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► To cite this version:

Mathias Choquer, Christine Rasclé, Isabelle R Gonçalves, Amélie de Vallée, Cécile Ribot, et al.. The infection cushion of *Botrytis cinerea*: a fungal ‘weapon’ of plant-biomass destruction. *Environmental Microbiology*, 2021, 23, pp.2293-2314. 10.1111/1462-2920.15416 . hal-03153197

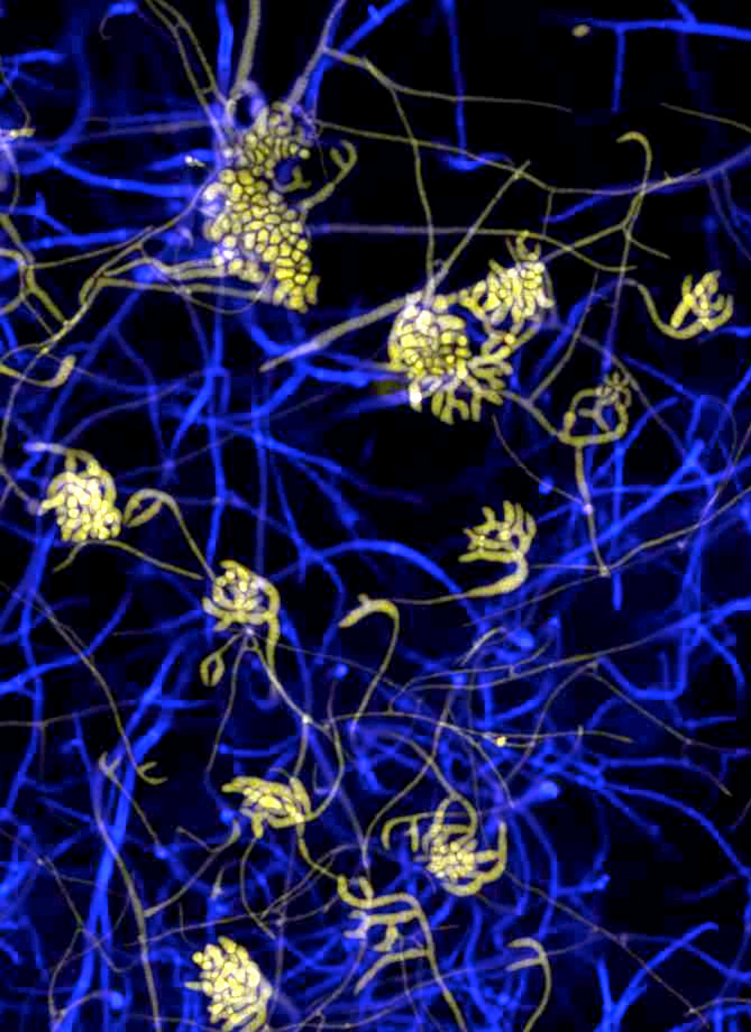
HAL Id: hal-03153197

<https://hal.science/hal-03153197v1>

Submitted on 10 Feb 2022

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2 **struction**

3 Running title: The infection cushion of *Botrytis cinerea*

4

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23 **Originality-Significance statement**

24 Fungi are the most devastating plant pathogens and the fight against them requires an under-
25 standing of their infectious process, and notably the first stages of their infection cycle. Invasion
26 of plants by fungi often involves differentiation of fungal structures called appressoria. Among
27 them, Infection Cushions (IC) are produced by major necrotrophic fungi, such as *Botrytis*,
28 *Sclerotinia*, *Rhizoctonia* and *Fusarium* species. Whether plant penetration would be their only
29 biological role is questionable. Besides, their function at the molecular level is poorly under-
30 stood. Our paper brings new data about IC of the gray mold causing agent *Botrytis cinerea*, a
31 major worldwide plant pathogen for which identification of new virulence factors and/or new
32 anti-fungal putative targets is of current significance. Based on a transcriptomic study, our work
33 provides molecular information that highlights biological processes specifically at work in ma-
34 ture IC of *B. cinerea*. The gene expression data are supported by a proteomic analysis of the IC
35 secretome, biochemical assays, microscopic observations and mutagenesis of two candidate
36 genes. Firstly, this paper supports the hypothesis that IC are structures dedicated to the massive
37 secretion of effectors important for plant penetration and early necrotrophic invasion, such as
38 phytotoxins, ROS, hydrolytic enzymes and plant cell death-inducing proteins. Secondly, this
39 paper suggests for the first time a role of IC in fungal nutrition. Thirdly, this paper reveals a
40 substantial remodeling of the fungal cell wall in the IC (melanin deposition and chitosan for-
41 mation) that suggests dynamic modifications at the fungal-host interface likely important for
42 infection. At last, this paper introduces fasciclin-like proteins as new players in *B. cinerea* pa-
43 thogenesis. In conclusion, our work brings new information about the biology and virulence of
44 this fungus. It could also be of interest to the community working on plant-pathogen interac-
45 tions.

46 **Summary**

47 The necrotrophic plant-pathogen fungus *Botrytis cinerea* produces multicellular appressoria
48 dedicated to plant penetration, named infection cushions (IC). A microarray analysis was per-
49 formed to identify genes up-regulated in mature IC. The expression data were validated by RT-
50 qPCR analysis performed *in vitro* and *in planta*, proteomic analysis of the IC secretome and
51 biochemical assays. 1,231 up-regulated genes and 79 up-accumulated proteins were identified.
52 The data support the secretion of effectors by IC: phytotoxins, ROS, proteases, cutinases, plant
53 cell wall degrading enzymes and plant cell death-inducing proteins. Parallel up-regulation of
54 sugar transport and sugar catabolism-encoding genes would indicate a role of IC in nutrition.
55 The data also reveal a substantial remodeling of the IC cell wall and suggest a role for melanin
56 and chitosan in IC function. Lastly, mutagenesis of two up-regulated genes in IC identified
57 secreted fasciclin-like proteins as actors in the pathogenesis of *B. cinerea*. These results support
58 the role of IC in plant penetration but also introduce other unexpected functions for this fungal
59 organ, in colonization, necrotrophy and nutrition of the pathogen.

60 **Introduction**

61 Many phytopathogenic fungi differentiate specific structures named appressoria that are dedi-
62 cated to the penetration of the host tissues (Emmet and Parbery, 1975; Deising *et al.*, 2000).
63 Appressoria facilitate the breaching of plant cuticles and cell walls through a mechanical and/or
64 chemical action. Early microscopy studies provided clear observations of these structures in
65 different fungal species and distinguished unicellular appressoria (UA) from multicellular ap-
66 pressoria, referred to as infection cushions (IC) (Emmet and Parbery, 1975). Later, UA have
67 attracted much attention and molecular understanding of their development and function has
68 tremendously increased (Ryder and Talbot, 2015). In contrast, IC remain far less understood at
69 the molecular level, even if a first transcriptomic study of IC in *Fusarium graminearum* has
70 recently been published (Mentges *et al.*, 2020).

71 *Botrytis cinerea* is an ascomycetous fungus that causes grey mould disease on more than 1000
72 plant species (Elad *et al.*, 2016). This disease affects fruits, vegetables and ornamental plants
73 around the world, causing considerable losses every year. It has been ranked among the ten
74 most severe fungal plant pathogens due to its negative impact on agronomically important crops
75 and on plant products in post-harvest storage. *B. cinerea* is considered a typical necrotrophic
76 fungus and has become a model to study plant infection (Dean *et al.*, 2012). It is characterized
77 by its ability to produce either UA or IC *in vitro* or *in planta* (Choquer *et al.*, 2007).

78 The *B. cinerea* IC are multicellular appressoria that develop *in planta* between 24 h to 48 h after
79 spore germination. Histological studies have revealed IC from *B. cinerea* on a wide variety of
80 infected plant hosts and organs: carrot roots (Sharman & Heale, 1977); bean or mung bean
81 hypocotyls (Garcia-Arenal & Sagasta, 1980; Backhouse & Willets, 1987), bean, cucumber or
82 oilseed rape leaves (Akutsu *et al.*, 1981; Van den Heuvel & Waterreus, 1983; Zhang *et al.*,
83 2010), stone fruit or waxflower flowers (Fourie & Holtz, 1994; Dinh *et al.*, 2011), lemon or
84 persimmon fruits (Fullerton *et al.*, 1999; Rheinländer *et al.*, 2013) and onion epidermis

85 (Choquer *et al.*, 2007). Lastly, *B. cinerea* IC also develop *in vitro*, in culture over hard surfaces
86 (Backhouse and Willetts, 1987).

87 IC have been described in several other Leotiomycetes fungi: *Sclerotinia sclerotiorum* (Tariq
88 & Jeffries, 1984), *Sclerotinia minor* (Lumsden & Wergin, 1980), *Sclerotinia trifoliorum* (Prior
89 & Owen, 1964), *Dumontinia tuberosa* (Pepin R, 1980), *Stromatinia cepivora* (Stewart *et al.*,
90 1989) and *Tapesia yallundae* (Daniels *et al.*, 1991). In parallel, IC have been described in the
91 Sordariomycetes *Fusarium graminearum* (Boenisch & Schäfer, 2011), and in the Basidiomy-
92 cota *Athelia rolfsii* (Smith *et al.*, 1986), *Rhizoctonia solani* (Demirci & Döken, 1998) and *Rhi-*
93 *zoctonia tuliparum* (Gladders & Coley-Smith, 1977).

94 The recent characterization of several avirulent mutants of *B. cinerea* revealed the importance
95 of IC in the infectious process of this necrotrophic fungus (De Vallée *et al.*, 2019) and hypo-
96 virulent strains of *B. cinerea* infected by mycoviruses are also deficient in IC formation (Zhang
97 *et al.*, 2010; Hao *et al.*, 2018). Although it has long been accepted that IC mediate penetration,
98 their differentiation as well as their functions are still poorly understood. By using a tran-
99 scriptomic approach, the aim of this study was to provide new molecular information that high-
100 lights biological processes specifically at work in mature IC of *B. cinerea*.

101 The transcriptomic results, supported by a secretome analysis, are consistent with IC being
102 structures dedicated to the secretion of fungal effectors important for plant penetration and col-
103 onization: phytotoxins, ROS, hydrolytic enzymes and plant cell death-inducing proteins. More-
104 over, the data reveal a deep remodeling of the IC cell wall composition suggesting the im-
105 portance of melanin and chitosan in the function of IC. The hypothesis of a role for IC in the
106 nutrition of the parasite is also proposed.

107

108 **Results**

109 **Microarray study of IC**

110 To gain information on the role of IC in the biology of *B. cinerea*, we identified genes expressed
111 in these structures. As IC develop onto solid surfaces, potato dextrose agar plates were used for
112 their production while potato dextrose broth was used to produce the control vegetative myce-
113 lium. Conidia were used as inoculum, and cellophane sheets were overlaid onto the plates to
114 increase the production of IC and to facilitate their harvest. To obtain as much mature IC as
115 possible, the samples were collected at 44 hpi. At that time, IC were fully differentiated and
116 hyperbranched (Fig. 1), and they covered about 40% of the plates surface (Fig. S1a) while the
117 liquid cultures produced only mycelium (Fig. S1b). Both biological materials were used to ex-
118 tract total RNA and to prepare cDNAs that were hybridized to microarrays carrying probes of
119 11,134 *B. cinerea* genes (Table S1).

120 Data processing, quality controls and differential expression analysis of the microarrays data
121 led to the listing of 1,231 up-regulated genes and 1,422 down-regulated Bcin genes (fold change
122 ≤ -2 or ≥ 2 ; FDR < 0.05 ; Table S1) in the IC-enriched sample (hereafter referred to as IC) when
123 compared to the control vegetative mycelium (13% and 15% of the 9,410 expressed genes,
124 respectively). The reliability of this result was tested by RT-qPCR analysis. For this, new IC
125 and new control mycelium were produced in order to extract new RNAs. The RT-qPCR reac-
126 tions were run on 39 genes selected among the up-, down- and non-regulated genes identified
127 by the microarray study (the selection covered genes with different fold-changes). The results
128 confirmed the microarrays data in 85% of the cases (33 genes) and showed no contradiction
129 with the transcriptomic data in the remaining 15% of the cases (Table S2). Altogether, this
130 granted the microarray data a good level of confidence.

131

132 **Functional enrichment analysis of differentially expressed genes in IC**

133 Enrichment analyses was performed on the up- and down-regulated genes in IC. By using the
134 GO biological process classification (Gene Ontology; Fig. S2), a significant enrichment was
135 revealed for carbohydrate metabolic process (58 genes), oxidation-reduction process
136 (136 genes), metabolism process (70 genes), transmembrane transport (76 genes) and proteol-
137 ysis (24 genes). For down-regulated genes, rRNA processing (15 genes), ribosome biogenesis
138 (9 genes) and tRNA splicing (5 genes) showed enrichment. In order to extract biological mean-
139 ing from the GO analysis, we used existing databases and published data to manually sort the
140 transcriptional data into 22 functional subcategories (Table S1). Fisher's exact tests confirmed
141 the enrichment of 18 subcategories relating to virulence: plant degradation, production of phy-
142 totoxins (and other secondary metabolites) and ROS, plant cell death induction, and fungal cell
143 wall remodeling, nutrition and secretion (Table 1).

144

145 **Fungal protein effectors: enzymes degrading the plant tissues and plant cell death-induc-** 146 **ing proteins**

147 The carbohydrate-active enzymes database (CAZy, Lombard *et al.*, 2014) and the classifica-
148 tions of Plant Cell Wall Degrading Enzymes (PCWDE; Van den Brink & de Vries, 2011; Glass
149 *et al.*, 2013) were used to subclassify 126 predicted PCWDE-encoding genes in *B. cinerea* ac-
150 cording to their putative specificity for cellulose (C), hemicellulose (H), pectin (P) or overlap-
151 ping specificity (H, P or C) (Table S1). As shown in Table 1, genes coding for hemicellulases,
152 pectinases, and PCWDE of overlapping specificity were significantly enriched among the up-
153 regulated genes in IC, while the genes coding for cellulases were not. Genes coding for cu-
154 tinases and proteases were also found significantly enriched among the up-regulated genes in
155 IC (Table 1). In particular, the aspartyl proteases (Ten Have *et al.*, 2010), sedolisins and metal-
156 lopeptidases subcategories were enriched. Altogether, these results suggest a role for IC in the

157 degradation of the plant barriers and tissues through the production and secretion of hydrolytic
158 enzymes.

159 In addition, 6 genes encoding plant cell death-inducing proteins (CDIP; Li *et al.*, 2020) were
160 up-regulated in IC: *BcPg2* and *BcPg3* (Kars *et al.*, 2005), *BcXyn11A* (Brito *et al.*, 2006),
161 *BcNep2* (Schouten *et al.*, 2008), *BcXyg1* (Zhu *et al.*, 2017) and the homolog of VmE02 (Nie *et*
162 *al.*, 2019). The subcategory of CDIP is significantly enriched among the up-regulated genes in
163 IC (Table 1) and this would suggest that IC actively participate in the triggering of plant cell
164 death.

165

166 **Fungal small molecule effectors: phytotoxins and reactive oxygen species (ROS).**

167 Detailed examination of the predicted 42 genes that code for Secondary Metabolism (SM) key
168 enzymes in *B. cinerea* B05.10 strain (Table S1; Collado & Viaud, 2016) showed that 11 were
169 up-regulated in IC (Table 1; Fig. S3). Noticeably, two SM gene clusters, responsible for the
170 production of botcinic acid and botrydial phytotoxins (Dalmais *et al.*, 2011; Porquier *et al.*,
171 2019), were both significantly enriched among the up-regulated genes in IC (subcategories “10”
172 and “11” in Table S1; Table 1; Fig. 2a). In addition, the *Bcreg1* gene (Bcin03g07420) encoding
173 the transcriptional regulator required for the synthesis of these toxins (Michielse *et al.*, 2011)
174 was also up-regulated (Table S1). At last, we observed that five remaining up-regulated SM
175 key enzyme-encoding genes are neighbored by genes whose expression was also up-regulated
176 in IC, and whose description is compatible with SM typical genes (Fig. S4). This suggests the
177 existence of four additional putative SM clusters transcriptionally induced in *B. cinerea* IC.
178 These gene clusters include the *Bcdtc3*, *Bcpks7*, *Bcpks8* or *Bcpks4/Bcnrps8* genes.

