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

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Botrytis cinerea can establish long-lived, symptomless, systemic infections in plant species. It is unclear how the fungus colonizes plant tissues without causing tissue damage and necrosis. Three hypotheses are: (i) the fungus state is similar in the two forms of infection, but the plant defences are more effective, leading to multiple small quiescent centres; (ii) excreted molecules that would trigger plant defences are suppressed; (iii) signal exchanges occur avoiding both extensive host cell death and complete spatial restriction of the pathogen. These hypotheses were tested by comparing transcript levels of a set of *B. cinerea* genes between symptomless and necrotizing infections. Four genes were analysed that participate in signalling pathways required for virulence, as well as five genes that directly participate in causing host cell death or degrading plant cell wall polysaccharides. In lettuce, necrotic infections on detached leaves (12–48 h after inoculation) had similar gene expression patterns to necrotic infections on leaves 44 days after inoculation of the seedlings. Symptomless infections on leaves that expanded after inoculation of young seedlings had similar fungal gene expression patterns at 14, 24 and 34 days after inoculation, which clearly differed from those in necrotizing infections. In *Arabidopsis thaliana*, there were differences in gene expression patterns between droplet inoculations on leaves, resulting in necrotic lesions, and symptomless infections in stems and leaves. The fungal gene expression patterns differed in detail between lettuce and *A. thaliana*. The observations suggest that the physiological state of *B. cinerea* during symptomless infection is distinct from necrotizing infections.

Keywords: botrydial, endophyte, *Lactuca sativa*, latent, systemic, transcription

Introduction

Botrytis cinerea is a plant pathogenic fungus causing grey mould disease and post-harvest losses in more than 1000 crops, ranging from ornamentals to vegetables and field crops (Elad *et al.*, 2016). Symptoms produced by *B. cinerea* range from restricted lesions to dry or spreading soft rots that often produce conspicuous sporulating colonies (Williamson *et al.*, 2007). In general, *B. cinerea* is considered to be a necrotroph, which draws nourishment from dead host tissue and produces initially local ('primary') necrotic lesions, which subsequently expand to actively cause plant tissue decomposition (Horst, 1983; Jarvis, 1994; Coertze & Holz, 2002; Elad *et al.*, 2004). In contrast, recent studies have revealed that *B. cinerea* can also cause symptomless systemic infection in several host plants including *Primula* spp., lettuce (*Lactuca sativa*), *Arabidopsis thaliana* and *Taraxacum vulgare* (Barnes & Shaw, 2003; Rajaguru & Shaw, 2010; Sowley *et al.*, 2010; Shaw *et al.*, 2016). In this type of infection the fungus grows along with the plant and enters newly expanding organs, without producing symptoms, until the plant becomes physiologically susceptible, typically at flowering. At this point extensive areas of host tissue death develop simultaneously, followed by sporulation of the fungus. Several species in the genus *Botrytis* are able to infect in this way (Shaw *et al.*, 2016). The physiological relationship between host and pathogen during symptomless systemic growth is unresolved. It is unclear how a fungus that can produce such a large arsenal of phytotoxic metabolites and proteins (van Kan, 2006) is able to grow inside plant tissue without causing extensive tissue damage and visual disease symptoms. The aim of the present study was to obtain preliminary insight into this question by comparing the expression of a set of fungal genes that participate in regulating virulence or in causing host cell death, between symptomless and necrotizing infections, in two host species.

A previous study illustrated that in some species a high proportion of symptomless plants can be infected with *B. cinerea*. The distribution of the fungus appeared to be discontinuous and scattered over distinct tissues

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(Shaw *et al.*, 2016). Given the unpredictable location of the fungus within plant tissue and the low fungal biomass at any given time point, the abundance of *Botrytis* mRNA in total RNA extracted from a symptomless plant is expected to be low. Such low abundance hampers a full transcriptome study by RNAseq throughout the plant in order to detect changes associated with infection, as only abundant fungal transcripts would be reliably quantified by direct sequencing. Therefore, quantitative RT-PCR was used to estimate relative concentrations of a selected set of nine fungal transcripts, encoding signalling components involved in virulence or proteins directly involved in necrotizing infection.

