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

Article

Accepted Version

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

C.J. Emmanuel[1,2]

J.A.L. van Kan[3]

M.W. Shaw[1*]

[1] School of Agriculture, Policy and Development, University of Reading, Whiteknights, Reading UK

[2] Current address: Department of Botany, Faculty of Science, University of Jaffna, Sri Lanka;

[3] Laboratory of Phytopathology, Wageningen University, Wageningen, The Netherlands

* Corresponding author. m.w.shaw@reading.ac.uk

Running head: Transcription in *Botrytis* infections

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

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: (1) the fungus state is similar in the two forms of infection, but the plant defences are more effective, leading to multiple small quiescent centres; (2) excreted molecules that would trigger plant defences are suppressed; (3) signal exchanges occur avoiding both extensive host cell death and complete spatial restriction of the pathogen. We tested these by comparing transcript levels of a set of *B. cinerea* genes between symptomless and necrotising 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 d 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 d after inoculation, which clearly differed from those in necrotising infections. In *A. 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 necrotising infections.

[238 words]

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 which 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, 1985; Jarvis, 1994; Coertze & Holz, 2002; Elad *et al*, 2004). In contrast, recent studies have revealed that *B. cinerea* also can cause symptomless systemic infection in several host plants including *Primula* spp., lettuce (*Lactuca sativa*), *Arabidopsis thaliana* and *Taraxacum vulgare* (Barnes & Shaw 2003; Sowley *et al*, 2010; Rajaguru & Shaw, 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 et al, 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 necrotising 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 (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 necrotising infection.

Materials and Methods

Plant growth

Lettuce (Lactuca sativa) 'Tom Thumb' (Thomson & Morgan, Ipswich UK) 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, UK), and in each tray 1 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 in 12 h day and 18°C in night, RH 65%. The compost was kept damp to touch all the time. Seven days after inoculation (14 days after seed sowing) 40 inoculated and 40 non-inoculated seedlings were transplanted into 1L pots filled with potting compost (John Innes 2 compost + 4g/L Osmacote). Later the seedlings were moved to a vented glasshouse under natural light in summer (May-Aug, 15-16.5 h day-length, temperature maintained at 18° C minimum at night, rising to about 30° C in day time. 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 5 to 6 times. After surface disinfection seed stratification was done at 4 °C for four 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 and 18 °C night

temperature, 65% relative humidity, 16 h light and 8 h dark period, 200-250 μmol/m2/s light intensity. The plants were watered from below every day sufficiently to keep the compost just moist up to two weeks from sowing, then at two-day intervals. Inside the isolation propagator, the average day time temperature was 26.5 °C and night 18.5 °C; relative humidity in day and night ranged between 80% to 85%. The dew point temperature within the covers during the day was about 22 °C and at night about 16 °C; light intensity was 170-220 μmol/m2/s.

Inoculation and sampling

Leaves were sampled from lettuce 'Tom Thumb' (Thomson & Morgan, Ipswich UK) and *Arabidopsis 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 sterilisation by immersion in 1% sodium hypochorite and detergent (JANGRO Bleach, Jangro Ltd, Bolton, UK) 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 48h 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 which 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.

A. thaliana plants were inoculated at early rosette stage, 21d 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 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 samples of lettuce were collected from two distinct sources: one was a necrotising infection resulting in about a day from inoculation of detached leaves with droplets of spore suspension; the second was the delayed necrotising infection which eventually developed in systemically infected lettuce following the approximately 40 d symptomless phase of infection. Rapidly necrotising 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) in 12g/L potato dextrose broth (Oxoid, UK). Leaves of lettuce with visible symptoms were sampled at

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 post inoculation.

RNA extraction and quantification

RNA extraction used RNeasy plant mini kits (Qiagen, Hilden, Germany) 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, USA). After extraction, Turbo DNase kits (Life Technologies) were used for further purification. cDNA preparation was carried out using High capacity RNA-to-cDNA kit s(Applied Biosystems, USA).