179 Detailed examination of the predicted 54 genes that code for ROS producing systems in *B. ci-*
180 *neria* (Siegmond & Viefhues, 2016) showed that 14 were up-regulated in IC, making this gene
181 family an enriched subcategory (Table 1; Table S1). These genes encode the catalytic subunit

182 BcNoxB of the NADPH oxidase and its regulator BcNoxR (Segmüller *et al.*, 2008), the glucose
183 oxidase BcGod1 (Rolke *et al.*, 2004), the galactose oxidase BcGox1, the quinone oxidoreduc-
184 tase BcNqo1 (An *et al.*, 2016), as well as 3 laccases (BcLcc6, 8, 13) and 6 glucose-methanol-
185 choline (GMC) oxidoreductases. As ROS play a role in the early phases of plant infection by
186 *B. cinerea* (Govrin and Levine, 2000), up-regulation of these genes suggested that IC could
187 secrete more ROS than vegetative mycelium. To test this hypothesis, *B. cinerea* was cultured
188 for 48 h on liquid PDB medium overlaid with cellophane sheets and the collected culture me-
189 dium was exposed to 3,3' diaminobenzidine (DAB) as previously described (Viefhues *et al.*,
190 2014). In comparison with the vegetative mycelium, a stronger oxidation of DAB was observed
191 with the IC-culture medium (Fig. 2b), indicating the presence of likely higher H₂O₂ concentra-
192 tion and secretion of peroxidase activity.

193

194 **Melanization and chitin deacetylation remodel the fungus IC cell wall**

195 In *B. cinerea*, dihydroxynaphthalene-(DHN)-melanins are produced and their biosynthesis re-
196 lies on a bipartite pathway operating in conidia or in sclerotia (Schumacher, 2016). In conidia,
197 the genes coding for the polyketide synthase BcPks13 and for the hydrolase BcYgh1 are in-
198 duced by the light transcription factor BcLft2. In sclerotia, the polyketide synthase BcPks12
199 encoding gene is induced by the transcription factor SMR1 and repressed by BcLft2 (Cohrs *et*
200 *al.*, 2016). In IC, *BcPks13*, *Bcygh1*, *Bcltf2* and the other downstream genes of the pathway were
201 up-regulated while *BcPks12* and *Bcsmr1* were down-regulated (Fig. 3a-c). This would indicate
202 that IC produce DHN-melanins by using the biosynthetic pathway at play in conidia. Schu-
203 macher (2016) proposed laccases as potential candidates catalyzing the polymerization of
204 DHN-melanins monomers in *B. cinerea*. As *BcLcc6* and *BcLcc8* laccases genes are up-regu-
205 lated in IC, they could eventually play this role (Sapmak *et al.*, 2015). In addition, thick dark
206 cell walls were observed in IC (Fig. 3d, top), suggesting that DHN-melanins are deposited at

207 the contact zones between the hyperbranched IC lobes. In the presence of tricyclazole, an in-
208 hibitor of the THN reductases BcBrn1 and BcBrn2 (Fig. 3b), hyperbranched IC lobes were still
209 formed but their dark cell walls were not visible anymore. Orange cell walls were observed
210 instead (Fig. 3d, bottom), likely due to accumulation of T4HN and T3HN and their autooxida-
211 tion products flaviolin and 2-hydroxyjuglone (Schumacher, 2016). These results confirm the
212 hyper-melanization of IC cell walls and suggest that melanization and hyperbranching are in-
213 dependent processes.

214 Chitin deacetylases (CDA) are enzymes that produce chitosan from chitin (Fig. 4a). Based on
215 conserved protein motifs identified in CDAs (Liu *et al.*, 2017), the genome of *B. cinerea* puta-
216 tively encodes five CDA (data not shown). Three of these genes were up-regulated in IC when
217 compared to the control mycelium (Fig. 4b; Table S1) while *in planta* RT-qPCR analysis
218 showed up-regulation of these 5 genes at the early phase of bean leaves infection (Fig. 4c).
219 Whether the up-regulation of CDA genes impacted the IC cell walls was addressed by using
220 confocal microscopy and differential staining of chitin and chitosan. This allowed the specific
221 visualization of chitosan in IC, hooks and their generating hyphae, but not in the vegetative
222 mycelium (Fig. 4d). This result confirms that some chitin is converted into chitosan in the IC
223 cell wall.

224

225 **Up-regulation of sugar uptake and catabolism in IC**

226 The “Sugar transporters” subcategory was explored by mining the BotPortal database
227 (<http://botbioger.versailles.inra.fr/botportal>) using the four InterPro domains IPR003663,
228 IPR005829, IPR007271, and IPR004689. This led to the listing of 86 predicted sugar transport-
229 ers in the genome of *B. cinerea* (Table S1), among which 22 were up-regulated in IC (Table 1).
230 This up-regulation suggests a possible activation of the sugar catabolic pathways and, noticea-
231 bly, two genes of the glycolysis pathway were up-regulated in IC, coding for the fructose-

232 bisphosphate aldolase (Bcin07g03760; Table S1) and the glucose-6-phosphate isomerase *Bcpgi*
233 (Bcin15g04970; Table S1). Besides, in the gluconeogenesis pathway, the *BcPck1* gene
234 (Bcin16g00630; Table S1) encoding the phosphoenolpyruvate carboxykinase was also up-reg-
235 ulated in IC. Liu *et al.* (2018) showed that this key gene is crucial for *B. cinerea* virulence and
236 for the formation of IC in the absence of glucose. In addition to the enriched metabolic pathways
237 that relate to the degradation of polysaccharides (pectin, glycans), these results suggest an acti-
238 vated sugar catabolism in IC.

239

240 **IC secretome analysis validates the transcriptome analysis**

241 Of the 1,231 up-regulated genes in IC, 266 were predicted to code for putative secreted proteins
242 with a N-terminal signal peptide (Table 1 and Table S1) To further investigate this data, the
243 secretomes of IC and control mycelium were compared. *B. cinerea* was grown on cellophane
244 sheets overlaying potato dextrose broth and the culture media were collected at 24 h (control
245 mycelium) or at 48 h when IC covered ~40% of the cellophane surface. The proteins were
246 extracted and subjected to a comparative and quantitative proteomics analysis. 79 proteins iden-
247 tified in 3 biological replicates with a minimum of 2 unique peptides were listed as up-accumu-
248 lated (Fold change ≥ 3 , FDR < 0.05) in IC (Table 2 and Table S3). Comparison of the prote-
249 omics and microarray data (Table S3) showed that 40 of the up-accumulated proteins (51%)
250 are encoded by genes up-regulated in IC. By using the GO biological process classification, we
251 observed that these secreted proteins are essentially involved in carbohydrate and polysaccha-
252 ride metabolic processes (27 proteins), proteolysis (16 proteins), oxidation-reduction process
253 (12 proteins), and other metabolic process (2 proteins). All these GO categories were found
254 enriched in the analysis of the genes up-regulated in IC (Fig. S2). We then used existing data-
255 bases and published data to manually sort the proteomics data into functional subcategories.
256 We identified virulence-related subcategories similar to those issued from the transcriptome

257 analysis: degradation of plant cuticle, plant cell wall and starch, production of ROS, other de-
258 grading enzymes (e.g. nucleases, phosphatases and esterases) and plant cell death-inducing pro-
259 teins. Besides, six CDIP were up-accumulated in the secretome of IC: BcXyn11A (Brito *et al.*,
260 2006), BcNep2 (Schouten *et al.*, 2008), BcSpl1 (Frias *et al.*, 2013), BcGs1 (Zhang *et al.*, 2015),
261 BcIeb1 (Frias *et al.*, 2016) and BcXyg1 (Zhu *et al.*, 2017). Lastly, a new subcategory enriched
262 in the IC secretome could be identified that corresponds to fungal enzymes implicated in the
263 crosslinking of cell wall polysaccharides. This suggests possible modifications of the covalent
264 links between chitin, glucans and proteins of the IC cell wall. Altogether, the proteomics data
265 support the transcriptomic data and strengthen the signatures of cell wall remodeling and secre-
266 tion of hydrolytic enzymes or effectors.

267

268 **Relevance of the microarray data to plant infection**

269 Fourteen up-regulated genes in IC whose predicted function relates to pathogenesis were se-
270 lected for a time course RT-qPCR analysis *in planta* (bean leaves infected with conidia of *B. ci-*
271 *neria*; Fig. 5a and 5b). All the selected genes were expressed *in planta* suggesting that the
272 microarray data are relevant to plant infection. Twelve of the studied genes were up-regulated
273 at days 2, 3 and/or 4 of infection, when IC were visible at the leaf surface. When only hooks
274 could be observed (day 1), most of these genes (11) were not expressed. Furthermore, expres-
275 sion peaked at day 2 for 7 genes, when IC were visible, but plant tissue maceration was not.
276 These results suggest that these genes expressed early in IC could play a role in events trigger-
277 ing plant cell death.

278

279 **Fasciclin-like genes up-regulated in IC are actors in the virulence of *B. cinerea*.**

280 Fasciclins play a role in the virulence of the rice pathogen *M. oryzae* (Liu *et al.*, 2009) via a
281 possible role in development or in autophagy (Seifert, 2018). In *B. cinerea*, two genes encode

282 fasciclin-like proteins (*Bcflp1*; Bcin04g05020 and *Bcflp2*; Bcin09g05010) and both these genes
283 were up-regulated in IC. Following confirmation of this differential expression by RT-qPCR *in*
284 *vitro* and *in planta* (Table S2; Fig. 5), and since BcFlp1 was up-accumulated in the secretome
285 of IC (Table 2 and Table S3), these two genes were selected for mutagenesis. By using a gene
286 replacement strategy, single mutant strains ($\Delta Bcflp1$ and $\Delta Bcflp2$) and the double mutant strain
287 ($\Delta Bcflp1::\Delta Bcflp2$) were constructed. These mutants were genetically purified, and the geno-
288 types were verified by PCR and Southern blotting (Fig. S5). Two independent transformants of
289 each mutant were then characterized. IC formation could be observed in all mutants (data not
290 shown), indicating that the two fasciclin-like proteins are not required for the differentiation of
291 hyphae into IC in *B. cinerea*. By contrast, bean leaves infection assays showed that the mutants
292 were affected in their pathogenesis (Fig. 6a-b). In the $\Delta Bcflp1$ mutant, the colonisation rate was
293 similar to that of the wild type, but apparition of the symptoms was delayed (estimated at 12h).
294 In the $\Delta Bcflp2$ and $\Delta Bcflp1::\Delta Bcflp2$ mutants, the colonisation rate was reduced and stopped
295 after 4 days. Noticeably, a dark ring was visible at the periphery of the macerated tissues. *In*
296 *vitro*, neither *Bcflp1* or *Bcflp2* deletion impaired hyphal growth on rich medium (data not
297 shown). On minimal medium, the $\Delta Bcflp2$ and $\Delta Bcflp1::\Delta Bcflp2$ mutants were moderately im-
298 paired in hyphal growth, but the $\Delta Bcflp1$ mutant was not (Fig. 6c). Altogether, the data could
299 indicate that Bcflp1 plays a role during the early stage of the infection process while Bcflp2
300 could play a role at a later stage. At last, the phenotype of the double mutant indicates a domi-
301 nant effect of the $\Delta Bcflp2$ mutation over $\Delta Bcflp1$.

302 **Discussion**

303 A role of IC in plant penetration and potentially in nutrition.

304 In plant pathogenic fungi, IC have been proposed to play an important role in host penetration.

305 In this study, four cutinase-encoding genes and 48 predicted PCWDE-encoding genes were
306 revealed as up-regulated in *B. cinerea* IC. These genes include the endopolygalacturonase
307 *BcPg2* (Bcin14g00610), the endoxylanase *BcXyn11A* (Bcin03g00480), and the endoarabi-
308 nanase *BcAra1* (Bcin02g07700) required for full virulence of *B. cinerea* (Kars *et al.*, 2005;
309 Brito *et al.*, 2006; Nafisi *et al.*, 2014). Besides, aspartyl proteases, sedolisins and metallopepti-
310 dases-encoding genes were also significantly up-regulated. A proteomic analysis of IC secre-
311 tome confirmed the up-accumulation of CAZymes and proteases when compared to vegetative
312 mycelium. Altogether these results support a role for IC in host penetration *via* enzymatic deg-
313 radation of plant cell walls.

314 Unexpectedly, 25 genes coding for sugar transporters and sugar metabolic enzymes were up-
315 regulated in IC. In connection with the up-regulation of PCWDE-encoding genes, this suggests
316 that IC could play a role in nutrition by importing and catabolizing sugar molecules originating
317 from plant polysaccharides degradation. This would compare to the unicellular appressorium
318 of the rice pathogen *M. oryzae* which, besides plant penetration, would also serve to feed on
319 host plant carbohydrates (Soanes *et al.*, 2012).

320

321 A role of IC in establishing necrotrophy ?

322 Two known SM gene clusters are up-regulated in IC. These clusters are responsible for the
323 production of botcinic acid and botrydial, two phytotoxins playing a role in plant infection by
324 *B. cinerea* (Cutler *et al.*, 1993 & 1996; Deighton *et al.*, 2001; Dalmais *et al.*, 2011; Massaroli
325 *et al.*, 2013; Collado & Viaud, 2016). Botrydial sesquiterpene and its derivatives botryanes are
326 proposed as fungal effectors manipulating plant host defenses, promoting cell death and thus

327 enabling *B. cinerea* to feed on necrotic tissues (Rossi *et al.*, 2011). Botcinic acid polyketide and
328 its structurally related botcinins and botrylactones cause plant chlorosis and necrosis (Cutler *et*
329 *al.*, 1993). Double inactivation of these SM clusters led to a defect of pathogenesis and demon-
330 strated the concerted action of the two toxins (Dalmais *et al.*, 2011). Interestingly, the co-ex-
331 pression of these clusters in IC supports this concerted action. In addition, four putative SM
332 clusters were also up-regulated in IC. The role these clusters might play in virulence and the
333 putative metabolites produced as a result of their activation await characterization. It is note-
334 worthy that several orphan SM have been reported in *B. cinerea* while their biosynthesis genes
335 remain unknown (Collado and Viaud, 2016). Additionally, it was proposed that secretion of
336 new sesquiterpenoid metabolites, called eremophilinols, could promote and regulate the pro-
337 duction of *B. cinerea* IC, perhaps acting as an endogenous signal (Pinedo *et al.*, 2016).

338 During the early phase of plant-fungal interaction, plants produce ROS as part of their defense
339 mechanisms, but ROS are also produced by the pathogen as cell death inducers. In order to
340 produce and to cope with ROS, *B. cinerea* is equipped with multiple oxidoreductases. Chemical
341 compounds or mutations that target some of these enzymes, or their encoding genes, impair
342 virulence (Rolke *et al.*, 2004; Segmüller *et al.*, 2008; An *et al.*, 2016; Siegmund & Viefhues,
343 2016). The analysis of the up-regulated genes in IC highlighted 136 genes related to the oxida-
344 tion-reduction process, among which 15 genes coding for putative ROS producing enzymes. In
345 addition, higher levels of H₂O₂ were detected in IC culture media compared to vegetative my-
346 celium control media. This suggests that IC secretes high amounts of ROS and would therefore
347 be consistent with the production of ROS observed in IC during penetration of onion epidermis
348 (Choquer *et al.*, 2007; Marschall & Tudzynski, 2016b). Several gene mutations which impair
349 IC formation in *B. cinerea* have been identified in recent years. Interestingly these genes are
350 often associated with oxidoreduction processes and play a role in the development and virulence
351 of *B. cinerea*. These genes encode the regulator of oxidative stress response BcSkn7 (Viefhues

352 *et al.*, 2015), the scaffold protein BcIqg1 involved in resistance against oxidative stress (Mar-
353 schall and Tudzynski, 2016a), the aquaporin BcAqp8 involved in ROS production (An *et al.*,
354 2016), the ER protein BcPdi1 involved in redox homeostasis (Marschall and Tudzynski, 2017)
355 and the H3K4 demethylase BcJar1 orchestrating ROS production (Hou *et al.*, 2020).

356 During the early phase of plant infection by *B. cinerea*, cell death inducing proteins (CDIP)
357 (Qutob *et al.*, 2007; Cuesta Arenas *et al.*, 2010) could trigger plant cell death from which the
358 fungus would benefit to achieve full virulence (Govrin and Levine, 2000; Govrin *et al.*, 2006).
359 Up-regulation of CDIP-encoding genes and/or secretion of CDIP have been recorded in *B. ci-*
360 *nerea* (Shlezinger *et al.*, 2011; González *et al.*, 2016; Noda *et al.*, 2010; Zhu *et al.*, 2017). Our
361 study reveals gene up-regulation and/or protein up-accumulation for 9 of the 14 known CDIPs
362 in *B. cinerea*. Altogether, the up-regulation of genes involved in the production of CDIP, phy-
363 totoxins and ROS argues for IC playing a role in the establishment of the necrotrophic lifestyle
364 of *B. cinerea*.

365 In *Colletotrichum* unicellular appressoria, genes encoding effectors and secondary metabolism
366 enzymes are induced before penetration and during biotrophy while genes encoding most hy-
367 drolases and transporters are up-regulated later, at the switch to necrotrophy (O'Connell *et al.*,
368 2012). In comparison, the concomitant expression of all these genes recorded herein could also
369 support a role of *B. cinerea* IC in necrotrophy.

