

Differences in the gene transcription state of Botrytis cinerea between necrotic and symptomless infections of lettuce and Arabidopsis thaliana

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1 Differences in the gene transcription state of *Botrytis cinerea*
2 between necrotic and symptomless infections of lettuce and
3 *Arabidopsis thaliana*

4

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13 Running head: Transcription in *Botrytis* infections

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15 *Botrytis cinerea* can establish long-lived, symptomless, systemic infections in plant species.
16 It is unclear how the fungus colonizes plant tissues without causing tissue damage and
17 necrosis. Three hypotheses are: (1) the fungus state is similar in the two forms of infection,
18 but the plant defences are more effective, leading to multiple small quiescent centres; (2)
19 excreted molecules that would trigger plant defences are suppressed; (3) signal exchanges
20 occur avoiding both extensive host cell death and complete spatial restriction of the pathogen.
21 We tested these by comparing transcript levels of a set of *B. cinerea* genes between
22 symptomless and necrotising infections. Four genes were analysed that participate in
23 signalling pathways required for virulence, as well as five genes that directly participate in
24 causing host cell death or degrading plant cell wall polysaccharides.

25 In lettuce, necrotic infections on detached leaves (12-48 h after inoculation) had similar gene
26 expression patterns to necrotic infections on leaves 44 d after inoculation of the seedlings.
27 Symptomless infections on leaves that expanded after inoculation of young seedlings had
28 similar fungal gene expression patterns at 14, 24 and 34 d after inoculation, which clearly
29 differed from those in necrotising infections. In *A. thaliana*, there were differences in gene
30 expression patterns between droplet inoculations on leaves, resulting in necrotic lesions, and
31 symptomless infections in stems and leaves. The fungal gene expression patterns differed in
32 detail between lettuce and *A. thaliana*. The observations suggest that the physiological state
33 of *B. cinerea* during symptomless infection is distinct from necrotising infections.

34 [238 words]

35 Introduction

36 *Botrytis cinerea* is a plant pathogenic fungus causing grey mould disease and post-harvest losses in
37 more than 1000 crops ranging from ornamentals to vegetables and field crops (Elad et al, 2016).
38 Symptoms produced by *B. cinerea* range from restricted lesions to dry or spreading soft rots which
39 often produce conspicuous sporulating colonies (Williamson *et al*, 2007). In general, *B. cinerea* is
40 considered to be a necrotroph which draws nourishment from dead host tissue and produces initially
41 local (“primary”) necrotic lesions, which subsequently expand to actively cause plant tissue
42 decomposition (Horst, 1985; Jarvis, 1994; Coertze & Holz, 2002; Elad *et al*, 2004). In contrast,
43 recent studies have revealed that *B. cinerea* also can cause symptomless systemic infection in several
44 host plants including *Primula* spp., lettuce (*Lactuca sativa*), *Arabidopsis thaliana* and *Taraxacum*
45 *vulgare* (Barnes & Shaw 2003; Sowley *et al*, 2010; Rajaguru & Shaw, 2010; Shaw *et al*, 2016). In
46 this type of infection the fungus grows along with the plant and enters newly expanding organs,
47 without producing symptoms until the plant becomes physiologically susceptible, typically at
48 flowering. At this point extensive areas of host tissue death develop simultaneously, followed by
49 sporulation of the fungus. Several species in the genus *Botrytis* are able to infect in this way (Shaw *et*
50 *al*. 2016). The physiological relationship between host and pathogen during symptomless systemic
51 growth is unresolved. It is unclear how a fungus that can produce such a large arsenal of phytotoxic
52 metabolites and proteins (van Kan et al, 2006) is able to grow inside plant tissue without causing
53 extensive tissue damage and visual disease symptoms. The aim of the present study was to obtain
54 preliminary insight into this question by comparing the expression of a set of fungal genes that
55 participate in regulating virulence or in causing host cell death, between symptomless and
56 necrotising infections, in two host species.

