Botrytis cinerea identifies host plants via the recognition of antifungal capsidiol to induce expression of a specific detoxification gene

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Abstract

The gray mold pathogen *Botrytis cinerea* has a broad host range, causing disease in over 400 plant species, but it is not known how this pathogen evolved this polyxenous nature. *B. cinerea* can metabolize a wide range of phytoalexins, including the stilbenoid, resveratrol, and the sesquiterpenoids capsidiol in tobacco, and rishitin in potato and tomato. In this study, we analyzed the metabolism of sesquiterpenoid phytoalexins by *B. cinerea*. Capsidiol was dehydrogenated to capsenone which was then further oxidized, while rishitin was directly oxidized to epoxy- or hydroxy-rishitins indicating that *B. cinerea* has separate mechanisms to detoxify structurally similar sesquiterpenoid phytoalexins. RNAseq analysis revealed that a distinct set of genes were induced in *B. cinerea* when treated with capsidiol or rishitin, suggesting that *B. cinerea* can distinguish structurally similar phytoalexins to activate appropriate detoxification mechanisms. The gene most highly upregulated by capsidiol treatment encoded a dehydrogenase, designated *Bccpdh*. Heterologous expression of *Bccpdh* in a capsidiol-sensitive plant symbiotic fungus, *Epichloë festucae*, resulted in an acquired tolerance of capsidiol and the ability to metabolize capsidiol to capsenone, while *B. cinerea Δbccpdh* mutants became relatively sensitive to capsidiol. The *Δbccpdh* mutant showed reduced virulence on the capsidiol producing *Nicotiana* and *Capsicum* species but remained fully pathogenic on potato and tomato. Homologs of *Bccpdh* are found in taxonomically distant Ascomycota fungi but not in other *Botrytis* species, suggesting that *B. cinerea* acquired the ancestral *Bccpdh* by horizontal gene transfer, thereby extending the pathogenic host range of this polyxenous pathogen to capsidiol-producing plant species.

Significance Statement

B. cinerea can metabolize a wide range of phytoalexins, however, the extent to which phytoalexin detoxification contributes to pathogenicity is largely unknown. In this study, we have shown that *B. cinerea* recognizes structurally resembling sesquiterpenoid phytoalexins, rishitin and capsidiol, to activate appropriate detoxification mechanisms. We identify *Bccpdh*, encoding a dehydrogenase for capsidiol detoxification, which is upregulated in *B. cinerea* exclusively during the infection of capsidiol producing plant species, and is required to exert full virulence. Analysis of the *Bccpdh* locus suggests that the gene was acquired via horizontal gene transfer. This work highlights that the polyxenous plant pathogen *B. cinerea* can distinguish its host plants by its anti-microbial compounds, to activate appropriate mechanisms for enhanced virulence.

Main Text

Introduction

The antimicrobial secondary metabolites produced in plants during the induction of disease resistance are collectively termed phytoalexins (1, 2). Several hundred phytoalexins of diverse structures have been identified from a wide range of plant species, which include terpenoids, flavonoids and indoles (3, 4). Many of these phytoalexins are considered to exhibit their antimicrobial activity by targeting the cell wall or cell membrane of pathogens (5, 6), but their mechanisms of action remain largely unknown.

In plants belonging to the Solanaceae family, the major phytoalexins are sesquiterpenoids, such as capsidiol in *Nicotiana* and *Capsicum* species and rishitin in *Solanum* species (2, 7, 8). In *Nicotiana* sp., production of capsidiol is strictly controlled by regulating the gene expression for capsidiol biosynthesis, such as *EAS* (5-*epi*-aristolochene synthase) and *EAH* (5-*epi*-aristolochene dihydroxylase), encoding the enzymes dedicated to the production of capsidiol (9, 10). In *N. benthamiana*, expression of *EAS* and *EAH* genes is hardly detected in healthy tissues, but their expression is rapidly induced during infection by pathogens or the treatment with elicitor protein (11, 12). Produced phytoalexins are secreted locally via ABC (ATP-binding cassette) transporters

at the site of pathogen attack (13, 14). For plants, such temporal and spatial control of phytoalexins is probably critical as the toxicity of phytoalexins is often not specific to microorganisms but is also harmful to plant cells (13, 15, 16). For example, rishitin affects the permeability of plant liposomal membranes and disrupts chloroplasts (17). Therefore, timely production and efficient transport of phytoalexins to the site of pathogen attack are important for plants to apply these double-edged weapons effectively.

It has been reported that various phytopathogenic fungi can metabolize and detoxify phytoalexins (18). Although many studies have shown an approximate correlation between the ability of strains to detoxify phytoalexins and their virulence (19–21), the importance of phytoalexin detoxification for pathogen virulence is largely unproven for most plant-pathogen interactions. The best-studied phytoalexin metabolizing enzyme is pisatin demethylase (PDA) of *Nectria haematococca*. Deletion of the *PDA* gene resulted in reduced virulence of *N. haematococca* on pea, directly proving the importance of PDA for the virulence of this pathogen (22). It has also been reported that sesquiterpenoid phytoalexins are metabolized by pathogenic fungi. Capsidiol is metabolized to less toxic capsenone via dehydration by pathogens such as the gray mold pathogen *Botrytis cinerea* and *Fusarium oxysporum* (23). *Gibberella pulicaris*, the dry rot pathogen of potato tubers, oxidizes rishitin to 13-hydroxy rishitin and 11,12-epoxyrishitin (24). However, the enzymes involved in the detoxification of sesquiterpenoid phytoalexins have not been isolated to date, and their importance for pathogenicity has not been demonstrated.

In this study, we first investigated some pathogens isolated from Solanaceae and non-Solanaceae plants on their tolerance of capsidiol and rishitin, as well as their ability to detoxify/metabolize these phytoalexins. Among the pathogens that can metabolize both capsidiol and rishitin, we chose *Botrytis cinerea* for further analysis to investigate the importance of its ability to detoxify capsidiol for the pathogenicity on plant species that produce capsidiol.

Results

Metabolization of Sesquiterpenoid Phytoalexins Capsidiol and Rishitin by Fungal Plant Pathogens.

Four oomycetes and 12 fungal species were evaluated for their ability to detoxify/metabolize sesquiterpenoid phytoalexins capsidiol and rishitin, produced by Solanaceae plant species. Four *Phytophthora* species isolated from Solanaceae host plants, including potato late blight pathogen *P. infestans*, *P. nicotiana* isolated from tobacco, *P. capsici* isolated from green pepper and *P. cryptogea* isolated from nipplefruit (*Solanum mammosum*), are all sensitive to capsidiol and rishitin. The amount of capsidiol and rishitin after the incubation with these oomycete pathogens did not decrease, indicating that they are neither capable of metabolizing nor tolerant to capsidiol and rishitin (Fig. 1 and Supplemental Fig. S1). Among 12 fungal plant pathogens (eight isolated from Solanaceae plants), seven and eight fungal strains can metabolize capsidiol and rishitin, respectively, and showed increased resistance to phytoalexins. In some cases, pathogens can metabolize phytoalexins that are not produced by their host, indicating that the ability to detoxify phytoalexins does not always correlate with their host range (Supplemental Figs. S1-3 and Supplementary Notes 1 and 2). *Botrytis cinerea* and *Sclerotinia sclerotiorum* are well-known as pathogens with a wide host range and are capable of metabolizing both capsidiol and rishitin. In this study, *B. cinerea* was selected as the pathogen to further investigate the role of detoxification of phytoalexins on the pathogenicity of this polyxenous pathogen.