Materials and methods

Plant growth

Lettuce (*L. sativa*) cv. Tom Thumb (Thomson & Morgan) was used in all experiments reported here. Two 80-cell modular seedling trays were filled with compost (Levington F1 seed and modular compost; Westland Ltd), and in each tray one seed per cell was sown, covered with a thin layer of compost. Seed germination and initial seedling growth was in controlled environment chambers: 20 °C day/18 °C night, 12 h photoperiod and RH 65%. The compost was kept damp to touch all the time. Seven days after inoculation (dai) (14 days after seed sowing), 40 inoculated and 40 noninoculated seedlings were transplanted into 1 L pots filled with potting compost (John Innes 2 compost + 4 g L^{-1} Osmocote). Later the seedlings were moved to a vented glasshouse under natural light in summer (May–Aug, 15–16.5 h daylength, temperature maintained at 18 °C minimum at night, rising to about 30 °C in the day). Plants were watered daily.

Arabidopsis thaliana (Col-0) seeds were surface disinfected in 70% ethanol for 2 min and then 20% bleach (1% NaOCl) for 5 min and finally thoroughly rinsed in sterile water five or six times. After surface disinfection, seed stratification was done at 4 °C for 4 days. Seeds were sown singly on the surface of the compost in pots covered with transparent polystyrene propagation covers with vents, and grown on in these propagators. The propagators were maintained at positive pressure via a pumped filtered airflow within a controlled environment cabinet at 22 °C day/18 °C night, 16 h light/8 h dark photoperiod, RH 65% and 200–250 μmol m^{-2} s^{-1} light intensity. The plants were sufficiently watered from below every day to keep the compost just moist up to 2 weeks from sowing, then at 2-day intervals. Inside the isolation propagator, the average temperature was 26.5 °C during the day and 18.5 °C at night; RH ranged between 80% and 85%. The dew point temperature within the covers was about 22 °C during the day and about 16 °C at night; light intensity was 170–220 μmol m^{-2} s^{-1}.

Inoculation and sampling

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

Production and sampling of symptomless infected plants

Internal infection of tissues was verified by isolation on selective agar (Edwards & Seddon, 2001), following surface sterilization by immersion in 1% NaOCl and detergent (JANGRO Bleach; Jangro Ltd) for 2–3 min followed by three rinses in sterile distilled water.

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

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

Arabidopsis thaliana plants were inoculated at early rosette stage, 21 days after sowing, also to a density of about 90 spores mm^{-2}. They were sampled 10 days after inoculation, at the start of flowering. Rosette leaves, flowering stem, root, stem leaves and flowers were collected separately, and half of each sampled tissue placed on selective medium (Edwards & Seddon, 2001) to detect the presence of symptomless *B. cinerea*

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 sterilization of tissue samples taken after destructive harvest of inoculated plants at three intervals after inoculation (days after inoculation, dai)

	Not surface sterilized			Surface sterilized		
Leaf number[a]	14 dai[b]	24 dai	34 dai	14 dai	24 dai	34 dai
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

At 44 dai, necrotizing lesions were common on older leaves.
[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 logistical reasons (symptomless infection was already common at the previous occasion).
[d]Leaf not yet expanded.

Table 2 Recovery of *Botrytis cinerea* from *Arabidopsis thaliana* Col-0 tissues following incubation on *Botrytis* selective medium, with or without prior surface sterilization of tissue samples

Tissue	Number sampled per plant	Number sampled per treatment[a]	Number of samples with *B. cinerea*	
			Not surface sterilized	Surface sterilized
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

Plants were inoculated 10 days after sowing and destructively harvested 10 days later.

[a]Equal numbers of samples were incubated with and without surface sterilization.

(Table 2). In cases where the plated tissue showed *B. cinerea* outgrowth, the remainder of each tissue sample was used for RNA extraction. Two *A. thaliana* plants that had the highest extent of internal symptomless infections were used as biological replicates for RNA extraction from rosette leaves and stem samples.