Transcript levels of ten *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 *Bcg*1 encoding a heterotrimeric Gα protein (Gronover et al. 2001); the gene *Bccnb*1 encoding the calcineurin β subunit (Harren *et al*, 2012); the adenylate cyclase gene *Bac* (Klimpel et al, 2002); and the MAP kinase gene *Bmp*1 (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 *Bcpg*1 and *Bcpg*2 (the latter gene was only studied in samples from lettuce); the botrydial biosynthetic gene *Bcbot*1; the superoxide dismutase gene *Bcsod*1; and the *Bcnep*1 gene encoding a phytotoxic necrosis- and ethylene-inducing protein (reviewed by van Kan, 2006). Transcript levels of these virulence-related genes were normalised to that of *Bcrpl*5, which is the most steadily expressed housekeeping gene, encoding ribosomal protein RPL5 (Zhang et al, 2011). The β-tubulin gene *Bctub*A (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 non-exponential pattern.

qPCR assays for the lettuce samples and *A. thaliana* samples were carried out separately. qPCR reactions 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 minimise effects due to inter-plate 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 tested nine were quantified in each plate. The assays were carried out in triplicate using the following cyclic conditions: 95 °C for 2 min, then 40 cycles of 95 °C for 15s, 60 °C for 30s, using StepOnePlus™ Real-Time PCR System (Applied Biosystems, USA). After each run, melting curves were acquired by heating to 95 °C for 15s, cooling to 60 °C for 1min and heating to 95 °C at 0.3 °C, before holding at 95°C for 15s 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.

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. 1).

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, we present only the results from 5th and 6th leaves, at successive time-periods.

Signalling genes.

There were no clearly significant differences in transcript levels of the *Bcg*1(P=0.4), *Bac* (P=0.09) or *Bccnb*1 (P=0.8) genes (Fig. 2, cyan, thicker, 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 dpi, than in other samples.

Genes involved in cell death or tissue degradation.

The relative concentrations of transcripts of *Bcpg*2 and *Bcnep*1 differed significantly at consecutive time points in detached lettuce leaves developing necrotic infection (Fig. 3, magenta symbols). *Bcpg*2 transcript first increased between 12 and 24 hpi and then decreased at 48 hpi, whereas *Bcnep*1 showed the highest transcript level at 12 hpi and strongly dropped at 24 and 48 hpi (Fig. 3, magenta symbols). In necrotic infections which developed following symptomless infection, levels of *Bcpg*2 and *Bcnep*1 transcripts were low (Fig. 3, black symbols). *Bcbot*1 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 *Bcpg*1 and *Bcsod*1were quite similar in both types of necrotic

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

Transcript patterns of genes were quite distinct in the non-symptomatic infection (Fig. 3, cyan, thicker, symbols) and the necrotic infections (Fig. 3, magenta and black symbols). Notably, *Bcbot*1 transcript was undetectable in non-symptomatic lettuce tissues, abundant in necrotic infections of detached leaves and scarce in delayed necrotic infection. By contrast, there was more *Bcnep*1 transcript in non-symptomatic tissues than in either type of necrotic infection ($P<0.001$). The level of *Bcpg*2 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. *Bcpg*1 transcript was consistently lower in non-symptomatic 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 *Bcsod*1 transcript.

Arabidopsis thaliana

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

Signalling genes.

Transcript levels of the signalling genes differed between infection types (Fig. 4). Transcript levels of *Bcg*1 were higher in symptomless stem samples (cyan, thicker, symbols) than in any necrotic stage (magenta symbols), or in symptomless rosette leaves (cyan symbols). Transcripts of the *Bac* gene were detected in only one of 4 samples from symptomless infections, suggesting a substantial depletion of cAMP signalling in the asymptomatic *A. thaliana* infection. The transcript levels of *Bccnb*1 were similar in symptomless leaves and stems (both cyan symbols), and in all necrotic stages (magenta symbols). Transcript levels of *Bmp*1 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 *Bcnep*1, *Bcbot*1, *and Bcpg*1 rose between 24 and 48 h old necrotising infections, coinciding with the appearance of necrotic lesions; *Bcsod*1 rose, but less extremely. The transcripts of *Bcnep*1 were not detected in any of the symptomless samples while *Bcbot*1 transcripts were (barely) above the detection threshold in only one of the samples (magenta symbols). By contrast, transcript levels of *Bcpg*1 and *Bcsod*1 in symptomless samples were comparable to those in the 48 hpi necrotising 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 non-symptomatic *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 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* Fig. 2 and Fig. 4); transcript levels of the toxin-producing gene *Bcbot*1 are similar and low in symptomless and late-appearing necrotic infections in lettuce (*cf* Fig. 3 and Fig. 5), but not in *A. thaliana*. Lettuce '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 non-symptomatic 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 *Bcnep*1 and *Bcbot*1..