Metabolization of Capsidiol and Rishitin by *B. cinerea*

Metabolization of capsidiol and rishitin by *B. cinerea* were evaluated by LC/MS. Under the experimental conditions of this study, capsidiol was metabolized to capsenone by dehydrogenation within 12 h, and oxidized capsenone was detected at 24 h, whereas oxidized capsidiol was not detected (Fig. 1C and Supplemental Figs. S4). In contrast, rishitin was directly oxidized within 6 h and at least four different forms of oxidized rishitin were detected within 24 h (Fig. 1C and

Supplemental Figs. S5), indicating that despite the structural similarity of these phytoalexins, *B. cinerea* detoxifies/metabolizes capsidiol and rishitin by different mechanisms (Fig. 1C).

Unique Sets of Genes Are Upregulated in *B. cinerea* Treated with Capsidiol, Rishitin or Resveratrol.

To identify *B. cinerea* genes upregulated during the detoxification of sesquiterpenoid phytoalexins, RNAseq analysis was performed for mycelia of *B. cinerea* cultured in minimal media supplemented with capsidiol or rishitin. A stilbenoid phytoalexin, resveratrol, produced in grape was also used for comparison. Mycelia of *B. cinerea* were grown in liquid CM media supplemented with either 500 μM rishitin, 500 μM resveratrol, or 100 μM capsidiol, as 500 μM capsidiol caused significant growth defects in *B. cinerea* (Fig. 1A, Supplemental Fig. S6). The mycelial tissue was then used to perform an RNAseq analysis. Among 11,707 predicted *B. cinerea* genes (25), 25, 27 or 23 genes were significantly upregulated (Log2 fold change >2, $p < 0.05$) by the treatment with capsidiol, rishitin or resveratrol, respectively. Unexpectedly, distinctive sets of genes were upregulated even between *B. cinerea* treated with capsidiol and rishitin which resemble each other structurally (Fig. 2A, Supplemental Table S1-3), indicating that *B. cinerea* can either distinguish the structural difference of capsidiol and rishitin or the damage caused by these sesquiterpenoid phytoalexins. For instance, two genes Bcin08g00930 and Bcin12g01750 encoding hypothetical proteins containing a motif for dehydrogenases were specifically induced by capsidiol, while the treatment of rishitin significantly induced Bcin07g05430, encoding a cytochrome P450 gene (Fig. 2B). Expression of *BcatrB* encoding an ABC transporter involved in the tolerance of *B. cinerea* against structurally unrelated phytoalexins resveratrol, camalexin and fungicides fenpiclonil and fludioxonil (26, 27), is upregulated by treatment with rishitin and resveratrol, but not by capsidiol. In contrast, Bcin15g00040, encoding a major facilitator superfamily (MFS)-type transporter was upregulated specifically by capsidiol (Fig. 2B). Interestingly, treatment of *B. cinerea* with phytoalexins also activated genes predicted to be involved in pathogenicity to plants. For example, capsidiol treatment induced expression of a hydrophobin gene Bcin06g00510.1 (Bhp3), while rishitin treatment activated the expression of Bcin01g00080.1 (Bcboa8), encoding an enzyme for biosynthesis of a phytotoxin, botcinic acid (Supplemental Tables S1 and S2). Given that capsidiol is metabolized to capsenone by a dehydrogenation reaction in *B. cinerea* (Fig. 1C, Supplemental Fig. S4), the function of Bcin08g00930 and Bcin12g01750, both encoding a predicted short-chain dehydrogenase/reductase (SDR) were further analyzed in this study.

Bcin08g00930 Encodes a Short-Chain Dehydrogenase for the Detoxification of Capsidiol.

To investigate the function of SDR genes induced by capsidiol treatment, an endophytic fungus *Epichloë festucae* was employed for the heterologous expression of these genes. Bcin08g00930 and Bcin12g01750 were expressed in *E. festucae* under the control of the constitutive *TEF* promoter (Vanden Wymelenberg et al., 1997). The growth of wild type and control *E. festucae* expressing DsRed was severely inhibited in the 100 μM capsidiol (Fig. 3A and B). *E. festucae* transformants expressing Bcin12g01750 also hardly grew in 100 μM capsidiol, however, *E. festucae* became tolerant to capsidiol by the expression of Bcin08g00930 (Fig. 3A and B). The amount of capsidiol was not altered by wild type, DsRed or Bcin12g01750 expressing *E. festucae*, while capsidiol was metabolized to capsenone by the *E. festucae* transformant expressing Bcin08g00930 (Fig. 3C). These results indicated that Bcin08g00930 encodes a dehydrogenase that can detoxify capsidiol, thus the SDR encoded by Bcin08g00930 was designated as BcCPDH standing for *B. cinerea* capsidiol dehydrogenase.

Expression of *Bccpdh* Is Specifically Induced by Capsidiol.

To further investigate the expression pattern of *Bccpdh*, *B. cinerea* was transformed with a reporter construct for GFP expression under the control of the 1 kb proximal *Bccpdh* promoter (P_*Bccpdh:GFP*). P_*Bccpdh:GFP* transformants showed no obvious expression of GFP in water, but a significant increase of GFP fluorescence was detected in 500 μM capsidiol (Fig. 4A). The P_*Bccpdh:GFP* transformant was incubated with other anti-microbial terpenoids produced by Solanaceae species, including rishitin, debneyol (29), sclareol (30) and capsidiol 3-acetate (31).

Among the terpenoids tested, treatment of capsidiol and capsidiol 3-acetate induced expression of GFP in the P_*Bccpdh:GFP* transformant. This result further indicated that *B. cinerea* specifically reacts to capsidiol and its derivative capsidiol 3-acetate. As the *Bccpdh* promoter was activated by capsidiol 3-acetate, we investigated whether BcCPDH could metabolize capsidiol 3-acetate to capsenone 3-acetate. However, capsidiol 3-acetate was not metabolized by *E. festucae* expressing *Bccpdh* (Bcin08g00930) (Supplemental Fig. S7A). Consistently, capsidiol 3-acetate was directly oxidized by *B. cinerea* and at least two different forms of oxidized capsidiol 3-acetate, but not capsenone 3-acetate, were detected (Supplemental Fig. S7B), indicating that capsidiol and capsidiol 3-acetate were metabolized in *B. cinerea* by distinctive pathways.

Analysis of different lengths of *Bccpdh* promoters indicated that 250 bp of promoter sequence are sufficient for specific activation of the promoter by capsidiol treatment, and the cis-element required for capsidiol-specific expression was shown to be within -250 and -200 bp upstream of the start codon (Supplemental Figs. S8 and 9). By further analysis using *B. cinerea* transformant P_*Bccpdh:Luc* using luciferase as a quantitative marker, it was shown that *Bccpdh* promoter was activated within 2 h of capsidiol treatment, and the expression activity decreased as capsidiol was metabolized (Supplemental Figs. S10, Supplementary Notes 3).