Production and sampling of necrotic infections

Samples of RNA from necrotic lettuce were collected from two distinct sources: one was a necrotizing infection resulting about a day after inoculation of detached leaves with droplets of a spore suspension; the second was the delayed necrotizing infection that eventually developed in systemically infected lettuce following the approximately 40 days symptomless phase of infection. Rapidly necrotizing infections of *A. thaliana* were obtained following droplet inoculation of attached leaves on intact plants. For the droplet infections, spore suspensions were prepared from 20-day-old *B. cinerea* cultures and applied to leaves as 10 μL droplets of spore suspension (2×10^5 spores mL^{-1}) in 12 g L^{-1} potato dextrose broth (Oxoid). Leaves of lettuce with visible symptoms were sampled at 12, 24 and 48 h post-inoculation. *Arabidopsis thaliana* leaves were sampled at 3, 6, 12, 24 and 48 h post-inoculation.

RNA extraction and quantification

RNA extraction was carried out using RNeasy Plant Mini kits (QIAGEN) following the manufacturer's protocol. DNA was removed in two stages. The first was column treatment during the extraction procedure using Pure-link DNase kits (Life Technologies). After extraction, Turbo DNase kits (Life Technologies) were used for further purification. cDNA preparation was carried out using High Capacity RNA-to-cDNA kits (Applied Biosystems).

Transcript levels of 10 *B. cinerea* genes were quantified in the RNA samples using the primers listed in Table 3. Four of the genes analysed encode proteins that act in signal transduction during the infection process: the gene *Bcg1* encoding a heterotrimeric Gα protein (Gronover *et al.*, 2001); the gene *Bccnb1* encoding the calcineurin β subunit (Harren *et al.*, 2012); the adenylate cyclase gene *Bac* (Klimpel *et al.*, 2002); and the MAP kinase gene *Bmp1* (Zheng *et al.*, 2000). Single deletion mutants in each of these genes results in reduction or complete loss of virulence (reviewed in Williamson *et al.*, 2007; Schumacher, 2016). A further five genes analysed encode proteins involved in host tissue degradation or cell death induction: endopolygalacturonase genes *Bcpg1* and *Bcpg2* (the latter gene was only studied in samples from lettuce); the botrydial biosynthetic gene

Table 3 Primers used for qRT-PCR of mRNA in tissue samples from symptomless systemic infections of lettuce or *Arabidopsis thaliana* with *Botrytis cinerea*

Gene	Gene product	Gene ID	NCBI accession number	Sequence (5′–3′)		Amplicon size (bp)
Bcg1	Heterotrimeric G-protein α subunit	Bcin05 g06770	Y18436.1	F	CAAGATGCTTCTTCTTGGAG	139
				R	TGATTGGACTGTGTTGCTGA	
Bmp1	Mitogen-activated protein kinase 1	Bcin02 g08170	AF205375.1	F	GCTTATGGTGTTGTCTGCTC	120
				R	TAGCTTCATCTCACGAAGTG	
Bac	Adenylate cyclase	Bcin15 g02590	AJ276473.1	F	GGTGAAGACGGATAGATCAAGTAG	121
				R	CTCCCGTGGGGACACATTAG	
Bccnb1	Calcineurin β subunit	Bcin03 g05990	KC935338.1	F	GTCGAATCCTCTAGCTACCAGAA	97
				R	GAATGCGCTGAGTCCACTG	
Bcsod1	Superoxide dismutase	Bcin03 g03390	AJ555872.1	F	ATTGAGCGTCATTGGCCGTA	77
				R	TGGACTCTTCGTTCTCTCCC	
Bcpg1	Endopolygalacturonase 1	Bcin14 g00850	EF195782.1	F	ACTCTGCTGGAGATGCTGGT	97
				R	TAGCGAGACAGTAATCTTGG	
Bcpg2	Endopolygalacturonase 2	Bcin14 g00610	AY665553.1	F	GGAACTGCCACTTTTGGTTAC	126
				R	TCCATCCCACCATCTTGCTC	
Bcbot1	Botrydial biosynthetic enzyme	Bcin12 g06380	AY277723.2	F	TTATGCCGCACTCCACGAGA	103
				R	TCCAGAGGAGTAGACCTCAT	
Bcnep1	Necrosis ethylene-inducing protein 1	Bcin06 g06720	DQ211824.1	F	GTAATGGTAACACCAGTGGT	96
				R	AGCCACCTCGGACATAGGTT	
Bcrpl5	Ribosomal protein large subunit 5	Bcin14 g04230	AL116000.1	F	GATGAGACCGTCAAATGGTTC	137
				R	CAGAAGCCCACGTTACGACA	

All primer pairs were designed to cross an exon-exon junction, except for *Bcpg*1, where no introns are present.