An obvious hypothesis as to how systemic infection can progress without visible symptoms is that transcription of necrosis-related genes is suppressed, since the production of enzymes or secondary metabolites that damage or kill plant cells would be detrimental in sustaining a non-symptomatic, 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 we observed high levels of transcripts from the *Bcnep*1, *Bcpg*1 and *Bcpg*2 genes in lettuce and *Bcnep1* and *Bcpg1* in *A. thaliana*. BcNEP1 protein can induce host programmed cell death in leaf tissue of all dicots tested (Schouten et al, 2007; 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 necrotising 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 makes it hard to observe the fungus within plant tissue, and work with fluorescently marked, but otherwise physiologically normal, *B. cinerea* is needed.

Acknowledgements

This work was paid for by a grant to CJE from the Commonwealth Scholarships Commission. We are extremely grateful for technical assistance provided by George Gibbings and Matthew Richardson.

References

Albert I, Böhm H, Albert M, Feiler CE, Imkampe J, Wallmeroth N, Brancato C, Raaymakers TM, Oome S, Zhang H, Krol E, Grefen C, Gust AA, Chai J, Hedrich R, van den Ackerveken G, Nürnberger T. 2015. An RLP23–SOBIR1–BAK1 complex mediates NLP-triggered immunity. *Nature Plants* **1**, 15140.

Barnes SE, Shaw MW. 2003. Infection of commercial hybrid *Primula* seed by *Botrytis cinerea* and latent disease spread through the plants. *Phytopathology* **93**, 573–578.

Benito EP, ten Have A, van't Klooster JW, van Kan, JAL 1998. Fungal and plant gene expression during synchronized infection of tomato leaves by *Botrytis cinerea. European Journal of. Plant Pathology* **104,** 207–220

Büttner P, Koch F, Voigt K, Quidde T, Risch S, Blaich R, Brückner B, Tudzynski P. 1994. Variations in ploidy among isolates of *Botrytis cinerea*: implications for genetic and molecular analyses. *Current Genetics* **25**, 445-450

Coertze S, Holz G. 2002. Epidemiology of *Botrytis cinerea* on grape: wound infection by dry, airborne conidia. *South African Journal of Enology and Viticulture* **23**, 72–77.

Cuesta Arenas Y, Kalkman RIC, Schouten A, Dieho M, Vredenbregt P, Uwumukiz B, Osés Ruiz M, van Kan JAL. 2010 Functional analysis and mode of action of phytotoxic Nep1-like proteins of *Botrytis cinerea*. *Physiological and Molecular Plant Pathology* **74**, 376-386

Edwards SG, Seddon B. 2001. Selective media for the specific isolation and enumeration of *Botrytis cinerea* conidia. *Letters in Applied Microbiology* **32**, 63–66.

Elad Y, Williamson B, Tudzynski P, Delen N. 2004 *Botrytis* spp. and diseases they cause in agricultural systems – an introduction in *Botrytis*, biology, pathology and control (Y. Elad, B. Williamson, Paul Tudzynski, Nafiz Delen eds) pp1-8. Dordrecht NL: Springer

Elad Y, Pertot I, Cotes Prado AM, Stewart, A. 2016 Plant Hosts of *Botrytis* spp. In: *Botrytis – The fungus, the pathogen and its management in agricultural systems*. (Fillinger, S. and Elad, Y. ed), pp413-486. Cham, Springer

Gronover CS, Kasulke D, Tudzynski P, Tudzynski B. 2001. The role of G protein alpha subunits in the infection process of the gray mold fungus *Botrytis cinerea*. *Molecular Plant-Microbe Interactions* **14**, 1293–1302.

Harren K, Schumacher J, Tudzynski B. 2012. The Ca 2^+ /calcineurin-dependent signalling pathway in the gray mold *Botrytis cinerea*: the role of calcipressin in modulating calcineurin activity. *PLoS ONE* **7**, doi: 10.1371/journal.pone.0041761

Horst K, 1985. Botrytis blight. In: *Compendium of Rose Diseases*. p186 St Paul, Minnesota: American Phytopathological Society,.

Jarvis WR. 1994. Latent infections in the pre- and postharvest environment. *HortScience* **29**, 749–751.

Kars I, Krooshof GH, Wagemakers L, Joosten R, Benen JAE, van Kan JAL. 2005. Necrotizing activity of five *Botrytis cinerea* endopolygalacturonases produced in *Pichia pastoris*. *The Plant Journal* **43**, 213-225

Klimpel A, Schulze Gronover C, Willliamson B, Stewart J, Tudzynski B. 2002. The adenylate cyclase (BAC) in *Botrytis cinerea* is required for full pathogenicity. *Molecular Plant Pathology* **3**, 439–450.

Rajaguru BAP, Shaw MW. 2010. Genetic differentiation between hosts and locations in populations of latent *Botrytis cinerea* in southern England. *Plant Pathology* **59**, 1081–1090.