57 A previous study illustrated that in some species a high proportion of symptomless plants can be
58 infected with *B. cinerea*. The distribution of the fungus appeared to be discontinuous and scattered
59 over distinct tissues (Shaw et al, 2016). Given the unpredictable location of the fungus within plant

60 tissue and the low fungal biomass at any given time point, the abundance of *Botrytis* mRNA in total
61 RNA extracted from a symptomless plant is expected to be low. Such low abundance hampers a full
62 transcriptome study by RNAseq throughout the plant in order to detect changes associated with
63 infection, as only abundant fungal transcripts would be reliably quantified by direct sequencing.
64 Therefore, quantitative RT-PCR was used to estimate relative concentrations of a selected set of nine
65 fungal transcripts, encoding signalling components involved in virulence or proteins directly
66 involved in necrotising infection.

67 **Materials and Methods**

68 ***Plant growth***

69 Lettuce (*Lactuca sativa*) ‘Tom Thumb’ (Thomson & Morgan, Ipswich UK) was used in all
70 experiments reported here. Two 80 cell modular seedling trays were filled with compost (“Levington
71 F1 seed and modular compost” Westland Ltd, UK), and in each tray 1 seed per cell was sown,
72 covered with a thin layer of compost. Seed germination and initial seedling growth was in controlled
73 environment chambers: 20° C in 12 h day and 18° C in night, RH 65%. The compost was kept damp
74 to touch all the time. Seven days after inoculation (14 days after seed sowing) 40 inoculated and 40
75 non-inoculated seedlings were transplanted into 1L pots filled with potting compost (John Innes 2
76 compost + 4g/L Osmacote). Later the seedlings were moved to a vented glasshouse under natural
77 light in summer (May-Aug, 15-16.5 h day-length, temperature maintained at 18° C minimum at
78 night, rising to about 30° C in day time. Plants were watered daily.

79 *Arabidopsis thaliana* (Col-0) seeds were surface disinfected in 70% ethanol for 2 min and then 20%
80 bleach (1% NaOCl) for 5 min and finally thoroughly rinsed in sterile water 5 to 6 times. After
81 surface disinfection seed stratification was done at 4 °C for four days. Seeds were sown singly on the
82 surface of the compost in pots covered with transparent polystyrene propagation covers with vents,
83 and grown on in these propagators. The propagators were maintained at positive pressure via a
84 pumped filtered airflow within a controlled environment cabinet at 22 °C day and 18 °C night

85 temperature, 65% relative humidity, 16 h light and 8 h dark period, 200-250 $\mu\text{mol}/\text{m}^2/\text{s}$ light
86 intensity. The plants were watered from below every day sufficiently to keep the compost just moist
87 up to two weeks from sowing, then at two-day intervals. Inside the isolation propagator, the average
88 day time temperature was 26.5 °C and night 18.5 °C; relative humidity in day and night ranged
89 between 80% to 85%. The dew point temperature within the covers during the day was about 22 °C
90 and at night about 16 °C; light intensity was 170-220 $\mu\text{mol}/\text{m}^2/\text{s}$.

91

92 ***Inoculation and sampling***

93 Leaves were sampled from lettuce ‘Tom Thumb’ (Thomson & Morgan, Ipswich UK) and
94 *Arabidopsis thaliana* Col-0 plants. In all cases, *B. cinerea* isolate B05.10 (van Kan et al, 2017) was
95 used for inoculation. This is an isolate sampled in Münster, Germany from an unknown source
96 (Büttner et al., 1994). In conventional droplet inoculations it is aggressively pathogenic on both
97 lettuce and *A. thaliana* and numerous other hosts.

98 ***Production and sampling of symptomless infected plants***

99 Internal infection of tissues was verified by isolation on selective agar (Edwards & Seddon, 2001),
100 following surface sterilisation by immersion in 1% sodium hypochlorite and detergent (JANGRO
101 Bleach, Jangro Ltd, Bolton, UK) for 2-3 min followed by three rinses in sterile distilled water.

102 Lettuce plants with symptomless infection were produced by inoculation at the 4-leaf stage using dry
103 dusting of spores diluted in talc at about 90 spores/ mm^2 of leaf, followed by 48h at high humidity
104 produced by enclosure in a polythene bag, but without direct wetting of plant surfaces. In a high
105 proportion of instances this resulted in endophytic, symptomless colonization (Shaw et al, 2016;
106 Table 1). Mock inoculated plants were sampled as control for pre-existing or background infection,
107 and used as negative controls in the RNA quantification. No amplification was seen with any primer
108 pair in mock inoculated plants.