Bcbot1; the superoxide dismutase gene *Bcsod1*; and the *Bcnep1* gene encoding a phytotoxic necrosis- and ethylene-inducing protein (reviewed by van Kan, 2006). Transcript levels of these virulence-related genes were normalized to that of *Bcrpl5*, which is the most steadily expressed housekeeping gene, encoding ribosomal protein RPL5 (Zhang *et al.*, 2011). The β-tubulin gene *BctubA* (Benito *et al.*, 1998) was also used as an internal standard, but its transcript was scarce and not detected consistently in symptomless samples. In pilot work, the actin gene *BcAct2* was initially tested but also failed in symptomless samples; the elongation factor *Bcef1a* amplified anomalously early with a nonexponential pattern.

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

Data analysis

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

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

Results

Lettuce

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

In lettuce, consistent qRT-PCR amplification could not be achieved from leaf pairs more distal to the inoculation site (leaves 1–4) than leaves 5–6. Therefore, only the results from fifth and sixth leaves, at successive time periods, are presented.

Signalling genes

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

Genes involved in cell death or tissue degradation

The relative concentrations of transcripts of *Bcpg2* and *Bcnep1* differed significantly at consecutive time points in detached lettuce leaves developing necrotic infection (Fig. 3, magenta/thin symbols on left). *Bcpg2* transcript first increased between 12 and 24 hpi and then decreased at 48 hpi, whereas *Bcnep1* showed the highest transcript level at 12 hpi and strongly dropped at 24 and 48 hpi (Fig. 3, magenta/thin symbols on left). In necrotic infections that developed following symptomless infection, levels of *Bcpg2* and *Bcnep1* transcripts were low (Fig. 3, black/thin symbols on right). *Bcbot1* was expressed at a steady level in detached leaf necrotic infections, but significantly less ($P = 0.007$) in the delayed necrotic infection. Transcript levels of the genes *Bcpg1* and *Bcsod1* were quite similar in both types of necrotic infections, both on detached lettuce leaves inoculated with spore suspensions (12, 24 and 48 hpi) and on the delayed necrotic lesions forming from symptomless colonization.

Transcript patterns of genes were quite distinct in the symptomless infection (Fig. 3, cyan/thick symbols) and the necrotic infections (Fig. 3, magenta and black/thin symbols). Notably, *Bcbot1* transcript was undetectable in symptomless lettuce tissues, abundant in necrotic infections of detached leaves and scarce in delayed necrotic infection. By contrast, there was more *Bcnep1* transcript in symptomless tissues than in either type of necrotic infection ($P < 0.001$). The level of *Bcpg2* transcript rose slightly over time in symptomless infection and was comparable with that in necrotic infections of detached leaves but much higher than in delayed necrotic infection. *Bcpg1* transcript was consistently lower in symptomless or delayed necrotic infections than in necrotic infection from direct inoculation ($P = 0.05$). There were no differences over time or between types of infection in levels of *Bcsod1* transcript.