Schouten A, Baarlen P, van Kan, JAL. 2008. Phytotoxic Nep1-like proteins from the necrotrophic fungus *Botrytis cinerea* associate with membranes and the nucleus of plant cells. *New Phytologist* **177**, 493–505.

Schumacher J. 2016 Signal transduction cascades regulating differentiation and virulence in *Botrytis cinerea.* In: *Botrytis – The fungus, the pathogen and its management in agricultural systems.* (Fillinger, S. and Elad, Y. ed), pp247-267 Cham, Springer

Shaw MW, Emmanuel CJ, Emilda D, Terhem RB, Shafia A, Tsamaidi D, Emblow M, van Kan, JAL. 2016. Analysis of cryptic, systemic *Botrytis* infections in symptomless hosts. *Frontiers in Plant Science* **7**: 625. doi: 10.3389/fpls.2016.00625

Sowley ENK, Dewey FM, Shaw MW. 2010. Persistent, symptomless, systemic, and seed-borne infection of lettuce by *Botrytis cinerea*. *European Journal of Plant Pathology* **126**, 61–71.

van Kan JAL. 2006. Licensed to kill : the lifestyle of a necrotrophic plant pathogen. *Trends in Plant Science* **11**, 247–253.

van Kan JAL, Stassen JHM, Mosbach A, van Der Lee TAJ, Faino L, Farmer A D, Papasotiriou DG, Zhou S, Seidl MF, Cottam E, Edel D, Hahn M, Schwartz DC, Dietrich RA, Widdison S, Scalliet G. 2017. A gapless genome sequence of the fungus *Botrytis cinerea*. *Molecular Plant Pathology* **18**: 75–89.

Williamson B, Tudzynski B, Tudzynski P, van Kan JAL. 2007. *Botrytis cinerea* : the cause of grey mould disease. *Molecular Plant Pathology* **8**, 561–580.

Zhang N, Zhang S, Borchert S, Richardson K, Schmid J. 2011. High levels of a fungal superoxide dismutase and increased concentration of a PR-10 plant protein in associations between the endophytic fungus *Neotyphodium lolii* and Ryegrass. *Molecular Plant-Microbe Interactions* **24**, 984–992.

Zhang L, Kars I, Essenstam B, Liebrand TWH, Wagemakers L, Elberse J, Tagkalaki P, Tjoitang D, van den Ackerveken G, van Kan JAL. 2014. Fungal endopolygalacturonases are recognized as microbe-associated molecular patterns by the *Arabidopsis* receptor-like protein RESPONSIVENESS TO BOTRYTIS POLYGALACTURONASES1 *Plant Physiology* **164**, 352-364

Zheng L, Campbell M, Murphy J, Lam S, Xu J, 2000. The BMP1 gene is essential for pathogenicity in the gray mold fungus *Botrytis cinerea*. *Molecular Plant-Microbe Interactions* **13**, 724–732.

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 cinereal*; (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 5^{th} and 6^{th} 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 *Bcrpl*5 and the detectable maximum C_t value of the reaction, which was always 40.
389 All values are relative to *Bcrpl*5 which therefore forms the 0-line on the y-axis (ie, the values are in
390 effect ΔC_t from *Bcrpl*5). 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

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 Fig. 2. Testing for differences between time-points by REML omitting samples with no RNA signal: *Bcsod*1, P=0.6, s.e. = 0.89; *Bcpg*1, P=0.05, s.e. = 1.3; *Bcpg*2, P=0.008, s.e. =1.5; *Bcnep*1, P<0.001, s.e. = 0.75; *Bcbot*1, P=0.007, s.e. = 0.9. All values are relative to *Bcrpl*5 as in Fig. 2. Where no detectable amplicon was formed, the possible range of ΔC_t is shown as a vertical line terminating at the threshold

Figure 4*:* Relative amount of mRNA of selected *Botrytis cinerea* signalling genes at different time points following inoculation of *Arabidopsis thaliana* col1 plants. Symbols, abbreviations and common x-axis as in Fig. 2. Infections were: (magenta) necrotic, resulting from droplet infection of attached rosette leaves; (cyan, thicker) symptomless 10 days after inoculation in newly produced rosette leaves; or (cyan) symptomless in stem and stipule samples 10 days after inoculation of the rosette . All values are relative to *Bcrpl*5 as in Fig. 2. Testing for differences between time-points by REML, omitting samples with no RNA signal: *Bccnb*1, 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.

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 Fig. 2; colour-coding and vertical lines as in Fig. 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.