370

371 Cell wall remodeling in IC.

372 In *B. cinerea*, cell walls are darkened by DHN-melanins. These pigments are secondary metab-
373 olites produced via a bipartite pathway in *B. cinerea*, so far identified as specific to conidia or
374 sclerotia (Schumacher, 2016). The gene expression data reported in this study indicate that the
375 conidial metabolic pathway is activated in IC, while the sclerotial pathway is not. This result
376 suggested that IC produce melanin, as observed in unicellular melanized appressoria (Soanes,

377 2012). Microscopic observation of thick dark cell walls in IC that are sensitive to DHN-mela-
378 nins inhibitors brought experimental support to this. Melanins biosynthesis is dispensable for
379 virulence in *B. cinerea* (Schumacher, 2016) and the reason of their increased production in IC
380 remains to be clarified. It could play a role in the fungus survival during the oxidative burst of
381 host plants (Govrin and Levine, 2000), but it could also play a role in the cross-linking of cell
382 wall components (Franzen *et al.*, 2006), the binding of proteins (Mani *et al.*, 2001) or that of
383 metals (Fogarty and Tobin, 1996).

384 Three genes coding for chitin deacetylases (CDA) are up-regulated in IC. Moreover, differential
385 staining of chitin and chitosan allowed microscopic observations of chitosan in IC and not in
386 vegetative hyphae. This indicates that some chitin is deacetylated into chitosan in the cell wall
387 of IC. Since CDA can play a role in *Aspergillus fumigatus* polar growth (Xie *et al.*, 2020) or in
388 *Magnaporthe oryzae* appressorium differentiation (Kuroki *et al.*, 2017), the transformation of
389 chitin into chitosan in *B. cinerea* might be involved in the change of hyphal growth that leads
390 to IC formation. Alternatively, the production of chitosan by CDA could play a role in cell wall
391 anchoring of melanins, cell wall integrity, osmotic stability, modulation of extra-cellular poly-
392 saccharide production and/or adhesion to surfaces (Baker *et al.*, 2007; Geoghegan and Gurr,
393 2016; Perez-Dulzaides *et al.*, 2018; Chrissian *et al.*, 2020). At last, chitin deacetylation could
394 prevent detection by the host immune system (Cord-Landwehr *et al.*, 2016; Upadhyya *et al.*,
395 2018; Lam *et al.*, 2019).

396 Another cell wall-related gene up-regulated in *B. cinerea* IC is *Bcsun1* (Bcin06g06040), a beta-
397 glucosidase-encoding gene that is required for full production of IC and full virulence of *B.*
398 *cinerea* (Pérez-Hernández *et al.*, 2017). This adds to the up-regulation of the DHN melanin-
399 biosynthesis and the CDA genes, and to the up-accumulation of 7 fungal cell wall polysaccha-
400 rides cross-linking enzymes in the IC secretome. Altogether, this argue for a remodeling of the
401 cell wall in IC.

402

403 Evidencing new putative virulence factors from the IC transcriptome.

404 Based on the up-regulation of their encoding genes in IC, a two-member family of fasciclin-
405 like proteins was selected for functional study. Plant colonisation was respectively delayed and
406 arrested in the *Bcflp1* and *Bcflp2* deletion mutants. This suggests a role for these genes in the
407 infection process of *B. cinerea*, at an early stage for *Bcflp1* and at a later stage for *Bcflp2*. As
408 fasciclins could be involved in autophagy in *M. oryzae* and *Schizosaccharomyces pombe* (Liu
409 *et al.*, 2009; Sun *et al.*, 2013), one may hypothesize a similar function for BcFlp1 and/or BcFlp2,
410 but this needs to be explored.

411 In a recent study, we collected transcriptomic and proteomic data from four non-pathogenic
412 mutants of *B. cinerea* that do not produce IC (De Vallée *et al.*, 2019). Comparison of these data
413 to those presented here showed that 40 of the first 100 up-regulated genes in IC are down-
414 regulated in all 4 mutants (Fig. S6). In addition, 47% of the proteins up-accumulated in the
415 culture medium of IC are down-accumulated in the secretome of the 4 IC-deficient mutants
416 (Table S3). These genes and proteins represent a list of potential virulence factors/effectors in
417 *Botrytis cinerea*.

418

419 Concluding remarks

420 This *in vitro* study of IC revealed several enriched categories of up-regulated genes (CAZymes,
421 putative effectors, secondary metabolism, ..) that were highlighted in dual-transcriptomes of
422 *B. cinerea* infecting lettuce, tomato, grapevine, cucumber or *Arabidopsis* (Blanco-Ulate *et al.*,
423 2014; Kelloniemi *et al.*, 2015; Kong *et al.*, 2015; Zhang *et al.*, 2019; Zhang *et al.*, 2020). It is
424 therefore relevant to plant infection by *B. cinerea*. Interestingly, the recent transcriptome anal-
425 yses of *F. graminearum* IC developed *in planta* also showed an enrichment of the same

426 categories of genes (Mentges *et al.*, 2020), and suggests that IC might share conserved virulence
427 functions in different plant pathogenic fungi.
428

429 **Experimental Procedures**

430 **Fungal strains and growth conditions**

431 *Botrytis cinerea* B05.10 conidia were collected in PDB medium (Difco) diluted $\frac{1}{4}$ (PDB $\frac{1}{4}$),
432 after ten days of culture on malt sporulation medium, at 21°C under near-UV light. All subse-
433 quent cultures were done in the dark at 21°C. For infection cushions formation, $2 \cdot 10^5$ conidia
434 were spread onto cellophane sheets overlaying PDB $\frac{1}{4}$ medium supplemented with agar (25 g.l⁻¹
435 ¹), and the plates were incubated for 44 h. For the control sample, $2 \cdot 10^5$ conidia were inoculated
436 in 50 ml PDB $\frac{1}{4}$ medium and the 250 ml-flasks were agitated (110 rpm) for 44 h. The flasks
437 were previously siliconized with Sigmacote (Sigma) to prevent mycelium adhesion and IC for-
438 mation on the glassware. For radial growth measurements, wild-type and mutant strains were
439 grown on minimal medium (NaNO₃ 2 g.l⁻¹, Glucose 20 g.l⁻¹, KH₂PO₄ 0.2 g.l⁻¹, MgSO₄, 7H₂O
440 0.1 g.l⁻¹, KCl 0.1 g.l⁻¹, FeSO₄, 7H₂O 4 mg.l⁻¹). For secretome analysis, $2 \cdot 10^5$ conidia were spread
441 onto cellophane membrane overlaying solid PDB $\frac{1}{4}$. After 6 h incubation (21°C), membranes
442 were transferred on 2 ml liquid PDB $\frac{1}{4}$ for 24h (Control mycelium condition) or 48 h (IC con-
443 dition) at 21°C. Liquid medium was then collected for proteomic analysis. For microscopy, 10^4
444 conidia or single mycelial plug served to inoculate PDB $\frac{1}{4}$ medium in 6-wells microplates ().
445 The plates were incubated 44h at 21°C in the dark.

446 **Microarray expression analysis**

447 To study the transcriptome of *B. cinerea*, NimbleGen 4-plex arrays containing 4 x 72,000 arrays
448 per slide were used (Roche, Mannheim, Germany). Construction of this chip was initially based
449 on combining two previous genome annotations (Amselem *et al.*, 2011), the one of the T4 strain
450 by URGI (BofuT4 gene references; www.urgi.versailles.inra.fr) and the one of the B05.10
451 strain by the Broad Institute (BC1G gene references; www.broadinstitute.org). Thus, 62,478
452 60-mer oligonucleotides were designed as specific probes covering 20,885 predicted gene

453 models and non-mapping Expressed Sequence Tags (EST) (3 oligonucleotides per gene or
454 EST) and 9,559 random probes were designed as negative controls.

455 The structural annotation used for this study was published by van Kan *et al.* (2017), displaying
456 11,710 predicted genes, associated to 13,749 predicted proteins, unlike the two previous anno-
457 tations showing more than 16,000 predicted genes (BofuT4 and BC1G). This annotation is con-
458 sidered better on the basis of RNA-seq data and is available at EnsemblFungi under the refer-
459 ence *Botrytis cinerea* B05.10 (ASM83294v1; Bcin gene references; http://fungi.ensembl.org/Botrytis_cinerea/). 11,710 Bcin genes are predicted but not all of them were analyzed
460 by the *B. cinerea* NimbleGen 4-plex arrays. In order to associate each BofuT4 and BC1G gene
461 from the chip to only one Bcin gene and reciprocally, we used the correspondence established
462 in the *B. cinerea* Portal (Simon & Viaud, 2018; <http://botbioger.versailles.inra.fr/botportal/>). In
463 a hundred of cases, BofuT4/BC1G genes showing correspondence with multiple Bcin genes
464 were checked by gene synteny. After this manual curation, we identified 11,630 BofuT4/ BC1G
465 genes out of 15,750 showing correspondence with only one Bcin gene. When a Bcin gene
466 showed correspondence with multiple BofuT4/ BC1G genes, we selected the BofuT4 or BC1G
467 gene giving the highest normalized intensities. After these two manual curations, we found that
468 11,134 Bcin genes were analyzed by the chip which represent 95% of the published Bcin genes
469 (11,710; Table S1). The EST and the small coding sequences (< 100 amino acids) lacking EST
470 support were excluded from our analysis.

472 Total RNA was extracted from 4 mg of ground lyophilized material using the RNeasy
473 Midi kit (Qiagen). A DNase treatment (Ambion) was performed to remove traces of genomic
474 DNA. RNA profiles were assessed using the Bioanalyzer RNA 6000 Nano kit (Agilent). Ten
475 micrograms of total RNA were converted into cDNA using the SuperScript II cDNA Conver-
476 sion Kit (Invitrogen). Double stranded cDNAs were then labelled with Cy3-nonamers using
477 NimbleGen One-Color DNA Labeling Kit before hybridization on the NimbleGen 4-plex

478 arrays. Microarrays were then scanned with an Agilent scanner at 532 nm (Cy3 absorption
479 peak) optimized for Nimblegen 4-plex arrays. All steps were performed following the proce-
480 dures established by NimbleGen. The entire microarray data set described in this article is avail-
481 able at the Gene Expression Omnibus (GEO) database under accession number GSE141822.

482 Data processing, quality controls, differential expression analysis and clustering were
483 performed using ANAIS methods (Simon and Biot, 2010). Hybridization signals of all probes,
484 comprising three and four independent replicates for mycelium condition and infection cushion
485 condition, respectively, were subjected to RMA-background correction, quantile normalization,
486 and gene summarization. Thresholds of gene expression were determined by referring the hy-
487 bridization signals to those of 9,559 random probes, calculated for each array using the R soft-
488 ware (R Core Team, 2019). Genes were considered expressed when their normalized intensity
489 was higher than the 99th percentile of random probes hybridization signals in at least one bio-
490 logical replicate. These genes were kept for differential expression analysis. Differentially ex-
491 pressed genes, between the infection cushion condition and the mycelium condition, were iden-
492 tified using a one-way ANOVA test. To deal with multiple testings, the ANOVA p-values were
493 submitted to a False Discovery Rate correction (FDR). Transcripts with a corrected p-value
494 <0.05 and for which a fold change ≤ -2 or ≥ 2 was observed between the two conditions were
495 considered to display significant differential expression. Clusters analyses of gene normalized
496 intensities were performed to highlight differentially expressed genes.

497 **Enrichment analysis and genes categorization**

498 Further analyses were performed to highlight biological processes potentially enriched in the
499 selected lists of up-regulated or down-regulated Bcin genes in infection cushion. Enrichment in
500 Gene Ontology (GO) Biological Process (BP) terms was assessed on the *Botrytis cinerea*
501 B05.10 (ASM83294v1) species using the Fungifun website (Priebe *et al.*, 2015;
502 <https://elbe.hki-jena.de/fungifun/>). The background dataset of genes used as reference was the

503 11134 Bcin genes which were associated to the chip (Table S1). Presence of a putative signal
504 peptide was predicted using the SignalP 5.0 Server (Almagro Armenteros *et al.*, 2019). The
505 CAZy database (www.cazy.org) was used for the manual curation of plant cell wall degrading
506 enzymes and the alpha-1,2-mannosidases. The InterPro database (www.ebi.ac.uk/interpro/;
507 Mitchell *et al.*, 2019) and the Botportal database (<http://botbioger.versailles.inra.fr/botportal/>;
508 Simon & Viaud, 2018) were used for the manual curation of cytochrome P450s, taurine catab-
509 olism dioxygenases, cutinases, sugar transporters, sedolisins, serine carboxypeptidases and
510 metallopeptidases. Functional categories with significant enrichment were identified using
511 Fisher's exact test with a p-value cutoff at 0.05 (only the Bcin genes analyzed by the chip were
512 used for this test).

513 **Expression profiling by quantitative PCR analysis**

514 Experiments were performed as described by Rasclé *et al.* (2018). RT-qPCR experiments were
515 performed using ABI-7900 Applied Biosystems (Applied Biosystems). Amplification reactions
516 were carried out using SYBR Green PCR Master Mix (Applied Biosystems). Relative quanti-
517 fication was based on the $2^{-\Delta\Delta C(T)}$ method (Livak & Schmittgen, 2001) using the *BcactA*
518 (*Bcin16g02020*), *Bcef1 α* gene (*Bcin09g05760*), and *Bcpd1* gene (*Bcin07g01890*) as normali-
519 zation internal controls. At least three independent biological replicates were analyzed. Primers
520 used for RT-qPCR are shown in Table S5.

521 **ROS and melanin production**

522 For visualization of melanin, a stock solution of the DHN melanogenesis inhibitor tricyclazole
523 (Sigma) was prepared in acetone (10 mg ml⁻¹). Three-day-old 2-mm mycelial plugs were de-
524 posited in 6-well plates. Droplets (50 μ l) of PDB^{1/4} (supplemented or not with 50 μ g ml⁻¹ tricy-
525 clazole) medium were added on the plugs. After 48 h incubation (21°C), IC were observed by
526 reverse microscopy. For visualization of ROS produced during IC formation, 100 μ l of medium
527 were added to 1 ml of DAB (Sigma) solution (0.05% in 100 mM citric acid buffer pH 3.7) and

528 incubated 20 h in darkness with gentle agitation. Controls were done by adding 1 μ l Horse
529 Radish Peroxydase (HRP - ThermoScientific) to 1 ml DAB in presence of different quantities
530 of H₂O₂.

531 **Quantitative proteomic analysis**

532 The steps of sample preparation and protein digestion were performed as previously described
533 (Dieryckx *et al.*, 2015) and online nanoLC-MS/MS analyses were performed using an Ultimate
534 3000 RSLC Nano-UPHLC system (Thermo Scientific) coupled to a nanospray Q Exactive hy-
535 brid quadrupole-Orbitrap mass spectrometer (Thermo Scientific). The parameters of the LC-
536 MS method used were as previously described (Pineda *et al.*, 2018). Protein identification and
537 Label-Free Quantification (LFQ) were done in Proteome Discoverer 2.3. MS Amanda 2.0, Se-
538 quest HT and Mascot 2.4 algorithms were used for protein identification in batch mode by
539 searching against the Ensembl *Botrytis cinerea* B05.10 database (ASM83294v1, 13 749 entries,
540 release 98.3). Two missed enzyme cleavages were allowed. Mass tolerances in MS and MS/MS
541 were set to 10 ppm and 0.02 Da. Oxidation (M), acetylation (K) and deamidation (N, Q) were
542 searched as dynamic modifications and carbamidomethylation (C) as static modification. Pep-
543 tide validation was performed using Percolator algorithm (Käll *et al.*, 2007) and only “high
544 confidence” peptides were retained, corresponding to a 1% false discovery rate at peptide level.
545 Minora feature detector node (LFQ) was used along with the feature mapper and precursor ions
546 quantifier. The normalization parameters were selected as follows: (1) Unique peptides, (2)
547 Precursor abundance based on intensity, (3) Normalization mode: total peptide amount, (4)
548 Protein abundance calculation: summed abundances, (5) Protein ratio calculation: pairwise ra-
549 tio based and (6) Hypothesis test: t-test (background based). Quantitative data were considered
550 for master proteins, quantified by a minimum of 2 unique peptides, a fold change ≥ 3 and a
551 statistical p-value lower than 0.05. The mass spectrometry proteomics data have been deposited

552 to the ProteomeXchange Consortium (<http://proteomecentral.proteomexchange.org>) via the
553 PRIDE partner repository (Perez-Riverol *et al.*, 2019) with the dataset identifier PXD016885.

