Article title:

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 ⊿bccpdh* mutants became relatively sensitive to capsidiol. The *⊿bccpdh* mutant showed reduced virulence on the capsidiol producing *Nicotiana* and *Capsicum* species but remained fully pathogenic on potato and tomato. Homologs of *Bccpdh* are not found in taxonomically distant Ascomycota fungi but not in related Leotiomycete 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 implicates 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.

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 (28). 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

198 SDR encoded by Bcin08g00930 was designated as BcCPDH standing for *B.*
199 *cinerea* capsidiol dehydrogenase.
200
201 **Expression of *Bccpdh* Is specifically induced by capsidiol.**
202 To further investigate the expression pattern of *Bccpdh*, *B. cinerea* was
203 transformed with a reporter construct for GFP expression under the control of the
204 1 kb proximal *Bccpdh* promoter (P_*Bccpdh:GFP*). P_*Bccpdh:GFP* transformants
205 showed no obvious expression of GFP in water, but a significant increase of GFP
206 fluorescence was detected in 500 μM capsidiol (Fig. 4A). The P_*Bccpdh:GFP*
207 transformant was incubated with other anti-microbial terpenoids produced by
208 Solanaceae species, including rishitin, debneyol (29), sclareol (30) and capsidiol
209 3-acetate (31). Among the terpenoids tested, treatment of capsidiol and capsidiol
210 3-acetate induced expression of GFP in the P_*Bccpdh:GFP* transformant. This
211 result further indicated that *B. cinerea* specifically reacts to capsidiol and its
212 derivative capsidiol 3-acetate. As the *Bccpdh* promoter was activated by capsidiol
213 3-acetate, we investigated whether BcCPDH could metabolize capsidiol 3-acetate
214 to capsenone 3-acetate. However, capsidiol 3-acetate was not metabolized by *E.*
215 *festucae* expressing *Bccpdh* (Bcin08g00930) (Supplemental Fig. S7A).
216 Consistently, capsidiol 3-acetate was directly oxidized by *B. cinerea* and at least
217 two different forms of oxidized capsidiol 3-acetate, but not capsenone 3-acetate,
218 were detected (Supplemental Fig. S7B), indicating that capsidiol and capsidiol 3-
219 acetate were metabolized in *B. cinerea* by distinctive pathways.
220 Analysis of different lengths of *Bccpdh* promoters indicated that 250 bp of
221 promoter sequence are sufficient for specific activation of the promoter by capsidiol
222 treatment, and the cis-element required for capsidiol-specific expression was
223 shown to be within -250 and -200 bp upstream of the start codon (Supplemental
224 Figs. S8 and 9). By further analysis using *B. cinerea* transformant P_*Bccpdh:Luc*
225 using luciferase as a quantitative marker, it was shown that *Bccpdh* promoter was
226 activated within 2 h of capsidiol treatment, and the expression activity decreased
227 as capsidiol was metabolized (Supplemental Figs. S10, Supplementary Notes 3).
228
229 **Expression of *Bccpdh* is specifically induced during the infection of**
230 **capsidiol producing plant species.**
231 Leaves of *Nicotiana benthamiana*, which produce capsidiol as the major
232 phytoalexin (13), were inoculated with conidia of the *B. cinerea* transformant
233 P_*Bccpdh:GFP*. No GFP signal was detected in germinating *B. cinerea* conidia
234 grown on the surface of *N. benthamiana* leaves 2 h after the inoculation, but
235 obvious GFP expression was detected in the appressoria at 8 h after the
236 inoculation (Fig. 4B). Growing hyphae in the leaf tissue of *N. benthamiana* showed
237 GFP fluorescence, and the intensity of GFP fluorescence in hyphae was stronger
238 near the edge of the lesion compared with that of hyphae growing in the areas of
239 dead tissue within the lesions (Fig. 4B), probably because capsidiol was eventually
240 detoxified in these areas, which are heavily infected with *B. cinerea*.
241 To examine the possibility whether polyxenous *B. cinerea* activates *Bccpdh* for
242 detoxification of other phytoalexin or anti-microbial compounds produced in other