109 Symptomless lettuce tissues were sampled at 14, 24 and 34 days after transplantation to their final
110 growing pots. Colonization by *B. cinerea* was determined by growth on selective medium as above.
111 Two plants which had internal *B. cinerea* infection in most of their sampled leaves were selected as
112 biological replicates and RNA extracted from samples frozen at 14, 24 and 34 days after inoculation.
113 Ten days after the third sampling, 44 days after inoculation, three of the dust-inoculated plants had
114 developed necrotic lesions, and mycelium of *B. cinerea* was visible on older leaves. RNA was
115 extracted from necrotic tissue of two of these plants.

116 *A. thaliana* plants were inoculated at early rosette stage, 21d after sowing, also to a density of about
117 90 spores/mm². They were sampled 10 days after inoculation, at the start of flowering. Rosette
118 leaves, flowering stem, root, stem leaves and flowers were collected separately, and half of each
119 sampled tissue placed on selective medium (Edwards & Seddon, 2001) to detect the presence of
120 symptomless *B. cinerea* (Table 2). In cases where the plated tissue showed *B. cinerea* outgrowth, the
121 remainder of each tissue sample was used for RNA extraction. Two *A. thaliana* plants that had the
122 highest extent of internal symptomless infections were used as biological replicates for RNA
123 extraction from rosette leaves and stem samples.

124 ***Production and sampling of necrotic infections***

125 Samples of RNA from necrotic samples of lettuce were collected from two distinct sources: one was
126 a necrotising infection resulting in about a day from inoculation of detached leaves with droplets of
127 spore suspension; the second was the delayed necrotising infection which eventually developed in
128 systemically infected lettuce following the approximately 40 d symptomless phase of infection.
129 Rapidly necrotising infections of *A. thaliana* were obtained following droplet inoculation of attached
130 leaves on intact plants. For the droplet infections, spore suspensions were prepared from 20 day old
131 *B. cinerea* cultures and applied to leaves as 10 µl droplets of spore suspension (2×10^5 spores/mL) in
132 12g/L potato dextrose broth (Oxoid, UK). Leaves of lettuce with visible symptoms were sampled at

133 12 h, 24 h and 48 h post inoculation. *A. thaliana* leaves were sampled at 3 h, 6 h, 12 h, 24 h and 48 h
134 post inoculation.

135 ***RNA extraction and quantification***

136 RNA extraction used RNeasy plant mini kits (Qiagen, Hilden, Germany) following the
137 manufacturer's protocol. DNA was removed in two stages. The first was column treatment during
138 the extraction procedure using Pure-link DNase kits (Life Technologies, USA). After extraction,
139 Turbo DNase kits (Life Technologies) were used for further purification. cDNA preparation was
140 carried out using High capacity RNA-to-cDNA kits (Applied Biosystems, USA).

141 Transcript levels of ten *B. cinerea* genes were quantified in the RNA samples using the primers listed
142 in Table 3. Four of the genes analysed encode proteins that act in signal transduction during the
143 infection process: the gene *Bcg1* encoding a heterotrimeric G α protein (Gronover et al. 2001); the
144 gene *Bccnb1* encoding the calcineurin β subunit (Harren *et al*, 2012); the adenylate cyclase gene *Bac*
145 (Klimpel et al, 2002); and the MAP kinase gene *Bmp1* (Zheng et al, 2000). Single deletion mutants
146 in each of these genes results in reduction or complete loss of virulence (reviewed in Williamson et
147 al, 2007; Schumacher, 2016). A further five genes analysed encode proteins involved in host tissue
148 degradation or cell death induction: endopolygalacturonase genes *Bcpg1* and *Bcpg2* (the latter gene
149 was only studied in samples from lettuce); the botrydial biosynthetic gene *Bcbot1*; the superoxide
150 dismutase gene *Bcsod1*; and the *Bcnep1* gene encoding a phytotoxic necrosis- and ethylene-inducing
151 protein (reviewed by van Kan, 2006). Transcript levels of these virulence-related genes were
152 normalised to that of *Bcrpl5*, which is the most steadily expressed housekeeping gene, encoding
153 ribosomal protein RPL5 (Zhang et al, 2011). The β -tubulin gene *BctubA* (Benito et al, 1998) was
154 also used as an internal standard, but its transcript was scarce and not detected consistently in
155 symptomless samples. In pilot work, the actin gene *BcAct2* was initially tested but also failed in
156 symptomless samples; the elongation factor *Bcef1a* amplified anomalously early with a non-
157 exponential pattern.