554 **Construction of deletion mutants in *Botrytis cinerea***

555 $\Delta Bcflp1$ and $\Delta Bcflp2$ deletion mutants were constructed using a gene replacement strategy
556 (Fig.S5). The replacement cassettes were generated by combining double-joint PCR (Yu *et al.*,
557 2004) and split-marker approach (Catlett *et al.*, 2003). All primers are listed in Table S4. The
558 three gene replacement cassettes were verified by sequencing. *B. cinerea* transformation was
559 carried out using protoplasts as previously described by Rascle *et al.* (2018), except that the
560 protoplasts were transformed with 1 μ g of each split-marker cassette DNA and plated on me-
561 dium containing 200 g.l⁻¹ saccharose and 2 g.l⁻¹ NaNO₃ supplemented with 70 μ g.ml⁻¹ hygro-
562 mycin (Invivogen, France) for single $\Delta Bcflp1$ or $\Delta Bcflp2$ mutants or 80 μ g.ml⁻¹ nourseothricin
563 (Werner BioAgents, Germany) for double replacement mutants. Diagnostic PCR was per-
564 formed to detect homologous recombination in the selected resistant transformants. Homokar-
565 yotic transformants were obtained after several rounds of single-spore isolation. Southern blot
566 analyses were performed to ensure single insertions. Genomic DNA digested with *EcoRI* was
567 hybridized with the 3'- flanking region of *Bcflp1* (amplified with Probe1For and Probe1Rev
568 primers) or the 5'-flanking region of *Bcflp2* (amplified with Probe2For and Probe2Rev primers)
569 using the PCR DIG Probe Synthesis Kit and the DIG Luminescent Detection Kit (Roche, Ger-
570 many) following manufacturer's instructions.

571 **Pathogenicity tests**

572 Infection assays were performed with one-week-old French bean (*Phaseolus vulgaris* var *Saxa*)
573 leaves using 4-mm 72-h-old mycelial plugs from *B. cinerea* WT, $\Delta Bcflp1$, $\Delta Bcflp2$ and
574 $\Delta Bcflp1::\Delta Bcflp2$ mutant strains grown on sporulation medium. Infected plants were incubated
575 at 21°C under 100% relative humidity and dark (10 h)-daylight (14 h) conditions. Necrosis zone

576 diameter was measured daily. All tests were assessed in three independent experiments, with at
577 least eight plants and thirty points of infection for each strain.

578 **Microscopy**

579 Confocal microscopy and imaging were performed with a Zeiss LSM510 confocal microscope
580 (Oberkochen, Germany) and its integrated ZEN software. For staining of chitin and chitosan,
581 the fungal culture medium was drained and replaced by 1 ml H₂O containing 20 µl KOH 10%,
582 10 µl Calcofluor (Fluka, 1g/L) and 10 µl eosin Y 0.5% (Sigma). Following 10 min incubation
583 in the dark, the staining solution was drained, the samples were washed 5 times with 1 ml H₂O
584 and overlaid with 0.5 ml H₂O. Fluorescent signals of calcofluor and eosin Y were respectively
585 captured using a 405 nm and 561 nm excitation wavelength.

586

587 **Acknowledgements**

588 We thank Adeline Simon, Lucile Albinet and Luna Nadjare for providing tools and help on
589 bioinformatic analyses. We thank Gwenlyn Fleury for her implication in the study of fasclicin
590 mutants, and Cindy Dieryckx and Vincent Girard for their advices on secretome analysis. We
591 are thankful to Glen Calvar for English editing.

592

593 **Conflict of Interest**

594 The authors have no conflict of interest to declare.

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| Functional subcategories (expert annotation manually curated) | Correspondence with the GO (Biological Process) | Proposed biological roles | Up-regulated genes (1231/11134) | Enrichment p-value | Sources used for curation | |
|--|---|--------------------------------|---------------------------------------|-----------------------|--|-------------------------------|
| 1: PCWDE*_Cellulose (C) | Carbohydrate metabolic process | Plant degradation Nutrition | 5/19 | >5E-2 | CAZy database (Lombard <i>et al.</i> , 2013); www.cazy.org/ Van den Brink & de Vries (2011) ; Glass (2013) | |
| 2: PCWDE*_Hemicellulose (H) | | | 17/32 | 5.6E-9 | | |
| 3: PCWDE*_Pectin (P) | | | 18/48 | 1.6E-6 | | |
| 4: PCWDE*_C_H_P | | 13/26 | 9.1E-7 | | | |
| 5: FCWE**_Chitin deacetylases (CDA) | | Fungal cell wall remodeling | 3/5 | 1.2E-2 | | Liu <i>et al.</i> (2017) |
| 6: Alpha-1,2-mannosidases | | unknown | 4/5 | 6.8E-4 | | GH92 family in CAZy database |
| 7: ROS producing systems | Oxidation-reduction process | ROS | 14/54 | 1.8E-3 | Siegmund and Viefhues (2016) ; Botportal database | |
| 8: ROS detoxification and scavenging | | | 7/41 | >5E-2 | | |
| 9: Cytochrome P450s | | Phytotoxins, others | 26/128 | 2.5E-3 | | IPR001128**** |
| 10: Botcinic acid biosynthesis genes (Boa) | | Phytotoxins | 10/10 | 2.7E-10 | | Porquier <i>et al.</i> , 2019 |
| 11: Botrydial biosynthesis genes (Bot) | | | 7/7 | 5.6E-8 | | Dalmais <i>et al.</i> , 2011 |
| 12: Taurine catabolism dioxygenase TauD/TfdA | | unknown | 4/14 | >5E-2 | | IPR003819**** |
| 13: Secondary metabolism key enzyme genes (e.g. PKS, NRPS, TS, DMATS)**** | Metabolic process | Phytotoxins, ROS, others | 11/42 | 4.9E-3 | Collado and Viaud (2016) | |
| 14: DHN-melanogenic genes | | Fungal cell wall remodeling | 6/11 | 5.1E-4 | Schumacher (2016); Cohrs <i>et al.</i> (2016) | |
| 15: Cutinases | | Plant degradation | 4/11 | 2.6E-2 | IPR000675**** | |
| 16: Sugar transporters | Transmembrane transport | Nutrition | 22/86 | 1.7E-4 | IPR003663; IPR005829; IPR007271; IPR004689**** | |
| 17: Aspartyl Proteases | Proteolysis | Plant degradation Nutrition | 6/14 | 2.5E-3 | ten Have <i>et al.</i> (2010) | |
| 18: Sedolisins | | | 5/10 | 2.6E-3 | IPR030400**** | |
| 19: Serine carboxypeptidases | | | 4/16 | >5E-2 | IPR001563, IPR008758, IPR001375**** | |
| 20: Metallopeptidases | | | 4/10 | 1.9E-2 | IPR024079**** | |
| 21: Putative secreted proteins (signalP) | | | All categories | Virulence | 266/1079 | 8.0E-41 |
| 22: Plant Cell Death-Inducing Proteins (CDIP) | 6/14 | 2.5E-3 | | | Liu <i>et al.</i> (2020) | |

Table 1: Functional enrichment analysis of up-regulated genes in the infection cushion of *Botrytis cinerea*.

| Functional category | Subcategory (expert annotation) | Description | Protein ID | Name | FC | p-value | Sources used for curation |
|---|--|---|---------------------|-----------------|------------|--------------------------------------|-----------------------------------|
| Metabolic process | Plant cuticle degradation | Cutinase (CE5) | Bcin15p00130 | | 54 | 2,2E-12 | IPR000675*** |
| | | | Bcin08p01580 | | 15 | 1,2E-06 | |
| Carbohydrate and polysaccharide metabolic processes | PCWDE* - Cellulose | Endo-beta-1,4-glucanase (GH5_5) | Bcin03p04010 | BcCel5A | 4 | 7,3E-03 | Espino <i>et al.</i> (2005) |
| | PCWDE* - Hemicellulose | Xylanase (GH11) (CDIP) | Bcin03p00480 | BcXyn11A | 40 | 7,1E-11 | Brito <i>et al.</i> (2006) |
| | | Xylanase (GH10,CBM1) | Bcin05p06020 | BcXyn10B | 5 | 4,3E-03 | Garcia <i>et al.</i> (2017) |
| | | Xylosidase (GH31) | Bcin11p06440 | | 3 | 2,8E-02 | CAZy DB**** |
| | PCWDE* - Cellulose and Hemicellulose | Xyloglucanase (GH12) (CDIP) | Bcin03p03630 | BcXyg1 | 54 | 2,0E-12 | Zhu <i>et al.</i> (2017) |
| | | Xyloglucanase (GH12 CBM1) | Bcin13p02320 | | 53 | 2,3E-12 | |
| | PCWDE* - Pectin | Rhamnogalacturonan acetyltransferase (CE12) | Bcin02p07100 | | 8 | 1,5E-04 | CAZy DB**** |
| | | Pectin methyl esterase (CE8) | Bcin01p11150 | | 1000 | 9,0E-17 | |
| | | | Bcin08p02970 | BcPme1 | 13 | 4,6E-06 | Kars <i>et al.</i> (2005) |
| | Starch degradation (nutrition) | Glucoamylase (GH15, CBM20) (CDIP) | Bcin04p04190 | BcGs1 | 164 | 9,0E-17 | Zhang <i>et al.</i>, 2015 |
| | | Glucoamylase (GH15) | Bcin04p00030 | | 19 | 1,7E-07 | CAZy DB**** |
| | | Alpha amylase (GH13) | Bcin02p01420 | | 94 | 2,0E-15 | |
| | | Lytic starch monooxygenase (AA13, CBM20) | Bcin06p05050 | | 33 | 5,4E-10 | Vu <i>et al.</i> (2014) |
| | | Beta-1,3-Glucan transferase GAS (GH72) | Bcin02p06940 | BcGas1 | 5 | 3,0E-03 | |
| | FCWE** remodeling - polysaccharides crosslinking | | Bcin13p02330 | | 3 | 2,7E-02 | |
| | | | Bcin14p03970 | | 3 | 2,8E-02 | |
| | | Beta-1,3-Glucan transferase BGL2 (GH17) | Bcin09p00200 | | 15 | 1,2E-06 | Patel and Free (2019) |
| | | | Bcin01p11220 | | 5 | 6,1E-03 | CAZy DB**** |
| | | Alpha-1,6-mannanase Dfg5/Dcw1 (GH76) | Bcin08p06110 | | 14 | 2,3E-06 | |
| | PCWDE* or FCWE** Glucan hydrolysis | Chitin / Glucan transglycosylase CRH (GH16) | Bcin01p06010 | | 8 | 1,9E-04 | |
| Beta Glucanase (GH131) | | Bcin12p06120 | | 8 | 2,4E-04 | | |
| Beta Glucanase (GH131, CBM1) | | Bcin09p01150 | | 7 | 3,2E-04 | CAZy DB**** | |
| Other fungal cell wall proteins | Beta-1,3-Glucanase (GH55) | Bcin10p00310 | | 3 | 4,6E-02 | | |
| | WSC domain protein | Bcin15p00810 | | 100 | 9,0E-17 | IPR002889*** | |
| Unknown | LysM effector (CBM50) - chitin binding | Bcin02p05630 | BcLysM1 | 28 | 4,2E-09 | Crumière <i>et al.</i> (unpublished) | |
| | Non classified Glycosyl Hydrolase (GHnc) | Bcin04p01310 | | 6 | 1,9E-03 | CAZy DB**** | |
| | Putative expansin (CBM63) | Bcin01p02460 | | 5 | 4,3E-03 | | |

Table 2: Up-accumulated proteins in the secretome from the infection cushion of *Botrytis cinerea*. (1/3)

| Functional category | Subcategory (expert annotation) | Description | Protein ID | Name | FC | p-value | Sources used for curation | | | |
|-----------------------------|-----------------------------------|---|-------------------------|--------------|--------------|--------------|-------------------------------|--|---------|---------|
| Proteolysis | Aspartic protease | | Bcin12p02040 | BcAp8 | 656 | 9,0E-17 | Ten Have <i>et al.</i> (2010) | | | |
| | | | Bcin05p05900 | BcAp5 | 25 | 9,9E-09 | | | | |
| | | | Bcin12p00180 | BcAp9 | 19 | 1,8E-07 | | | | |
| | | | Bcin04p02060 | BcAp13 | 4 | 1,2E-02 | | | | |
| | Sedolisin | | | Bcin08p01020 | | 1000 | 9,0E-17 | IPR030400*** | | |
| | | | | Bcin15p03150 | | 172 | 9,0E-17 | | | |
| | | | | Bcin15p04670 | BcSer8 | 44 | 2,1E-11 | | | |
| | | | | Bcin06p00620 | BcTpp2 | 22 | 4,1E-08 | | | |
| | | | | Bcin06p00330 | | 18 | 3,2E-07 | | | |
| | Serine carboxypeptidase | | | Bcin10p01890 | | 12 | 1,0E-05 | IPR001563*** IPR008758*** IPR001375*** | | |
| | | | | Bcin08p00280 | | 269 | 9,0E-17 | | | |
| | | | | Bcin08p02390 | | 10 | 3,7E-05 | | | |
| | | | | Bcin06p02510 | | 8 | 2,0E-04 | | | |
| | | Metallopeptidase | | Bcin16p02770 | BcMp1 | 276 | 9,0E-17 | IPR024079*** | | |
| | | Glutamic protease | | Bcin15p02380 | BcAcp1 | 997 | 9,0E-17 | Billon-Grand <i>et al.</i> (2012) | | |
| | Putative peptidase S41 | | Bcin07p04370 | | 48 | 8,6E-12 | | | | |
| Oxidation-reduction process | ROS producing systems | Glucose-methanol-choline oxidoreductase | | | Bcin03p01540 | 53 | 2,7E-12 | Siegmund and Viefhues (2016) Botportal DB Simon & Viaud (2018) | | |
| | | | | | Bcin02p07080 | 16 | 6,3E-07 | | | |
| | | | | | Bcin12p02910 | 7 | 7,2E-04 | | | |
| | Laccase | | | Bcin14p02510 | BcLcc2 | 1000 | 9,0E-17 | | | |
| | | | | Bcin01p00800 | BcLcc8 | 6 | 1,2E-03 | | | |
| | ROS detoxification and scavenging | Dyp-type peroxidase | | | Bcin13p05720 | BcPrd1 | 1000 | | 9,0E-17 | |
| | | | | | Bcin03p07850 | BcPrd10 | 26 | | 8,0E-09 | |
| | Other oxydases | Putative oxidase | Glyoxal oxidase 2 (AA5) | | | Bcin12p01520 | BcGo2 | | 1000 | 9,0E-17 |
| | | | | | | Bcin02p00280 | 18 | | 3,0E-07 | |
| | | | | | | Bcin02p00220 | 12 | | 9,5E-06 | |
| | | | | | Bcin05p00580 | 12 | 1,2E-05 | | | |
| | | | | | Bcin03p00380 | 4 | 1,7E-02 | | | |

Table 2: Up-accumulated proteins in the secretome from the infection cushion of *Botrytis cinerea*. (2/3)

| Functional category | Subcategory (expert annotation) | Description | Protein ID | Name | FC | p-value | Sources used for curation | |
|----------------------------|---------------------------------|--|---------------------|---------------|------------|----------------|--------------------------------------|-------------------------------|
| Other or unknown functions | Nucleases | S1/P1 Nuclease | Bcin07p06590 | | 81 | 1,2E-14 | Botportal DB Simon & Viaud (2018) | |
| | | Ribonuclease T2 | Bcin03p07320 | | 73 | 4,2E-14 | | |
| | Phosphatases | Acid phosphatase | Bcin04p03620 | | 3 | 3,3E-02 | | |
| | | Phytase subfamily of histidine acid phosphatase | Bcin15p03320 | | 15 | 1,2E-06 | | |
| | Esterases | Carboxylesterase | Bcin07p04290 | BcHap1 | 7 | 4,8E-04 | | |
| | | Putative esterase | Bcin16p02130 | | 8 | 1,9E-04 | | |
| | Other enzymes | Carboxymuconate cyclase | Bcin02p08230 | | 7 | 6,0E-04 | | |
| | | Glutamate dehydrogenase | Bcin12p03690 | | 9 | 1,0E-04 | | |
| | Other proteins | Necrosis & ethylene-inducing protein (CDIP) | Bcin16p04050 | | 5 | 5,6E-03 | | |
| | | Glycoprotein necrosis inducer (CDIP) | Bcin02p07770 | BcNep2 | 137 | 9,0E-17 | | Schouten <i>et al.</i> (2008) |
| | | Cerato-platanin family protein (CDIP) | Bcin15p00100 | BcIeb1 | 112 | 9,0E-17 | | Frias <i>et al.</i> (2016) |
| | | Cerato-platanin family protein (CDIP) | Bcin03p00500 | BcSpl1 | 78 | 1,7E-14 | | Frias <i>et al.</i> (2013) |
| | | Ubiquitin 3 binding protein But2 | Bcin09p05250 | | 40 | 6,4E-11 | | Botportal DB |
| | | Putative Ferritin | Bcin04p05020 | BcFlp1 | 16 | 8,8E-07 | | |
| | | Hypothetical proteins | | Bcin14p00810 | | 1000 | | 9,0E-17 |
| | | | Bcin06p06670 | | 34 | 5,0E-10 | | |
| | | | Bcin09p02890 | | 19 | 1,6E-07 | | |
| | | | Bcin01p06060 | | 18 | 3,5E-07 | | |
| | | | Bcin11p02610 | | 5 | 3,8E-03 | | |
| | | | Bcin09p00220 | | 4 | 7,5E-03 | | |
| | Bcin12p00750 | | | 3 | 3,3E-02 | | | |

Table 2: Up-accumulated proteins in the secretome from the infection cushion of *Botrytis cinerea*. (3/3)

Table 1: Functional enrichment analysis of up-regulated genes in the infection cushion of *Botrytis cinerea*.