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.

	Not surface sterilised			Surface sterilised		
Leaf number[a]	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.

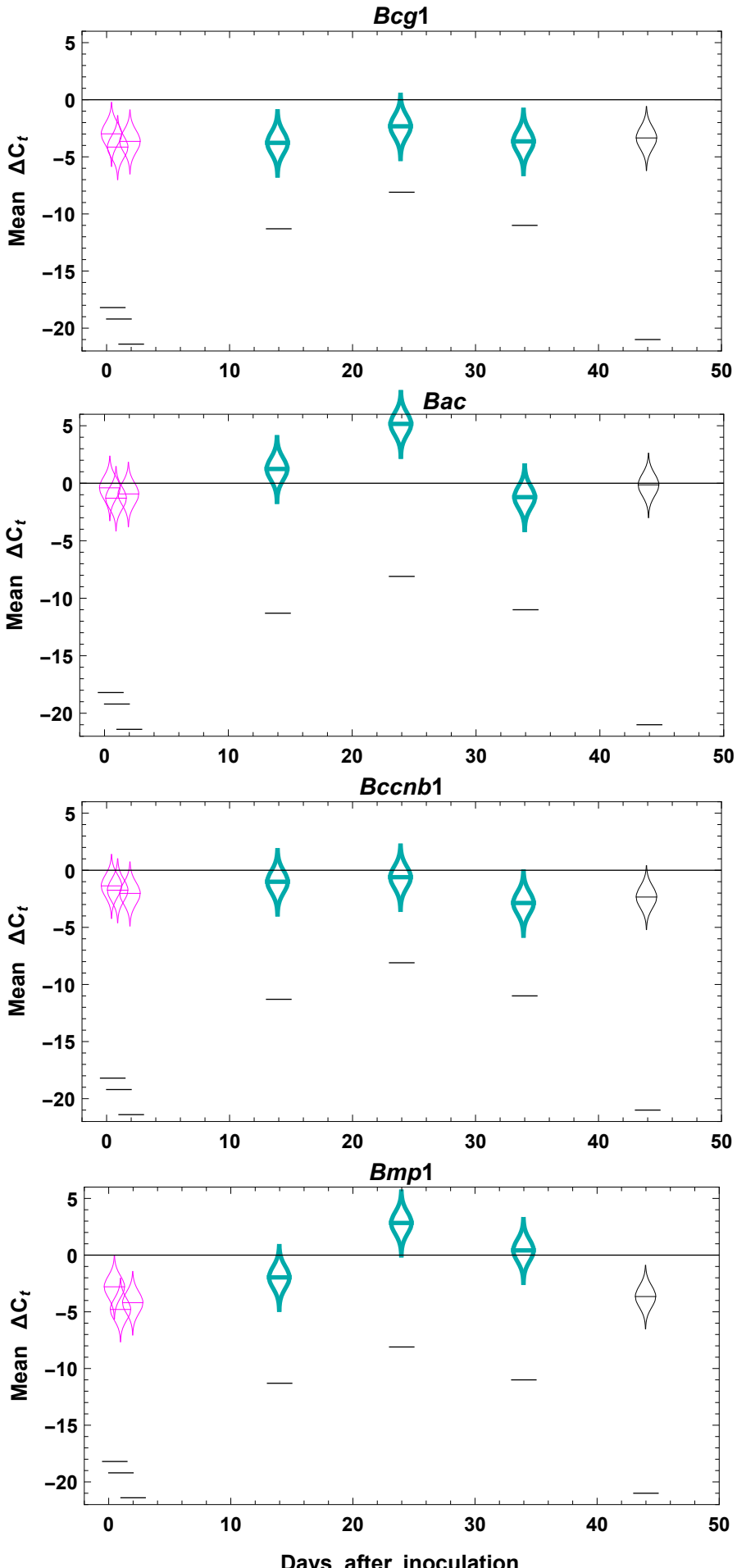
Tissue	Number sampled per plant	Number sampled per treatment[a]	Number of samples with *B. cinerea*	
			Not surface sterilised	Surface sterilised
Root	1	10	0	1
Rosette leaf	3	30	23	16
Stem	2	20	12	6
Stem leaf	2	20	17	5
Flower	2	20	16	4