158 qPCR assays for the lettuce samples and *A. thaliana* samples were carried out separately. qPCR
159 reactions were carried out in a partially balanced block experimental design to reduce experimental
160 error; the reactions for each technical replicate of a sample were carried out in separate 96 well
161 plates, and in each plate a subset of genes were tested using all the test extracts, so as to balance
162 comparisons between genes over the whole experiment and minimise effects due to inter-plate
163 differences. Each plate included negative and positive controls and cDNA from all symptomless and
164 necrotic samples from two biological replicates. The amounts of housekeeping gene (*Bcrpl5*) and
165 four genes out of tested nine were quantified in each plate. The assays were carried out in triplicate
166 using the following cyclic conditions: 95 °C for 2 min, then 40 cycles of 95 °C for 15s, 60 °C for
167 30s, using StepOnePlus™ Real-Time PCR System (Applied Biosystems, USA). After each run,
168 melting curves were acquired by heating to 95 °C for 15s, cooling to 60 °C for 1min and heating to
169 95 °C at 0.3 °C, before holding at 95°C for 15s with data collection.

170 ***Data analysis***

171 The contrasts between the C_t of *Bcrpl5* and each gene studied under the two (*A. thaliana*:
172 symptomless, necrotic after inoculation with spore suspension) or three (lettuce: symptomless,
173 delayed necrotic, necrotic after inoculation with spore suspension) types of infection were estimated
174 by REML (restricted maximum likelihood) separately for each mRNA species and host, using
175 Genstat (VSN International, www.vsni.co.uk). Sample origin (including sampling time) was treated
176 as a fixed effect. Random effects were plate, biological replicate, and technical replicate nested
177 within the interaction term between sample origin and biological replicate.

178 Results

179 Lettuce

180 As the host lettuce plants grew, *B. cinerea* spread into newly expanded leaves and was recovered
181 from the majority of (uninoculated) symptomless lettuce leaf tissues plated on selective medium
182 (Table 1; Fig. 1).

183 In lettuce, consistent qRT-PCR amplification could not be achieved from leaf-pairs more distal to the
184 inoculation site (leaves 1-4) than leaves 5-6. Therefore, we present only the results from 5th and 6th
185 leaves, at successive time-periods.

186 *Signalling genes.*

187 There were no clearly significant differences in transcript levels of the *Bcg1* (P=0.4), *Bac* (P=0.09) or
188 *Bccnb1* (P=0.8) genes (Fig. 2, cyan, thicker, symbols) between time-points or types of infection. The
189 level of the signalling gene *Bmp1* transcript was higher (P=0.004) in late symptomless stages, 24 and
190 34 dpi, than in other samples.

191 *Genes involved in cell death or tissue degradation.*

192 The relative concentrations of transcripts of *Bcpg2* and *Bcnep1* differed significantly at consecutive
193 time points in detached lettuce leaves developing necrotic infection (Fig. 3, magenta symbols).
194 *Bcpg2* transcript first increased between 12 and 24 hpi and then decreased at 48 hpi, whereas *Bcnep1*
195 showed the highest transcript level at 12 hpi and strongly dropped at 24 and 48 hpi (Fig. 3, magenta
196 symbols). In necrotic infections which developed following symptomless infection, levels of *Bcpg2*
197 and *Bcnep1* transcripts were low (Fig. 3, black symbols). *Bcbot1* was expressed at a steady level in
198 detached leaf necrotic infections, but significantly less (P=0.007) in the delayed necrotic infection.
199 Transcript levels of the genes *Bcpg1* and *Bcsod1* were quite similar in both types of necrotic

200 infections, both on detached lettuce leaves inoculated with spore suspensions (12, 24 and 48hpi) and
201 the delayed necrotic lesions forming from symptomless colonization.