Functional subcategories were determined according to a GO biological process analysis followed by a manual curation using existing databases and published data. Categories with significant enrichment were identified using Fisher's exact test with a p-value cutoff at 0.05 (only the Bcin genes analyzed by the chip were used for this test); Number of differentially regulated genes (in bold) and p-values are indicated. *PCWDE, Plant Cell Wall Degrading Enzymes; **FCWE, Fungus Cell Wall Enzymes; *** Bcin proteins displaying an "IPR" InterPro domain (www.ebi.ac.uk/interpro/; Mitchell *et al.*, 2019) were searched on the Botportal database (<http://botbioger.versailles.inra.fr/botportal/>; Simon & Viaud, 2018); **** PKS, Polyketide synthases; NRPS, Non-Ribosomal Peptide Synthetases; TS, Terpen cyclases; DMATS, Dimethylallyl tryptophan synthases.

Table 2: Up-accumulated proteins in the secretome from the infection cushion of *Botrytis cinerea*.

Comparison of IC secretome versus mycelium secretome revealed 79 proteins up-accumulated. For quantification, all unique peptides of an identified protein were included, and the total cumulative abundance was calculated by summing the abundances of all peptides allocated to the respective protein. ANOVA test was applied at the protein level. Three independent biological experiments were conducted and analyzed. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD016885. Gene ID and protein accession numbers can be found in Ensembl Fungi release database (http://fungi.ensembl.org/Botrytis_cinerea/Info/Index). The fold change values (FC) between the average protein abundances in the IC and the vegetative mycelia are indicated with the associated p-values. The up-accumulated proteins, identified in 3 biological

replicates with a minimum of 2 unique peptides, are listed and classified according to their functional category (manual annotation). *PCWDE, Plant Cell Wall Degrading Enzymes according to Van den Brink & de Vries (2011) and Glass (2013); **FCWE, Fungus Cell Wall Enzymes; *** Bcin proteins displaying an "IPR" InterPro domain (www.ebi.ac.uk/interpro/; Mitchell et al., 2019) were searched on the Botportal database (<http://botbioger.ver-sailles.inra.fr/botportal/>; Simon & Viaud, 2018); **** CAZy database (Lombard *et al.*, 2014 ; www.cazy.org/). Six plant cell death-inducing proteins (CDIP; Li *et al.*, 2020) are shown in bold. (Additional information is provided in Table S3).

Figure legends

Figure 1: Scanning Electron Microscopy of *Botrytis cinerea* infection cushion.

Mature infection cushion, produced on glass surface, developing multiple and successive ramifications of hyphae (2 dpi).

Figure 2: Up-regulation of phytotoxins and ROS production in the infection cushion of *Botrytis cinerea*

(a) Hierarchical clustering of the expression of the Botcinic acid genes (*Bcboa*) and botrydial genes (*Bcbot*) in infection cushion. (IC, 4 biological replicates) and control mycelium (Myc, 3 biological replicates). The normalized expression intensities are clustered and represented by color-coded squares; Shades of green and red depict down- and up-regulation in IC, respectively (fold change (FC) ≤ -2 or ≥ 2 and FDR < 0.05). (b) Botcinic acid (boa) and botrydial (bot) structures (left; Pinedo *et al.*, 2016) and biosynthesis gene clusters (right; Porquier *et al.*, 2019 and Dalmais *et al.*, 2011). Genes up-regulated in IC are colored in red. Genes with no chip reference and whose expression could hence not be measured are colored in white. (c) Visualization of ROS produced by IC. 100 μ l of medium were added to 1ml of DAB and incubated

20h. Controls were done by adding 1µl Horse Radish Peroxydase (HRP) to 1ml DAB in presence of different quantities of H₂O₂. The growth medium of 48 h cellophane-overlaid liquid cultures (IC) or agitated liquid cultures (Myc) was mixed to a DAB solution. The oxidation of DAB by H₂O₂ in the presence of peroxidases is revealed by a brown coloration.

Figure 3: Regulation of the DHN melanogenesis bipartite pathway in the infection cushion of *Botrytis cinerea*.

This figure is adapted from Schumacher *et al.* (2016) and Cohrs *et al.* (2016). (a) Hierarchical clustering of the expression of DHN-melanogenic genes in IC (IC, 4 replicates) and control mycelium (Myc, 3 replicates) as presented in figure 2. Up-regulated genes that co-localize with the DHN-melanogenic genes are added for information (*), but their role in melanin biosynthesis remains to be established. Similarly, 2 laccases up-regulated genes (*Bclcc6* and *Bclcc8*) are listed (**), but their role in melanin biosynthesis remains to be established. (b) DHN-melanins biosynthesis putative gene clusters. (c) DHN-melanin metabolic pathway and targets of tricyclazole (lightnings). Genes up- or down-regulated in IC and corresponding proteins are colored in red and green, respectively. (d) Light microscopy images of hyphae and IC of *B. cinerea* produced on plastic surface in the absence (top) and presence (bottom) of tricyclazole. Pigmented IC versus hyaline hyphae (left) and dark versus orange-brown thick cell walls inside IC (right) are shown. Bars represent 10 µm.

Figure 4: Cell wall chitin deacetylation in the infection cushion of *Botrytis cinerea*.

(a) Representation of the transformation of chitin into chitosan by chitin deacetylases (*cda*). (b) Hierarchical clustering of the expression of 5 putative *cda* genes in infection cushion (IC) and control mycelium (Myc), as presented in figure 2. (c) Expression of *Bccda* genes during a kinetics of bean leaves infection by *B. cinerea* (dpi; days post inoculation). Expression levels

were calculated following the $2^{-\Delta\Delta CT}$ method using constitutively expressed actin gene *BcactA* (Bcin16g02020) as a reference. The use of two other housekeeping genes, elongation factor *Bceflα* (Bcin09g05760) and pyruvate dehydrogenase *Bcpda1* (Bcin07g01890) gave similar results (data not shown). Three independent biological replicates were assessed for each experiment. Standard errors are displayed, and asterisks indicate a significant difference in gene expression compared with the previous time point (Student's *t* test, * p-value <0.05). (d) Confocal microscopy of mycelium and mature IC produced onto a plastic surface at 44 hpi and double-stained with Calcofluor targeting mainly chitin (blue) and Eosin Y targeting mainly chitosan (yellow).

Figure 5: *In planta* RT-qPCR validation of microarray up-regulated genes.

(a) and (b) Infection cushions development on leaves infected by *B. cinerea*. Primary bean leaves were inoculated with conidia and fungal development (1-4 days post inoculation (dpi)) was monitored using a Stereomicroscope (Zeiss) and cotton blue to stain the fungal cells at the plant surface. IC are pointed by white arrows. Bars represent 5 mm (a) or 50 μm (b). (c) Expression of selected genes during infection. Infected bean leaves were collected to prepare RNAs and RT-qPCR was used to measure the expression of 14 genes up-regulated in IC produced *in vitro* conditions (microarray data) and whose predicted function relates to virulence. The red bar indicates the peak of expression for each gene (when 2 or 3 bars are in red for the same gene, statistics cannot distinguish them). Gene expression levels were calculated following the $2^{-\Delta\Delta CT}$ method using constitutively expressed actin gene *BcactA* (Bcin16g02020) as a reference. The use of two other housekeeping genes, elongation factor *Bceflα* (Bcin09g05760) and pyruvate dehydrogenase *Bcpda1* (Bcin07g01890) showed similar results (data not shown). Standard errors of three independent biological replicates are displayed and asterisks indicate a

significant difference in gene expression compared with the previous time point (Student's *t* test, p-value <0.05).

Figure 6: Characterization of fasciclin-like deletion mutants in *Botrytis cinerea*.

(a) Infection kinetics - French bean leaves were inoculated with mycelial plugs of the WT and deletion strains. The necrosis of the plant tissues was measured every 24 h (at least eight plants and 32 infection points) over 4 days in 3 independent experiments. Bars indicate standard errors. (b) Visualization of the necrotic lesions. At 72 h post inoculation, typical images of bean leaves infected by the WT and mutant strains are presented. White arrows indicate dark rings at the edge of the necrotic zones. (c) Growth *in vitro*: The WT strain, the single mutant strains ($\Delta Bcflp1$ and $\Delta Bcflp2$) and the double mutant strain ($\Delta Bcflp1::\Delta Bcflp2$) were grown on minimal medium for 72h. The diameters of the colonies were measured on 4 independent experiments conducted with 5 plates for each strain. Bars indicate standard errors and stars indicate significant differences (Student *t*-test, p-value <0.05) between the WT and the mutants. Similar results were recorded for all experiments on two independent strains of each mutant.

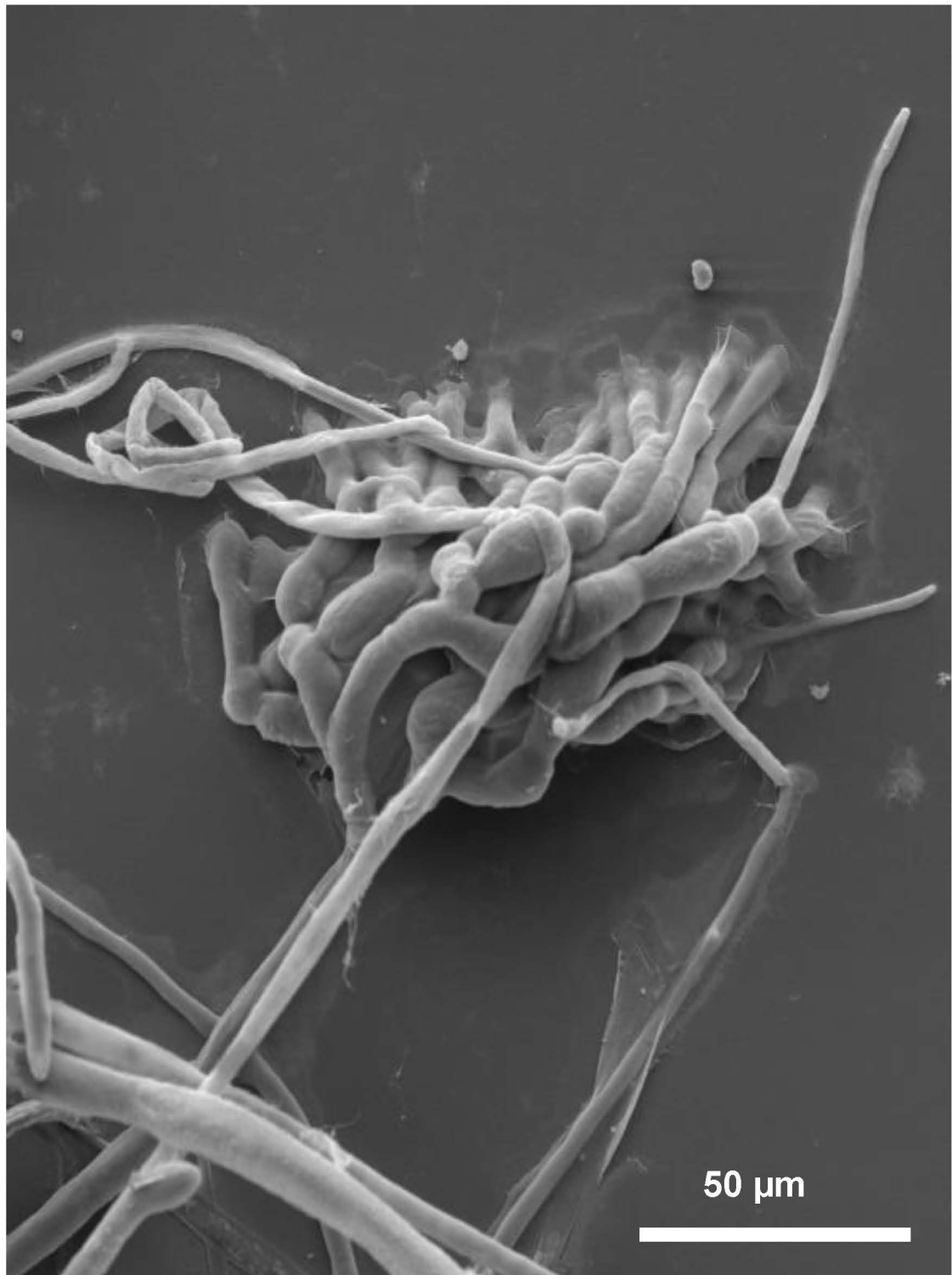
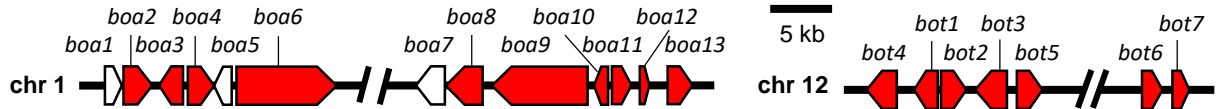
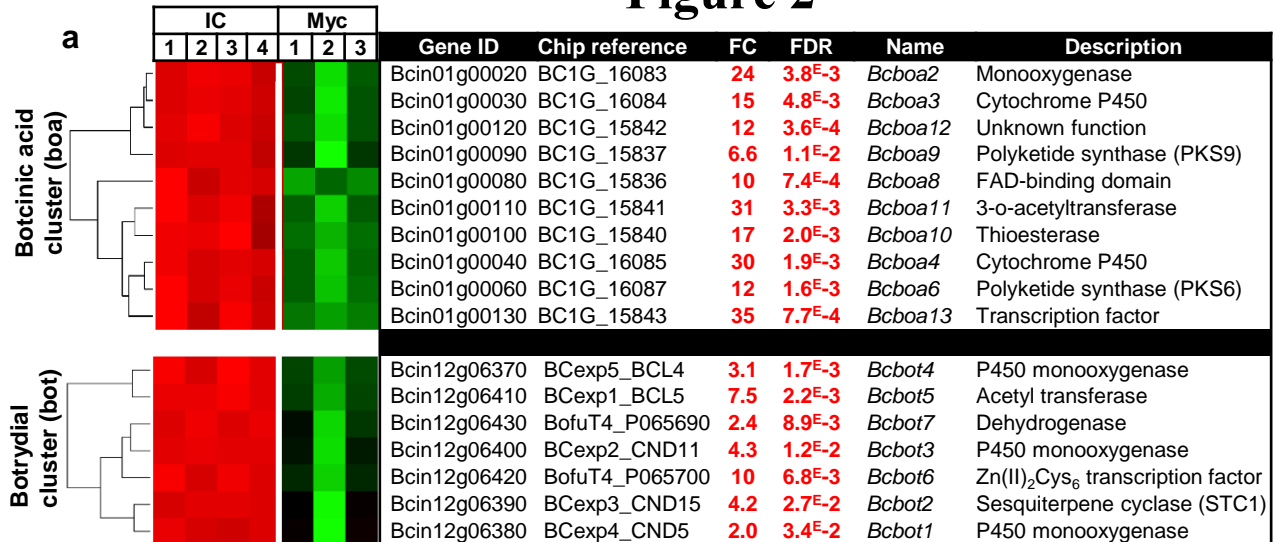


