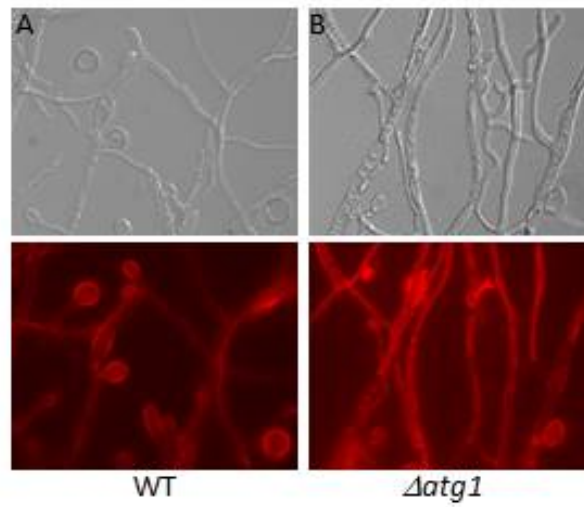


Down regulated



■ VsSf ■ VsSm ■ VI

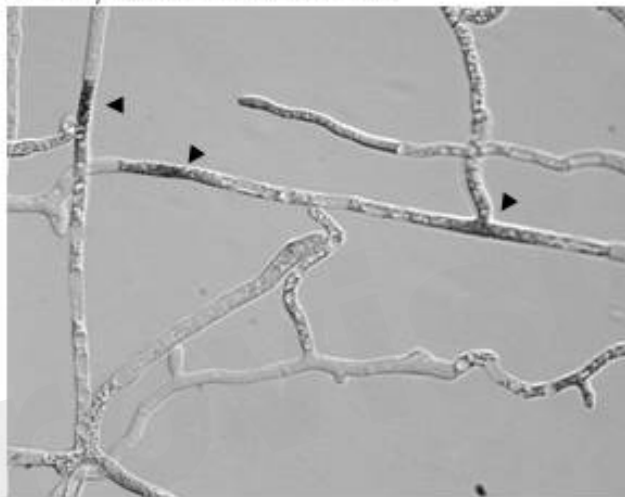
Figure 07.JPEG



+NBT, vs *S. fonticola*



+NBT, vs *S. marcescens*



+NBT, no bacteria