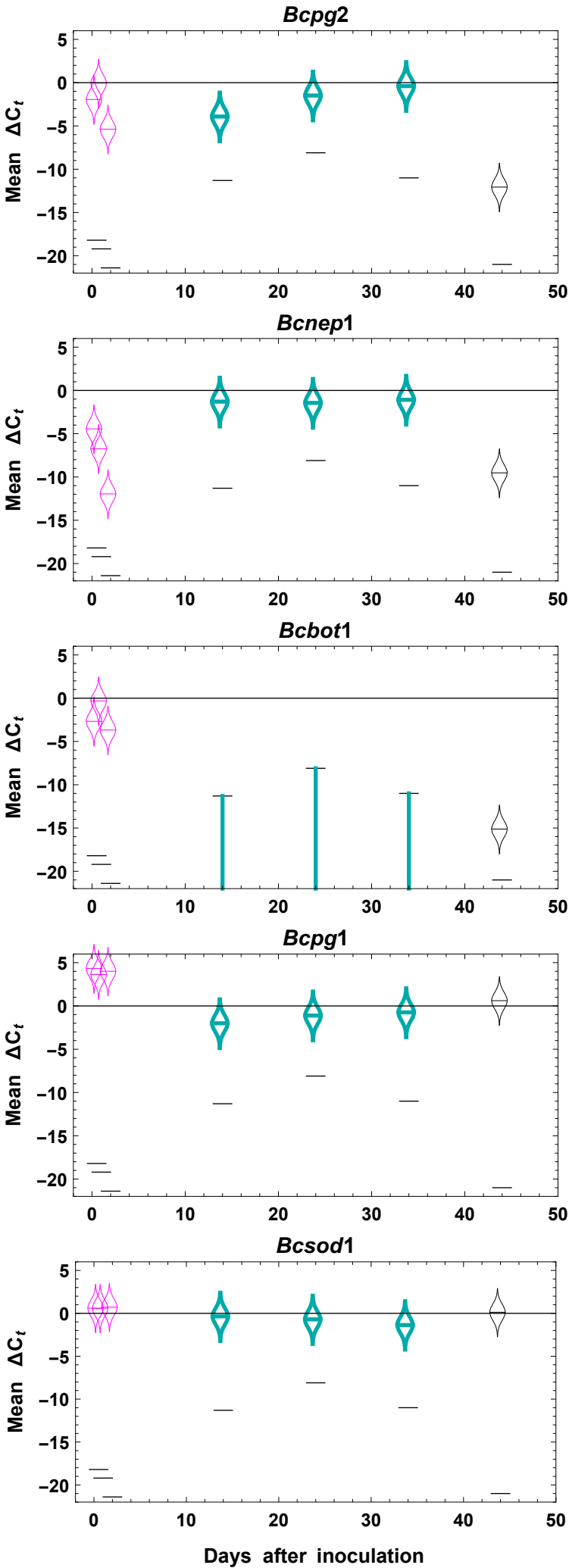
[a] Equal numbers of samples were incubated with and without surface sterilisation.