Figure 2



b

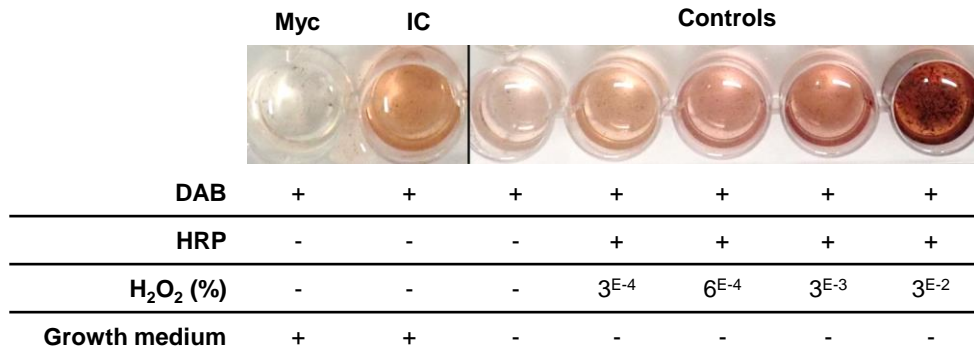
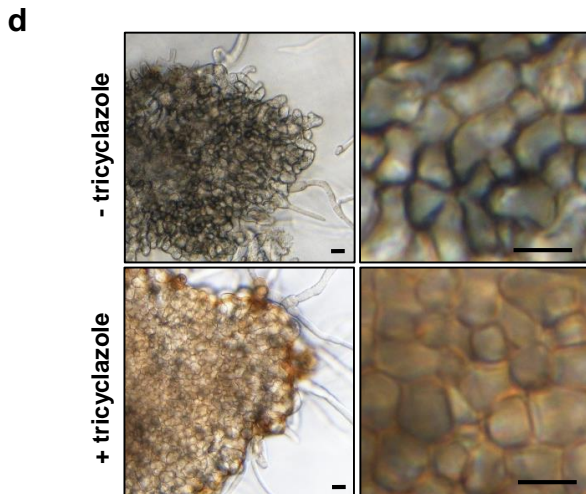
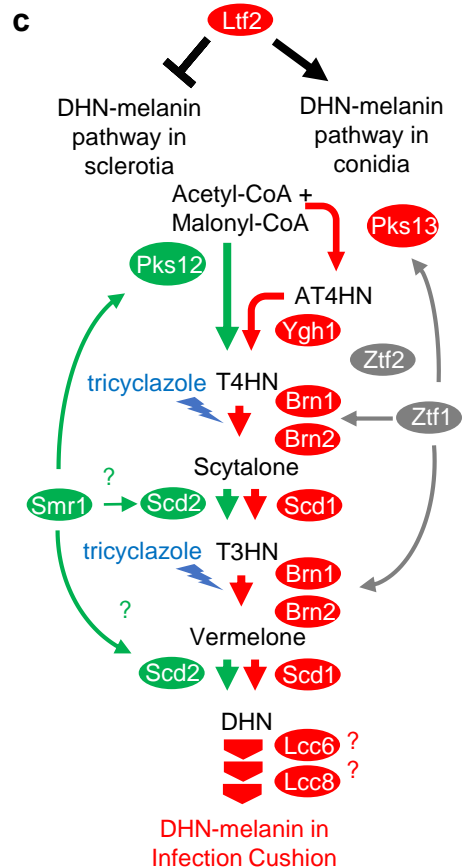
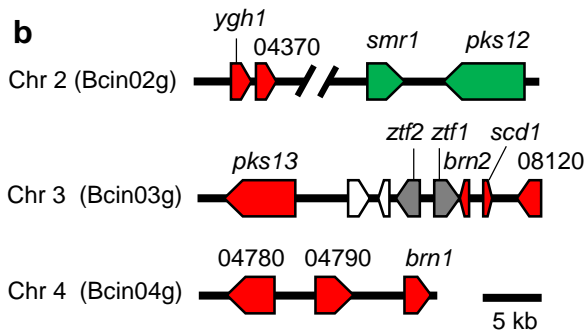
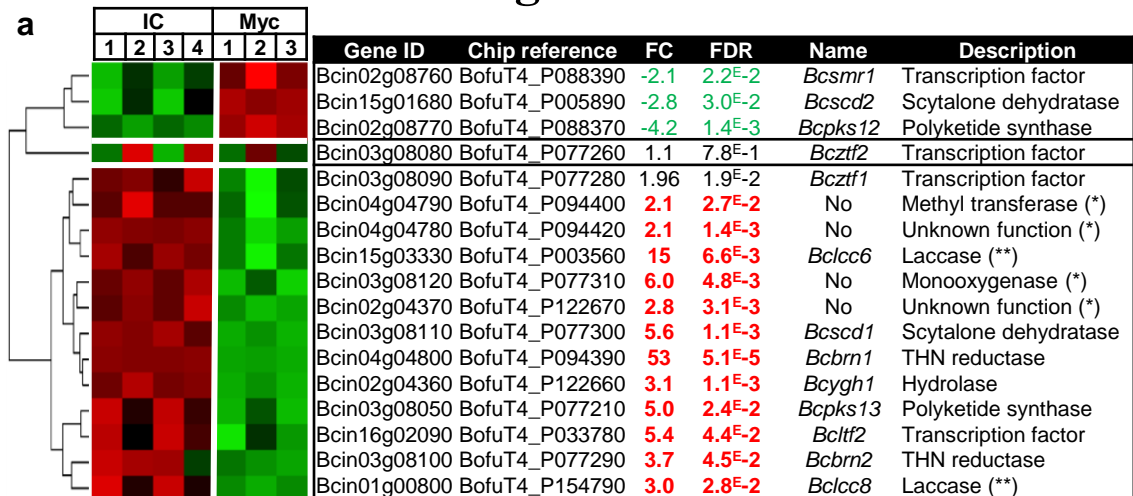
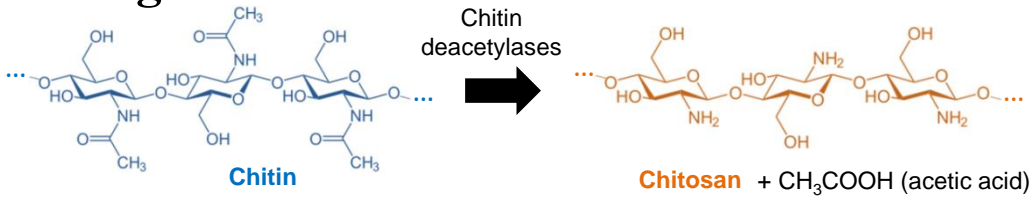


Figure 3



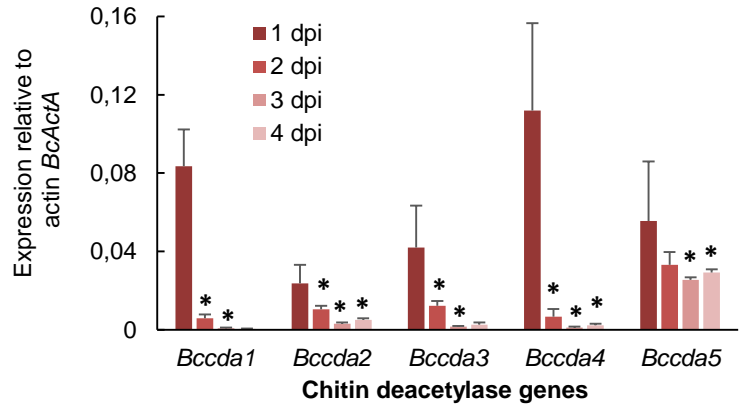
a Figure 4



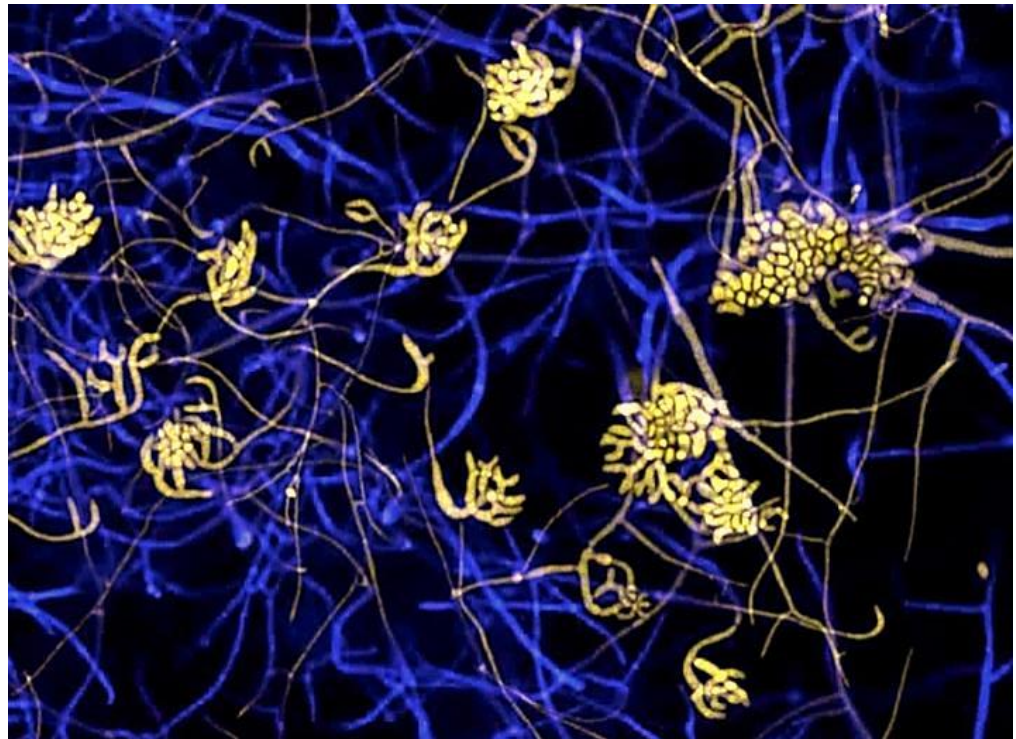
b

| | IC | | | Myc | | | Gene ID | Chip reference | FC | FDR | Name |
|--|-----|-----|-----|-----|-------|-------|--------------|----------------|-----|--------------------|---------------|
| | 1 | 2 | 3 | 4 | 1 | 2 | | | | | |
| | Red | Red | Red | Red | Green | Green | Bcin09g00620 | BofuT4_P101030 | 4.1 | 2.9 ^{E-2} | <i>Bccda4</i> |
| | Red | Red | Red | Red | Green | Green | Bcin11g04800 | BofuT4_P079220 | 1.7 | 3.9 ^{E-2} | <i>Bccda2</i> |
| | Red | Red | Red | Red | Green | Green | Bcin03g02970 | BofuT4_P051770 | 18 | 3.6 ^{E-4} | <i>Bccda3</i> |
| | Red | Red | Red | Red | Green | Green | Bcin03g05710 | BofuT4_P018380 | 55 | 4.1 ^{E-3} | <i>Bccda1</i> |
| | Red | Red | Red | Red | Green | Green | Bcin01g08990 | BofuT4_P050320 | 1.2 | 3.3 ^{E-1} | <i>Bccda5</i> |

c



d



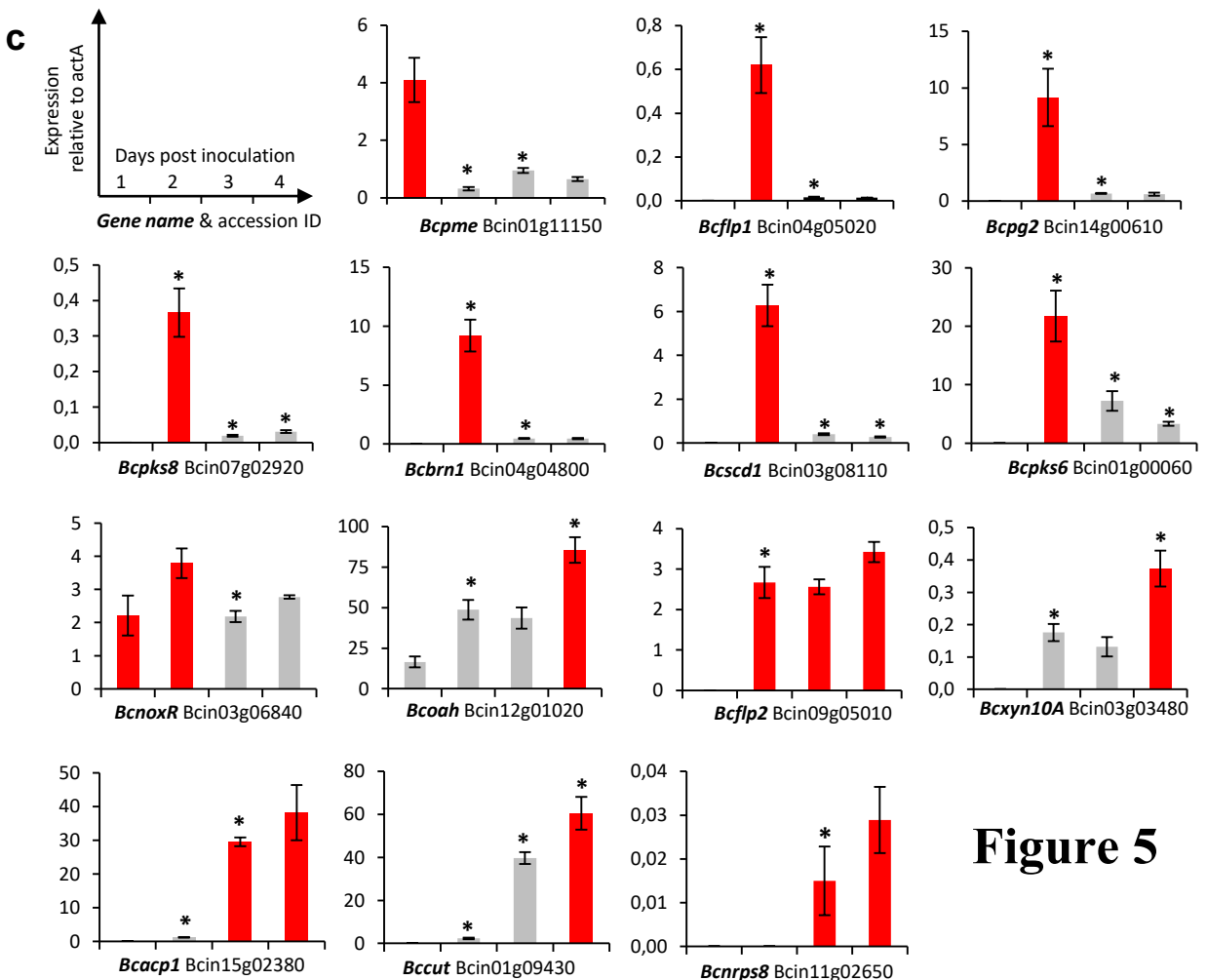
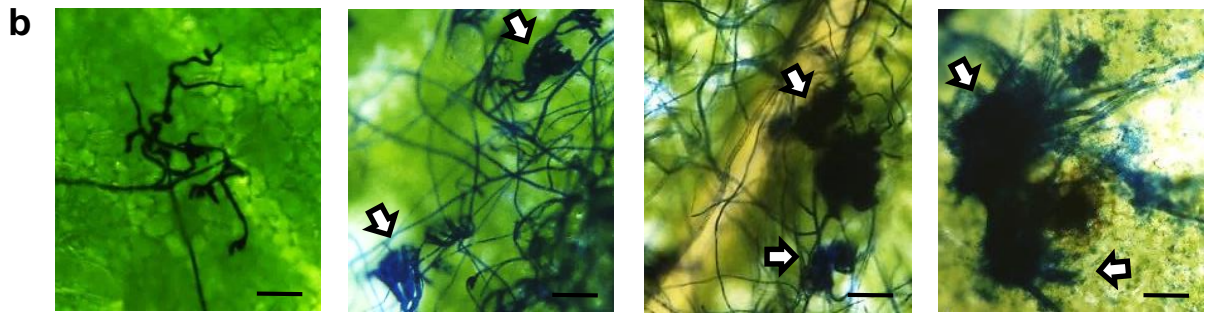
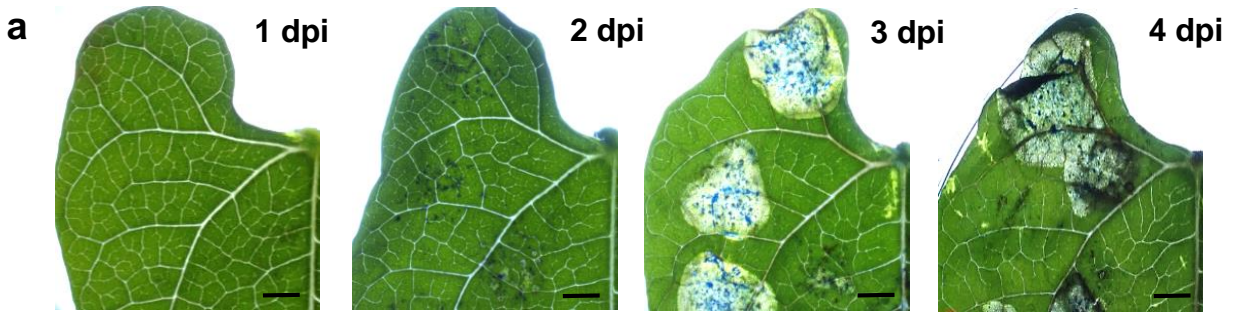
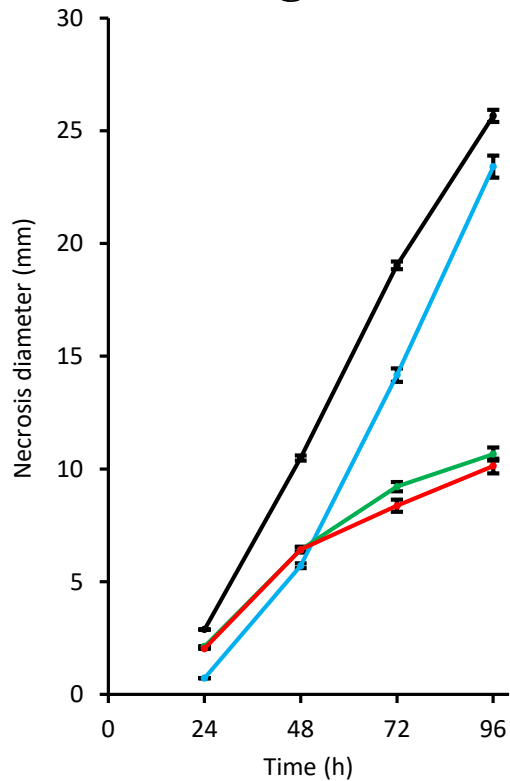


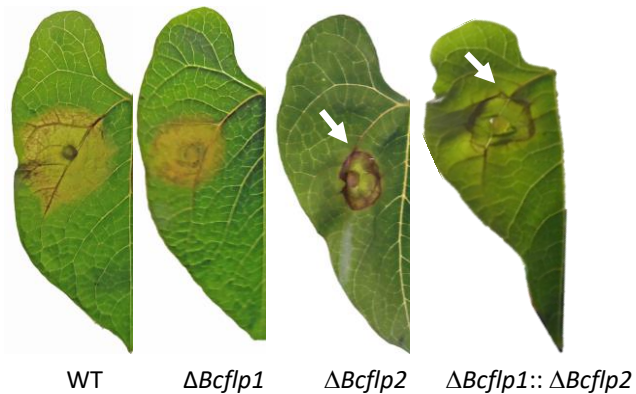
Figure 5

Figure 6

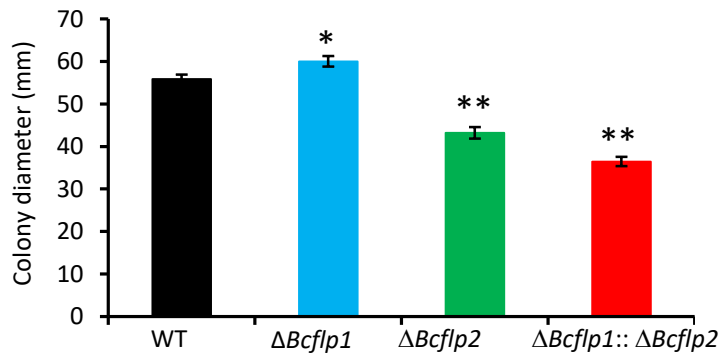
a



b



c



Supporting Information

Article title: **The infection cushion of *Botrytis cinerea*: a fungal “weapon” of plant-biomass destruction**

Authors: Mathias Choquer, Christine Rascle, Isabelle R Gonçalves, Amélie de Vallée, Cécile Ribot, Elise Loisel, Pavlé Smilevski, Jordan Ferria, Mahamadi Savadogo, Eytham Souibgui, Marie-Josèphe Gagey, Jean-William Dupuy, Jeffrey A Rollins, Riccardo Marcato, Camille Noûs, Christophe Bruel and Nathalie Poussereau

The following Supporting Information is available for this article:

Fig. S1 *In vitro* production of infection cushions of *Botrytis cinerea*.