Expression of Bccpdh Is Specifically Induced During the Infection of Capsidiol Producing Plant Species.

Leaves of *Nicotiana benthamiana*, which produce capsidiol as the major phytoalexin (13), were inoculated with conidia of the *B. cinerea* transformant P_*Bccpdh:GFP*. No GFP signal was detected in germinating *B. cinerea* conidia grown on the surface of *N. benthamiana* leaves 2 h after the inoculation, but obvious GFP expression was detected in the appressoria at 8 h after the inoculation (Fig. 4B). Growing hyphae in the leaf tissue of *N. benthamiana* showed GFP fluorescence, and the intensity of GFP fluorescence in hyphae was stronger near the edge of the lesion compared with that of hyphae growing in the areas of dead tissue within the lesions (Fig. 4B), probably because capsidiol was eventually detoxified in these areas, which are heavily infected with *B. cinerea*.

To examine the possibility whether polyxenous *B. cinerea* activates *Bccpdh* for detoxification of other phytoalexin or anti-microbial compounds produced in other plant species, the *B. cinerea* P_*Bccpdh:GFP* transformant was inoculated on a wide variety of plants. Over 50 plant species were used for the inoculation test including 9 Solanaceae, 6 Brassicaceae, 6 Rosaceae, 5 Fabaceae and 6 Asteraceae plants, and development of disease symptoms was observed in all tested plant species. Among the tested plants, expression of GFP under the control of *Bccpdh* promoter was only detected during the infection in three *Nicotiana* and two *Capsicum* species (Fig. 4C, Supplemental Fig. S11 and Table S4), all of which are reported to produce capsidiol (8, 32–34). These results further indicated that *B. cinerea* specifically recognizes capsidiol for the induction of *Bccpdh*.

BcCPDH Metabolizes and Detoxifies Capsidiol to Capsenone in B. cinerea.

B. cinerea Bccpdh KO mutant (*Δbccpdh*) was produced to investigate the function of BcCPDH (Supplemental Fig. S12). Mycelia of *B. cinerea* wild type and *Δbccpdh* were incubated with capsidiol and the metabolites were detected by LC/MS. While capsidiol was metabolized to capsenone in the wild type strain, most of the capsidiol remained unmetabolized two days after incubation with the *Δbccpdh* strain (Fig. 5A). Instead, oxidized capsidiol, which was not detectable in the wild type strain, was detected in *Δbccpdh* incubations (Supplemental Fig. S13 and Supplementary Notes 4). While the growth of *B. cinerea* hyphae was not affected by the disruption of the *Bccpdh* gene in 100 μM capsidiol, growth of *Δbccpdh* was diminished compared with that of wild type *B. cinerea* at higher concentrations of capsidiol (Fig. 5B). These results confirmed that BcCPDH is the enzyme responsible for the detoxification of capsidiol in *B. cinerea*.

Epoxidation of Capsidiol and Capsenone is Mediated via Bcin16g01490

The incubation of the *Δbccpdh* knockout strain in capsidiol revealed the presence of a potential secondary capsidiol degradation pathway, which resulted in the accumulation of oxidized capsidiol. Since we were unable to find any indication for such a pathway in our RNAseq results for 100 μM

231 capsidiol treated incubations, we extended our search to a preliminary RNAseq analysis which
232 used 500 μM capsidiol treatments. We identified the gene Bcin16g01490 encoding a cytochrome
233 P450, which was significantly upregulated under these elevated capsidiol concentrations
234 (Supplemental Table S1 and Fig. S14A). Heterologous expression of Bcin16g01490 in *E. festucae*,
235 resulted in the conversion of capsidiol to oxidized capsidiol as detected during *Δbccpdh* strain
236 incubations (Supplemental Figs. S13 and 14, Supplementary Notes 4). Moreover, sequential
237 incubation of capsidiol with the *Bccpdh* expressing *E. festucae* transformant followed by a
238 Bcin16g01490 expressing transformant reproduced the reactions detected in *B. cinerea*
239 (Supplemental Figs. S4 and S14B). Structural analysis of oxidized capsenone indicated that the
240 end product of these reactions is capsenone 11,12-epoxide (Fig. 1 and Supplemental Fig. S15-17,
241 Supplementary Notes 5).
242
243 **BcCPDH Is Required for the Pathogenicity of *B. cinerea* on Plant Species Producing**
244 **Capsidiol.**
245 Pathogenicity of *B. cinerea Δbccpdh* transformants was tested for several plant species. On plant
246 species that produce capsidiol, such as *N. benthamiana*, *N. tabacum* and *C. annuum*, the
247 development of disease symptoms by *Δbccpdh* was significantly reduced compared with those
248 caused by wild type *B. cinerea*. In contrast, both wild type and *Δbccpdh* strains caused comparable
249 symptoms in potato, tomato, grape and eggplant, which is consistent with the finding that
250 expression of *Bccpdh* is not induced during the infection in these plant species (Fig. 5C,
251 Supplemental Fig. S18). In the complementation strain, the ability to metabolize capsidiol to
252 capsenone was restored, as was virulence against capsidiol producing plants (Supplemental Fig.
253 S19). These results indicated that BcCPDH is a dedicated enzyme for the virulence of *B. cinerea*
254 on capsidiol-producing plant species.
255
256 **Distribution of *Bccpdh* Homologues in the Fungal Kingdom**
257 The distribution of *Bccpdh* homologs in taxonomically related fungal species (Ascomycota,
258 Leotiomycetes) was investigated. A search for *Bccpdh* homologs in the genome sequences of 6
259 host-specialized phytopathogenic *Botrytis* species, such as *B. tulipae* (pathogen of tulip), *B.*
260 *hyacinthi* (hyacinth) and *B. paeoniae* (peony), indicated that among *Botrytis* species, *Bccpdh* is a
261 unique gene only found in *B. cinerea* (Supplemental Fig. S20). Consistently, 4 *Botrytis* species, not
262 including *B. cinerea*, were incubated with capsidiol, but no CPDH activity was detected for the
263 tested strains (Supplemental Fig. S21). Search in 11 draft genomes of Leotiomycete fungi also
264 indicated the absence of *Bccpdh* homologs in these species. Comparison of the corresponding
265 genome regions between *Botrytis* species and *S. sclerotiorum* (another polyxenous pathogen
266 belonging to Leotiomycetes) revealed that the approx. 4.9 kb sequence surrounding *Bccpdh* is
267 unique to *B. cinerea* (Fig. 6). This unique sequence shows a lower GC content compared to the
268 surrounding region (Supplementa Fig. S22), suggesting that *Bccpdh* might have been obtained via
269 horizontal gene transfer (35). A blast search using BcCPDH as query sequence revealed that
270 probable orthologs can be found only in some Pezizomycotina fungi belonging to Ascomycota
271 (Supplemental Fig. S20). Orthologs were found from a taxonomically diverse range of fungal
272 species, including animal and insect pathogens, and there was no correlation between their
273 homology and taxonomic relationship, which might indicate multiple horizontal gene transfer events
274 of the *cpdh* gene in the diversification of Ascomycota fungi (Supplemental Figs. 20, 23-25,
275 Supplementary Notes 6). Among plant pathogenic fungi, *Bccpdh* homologs were found in *Fusarium*
276 species, all of which are pathogens that infect plants that do not produce capsidiol. This result is
277 consistent with a previous report showing that some *Fusarium* species can metabolize capsidiol to
278 capsenone (36).
279
280 **CPDH Activity Is Conserved Among *Botrytis cinerea* Strains**
281 Although *B. cinerea* is a polyxenous fungus, it has been reported that different strains of *B. cinerea*
282 exhibit various degrees of virulence on different host plants (37, 38). Hence, we investigated
283 whether BcCPDH is conserved among strains isolated from diverse plant species. Twenty-four *B.*
284 *cinerea* strains isolated from 14 different plant species were incubated with capsidiol and resultant