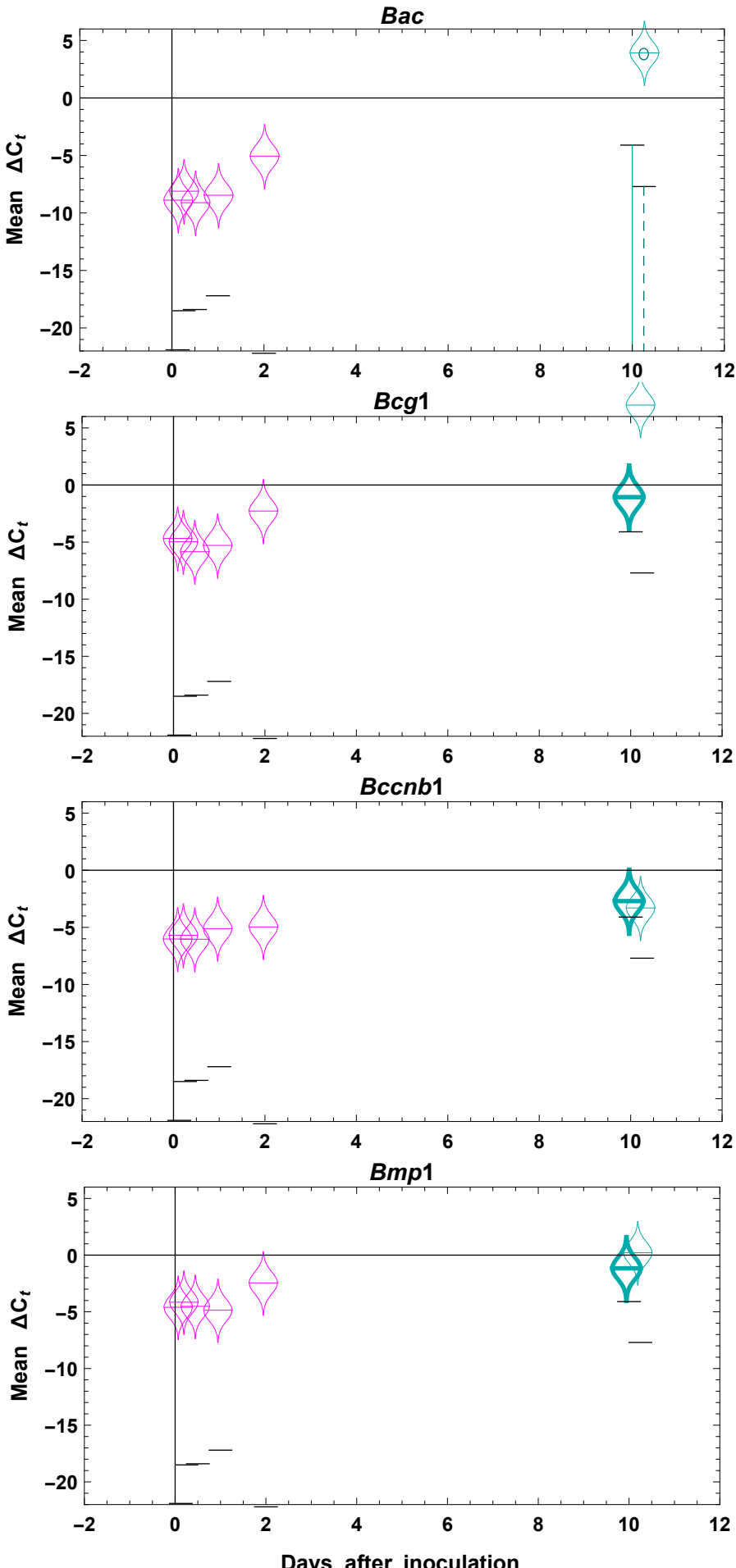
Table 3: Primers used for qRT-PCR of mRNA in tissue samples from symptomless systemic infections of lettuce or *Arabidopsis thaliana* with *Botrytis cinerea*. All primer pairs were designed to cross an exon-exon junction, except for *Bcpg*1, where no introns are present.