202 Transcript patterns of genes were quite distinct in the non-symptomatic infection (Fig. 3, cyan,
203 thicker, symbols) and the necrotic infections (Fig. 3, magenta and black symbols). Notably, *Bcbot1*
204 transcript was undetectable in non-symptomatic lettuce tissues, abundant in necrotic infections of
205 detached leaves and scarce in delayed necrotic infection. By contrast, there was more *Bcnepl*
206 transcript in non-symptomatic tissues than in either type of necrotic infection ($P<0.001$). The level of
207 *Bcpg2* transcript rose slightly over time in symptomless infection and was comparable with that in
208 necrotic infections of detached leaves but much higher than in delayed necrotic infection. *Bcpg1*
209 transcript was consistently lower in non-symptomatic or delayed necrotic infections than in necrotic
210 infection from direct inoculation ($P=0.05$). There were no differences over time or between types of
211 infection in levels of *Bcsod1* transcript.

212 ***Arabidopsis thaliana***

213 Dry spore inoculated plants remained symptomless over the entire length of the experiment (Fig. 1).

214 *Signalling genes.*

215 Transcript levels of the signalling genes differed between infection types (Fig. 4). Transcript levels
216 of *Bcg1* were higher in symptomless stem samples (cyan, thicker, symbols) than in any necrotic
217 stage (magenta symbols), or in symptomless rosette leaves (cyan symbols). Transcripts of the *Bac*
218 gene were detected in only one of 4 samples from symptomless infections, suggesting a substantial
219 depletion of cAMP signalling in the asymptomatic *A. thaliana* infection. The transcript levels of
220 *Bccnb1* were similar in symptomless leaves and stems (both cyan symbols), and in all necrotic stages
221 (magenta symbols). Transcript levels of *Bmp1* were marginally higher in symptomless infections
222 than in early necrotic infections.

223 *Genes involved in cell death or tissue degradation.*

224 Transcript levels of virulence-related genes also differed between sample timings and infection types
225 (Fig. 5). The transcript levels of *Bcnep1*, *Bcbot1*, and *Bcpq1* rose between 24 and 48 h old
226 necrotising infections, coinciding with the appearance of necrotic lesions; *Bcsod1* rose, but less
227 extremely. The transcripts of *Bcnep1* were not detected in any of the symptomless samples while
228 *Bcbot1* transcripts were (barely) above the detection threshold in only one of the samples (magenta
229 symbols). By contrast, transcript levels of *Bcpq1* and *Bcsod1* in symptomless samples were
230 comparable to those in the 48 hpi necrotising samples.

231 Discussion

232 The experiments reported here show that the transcriptional state of *B. cinerea* in a symptomless,
233 systemic growth phase in lettuce cv. Tom Thumb and *A. thaliana* is distinct from that of a
234 necrotizing infection, either developing on detached leaves following inoculation with spore
235 suspensions or developing from non-symptomatic *B. cinerea*-infected plants obtained by dusting low
236 amounts of dry spores onto seedlings.

237 It would be useful to know how general the results are. In lettuce Tom Thumb and *A. thaliana* col-0
238 there are some clear differences: in *A. thaliana* the signalling gene *Bac* is much more transcribed in
239 the symptomless samples than in the necrotic, which is not the case in lettuce (*cf* Fig. 2 and Fig. 4);
240 transcript levels of the toxin-producing gene *Bcbot1* are similar and low in symptomless and late-
241 appearing necrotic infections in lettuce (*cf* Fig. 3 and Fig. 5), but not in *A. thaliana*. Lettuce ‘All the
242 Year Round’ and several other wild and cultivated plant species (Shaw *et al.*, 2016) support
243 symptomless and systemic infection. It would be of great interest to see whether transcript pool
244 patterns in *B. cinerea* fell into distinct groups; it would also be extremely interesting to see whether
245 the other *Botrytis* species found to establish symptomless systemic infections (Shaw *et al.*, 2016)
246 have similar patterns of up- and down- regulation of transcript pools.