capsenone was detected by GC/MS. All tested *B. cinerea* strains can metabolize capsidiol to capsenone, indicating that BcCPDH is highly conserved among *B. cinerea* strains even though their (most recent) host was not a producer of capsidiol (Fig. 7A, Supplemental Table S5). Similarly, 17 strains of *F. oxysporum* isolated from 7 plant species were subjected to the analysis for CPDH activity. Eight out of 17 *F. oxysporum* strains showed CPDH activity, and the activity was not related to the natural host of the strains (Fig. 7B, Supplemental Table S6). This result is consistent with the finding that *Bccpdh* orthologues can be found in 5 out of 14 available genomes of *F. oxysporum* (Supplemental Fig. S25, data not shown). These results indicate that the cpdh gene is randomly distributed in *F. oxysporum* strains, whereas it is highly conserved among *B. cinerea* strains.

Discussion

To survive the exposure to microorganisms in the environment, different plant species have developed diverse resistance mechanisms over the course of evolution. The structural variety of phytoalexins is a prime example of such diversity: different plant families produce phytoalexins with relatively similar basic structures, but their side-chain structures often differ from species to species (4). These differences in structure between species may have contributed to the determination of host specificity between plants and pathogens, as in some cases a pathogen may have acquired resistance to a particular phytoalexin, but an analogous substance is not overcome by the same resistance mechanism (39). However, plant pathogens with a broad host range such as *B. cinerea* must employ strategies to counter a multitude of diverse phytoalexins. The prompt killing of plant cells, or the presence of efflux pumps of extremely low specificity may present effective strategies for such pathogens. Indeed, *B. cinerea* produces host-nonspecific phytotoxins (such as botrydial and botcinic acid, 40, 41) upon infection, and activates transporters capable of effluxing diverse substances such as PDR-type ABC transporter BcatrB and MFS transporter Bcmfs1 (26, 27, 42). More recently, it has been shown that *B. cinerea* suppresses the immune response of different plant species via gene silencing by delivering various small RNAs into host cells (43, 44).

In addition to effective infection mechanisms which generally facilitate infection of a wide range of plants, this study revealed that *B. cinerea* responds to chemical cues from the host plant to adapt its infection strategy to overcome specific plant resistance mechanisms. *B. cinerea* strictly distinguishes structurally similar phytoalexins, such as capsidiol and rishitin, and activates appropriate detoxification responses. Interestingly, treatment of *B. cinerea* with phytoalexins activated not only genes involved in detoxification and efflux of toxic compounds, but also genes predicted to be involved in pathogenicity to plants. These results support the notion that *B. cinerea* utilizes phytoalexins as a means to identify a given host and adjust the method of infection.

Expression of the *Bccpdh* gene is activated specifically during infection of capsidiol-producing plants, and the pathogenicity of the *bccpdh* mutant strains is compromised on plants producing capsidiol, but does not suffer any disadvantages on plants that do not produce capsidiol. These results suggest that BcCPDH is a critical component that specifically enables *B. cinerea* to infect capsidiol producing plants. Despite capsidiol producing plants representing only a small fraction of the over 400 host plants of *B. cinerea*, CPDH activity was maintained in all investigated *B. cinerea* strains isolated from plants that do not produce capsidiol. This may hint at the presence of a selection pressure against the loss of CPDH, despite it only affecting a limited number of host plants. This poses the question of whether *B. cinerea* is able to maintain acquired resistances for a prolonged time, which may explain how it evolved and establish itself as the polyxenous pathogen that it is.

How does *B. cinerea* Recognize Phytoalexins with Similar Structures?

Although *B. cinerea* is known to have the ability to metabolize a wide variety of phytoalexins (18), only a small portion of these detoxification mechanisms are required for the infection of any particular plant. Thus, *B. cinerea* needs to strictly control those various detoxification mechanisms, since maintaining sufficient levels of all these detoxification mechanisms represents a considerable waste of resources. Elucidating the mechanism by which *B. cinerea* recognizes phytoalexins and activates a specific set of genes is one of the important subjects for further research. Two major