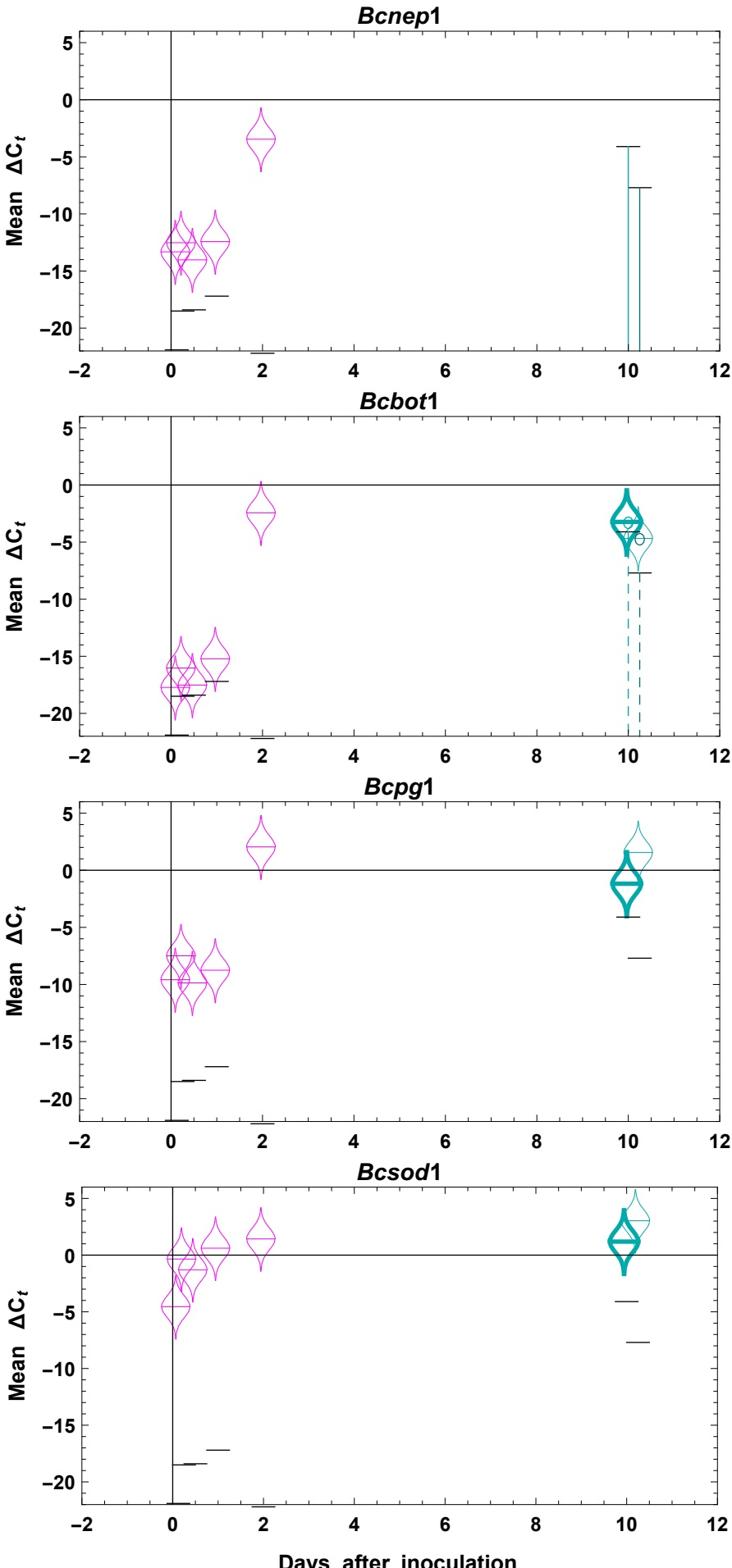
Gene	Gene product	Gene ID	NCBI accession numbers	Sequence (5'-3')	Amplicon size (bp)
Bcg1	Heterotrimeric G-protein α subunit	Bcin05g06770	Y18436.1	F- CAAGATGCTTCTTCTTGGAG R- TGATTGGACTGTGTTGCTGA	139
Bmp1	Mitogen-activated protein kinase 1	Bcin02g08170	AF205375.1	F- GCTTATGGTGTTGTCTGCTC R- TAGCTTCATCTCACGAAGTG	120
Bac	Adenylate cyclase	Bcin15g02590	AJ276473.1	F- GGTGAAGACGGATAGATCAAGTAG R- CTCCCGTGGGGACACATTAG	121
Bccnb1	Calcineurin β subunit	Bcin03g05990	KC935338.1	F- GTCGAATCCTCTAGCTACCAGAA R- GAATGCGCTGAGTCCACTG	97
Bcsod1	Superoxide dismutase	Bcin03g03390	AJ555872.1	F- ATTGAGCGTCATTGGCCGTA R- TGGACTCTTCGTTCTCTCCC	77
Bcpg1	Endopolygalacturonase 1	Bcin14g00850	EF195782.1	F- ACTCTGCTGGAGATGCTGGT R- TAGCGAGACAGTAATCTTGG	97
Bcpg2	Endopolygalacturonase 2	Bcin14g00610	AY665553.1	F– GGAACTGCCACTTTTGGTTAC R- TCCATCCCACCATCTTGCTC	126
Bcbot1	Botrydial biosynthetic enzyme	Bcin12g06380	AY277723.2	F– TTATGCCGCACTCCACGAGA R- TCCAGAGGAGTAGACCTCAT	103
Bcnep1	Necrosis ethylene-inducing protein 1	Bcin06g06720	DQ211824.1	F- GTAATGGTAACACCAGTGGT R- AGCCACCTCGGACATAGGTT	96
Bcrpl5	Ribosomal protein large subunit 5	Bcin14g04230	AL116000.1	F-GATGAGACCGTCAAATGGTTC R- CAGAAGCCCACGTTACGACA	137


a
14 dpi
b
24dpi
c
34 dpi
d
44 dpi
e
48 hpi
f
0 dpi
g
10 dpi


Bcg1
Bac
Bccnb1
Bmp1
Mean ΔC_t
5
0
−5
−10
−15
−20
0
10
20
30
40
50
Days after inoculation


Bcpg2
Bcnep1
Bcbot1
Bcpg1
Bcsod1
Mean ΔCt
5
0
−5
−10
−15
−20
0
10
20
30
40
50
Days after inoculation


Bac
Bcg1
Bccnb1
Bmp1
Mean ΔCt
5
0
−5
−10
−15
−20
−2
0
2
4
6
8
10
12
Days after inoculation


Bcnep1
Bcbot1
Bcpg1
Bcsod1
Mean ΔCt
5
0
−5
−10
−15
−20
−2
0
2
4
6
8
10
12
Days after inoculation