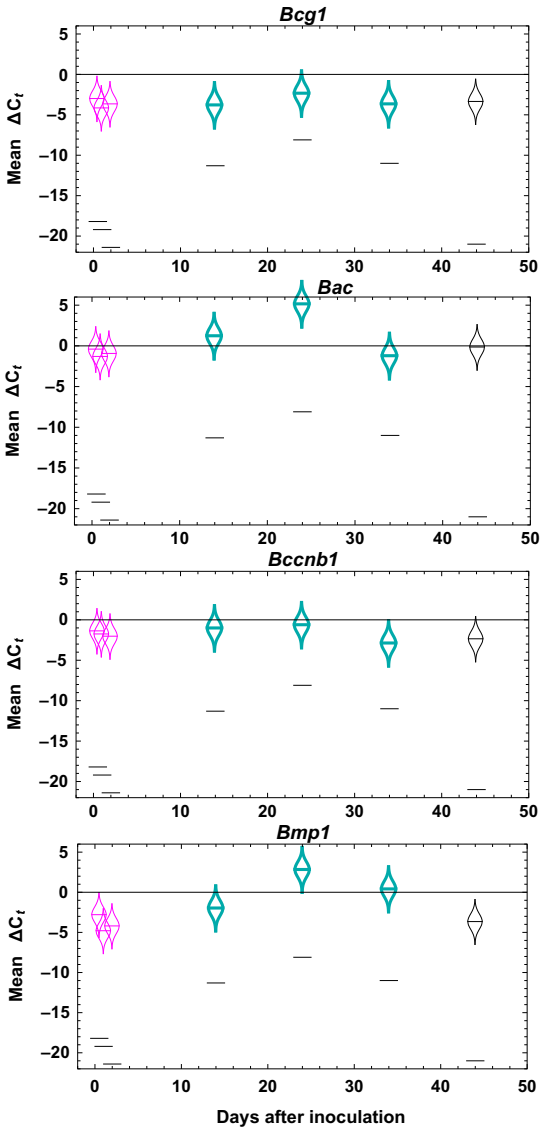

Figure 2 Relative amount of mRNA of selected *Botrytis cinerea* signalling-related genes at successive time points following inoculation of lettuce plants (indicated on the common *x*-axis as days after inoculation). Width of symbols shows the likelihood of C_t values around the mean, assuming normality, with the observed SEM between biological replicates, based on all time points. Horizontal bars in the symbol show the estimated mean. Infections were: (magenta/thin) necrotic resulting from droplet infection of detached leaves; (cyan/thick) symptomless in the 5th and 6th leaves, or (black/thin) necrotic developed in the 5th and 6th leaves following symptomless infection. Black horizontal lines show the threshold detection level, calculated as the difference between the mean C_t value of *Bcrpl5* and the detectable maximum C_t value of the reaction, which was always 40. All values are relative to *Bcrpl5* which therefore forms the 0-line on the *y*-axis (i.e. the values are in effect ΔC_t from *Bcrpl5*). Testing for differences between time points by REML, omitting samples 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; *Bmp1*, $P = 0.004$, s.e. = 0.86. Where no detectable amplicon was formed, the possible range of ΔC_t is shown as a vertical line terminating at the threshold level. [Colour figure can be viewed at wileyonlinelibrary.com].