Fig. S2 Gene ontology (GO) term enrichment analysis of genes differentially expressed in the infection cushion of *Botrytis cinerea*.

Fig. S3 Regulation of secondary metabolism biosynthesis key genes in the infection cushion of *Botrytis cinerea*.

Fig. S4 Putative secondary metabolism gene clusters up-regulated in the infection cushion of *Botrytis cinerea*.

Fig. S5 Mutagenesis of genes encoding fasciclin-like proteins in *Botrytis cinerea*.

Fig. S6 Comparison of the *Botrytis cinerea* infection cushion (IC) transcriptome and the transcriptomes of four IC-deficient mutants.

Table S1 Microarray analysis of the infection cushion of *Botrytis cinerea*.

Table S2 RT-qPCR validation of microarray expression profiles from the infection cushion of *Botrytis cinerea* produced *in vitro*.

Table S3 Up-accumulated proteins in the secretome of the infection cushion of *Botrytis cinerea*.

Table S4 Constructs and primers used in this study.

Fig. S1 *In vitro* production of infection cushions of *Botrytis cinerea*. (a) Light microscopy of mycelia produced from conidia of *B. cinerea* spread onto PDA plates overlaid with cellophane and culture for 44h. Mature and melanized infection cushions (dark patches) were fully differentiated and hyperbranched and they covered about 40% of the PDA plates surfaces (b) Light microscopy of mycelia produced from conidia inoculated in PDB-containing flasks and cultured under agitation for 44h. The liquid cultures produced only mycelium and no infection cushion. Magnifications are indicated.

Supplementary Figure S1

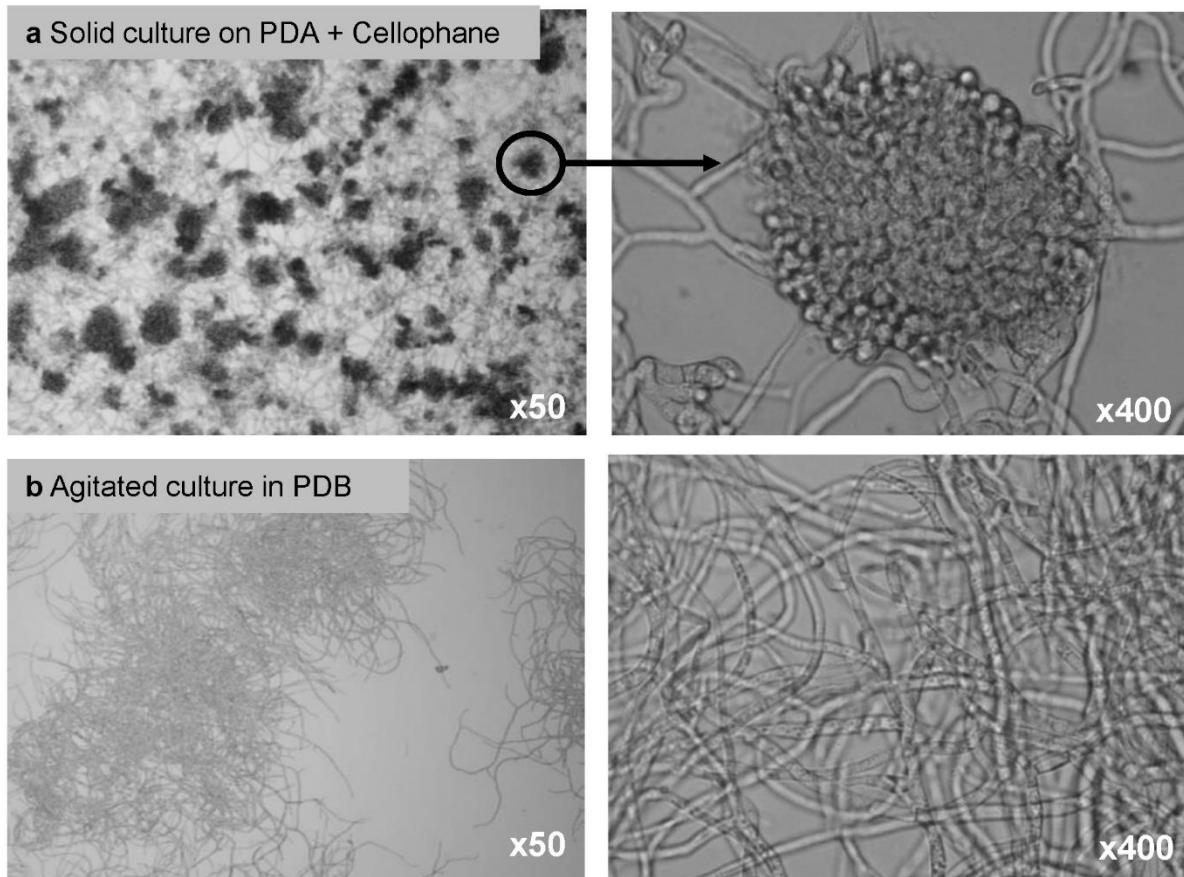


Fig. S2 Gene Ontology (GO) term enrichment analysis of genes differentially expressed in the infection cushion of *Botrytis cinerea*. Significantly (Fisher's exact test; p -values < 0.05) over-represented biological processes (BP) for the (a) 1,231 genes up-regulated in IC and (b) 1,422 genes down-regulated in IC. Enriched categories are classified according to p -values. Ratios inside the pie charts indicate the number of differentially expressed genes (underlined) relative to the total number of genes in each BP category (*italics*).

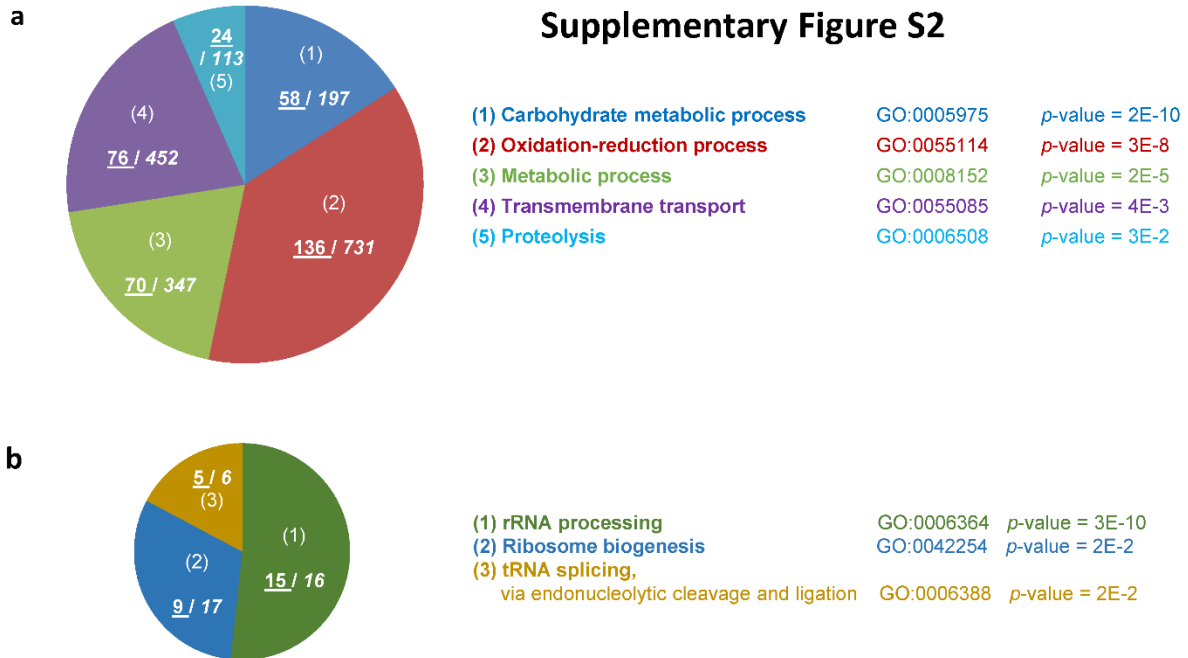


Fig. S3 Regulation of secondary metabolism biosynthesis key genes in the infection cushion of *Botrytis cinerea*.

(a) Inventory of secondary metabolism (SM) biosynthesis key enzymes. The names, acronyms and number of genes coding for the SM key enzymes in *B. cinerea* are presented. (b) Hierarchical clustering of the expression of the 42 predicted SM key enzymes-encoding genes in IC (IC, 4 replicates) and control mycelium (Myc, 3 replicates). The normalized expression intensities are clustered and represented by color-coded squares; Shades of green and red depict down- and up-regulation in IC, respectively (Fold change ≤ -2 or ≥ 2 and FDR <0.05). Background corresponds to genes considered not expressed (with normalized intensities lower than the 99th percentile of random probes hybridization signals in all biological replicates).

Supplementary Figure S3

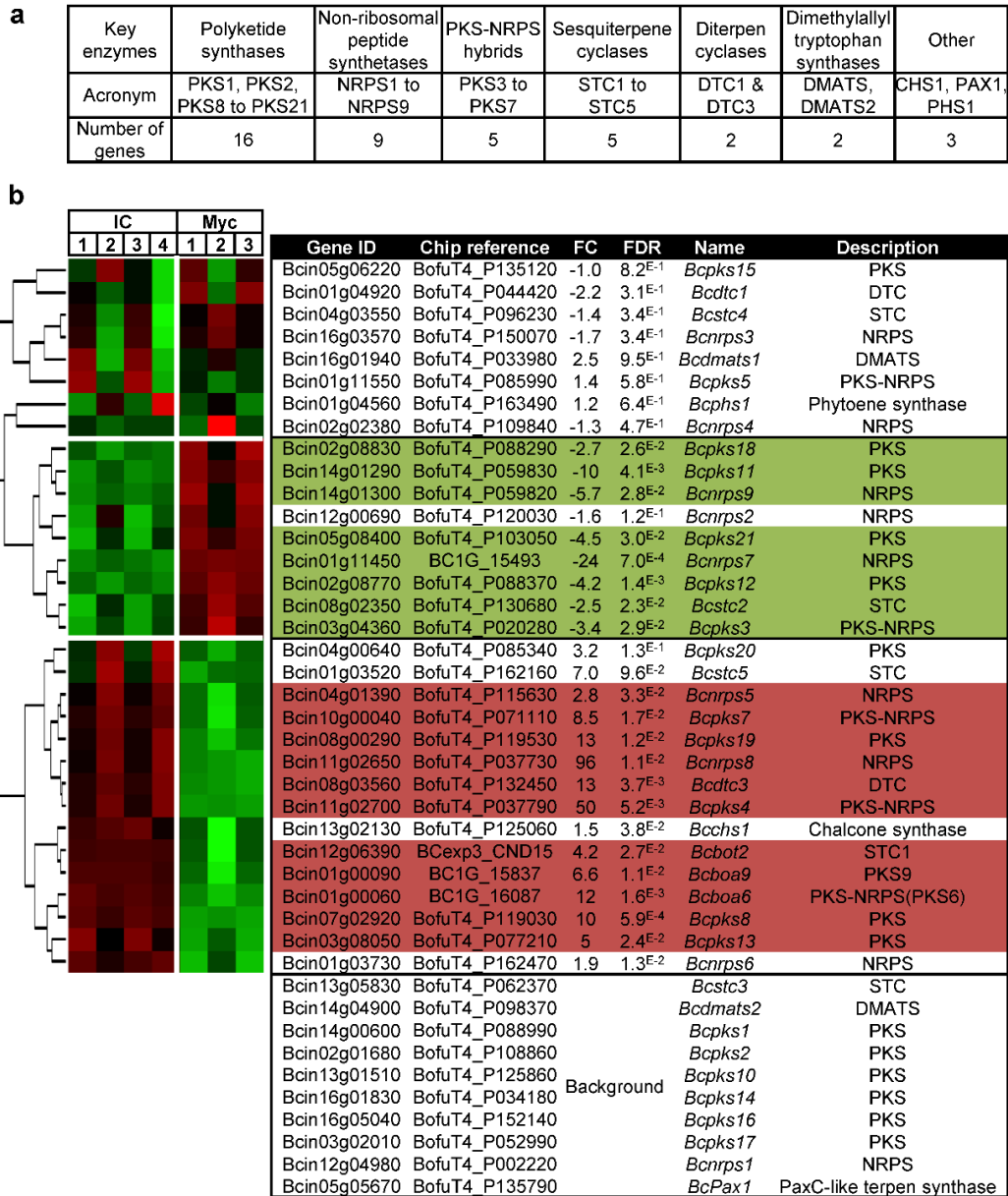


Fig. S4 Putative secondary metabolism gene clusters up-regulated in the infection cushion of *Botrytis cinerea*. (a)

Hierarchical clustering of the expression of SM key enzymes-encoding genes and surrounding genes in IC (IC, 4 replicates) and control mycelium (Myc, 3 replicates). The normalized expression intensities are represented by color-coded squares; Shades of green and red depict down- and up-regulation in IC, respectively (Fold change ≤ -2 or ≥ 2 and $FDR < 0.05$). (b) Genomic localization and representation of the 4 putative SM gene clusters DTC3, PKS7, PKS8 and PKS4-NRPS8. The genes up-regulated in IC are indicated in red and the supposed ends of each cluster are represented by the first neighboring genes non-regulated in IC (grey).