339 scenarios can be envisioned: first, *B. cinerea* may possess receptors that can identify the chemical
340 structures of phytoalexins. In plants, various terpenes with diverse structures are employed as
341 hormones recognized by specific receptors, such as gibberellins, cytokinins, and abscisic acid (39).
342 However, the possibility that *B. cinerea* maintains receptors to perceive all of the myriad of
343 phytoalexins may not be realistic. The second scenario would be that *B. cinerea* recognizes the
344 damage caused by phytoalexins. However, as for capsidiol and rishitin, both are presumed to have
345 cell membranes as their targets, and these phytoalexins are relatively unspecific toxicants that
346 cause damage even to plant cells (5, 13, 17, 45), so it is not certain whether there exists a specific
347 target by which these two compounds can be distinguished. Given that some genes, such as
348 *BcatrB* encoding a multidrug resistance pump, are commonly induced by structurally unrelated
349 phytoalexins and fungicides treatments (26, 27), *B. cinerea* may possess both induction
350 mechanisms. Using the reporter system developed in this study, mutant strains defective in
351 capsidiol response could be isolated to elucidate the mechanism by which *B. cinerea* distinctly
352 identifies phytoalexins.
353
354 **Does *B. cinerea* Possess Other Capsidiol Resistance Mechanism besides Detoxification by**
355 **BcCPDH?**
356 Although the *bccpdh* knockout mutants showed reduced virulence on plant species producing
357 capsidiol, the mutant can develop the disease symptom on these plants and showed tolerance to
358 100 μM capsidiol, same as the wild type. In contrast, *Phytophthora* spp., *Alternaria* spp. and *E.*
359 *festucae* are sensitive to the same concentration of capsidiol. Although we also isolated
360 Bcin16g01490, which we found to oxidize capsidiol, its activity is fairly limited, indicating that it is
361 unlikely to confer sufficient protection against capsidiol. We therefore presumed that *B. cinerea* has
362 another mechanism for capsidiol tolerance other than detoxification. RNAseq analysis of *B. cinerea*
363 genes upregulated by capsidiol treatment identified 2 genes (Bcin15g00040.1 and
364 Bcin14g02870.1/Bcmfs1) encoding MFS transporters and a gene (Bcin01g05890.1/Bcbmr1)
365 encoding an ABC transporter (Supplemental Table S1). Bcmfs1 has been reported to be involved
366 in the resistance of *B. cinerea* to structurally different natural toxicants (camptothecin produced by
367 the plant *Camptotheca acuminata* and cercosporin produced by the plant pathogenic fungus
368 *Cercospora kikuchii*) and fungicides (sterol demethylation inhibitors, DMIs) (42). The *bcbmr1*
369 mutants showed an increased sensitivity to fungicides, polyoxin (a chitin synthetase inhibitor) and
370 iprobenfos (a choline biosynthesis inhibitor) (46). The expression of one or more of these
371 transporters may be involved in capsidiol efflux from B. cinerea cells, and regulated by signaling
372 that is common to *Bccpdh*.
373
374 **Why and How Can *B. cinerea* Strains Stably Maintain BcCPDH?**
375 CPDH activity was detected in all tested *B. cinerea* strains isolated from plants that do not produce
376 capsidiol, and the *Bccpdh* gene is conserved in the genomes of published *B. cinerea* strains. In *F.*
377 *oxysporum*, in contrast, there were strains with and without CPDH activity regardless of the host
378 plant, and consistently, some publicly available *F. oxysporum* genomes contain *cpdh* homologs
379 and others do not. As shown in this study, metabolic capacity (and tolerance) to capsidiol and
380 rishitin can be detected in some fungal strains regardless of the natural host of these pathogens,
381 and perhaps unused detoxification activities are maintained in the population of phytopathogenic
382 filamentous fungi for future use.
383 It is theoretically implausible that all *B. cinerea* strains maintain an enzyme required only upon
384 infection of capsidiol-producing plants, even if said gene is completely repressed in its expression
385 under normal circumstances. Given that *B. cinerea* has an extremely wide host range, some strains
386 will go for long periods where capsidiol detoxification ability is not a selection pressure. One
387 possibility is that BcCPDH has functions other than capsidiol degradation. For example, BcCPDH
388 might be involved in the detoxification of antimicrobial substances produced by insects, because it
389 has been reported that spores of *B. cinerea* are transmitted between plants via insects such as
390 thrips (47). Since homologous genes for *Bccpdh* are found in several insect-infecting fungi, it is
391 expected that CPDH-like enzymes in these species can metabolize insect-derived substances. We,
392 therefore, performed preliminary experiments to determine whether *Bccpdh* expression can be

induced by inoculation of *B. cinerea* P_*Bccpdh:GFP* transformant with several insects, but to date, no induction of GFP expression has been detected (data not shown). Alternatively, *B. cinerea* may have acquired the trait of having many hosts because of its ability to maintain rarely used virulence factors. Future clarification of the phytoalexin recognition mechanisms and comparative analysis of the genome with that of closely related *Botrytis* species are anticipated to elucidate unknown features of *B. cinerea* that have led to its evolution as a polyxenous fungi.

Materials and Methods

Comprehensive descriptions of the materials and methods used in this study, including, biological materials, vector construction, transformation of *E. festucae* and *B. cinerea*, confocal microscopy, pathogenicity tests and detection and structural analysis of phytoalexins and their derivatives, are available in Supplemental data.

Data Availability

RNA-seq data reported in this work are available in GenBank under the accession number DRA013980.

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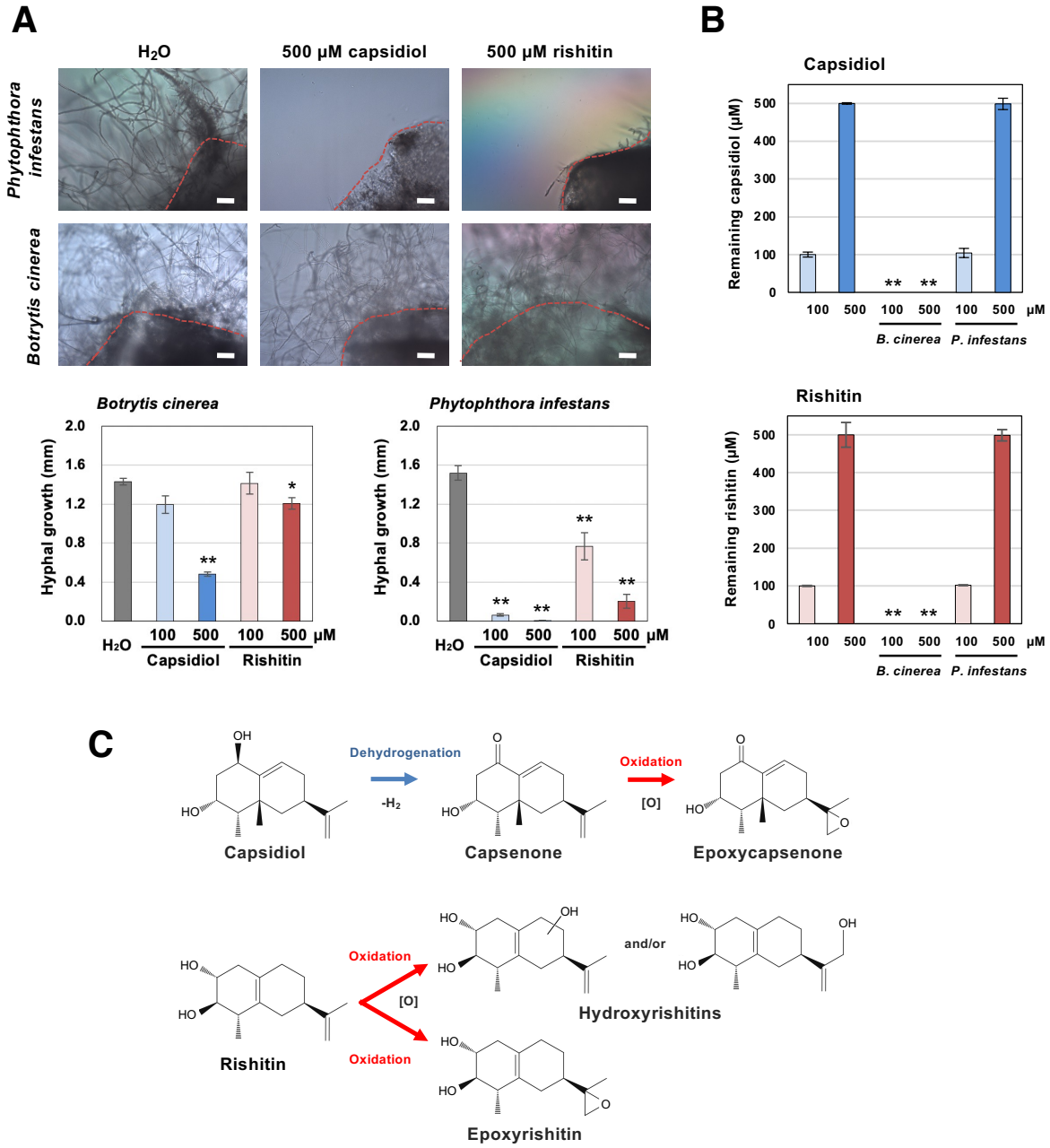
545 **Figures and Tables**