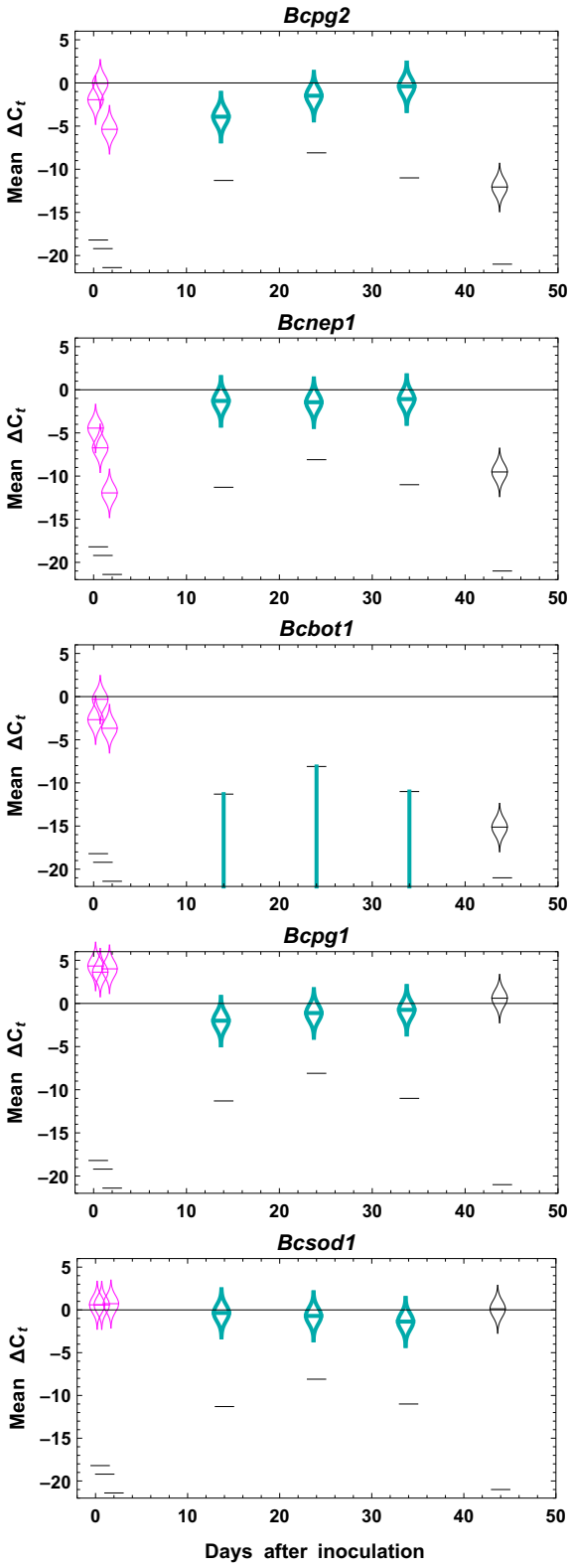

Figure 3 Relative amount of mRNA of selected *Botrytis cinerea* pathogenicity-related genes at different time points following inoculation of lettuce plants. Symbols, colour coding, thresholds and abbreviations as in Figure 2. Testing for differences between time points by REML, omitting samples with 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. = 1.5; *Bcnep1*, $P < 0.001$, s.e. = 0.75; *Bcbot1*, $P = 0.007$, s.e. = 0.9. All values are relative to *Bcrpl5* as in Figure 2. Where no detectable amplicon was formed, the possible range of ΔC_t is shown as a vertical line terminating at the threshold. [Colour figure can be viewed at wileyonlinelibrary.com].

Arabidopsis thaliana

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

Signalling genes

Transcript levels of the signalling genes differed between infection types (Fig. 4). Transcript levels of *Bcg1* were higher in symptomless stem samples (cyan/thick symbols) than in any necrotic stage (magenta symbols on left), or in symptomless rosette leaves (cyan symbols on right). Transcripts of the *Bac* gene were detected in only one of four samples from symptomless infections, suggesting a substantial depletion of cAMP signalling in the symptomless *A. thaliana* infection. The transcript levels of *Bccnb1* were similar in symptomless leaves and stems (both cyan symbols), and in all necrotic stages (magenta symbols). Transcript levels of *Bmp1* were marginally higher in symptomless infections than in early necrotic infections.

Genes involved in cell death or tissue degradation

Transcript levels of virulence-related genes also differed between sample timings and infection types (Fig. 5). The transcript levels of *Bcnep1*, *Bcbot1* and *Bcpg1* rose between 24 and 48 hpi in necrotizing infections, coinciding with the appearance of necrotic lesions; *Bcsod1* rose, but less extremely. The transcripts of *Bcnep1* were not detected in any of the symptomless samples while *Bcbot1* transcripts were (barely) above the detection threshold in only one of the samples (magenta symbols on left). By contrast, transcript levels of *Bcpg1* and *Bcsod1* in symptomless samples were comparable to those in the 48 hpi necrotizing samples.

Discussion

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

It would be useful to know how general the results are. In lettuce cv. Tom Thumb and *A. thaliana* Col-0 there are some clear differences: in *A. thaliana* the signalling gene *Bac* is much more transcribed in the symptomless samples than in the necrotic, which is not the case in lettuce (cf. Figs 2 & 4); transcript levels of the toxin-producing gene *Bcbot1* are similar and low in