247 For the genes involved in virulence-related signal transduction, it was not particularly surprising to
248 note that their transcript levels were mostly similar between necrotizing *B. cinerea* infections and
249 non-symptomatic infected tissue. Although these genes are essential to establish necrotic infections,
250 signal transduction pathways involving heterotrimeric G proteins, cAMP, calcium and/or MAP
251 kinase activity are also essential in various developmental stages in the fungal life cycle
252 (Schumacher, 2016). These signalling pathways affect many post-transcriptional and post-
253 translational feedback mechanisms. With the exception of the *Bac* transcript in symptomless *A.*
254 *thaliana* tissues, the transcript levels of these genes are similar. It is therefore difficult to infer from
255 these observations any regulatory pathways in the fungus that may explain the differences between
256 the types of infection. The extremely low level of the *Bac* transcript in symptomless *A. thaliana*
257 implies that the fungus is experiencing a depletion of cAMP, however, the resulting impact on the
258 production of enzymes or secondary metabolites that damage or kill plant cells remains unknown.
259 There are no reports of studies of a functional link between cAMP levels and the expression of
260 *Bcnep1* and *Bcbot1*..

261 An obvious hypothesis as to how systemic infection can progress without visible symptoms is that
262 transcription of necrosis-related genes is suppressed, since the production of enzymes or secondary
263 metabolites that damage or kill plant cells would be detrimental in sustaining a non-symptomatic,
264 endophytic interaction between a fungus and its host plant. Consistent with this hypothesis, the
265 *Bcbot1* transcript, coding for a crucial enzyme in the biosynthesis of the toxin botrydial, was
266 undetectable in symptomless infections (both in lettuce and *A. thaliana*). However, in plants
267 containing a symptomless *B. cinerea* infection we observed high levels of transcripts from the
268 *Bcnep1*, *Bcpg1* and *Bcpg2* genes in lettuce and *Bcnep1* and *Bcpg1* in *A. thaliana*. BcNEP1 protein
269 can induce host programmed cell death in leaf tissue of all dicots tested (Schouten et al, 2007; Cuesta
270 Arenas et al, 2010). In symptomless infections, its cell death-inducing capacity may be mitigated by
271 other fungal (suppressor) proteins, or by a reduction of protein excretion. Alternatively,

272 physiological changes in the plant might make it locally insensitive to BcNEP1-induced death.
273 Although receptors required for NEP1-like-protein (NLP)-mediated immune response activation (but
274 not death) have been identified (Albert et al, 2015), the mechanisms underlying plant cell death
275 induction by NLPs remain to be unravelled. Besides production of phytotoxic proteins like BcNEP1,
276 secretion of endopolygalacturonases BcPG1 and BcPG2 by *B. cinerea* is potentially damaging to
277 plants, as these enzymes hydrolyse pectin and thereby affect plant cell wall architecture and integrity.
278 Both enzymes, when infiltrated into leaf mesophyll tissue, may cause rapid tissue collapse (Kars et
279 al, 2005). More recently, *B. cinerea* endopolygalacturonases have been shown to act as MAMPs that
280 in *A. thaliana* can be recognized by an LRR-type receptor (Zhang et al, 2014). It remains elusive
281 why the relatively high expression of the above three genes does not result in visible tissue damage.
282 Whether the transcripts are actually translated into proteins that are secreted into the host tissue could
283 not be investigated for lack of a sufficiently sensitive detection method.

284 Taken together, the observations clearly suggest that host and pathogen do interact during
285 symptomless infection. The results reject the hypothesis that the symptomless state is due to many
286 very small, spatially restricted necrotic infections. The symptomless state involves pathogen growth
287 in association with the host and involves exchanges between host and pathogen, but the outcome of
288 this exchange differs from a necrotising infection. Histological understanding of the interaction
289 would be desirable. However the extreme relative scarcity of fungal RNA in samples from
290 symptomless infected tissue - and therefore low density of fungal cells - makes makes it hard to
291 observe the fungus within plant tissue, and work with fluorescently marked, but otherwise
292 physiologically normal, *B. cinerea* is needed.

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370

371 **Figure Legends**

372 Figure 1. (a-d) Successive stages of growth (14, 24, 34 and 44 d post inoculation (dpi)) of lettuce
373 inoculated at the 4-leaf stage with dry spores of *Botrytis cinerea*; (d) shows severe internal necrosis.
374 Apparent variation in colour is due to varying natural light conditions at the time of photography. Pot
375 diameter is 13 cm in each picture. (e) Detached lettuce leaf 48 h after inoculation with droplets of a
376 spore suspension of the same isolate of *B. cinerea*. (f) *Arabidopsis thaliana* growing in an isolation
377 propagator at the time of inoculation with dry spores of *B. cinerea* (photograph was taken through
378 the polystyrene propagator cover); (g) *A. thaliana* 10 dpi, with the propagator lid removed for
379 sampling.