546
547 **Figure 1.** Sensitivities and metabolic capacities of *Botrytis cinerea* and *Phytophthora infestans* to
548 sesquiterpenoid phytoalexins.
549 **(A)** Mycelial blocks (approx. 1 mm^3) of indicated pathogen were incubated in 50 µl water, 500 µM
550 capsidiol or 500 µM rishitin. Outgrowth of hyphae from the mycelial block (outlined by dotted red
551 lines) was measured after 24 h of incubation (n = 6). Bars = 100 µm. **(B)** Residual capsidiol and
552 rishitin was quantified by GC/MS 2 days after the incubation. **(C)** Predicted metabolism of capsidiol
553 and rishitin by *B. cinerea*. Note that the structure of oxidized capsenone was determined in this
554 study. Data marked with asterisks are significantly different from control as assessed by the two-
555 tailed Student's *t*-test: **P < 0.01, *P < 0.05.
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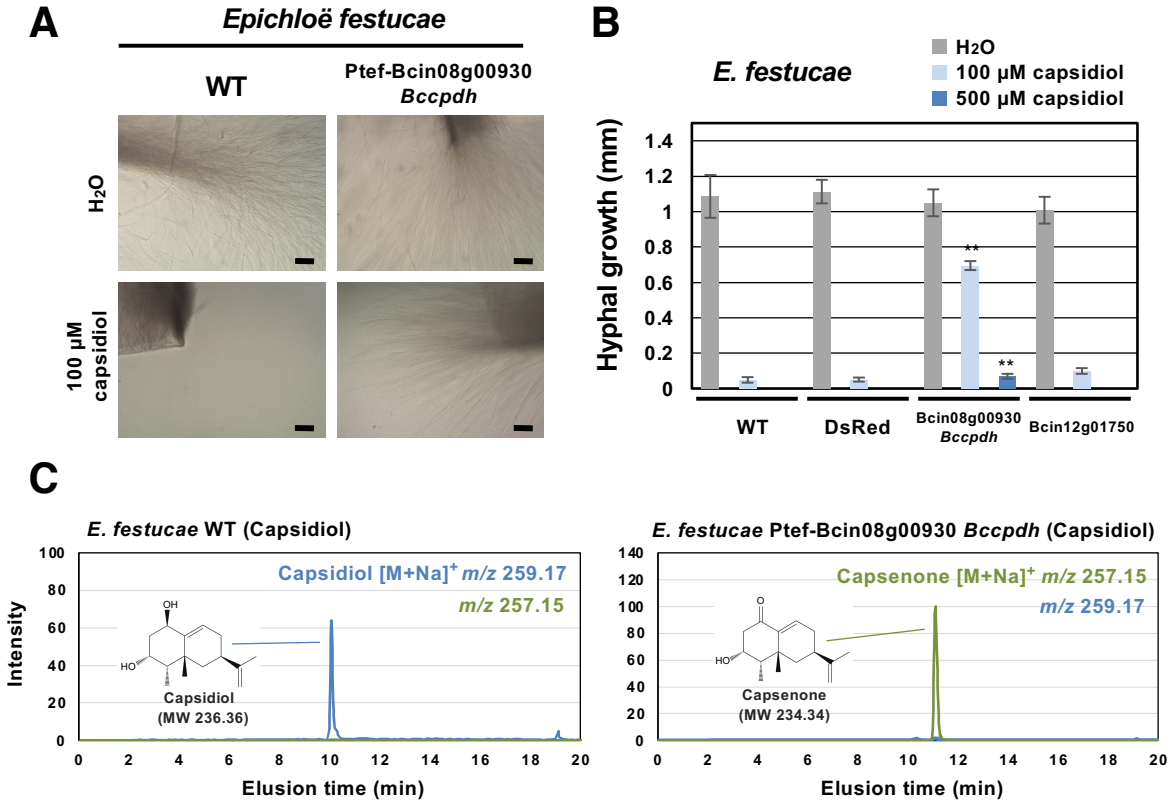

558

559 **Figure 3.** Bcin08g00930 encodes a capsidiol detoxification enzyme, capsidiol dehydrogenase
560 BcCPDH.
561 **(A)** Mycelia of *Epichloë festucae* wild type (WT) or a transformant expressing Bcin08g00930 were
562 incubated in water or 100 µM capsidiol and outgrowth of mycelia was observed 7 days after
563 inoculation. Bars = 100 µm. **(B)** Hyphal outgrowth of *E. festucae* WT, transformants expressing
564 DsRed, Bcin08g00930 (BcCPDH) or Bcin12g01750 in water, 100 µM or 500 µM capsidiol was
565 measured after 24 h of incubation. Data are mean ± SE (n = 6). Asterisks indicate a significant
566 difference from WT as assessed by two-tailed Student's *t*-test, **P < 0.01. **(C)** Mycelia of *E. festucae*
567 WT or transformant expressing Bcin08g00930 (*Bccpdh*) were incubated in 100 µM capsidiol or for
568 48 h. Capsidiol and capsenone were detected by LC/MS.
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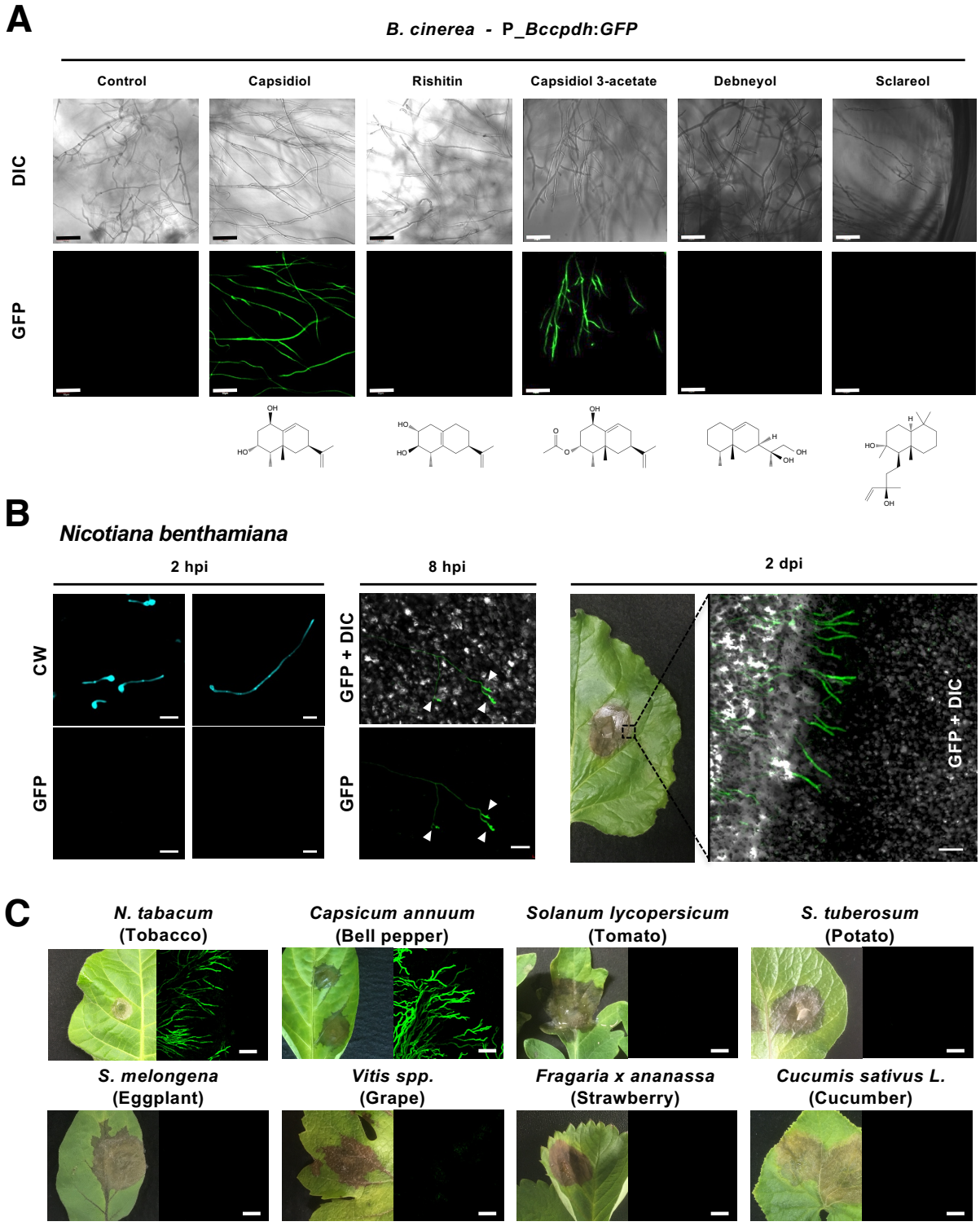