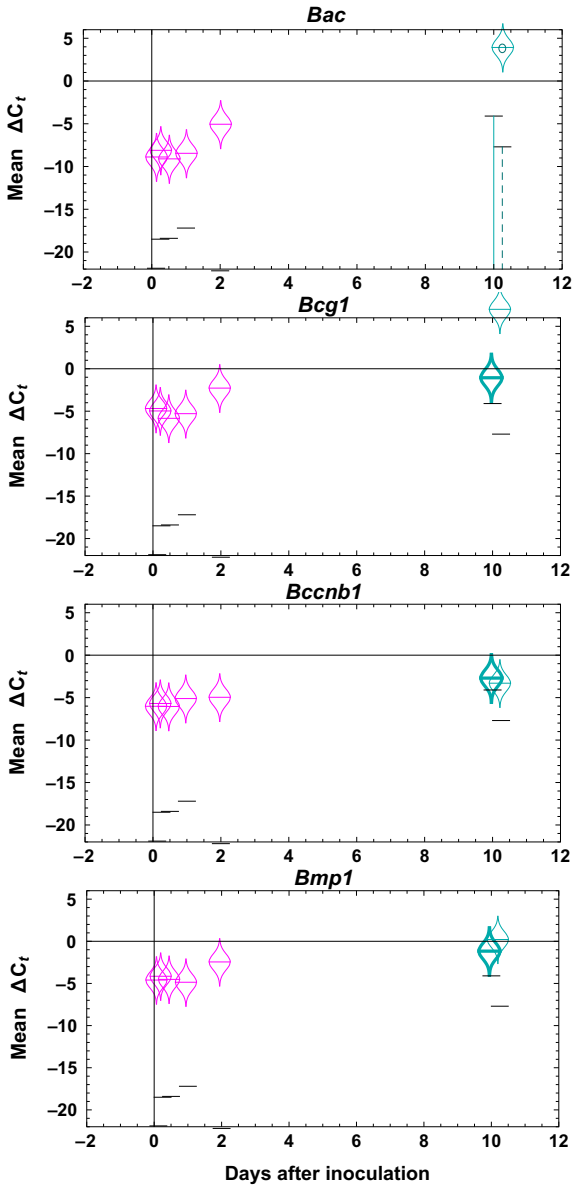

Figure 4 Relative amount of mRNA of selected *Botrytis cinerea* signalling genes at different time points following inoculation of *Arabidopsis thaliana* Col-0 plants. Symbols, abbreviations and common *x*-axis as in Figure 2. Infections were: (magenta symbols on left) necrotic, resulting from droplet infection of attached rosette leaves; (cyan symbols on right/thick) symptomless 10 days after inoculation in newly produced rosette leaves; or (cyan symbols on right/thin) symptomless in stem and stipule samples 10 days after inoculation of the rosette. All values are relative to *Bcrpl5* as in Figure 2. Testing for differences between time points by REML, omitting samples with no RNA signal: *Bccnb1*, $P = 0.06$, s.e. = 0.71; *Bcac*, $P < 0.001$, s.e. = 0.5; *Bcg1*, $P = 0.001$, s.e. = 0.92; *Bmp1*, $P = 0.01$, s.e. = 0.77. Where no detectable amplicon was formed, the possible range of ΔC_t is shown as a vertical line terminating at the threshold detection limit. A dashed line indicates one replicate had no detectable amplicon; the other replicate is shown as a small circle. [Colour figure can be viewed at wileyonlinelibrary.com].