243 plant species, the *B. cinerea* P_*Bccpdh:GFP* transformant was inoculated on a
244 wide variety of plants. Over 50 plant species were used for the inoculation test
245 including 9 Solanaceae, 6 Brassicaceae, 6 Rosaceae, 5 Fabaceae and 6
246 Asteraceae plants, and development of disease symptoms was observed in all
247 tested plant species. Among the tested plants, expression of GFP under the control
248 of *Bccpdh* promoter was only detected during the infection in three *Nicotiana* and
249 two *Capsicum* species (Fig. 4C, Supplemental Fig. S11 and Table S4), all of which
250 are reported to produce capsidiol (8, 32–34). These results further indicated that
251 *B. cinerea* specifically recognizes capsidiol for the induction of *Bccpdh*.
252
253 **BcCPDH metabolizes and detoxifies capsidiol to capsenone in *B. cinerea*.**
254 *B. cinerea Bccpdh* KO mutant (*Δbccpdh*) was produced to investigate the function
255 of BcCPDH (Supplemental Fig. S12). Mycelia of *B. cinerea* wild type and *Δbccpdh*
256 were incubated with capsidiol and the metabolites were detected by LC/MS. While
257 capsidiol was metabolized to capsenone in the wild type strain, most of the
258 capsidiol remained unmetabolized two days after incubation with the *Δbccpdh*
259 strain (Fig. 5A). Instead, oxidized capsidiol, which was not detectable in the wild
260 type strain, was detected in *Δbccpdh* incubations (Supplemental Fig. S13 and
261 Supplementary Notes 4). While the growth of *B. cinerea* hyphae was not affected
262 by the disruption of the *Bccpdh* gene in 100 μM capsidiol, growth of *Δbccpdh* was
263 diminished compared with that of wild type *B. cinerea* at higher concentrations of
264 capsidiol (Fig. 5B). These results confirmed that BcCPDH is the enzyme
265 responsible for the detoxification of capsidiol in *B. cinerea*.
266
267 **Epoxidation of capsidiol and capsenone is mediated via Bcin16g01490.**
268 The incubation of the *Δbccpdh* knockout strain in capsidiol revealed the presence
269 of a potential secondary capsidiol degradation pathway, which resulted in the
270 accumulation of oxidized capsidiol. Since we were unable to find any indication for
271 such a pathway in our RNAseq results for 100 μM capsidiol treated incubations,
272 we extended our search to a preliminary RNAseq analysis which used 500 μM
273 capsidiol treatments. We identified the gene Bcin16g01490 encoding a
274 cytochrome P450, which was significantly upregulated under these elevated
275 capsidiol concentrations (Supplemental Table S1 and Fig. S14A). Heterologous
276 expression of Bcin16g01490 in *E. festucae*, resulted in the conversion of capsidiol
277 to oxidized capsidiol as detected during *Δbccpdh* strain incubations (Supplemental
278 Figs. S13 and 14, Supplementary Notes 4). Moreover, sequential incubation of
279 capsidiol with the *Bccpdh* expressing *E. festucae* transformant followed by a
280 Bcin16g01490 expressing transformant reproduced the reactions detected in *B.*
281 *cinerea* (Supplemental Figs. S4 and S14B). Structural analysis of oxidized
282 capsenone indicated that the end product of these reactions is capsenone 11,12-
283 epoxide (Fig. 1 and Supplemental Fig. S15-17, Supplementary Notes 5).
284