Figure 4. Specific activation of the *B. cinerea Bccpdh* promoter by capsidiol and its derivative. **(A)** Mycelia of *B. cinerea* transformant containing *GFP* gene under the control of 1 kb *Bccpdh* promoter (P_*Bccpdh:GFP*) was incubated in CM media containing 500 μM of anti-microbial terpenoids. Bars = 50 μm. **(B)** (left and middle) Leaves of *N. benthamiana* were inoculated with

575 conidia of *B. cinerea* P_*Bccpdh:GFP* transformant. Expression of GFP in germinating conidia on
576 the leaf surface was observed by confocal laser microscopy 2 or 8 h after inoculation (hpi). CW,
577 stained with calcofluor white. Arrowheads indicate the appressoria of *B. cinerea.* Bars = 20 μm.
578 (right) Leaves of *N. benthamiana* were inoculated with mycelia of *B. cinerea* P_*Bccpdh:GFP*
579 transformant and the edge of the lesion was observed by confocal laser microscopy 2 d after the
580 inoculation (2 dpi). Bar = 100 μm. **(C)** Leaves of indicated plant were inoculated with mycelia of *B.*
581 *cinerea* P_*Bccpdh:GFP* transformant and the edge of the lesion was observed by confocal laser
582 microscopy 2 d after the inoculation. Bars = 100 μm.

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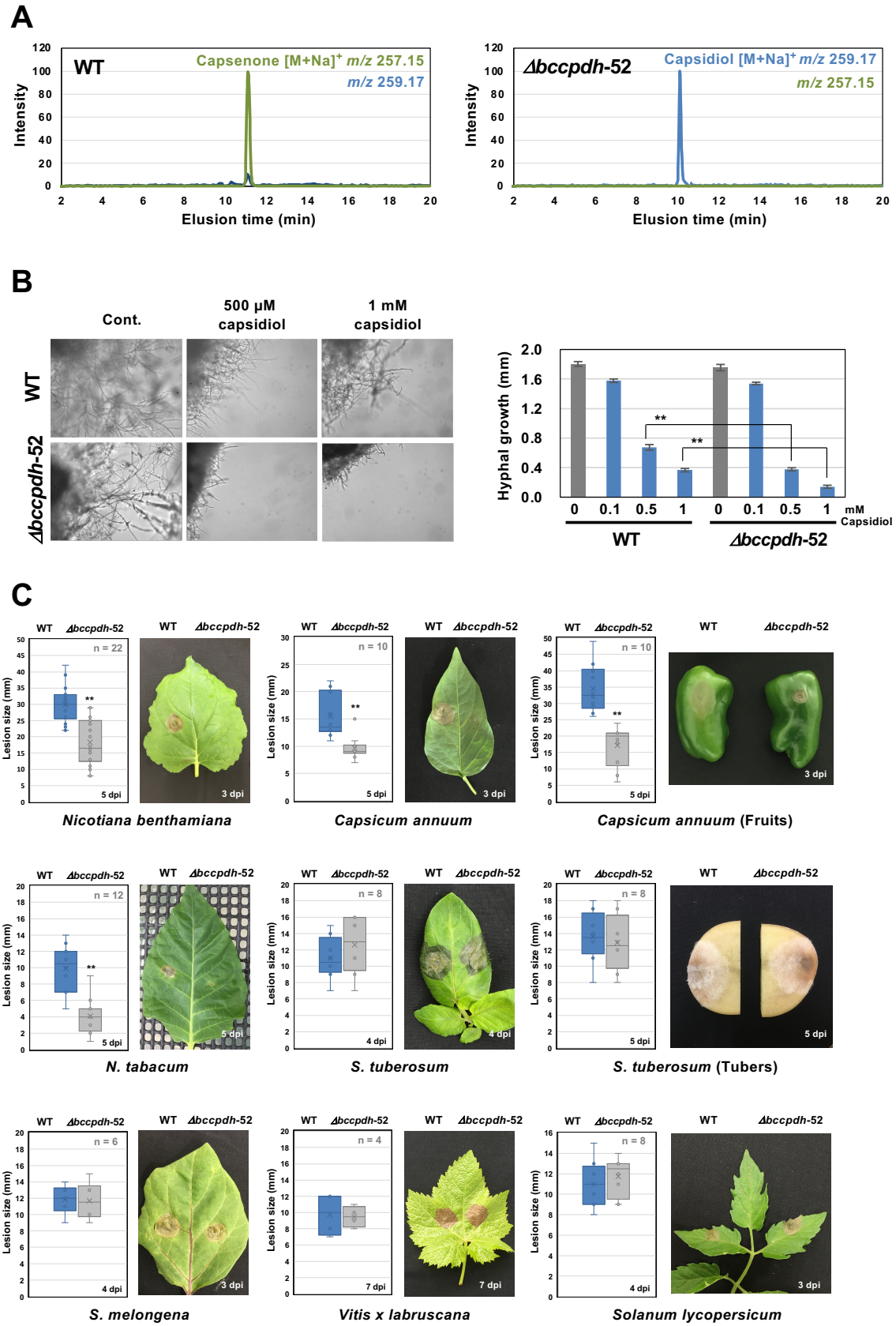
A
WT
Capsenone [M+Na]+ m/z 257.15
m/z 259.17
Intensity
Elusion time (min)
Δbccpdh-52
Capsidiol [M+Na]+ m/z 259.17
m/z 257.15
B
Cont.
500 µM capsidiol
1 mM capsidiol
WT
Δbccpdh-52
Hyphal growth (mm)
mM Capsidiol
C
Lesion size (mm)
n = 22
n = 10
n = 12
n = 8
n = 6
n = 4
5 dpi
3 dpi
4 dpi
7 dpi
Nicotiana benthamiana
Capsicum annuum
Capsicum annuum (Fruits)
N. tabacum
S. tuberosum
S. tuberosum (Tubers)
S. melongena
Vitis x labruscana
Solanum lycopersicum