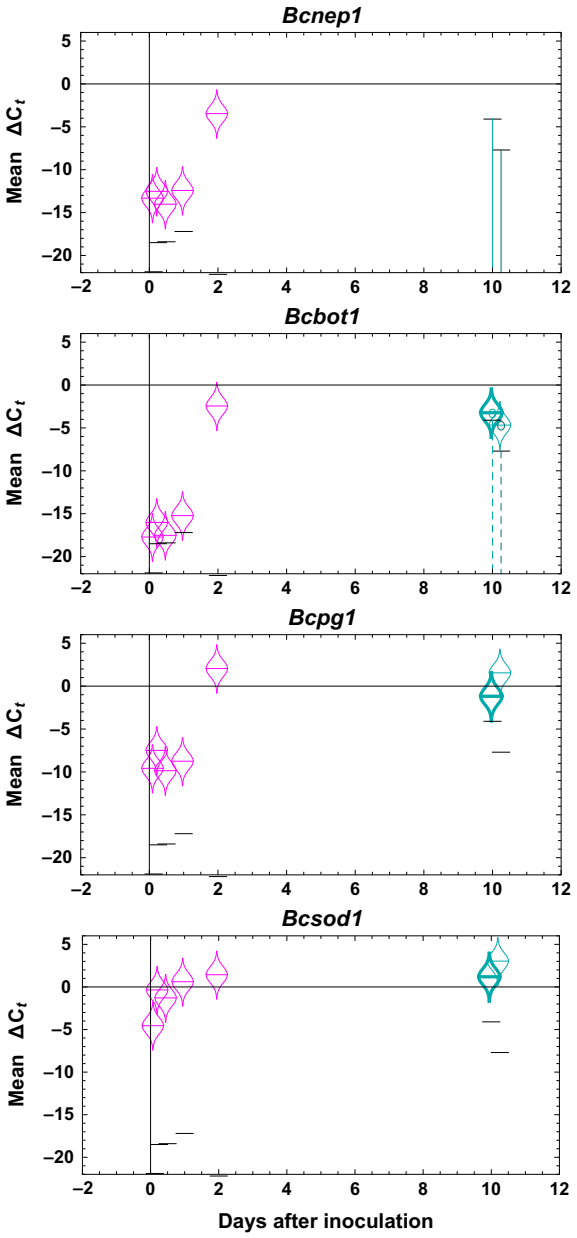

Figure 5 Relative amount of mRNA of selected *Botrytis cinerea* pathogenicity-related genes at different time points following inoculation of *Arabidopsis thaliana* Col-0 plants. Symbols and abbreviations as in Figure 2; colour coding and vertical lines as in Figure 4. Testing for differences between time points by REML, omitting samples with no RNA signal: *Bcsod1*, $P = 0.004$, s.e. = 0.78; *Bcpg1*, $P < 0.001$, s.e. = 0.92; *Bcnep1*, $P < 0.001$, s.e. = 0.46; *Bcbot1*, $P = 0.006$, s.e. = 2.2. [Colour figure can be viewed at wileyonlinelibrary.com].

symptomless and late-appearing necrotic infections in lettuce (cf. Figs 3 & 5), but not in *A. thaliana*. Lettuce cv. All the Year Round and several other wild and cultivated plant species (Shaw *et al.*, 2016) support symptomless and systemic infection. It would be of great interest to

see whether transcript pool patterns in *B. cinerea* fell into distinct groups; it would also be extremely interesting to see whether the other *Botrytis* species found to establish symptomless systemic infections (Shaw *et al.*, 2016) have similar patterns of up- and down-regulation of transcript pools.

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

An obvious hypothesis as to how systemic infection can progress without visible symptoms is that transcription of necrosis-related genes is suppressed, because the production of enzymes or secondary metabolites that damage or kill plant cells would be detrimental in sustaining a symptomless, endophytic interaction between a fungus and its host plant. Consistent with this hypothesis, the *Bcbot1* transcript, coding for a crucial enzyme in the biosynthesis of the toxin botrydial, was undetectable in symptomless infections, both in lettuce and *A. thaliana*. However, in plants containing a symptomless *B. cinerea* infection, high levels of transcripts from the *Bcnep1*, *Bcpg1* and *Bcpg2* genes in lettuce and *Bcnep1* and *Bcpg1* in *A. thaliana* were observed. BcNEP1 protein can induce host programmed cell death in leaf tissue of all dicots tested (Schouten *et al.*, 2008 Cuesta Arenas *et al.*, 2010). In symptomless infections, its cell death-inducing capacity may be mitigated by other fungal (suppressor) proteins, or by a reduction of protein excretion. Alternatively, physiological changes in the plant might make it locally insensitive to BcNEP1-induced death. Although receptors required for NEP1-like-protein (NLP)-mediated immune response activation (but not death) have been identified (Albert *et al.*, 2015), the mechanisms underlying plant cell death induction by NLPs remain to be unravelled. Besides production of phytotoxic proteins like BcNEP1, secretion of endopolygalacturonases BcPG1 and BcPG2 by *B. cinerea* is potentially damaging to plants, as these enzymes hydrolyse pectin and thereby affect plant cell wall architecture and integrity. Both enzymes, when infiltrated into leaf mesophyll tissue, may cause rapid tissue collapse (Kars *et al.*, 2005). More recently, *B. cinerea* endopolygalacturonases have been shown to act as MAMPs that in *A. thaliana* can be recognized by an LRR-type receptor (Zhang *et al.*, 2014). It remains elusive why the relatively high expression of the above three genes does not result in visible tissue damage. Whether the transcripts are actually translated into proteins that are secreted into the host tissue could not be investigated for lack of a sufficiently sensitive detection method.

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

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