285 **BcCPDH is required for the pathogenicity of *B. cinerea* on plant species**
286 **producing capsidiol**
287 Pathogenicity of *B. cinerea ⊿bccpdh* transformants was tested for several plant
288 species. On plant species that produce capsidiol, such as *N. benthamiana*, *N.*
289 *tabacum* and *C. annuum*, the development of disease symptoms by *⊿bccpdh* was
290 significantly reduced compared with those caused by wild type *B. cinerea*. In
291 contrast, both wild type and *⊿bccpdh* strains caused comparable symptoms in
292 potato, tomato, grape and eggplant, which is consistent with the finding that
293 expression of *Bccpdh* is not induced during the infection in these plant species
294 (Fig. 5C, Supplemental Fig. S18). In the complementation strain, the ability to
295 metabolize capsidiol to capsenone was restored, as was virulence against
296 capsidiol producing plants (Supplemental Fig. S19). These results indicated that
297 BcCPDH is a dedicated enzyme for the virulence of *B. cinerea* on capsidiol-
298 producing plant species.
299
300 **Distribution of *Bccpdh* homologues in the fungal kingdom**
301 The distribution of *Bccpdh* homologs in taxonomically related fungal species
302 (Ascomycota, Leotiomycetes) was investigated. A search for *Bccpdh* homologs in
303 the genome sequences of 6 host-specialized phytopathogenic *Botrytis* species,
304 such as *B. tulipae* (pathogen of tulip), *B. hyacinthi* (hyacinth) and *B. paeoniae*
305 (peony), indicated that among *Botrytis* species, *Bccpdh* is a unique gene only
306 found in *B. cinerea* (Supplemental Fig. S20). Consistently, 4 *Botrytis* species, not
307 including *B. cinerea*, were incubated with capsidiol, but no CPDH activity was
308 detected for the tested strains (Supplemental Fig. S21). Search in 11 draft
309 genomes of Leotiomycete fungi also indicated the absence of *Bccpdh* homologs
310 in these species. Comparison of the corresponding genome regions between
311 *Botrytis* species and *S. sclerotiorum* (another polyxenous pathogen belonging to
312 Leotiomycetes) revealed that the approx. 4.9 kb sequence surrounding *Bccpdh* is
313 unique to *B. cinerea* (Fig. 6). This unique sequence shows a lower GC content
314 compared to the surrounding region (Supplemental Fig. S22), suggesting that
315 *Bccpdh* might have been obtained via horizontal gene transfer (35). A blast search
316 using BcCPDH as query sequence revealed that probable orthologs can be found
317 only in some Pezizomycotina fungi belonging to Ascomycota (Supplemental Fig.
318 S20). Orthologs were found from a taxonomically diverse range of fungal species,
319 including animal and insect pathogens, and there was no correlation between their
320 homology and taxonomic relationship, which might indicate multiple horizontal
321 gene transfer events of the *cpdh* gene in the diversification of Ascomycota fungi
322 (Supplemental Figs. 20, 23-25, Supplementary Notes 6). Among plant pathogenic
323 fungi, *Bccpdh* homologs were found in *Fusarium* species, all of which are
324 pathogens that infect plants that do not produce capsidiol. This result is consistent
325 with a previous report showing that some *Fusarium* species can metabolize
326 capsidiol to capsenone (36).
327

328 **CPDH activity is conserved among *Botrytis cinerea* strains**
329 Although *B. cinerea* is a polyxenous fungus, it has been reported that different
330 strains of *B. cinerea* exhibit various degrees of virulence on different host plants
331 (37, 38). Hence, we investigated whether BcCPDH is conserved among strains
332 isolated from diverse plant species. Twenty-four *B. cinerea* strains isolated from
333 14 different plant species were incubated with capsidiol and resultant capsenone
334 was detected by GC/MS. All tested *B. cinerea* strains can metabolize capsidiol to
335 capsenone, indicating that BcCPDH is highly conserved among *B. cinerea* strains
336 even though their (most recent) host was not a producer of capsidiol (Fig. 7A,
337 Supplemental Table S5). Similarly, 17 strains of *F. oxysporum* isolated from 7 plant
338 species were subjected to the analysis for CPDH activity. Eight out of 17 *F.*
339 *oxysporum* strains showed CPDH activity, and the activity was not related to the
340 natural host of the strains (Fig. 7B, Supplemental Table S6). This result is
341 consistent with the finding that *Bccpdh* orthologues can be found in 5 out of 14
342 available genomes of *F. oxysporum* (Supplemental Fig. S25, data not shown).
343 These results indicate that the cpdh gene is randomly distributed in *F. oxysporum*
344 strains, whereas it is highly conserved among *B. cinerea* strains.
345
346 **Discussion**
347
348 To survive the exposure to microorganisms in the environment, different plant
349 species have developed diverse resistance mechanisms over the course of
350 evolution. The structural variety of phytoalexins is a prime example of such
351 diversity: different plant families produce phytoalexins with relatively similar basic
352 structures, but their side-chain structures often differ from species to species (4).
353 These differences in structure between species may have contributed to the
354 determination of host specificity between plants and pathogens, as in some cases
355 a pathogen may have acquired resistance to a particular phytoalexin, but an
356 analogous substance is not overcome by the same resistance mechanism (39).
357 However, plant pathogens with a broad host range such as *B. cinerea* must employ
358 strategies to counter a multitude of diverse phytoalexins. The prompt killing of plant
359 cells, or the presence of efflux pumps of extremely low specificity may present
360 effective strategies for such pathogens. Indeed, *B. cinerea* produces host-
361 nonspecific phytotoxins (such as botrydial and botcinic acid, 40, 41) upon infection,
362 and activates transporters capable of effluxing diverse substances such as PDR-
363 type ABC transporter BcatrB and MFS transporter Bcmfs1 (26, 27, 42). More
364 recently, it has been shown that *B. cinerea* suppresses the immune response of
365 different plant species via gene silencing by delivering various small RNAs into
366 host cells (43, 44).
367 In addition to effective infection mechanisms which generally facilitate infection of
368 a wide range of plants, this study revealed that *B. cinerea* responds to chemical
369 cues from the host plant to adapt its infection strategy to overcome specific plant
370 resistance mechanisms. *B. cinerea* strictly distinguishes structurally similar
371 phytoalexins, such as capsidiol and rishitin, and activates appropriate
372 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 scenarios can be envisioned: first, *B. cinerea* may possess receptors that can identify the chemical structures of phytoalexins. In plants, various terpenes with diverse structures are employed as hormones recognized by specific receptors, such as gibberellins, cytokinins, and abscisic acid (39). However, the possibility that *B. cinerea* maintains receptors to perceive all of the myriad of phytoalexins may not be realistic. The second scenario would be that *B. cinerea* recognizes the damage caused by phytoalexins. However, as for capsidiol and rishitin, both are presumed to have cell membranes as their targets, and these phytoalexins are relatively unspecific toxicants that cause damage even to plant cells (5, 13, 17, 45), so it is not certain whether there exists a specific target by which these two compounds can be distinguished. Given that some genes, such as *BcatrB* encoding a multidrug resistance pump, are commonly induced by structurally unrelated phytoalexins and fungicides treatments (26, 27), *B. cinerea* may possess both induction mechanisms. Using the reporter system developed in this study, mutant strains defective in capsidiol response could be isolated to elucidate the mechanism by which *B. cinerea* distinctly identifies phytoalexins.