380 **Figure 2.** Relative amount of mRNA of selected *Botrytis cinerea* signalling-related genes at
381 successive time points following inoculation of lettuce plants (indicated on the common x-axis as
382 days after inoculation). Width of symbols shows the likelihood of C_t values around the mean,
383 assuming normality, with the observed SEM between biological replicates, based on all time points.
384 Horizontal bars in the symbol show the estimated mean. Infections were: (magenta) necrotic
385 resulting from droplet infection of detached leaves; (cyan and thicker) symptomless in the 5th and
386 6th leaves, or (black) necrotic developed in the 5th and 6th leaves following symptomless infection.
387 Black horizontal lines show the threshold detection level, calculated as the difference between the
388 mean C_t value of *Bcrp15* and the detectable maximum C_t value of the reaction, which was always 40.
389 All values are relative to *Bcrp15* which therefore forms the 0-line on the y-axis (ie, the values are in
390 effect ΔC_t from *Bcrp15*). Testing for differences between time-points by REML, omitting samples
391 with no RNA signal: *Bccnp1*, $P=0.8$, s.e. = 1.1; *Bac*, $P=0.09$, s.e. = 1.1; *Bcg1*, $P=0.4$, s.e. = 0.55;
392 *Bmp1*, $P=0.004$, s.e. = 0.86. Where no detectable amplicon was formed, the possible range of ΔC_t is
393 shown as a vertical line terminating at the threshold level.

394

395 **Figure 3:** Relative amount of mRNA of selected *Botrytis cinerea* pathogenicity-related genes at
396 different time points following inoculation of lettuce plants. Symbols, colour-coding, thresholds and
397 abbreviations as in Fig. 2. Testing for differences between time-points by REML omitting samples
398 with no RNA signal: *Bcsod1*, P=0.6, s.e. = 0.89; *Bcpg1*, P=0.05, s.e. = 1.3; *Bcpg2*, P=0.008, s.e.
399 =1.5; *Bcnep1*, P<0.001, s.e. = 0.75; *Bcbot1*, P=0.007, s.e. = 0.9. All values are relative to *Bcrp15* as
400 in Fig. 2. Where no detectable amplicon was formed, the possible range of ΔC_t is shown as a vertical
401 line terminating at the threshold

402 **Figure 4:** Relative amount of mRNA of selected *Botrytis cinerea* signalling genes at different time
403 points following inoculation of *Arabidopsis thaliana* col1 plants. Symbols, abbreviations and
404 common x-axis as in Fig. 2. Infections were: (magenta) necrotic, resulting from droplet infection of
405 attached rosette leaves; (cyan, thicker) symptomless 10 days after inoculation in newly produced
406 rosette leaves; or (cyan) symptomless in stem and stipule samples 10 days after inoculation of the
407 rosette . All values are relative to *Bcrp15* as in Fig. 2. Testing for differences between time-points
408 by REML, omitting samples with no RNA signal: *Bccnb1*, P=0.06, s.e. = 0.71; *Bcac*, P<0.001, s.e. =
409 0.5; *Bcg1*, P=0.001, s.e. = 0.92; *Bmp1*, P=0.01, s.e. = 0.77. Where no detectable amplicon was
410 formed, the possible range of ΔC_t is shown as a vertical line terminating at the threshold detection
411 limit. A dashed line indicates one replicate had no detectable amplicon; the other replicate is shown
412 as a small circle.

413
414 **Figure 5:** Relative amount of mRNA of selected *Botrytis cinerea* pathogenicity-related genes at
415 different time points following inoculation of *Arabidopsis thaliana* col-0 plants. Symbols and
416 abbreviations as in Fig. 2; colour-coding and vertical lines as in Fig. 4. Testing for differences
417 between time-points by REML, omitting samples with no RNA signal: *Bcsod1*, P=0.004, s.e.=0.78;
418 *Bcpg1*, P<0.001, s.e. = 0.92; *Bcnep1*, P<0.001, s.e. = 0.46; *Bcbot1*, P=0.006, s.e. = 2.2.