Supplementary Figure S4

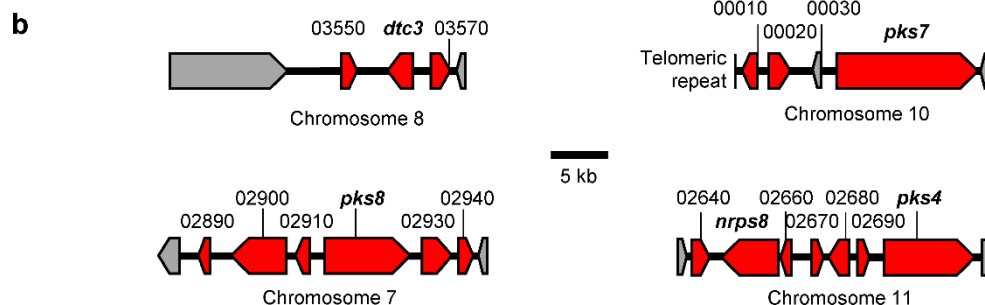
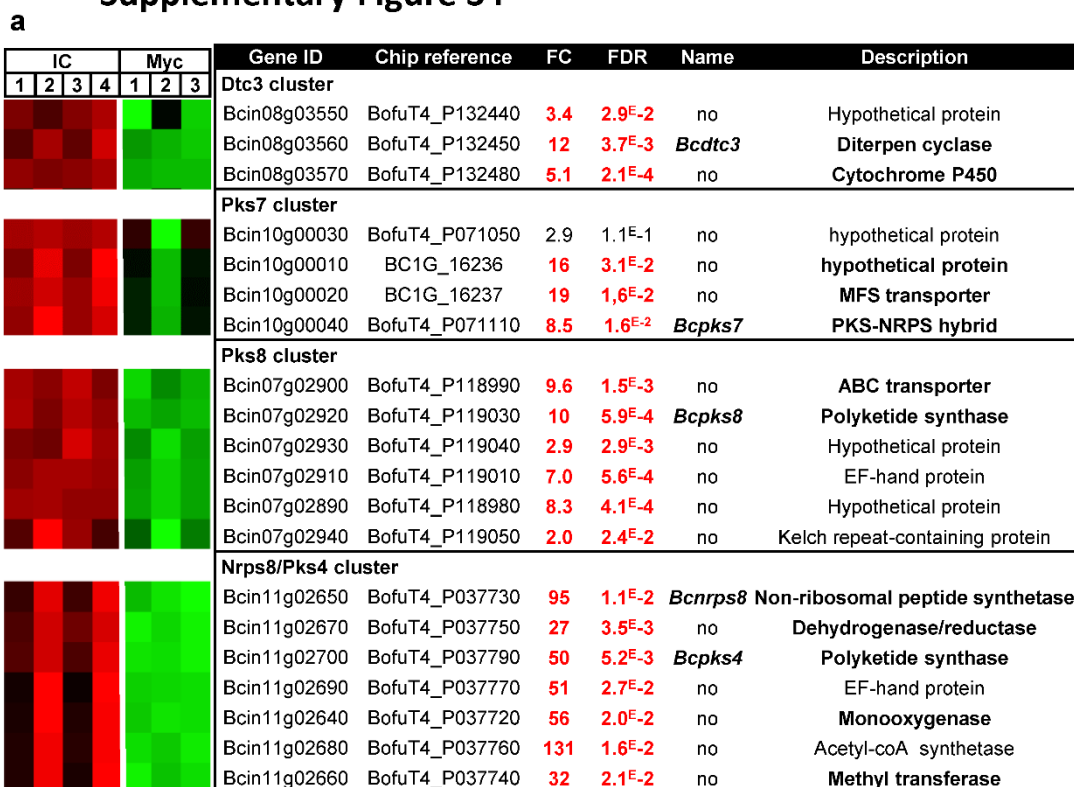


Fig. S5 Mutagenesis of genes encoding fasciclin-like proteins in *Botrytis cinerea*. (a) Creation of the $\Delta Bcflp1$ deletion mutant (left) and Southern analysis (right). A split-marker strategy was used to replace the *Bcflp1* gene of the *B. cinerea* B05.10 strain by a hygromycin resistance gene (Hygro). The promoter and terminator DNA regions of *Bcflp1* (5'- and 3'-Flank) were inserted to the overlapping semi-replacement DNA cassettes in order to target them to the appropriate genomic locus. Homologous recombination (dashed lines) led to gene replacement. Genomic DNA of two hygromycin resistant clones (T2, T3) and the parental strain (WT) was digested by EcoRI (E) and subjected to Southern blot analysis using the 3'-flanking region as DNA probe (blue bar). (b) Creation of the $\Delta Bcflp2$ deletion mutant (left) and Southern analysis (right). Following the same strategy described in (a), six transformants (T6-12) were analyzed by using the 5'-flanking region as DNA probe (green bar). (c) Creation of the $\Delta Bcflp1::\Delta Bcflp2$ double deletion mutant (left) and Southern analysis (right). The same strategy described in (a) was used with the nourseothricin resistance gene (Nourseo) replacing the hygromycin resistance gene and the $\Delta Bcflp1$ strain being the recipient strain for the transformation experiment. Three transformants (T) were analyzed by using the 3'-flanking region as DNA probe (blue bar). Gene replacements in the deletion strains were also verified by diagnostic PCR (data not shown).

Supplementary Figure S5

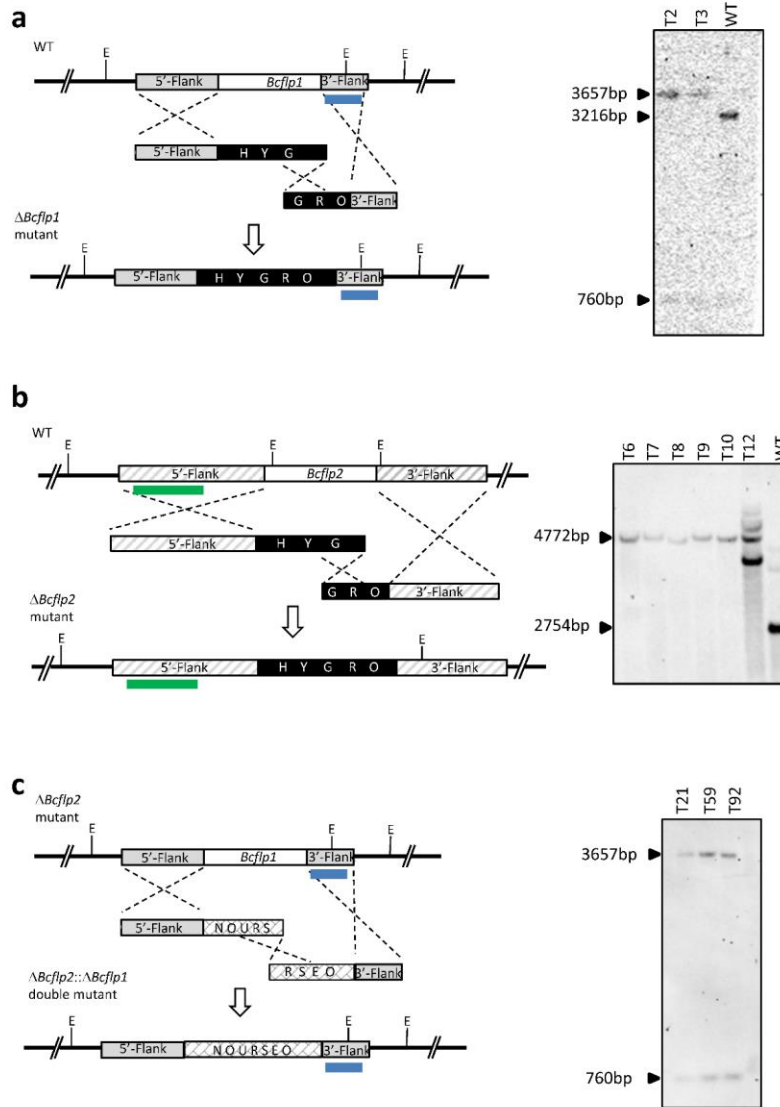
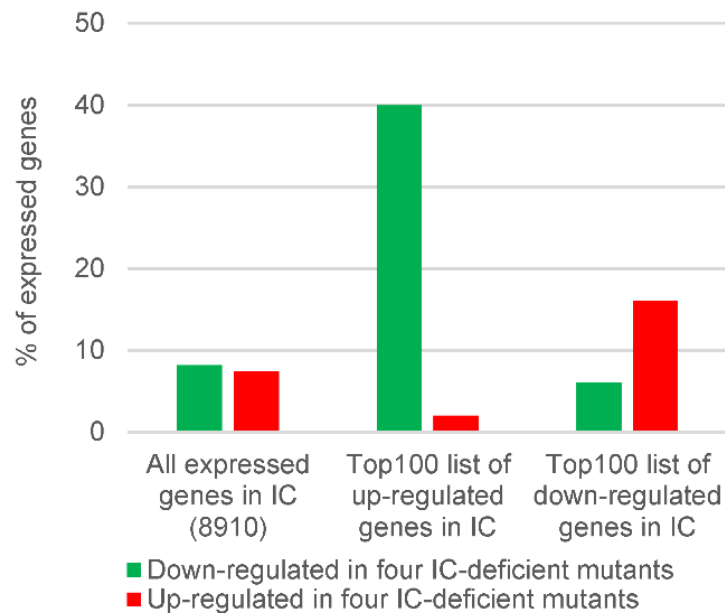


Fig. S6 Comparison of the *Botrytis cinerea* infection cushion (IC) transcriptome and the transcriptomes of four IC-deficient mutants. The transcriptomes (RNAseq) of four *B. cinerea* mutants impaired in the formation of IC were published (De Vallée *et al.*, 2019) and used for data comparison with the transcriptome of IC (this study). Genes repressed in these IC-deficient mutants were assumed to be induced in the infection cushion of the wild-type strain. This inverted correlation was confirmed for 40 genes among the top 100 upregulated genes in IC as they showed a common down-regulation in the four IC-deficient mutants.

De Vallée, A., Bally, P., Bruel, C., Chandat, L., Choquer, M., Dieryckx, C., *et al.* (2019) A Similar Secretome Disturbance as a Hallmark of Non-pathogenic *Botrytis cinerea* ATMT-Mutants? *Front Microbiol* **10**: 2829.

Supplementary Figure S6



Legends of supplementary Tables

Table S1 Microarray analysis of the infection cushion of *Botrytis cinerea*. Table showing all Bcin genes (N=11,710) corresponding to the genome of B05.10 strain (ASM83294v1) structurally annotated by Van Kan *et al.* (2017) and available at http://fungi.ensembl.org/Botrytis_cinerea. N represents the total number of genes annotated on the genome and n represents the number of genes present on the chip. For each Bcin gene, the indicated Microarray chip reference is the corresponding gene from two previous genome annotations BofuT4 or BC1G (Amselem *et al.*, 2011). Usual names were imported from the *Botrytis cinerea* Portal (<http://botbioger.versailles.inra.fr/botportal/>). 11,134 Bcin genes were analyzed by the Nimblegen Microarray chip which represent 95% of the published Bcin genes. They include Bcin genes up-regulated in IC (n=1,231), Bcin genes down-regulated in IC (n=1,422), Bcin genes expressed but not differentially regulated in IC (n=6,757), and Bcin genes not expressed in IC nor mycelium control (n=1,724). Fold change is the ratio between the mean of IC normalized intensities and the mean of mycelium control normalized intensities. ANOVA p-values were submitted to a False Discovery Rate correction (FDR). Transcripts with a corrected p-value < 0.05 and for which a fold change ≤ -2 or ≥ 2 was observed between the two conditions were considered to display significant differential expression. Normalized intensities are given for the infection cushion (4 biological replicates) and the mycelium control (3 biological replicates). Genes were considered not expressed when their normalized intensity was smaller than the 99th percentile of random probes hybridization signals in all biological replicates. For IC replicates 1 to 4, the 99th percentile of random probes hybridization signals was: 752, 572, 532 and 995, respectively. For mycelium replicates 1 to 3, the 99th percentile of random probes hybridization signals was: 752, 634 and 599, respectively (as determined by R software; R Core Team, 2019). Genes were manually sorted into 22 functional categories (see Table 1 for sources used for curation). Presence for a putative signal peptide was predicted using the SignalP 5.0 Server (Almagro Armenteros *et al.*, 2019).

Almagro Armenteros, J.J., Tsirigos, K.D., Sønderby, C.K., Petersen, T.N., Winther, O., Brunak, S., *et al.* (2019) SignalP 5.0 improves signal peptide predictions using deep neural networks. *Nature Biotechnology* **37**: 420–423.

Amselem, J., Cuomo, C.A., van Kan, J.A.L., Viaud, M., Benito, E.P., Couloux, A., *et al.* (2011) Genomic Analysis of the Necrotrophic Fungal Pathogens *Sclerotinia sclerotiorum* and *Botrytis cinerea*. *PLoS Genetics* **7**: e1002230.

R Core Team (2019) R: A Language and Environment for Statistical Computing, Vienna, Austria: R Foundation for Statistical Computing.

Van Kan, J.A.L., Stassen, J.H.M., Mosbach, A., Van Der Lee, T.A.J., Faino, L., Farmer, A.D., *et al.* (2017) A gapless genome sequence of the fungus *Botrytis cinerea*: Gapless Genome Sequence of *B. cinerea*. *Molecular Plant Pathology* **18**: 75–89.

Table S2 In vitro RT-qPCR validation of microarray expression profiles from the infection cushion of *Botrytis cinerea*. Forty genes were chosen to test the differential expression recorded by the microarray analysis in the IC versus control mycelium. New biological replicates (n=3) were prepared and the expression of 24 up-regulated genes, 6 down-regulated genes and 10 non-regulated genes was measured by RT-qPCR. Gene expression levels were calculated following the $2^{-\Delta\Delta CT}$ method using constitutively expressed elongation factor *Bcef1 α* as a reference (the use of the house-keeping actin (*BcactA*) and pyruvate dehydrogenase (*Bcpda1*) genes gave similar results; data not shown). Genes are ordered in the table according to their fold change in the microarray analysis and color codes corresponding to different fold change intensities are indicated. FC, fold change; FDR, false discovery rate; DE, differential expression.

Table S3 Up-accumulated proteins in the secretome of the infection cushion of *Botrytis cinerea*. (See legend of Table 2).

Table S3 corresponds to table 2 with additional information. SignalP and ApoplastP prediction softwares were respectively used to predict the presence of a signal peptide in these proteins sequence and the presence of these proteins in plant apoplast (<http://apoplastp.csiro.au/>; Sperschneider *et al.*, 2017). The microarray data (Chip reference, fold change and FDR) of these proteins-encoding genes are indicated. The behaviour of these proteins and corresponding genes in 4 IC-deficient mutants (De Vallée *et al.*, 2019) are also indicated (with the fold change values (FC) and the associated p-values). up-regulated genes and up-accumulated proteins are colored in red. Down-regulated genes and down-accumulated proteins are colored in green.

De Vallée, A., Bally, P., Bruel, C., Chandat, L., Choquer, M., Dieryckx, C., *et al.* (2019) A Similar Secretome Disturbance as a Hallmark of Non-pathogenic *Botrytis cinerea* ATMT-Mutants? *Front Microbiol* **10**: 2829.

Sperschneider, J., Dodds, P.N., Singh, K.B., Taylor, J.M. (2018) APOPLASTP: prediction of effectors and plant proteins in the apoplast using machine learning. *New Phytologist*. **217**: 1764-1778.

Table S4 Primers used in this study for RT-qPCR analysis and constructs. For *Bcflp1* deletion construct, the 5'- and 3'- flanking regions of *Bcflp1* (1.115 kb and 0.624 kb) were amplified from *B. cinerea* genomic DNA using the primers pairs flp1-H1/flp1-H2 and flp1-H3/flp1-H4, respectively. flp1-H2 and flp1-H3 also contained sequences homologous to the hygromycin resistance cassette containing the hph gene under control of the oliC promoter. This cassette was generated using primers Hyg-H5 and Hyg-H6 and plasmid pFV8 as a template (Villalba *et al.*, 2008). The purified amplicons were fused in a second round of PCR without any primer. Finally, a third step of PCR led to the amplification of two overlapping semi- replacement cassettes using nested primers flp1-H7/HYG-H7 and GRO-H8/flp1-H8 respectively. For *Bcflp2* deletion construct, the same strategy was applied using primers pairs flp2-H1/flp2-H2 for 5'- flanking region of *Bcflp2* (1.962 kb) and flp2-H3/flp2-H4 for 3'- flanking region of *Bcflp2* (1.468 kb) and resulted in two overlapping semi-replacement cassettes using nested primers flp2-H7/HYG-H7 and GRO-H8/flp2-H8 respectively. The development of double replacement mutants was achieved by transforming the previously generated $\Delta Bcflp2$ mutant. Double deletion mutants $\Delta Bcflp2::\Delta Bcflp1$ were constructed with the same strategy excepted that nourseothricin resistance was used instead of hygromycin. The 5'- and 3'- flanking regions of *Bcflp1* (1.095 kb and 0.56 kb) were amplified from *B. cinerea* genomic DNA using the primers pairs flp1-N1/flp1-N2 and flp1-N3/flp1-N4, respectively. The nourseothricin resistance cassette was amplified using flp1-N5 and flp1-N6 primers before fusion with the 5' and 3' flanking regions. Finally, two semi-cassettes were amplified using nested primers pairs flp1-N7/NOU-N7 and URS-N8/flp1-N8 respectively. *Bcflp1* replacement by the hygromycin cassette was verified using the primers pairs flp1-F5'/ hygro5' and hygro3'/flp1-F3' and the primers pairs flp1-N4/nour5' and nour3'/flp1-N1 for *Bcflp1* replacement by the nourseothricin cassette. *Bcflp2* replacement by the hygromycin cassette was verified using the primers pairs flp2-F5'/ hygro5' and hygro3'/flp2-F3'. Villalba, F., Collemare, J., Landraud, P., Lambou, K., Brozek, V., Cirer, B., *et al.* (2008). Improved gene targeting in *Magnaporthe grisea* by inactivation of MgKU80 required for non-homologous end joining. *Fungal Genetics and Biology*. **45**: 68-75.