585 **Figure 5.** *B. cinerea* BcCPDH is essential for the detoxification of capsidiol and virulence in the
586 plant species producing capsidiol.
587 **(A)** Mycelial block (approx. 1 mm^3) of *B. cinerea* wild type (WT) or *Bccpdh* KO mutant strain
588 (*Δbccpdh*-52) was incubated in 100 μM capsidiol for 4 d. Capsenone and capsidiol was detected
589 by LC/MS. **(B)** Mycelial blocks of *B. cinerea* WT or *Δbccpdh*-52 were incubated in capsidiol and
590 outgrowth of hyphae was measured after 36 h of incubation. Data are mean ± SE (n = 10). Asterisks
591 indicate a significant difference from WT as assessed by two-tailed Student's *t*-test, **P < 0.01. **(C)**
592 Leaves, tubers or fruits of indicated plant were inoculated with mycelial block (5 mm^3) of *B. cinerea*
593 WT or *Δbccpdh*-52 and lesion size was measured between 4 and 7 days after inoculation (dpi).
594 Asterisks indicate a significant difference from WT as assessed by two-tailed Student's *t*-test. **P
595 < 0.01. Lines and crosses (x) in the columns indicate the median and mean values, respectively.

596

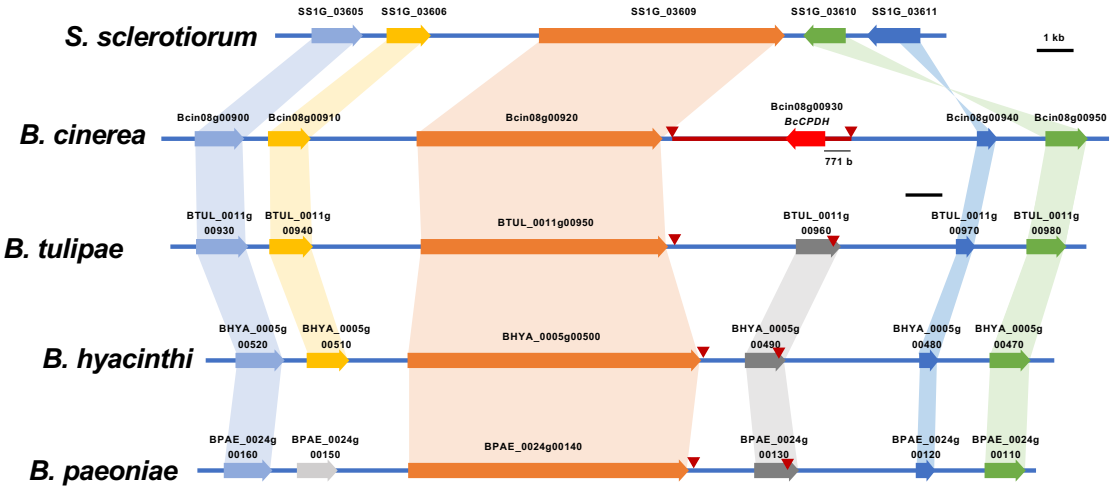

Figure 6. Comparison of the *B. cinerea Bccpdh* gene locus with corresponding genome region of other *Botrytis* species and *Sclerotinia sclerotiorum*. Edge of conserved region among *Botrytis species* and specific region for *B. cinerea* (red line) were indicated by red arrowheads.

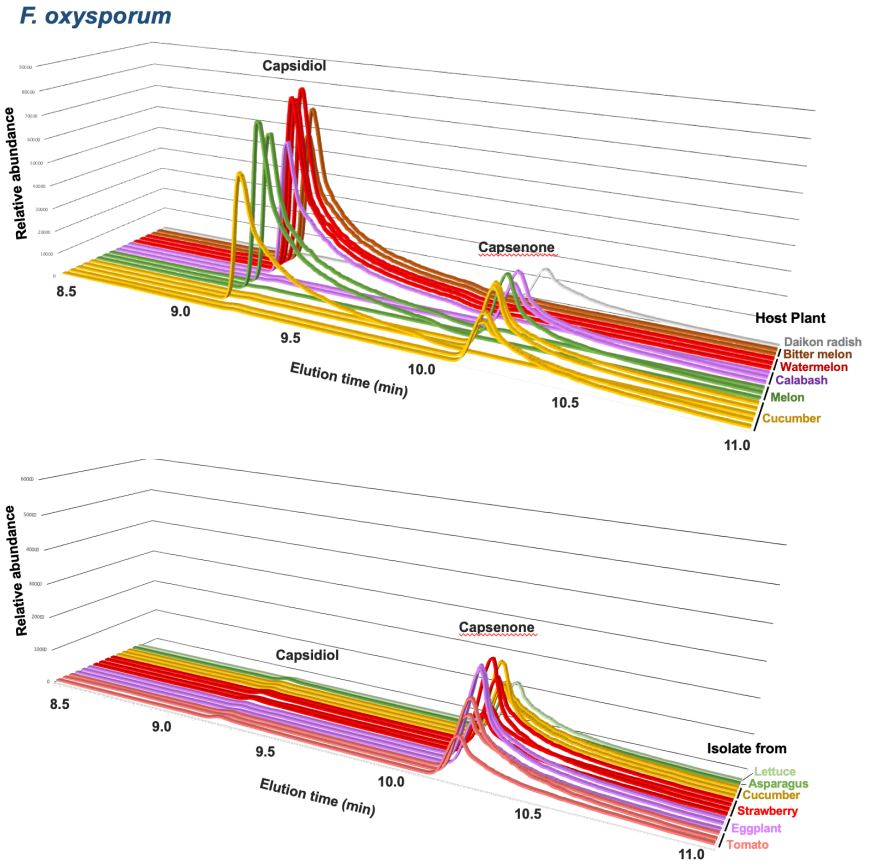

Figure 7. CPDH activity in *F. oxysporum* and *B. cinerea* strains isolated form a variety of plants. CPDH activity in *F. oxysporum* and *B. cinerea* strains isolated form a variety of plants. Mycelial blocks (approx. 1 mm^3) were incubated in 500 μM capsidiol for 2 days and capsidiol or capsenone were detected by GC/MS. Each elution profile describes the capsidiol and capsenone content in the solution after incubation of the strain with capsidiol. Strains with a substantial peak for capsidiol indicate the absence of CPDH activity.