Table 1. Numbers of samples of lettuce leaf tissue (n=10) from which *Botrytis cinerea* was recovered following incubation on Botrytis selective medium, with or without surface sterilisation of tissue samples taken after destructive harvest of inoculated plants at three intervals after inoculation. At 44d post inoculation necrotising lesions were common on older leaves.

Leaf number ^a	Not surface sterilised			Surface sterilised		
	14dai ^b	24dai	34dai	14dai	24dai	34dai
5-6	10	nt ^c	nt	3	nt	nt
7-8	10	nt	nt	5	nt	nt
9-10	10	7	nt	4	9	nt
11-12	- ^d	7	6	-	7	6
13-14	-	7	4	-	7	4
15-16	-	-	3	-	-	3
17-18	-	-	2	-	-	2
Stem		10	6		10	6
Root	10	8	6	1	8	6

^a Leaf pairs numbered in order of expansion; lettuce has opposite phyllotaxis

^b Days after inoculation with a dust of *Botrytis* spores at the 4 leaf stage

^c Not tested, for logistic reasons (symptomless infection was already common at the previous occasion)

^d Leaf not yet expanded

Table 2. Recovery of *Botrytis cinerea* from *Arabidopsis thaliana* col0 tissues following incubation on Botrytis selective medium, with or without prior surface sterilisation of tissue samples. Plants were inoculated 10 d after sowing and destructively harvested 10 d later.

Tissue	Number sampled per plant	Number sampled per treatment ^a	Number of samples with <i>B. cinerea</i>	
			Not surface sterilised	Surface sterilised
Root	1	10	0	1
Rosette leaf	3	30	23	16
Stem	2	20	12	6
Stem leaf	2	20	17	5
Flower	2	20	16	4

^a Equal numbers of samples were incubated with and without surface sterilisation.

Table 3: Primers used for qRT-PCR of mRNA in tissue samples from symptomless systemic infections of lettuce or *Arabidopsis thaliana* with *Botrytis cinerea*. All primer pairs were designed to cross an exon-exon junction, except for *Bcpg1*, where no introns are present.

Gene	Gene product	Gene ID	NCBI accession numbers	Sequence (5'-3')	Amplicon size (bp)
<i>Bcg1</i>	Heterotrimeric G-protein α subunit	Bcin05g06770	Y18436.1	F- CAAGATGCTTCTTCTTGGAG R- TGATTGGACTGTGTTGCTGA	139
<i>Bmpl</i>	Mitogen-activated protein kinase 1	Bcin02g08170	AF205375.1	F- GCTTATGGTGTGTTGTCTGCTC R- TAGCTTCATCTCACGAAGTG	120
<i>Bac</i>	Adenylate cyclase	Bcin15g02590	AJ276473.1	F- GGTGAAGACGGATAGATCAAGTAG R- CTCCCGTGGGGACACATTAG	121
<i>Bccnb1</i>	Calcineurin β subunit	Bcin03g05990	KC935338.1	F- GTCGAATCCTCTAGCTACCAGAA R- GAATGCGCTGAGTCCACTG	97
<i>Bcsod1</i>	Superoxide dismutase	Bcin03g03390	AJ555872.1	F- ATTGAGCGTCATTGGCCGTA R- TGGACTCTTCGTTCTCTCCC	77
<i>Bcpg1</i>	Endopolygalacturonase 1	Bcin14g00850	EF195782.1	F- ACTCTGCTGGAGATGCTGGT R- TAGCGAGACAGTAATCTTGG	97
<i>Bcpg2</i>	Endopolygalacturonase 2	Bcin14g00610	AY665553.1	F- GGAAGTCCACTTTTGGTTAC R- TCCATCCCACCATCTTGCTC	126
<i>Bcbot1</i>	Botrydial biosynthetic enzyme	Bcin12g06380	AY277723.2	F- TTATGCCGCACTCCACGAGA R- TCCAGAGGAGTAGACCTCAT	103
<i>Bcnep1</i>	Necrosis ethylene-inducing protein 1	Bcin06g06720	DQ211824.1	F- GTAATGGTAACACCAGTGGT R- AGCCACCTCGGACATAGGTT	96
<i>Bcrpl5</i>	Ribosomal protein large subunit 5	Bcin14g04230	AL116000.1	F-GATGAGACCGTCAAATGGTTC R- CAGAAGCCCACGTTACGACA	137