416 **Does *B. cinerea* possess other capsidiol resistance mechanism besides**
417 **detoxification by BcCPDH?**
418 Although the *bccpdh* knockout mutants showed reduced virulence on plant
419 species producing capsidiol, the mutant can develop the disease symptom on
420 these plants and showed tolerance to 100 μM capsidiol, same as the wild type. In
421 contrast, *Phytophthora* spp., *Alternaria* spp. and *E. festucae* are sensitive to the
422 same concentration of capsidiol. Although we also isolated Bcin16g01490, which
423 we found to oxidize capsidiol, its activity is fairly limited, indicating that it is unlikely
424 to confer sufficient protection against capsidiol. We therefore presumed that *B.*
425 *cinerea* has another mechanism for capsidiol tolerance other than detoxification.
426 RNAseq analysis of *B. cinerea* genes upregulated by capsidiol treatment identified
427 2 genes (Bcin15g00040.1 and Bcin14g02870.1/Bcmfs1) encoding MFS
428 transporters and a gene (Bcin01g05890.1/Bcbmr1) encoding an ABC transporter
429 (Supplemental Table S1). Bcmfs1 has been reported to be involved in the
430 resistance of *B. cinerea* to structurally different natural toxicants (camptothecin
431 produced by the plant *Camptotheca acuminata* and cercosporin produced by the
432 plant pathogenic fungus *Cercospora kikuchii*) and fungicides (sterol demethylation
433 inhibitors, DMIs) (42). The *bcbmr1* mutants showed an increased sensitivity to
434 fungicides, polyoxin (a chitin synthetase inhibitor) and iprobenfos (a choline
435 biosynthesis inhibitor) (46). The expression of one or more of these transporters
436 may be involved in capsidiol efflux from *B. cinerea* cells, and regulated by signaling
437 that is common to *Bccpdh*.
438
439 **Why and how can *B. cinerea* strains stably maintain BcCPDH?**
440 CPDH activity was detected in all tested *B. cinerea* strains isolated from plants
441 that do not produce capsidiol, and the *Bccpdh* gene is conserved in the genomes
442 of published *B. cinerea* strains. In *F. oxysporum*, in contrast, there were strains
443 with and without CPDH activity regardless of the host plant, and consistently, some
444 publicly available *F. oxysporum* genomes contain *cpdh* homologs and others do
445 not. As shown in this study, metabolic capacity (and tolerance) to capsidiol and
446 rishitin can be detected in some fungal strains regardless of the natural host of
447 these pathogens, and perhaps unused detoxification activities are maintained in
448 the population of phytopathogenic filamentous fungi for future use.
449 It is theoretically implausible that all *B. cinerea* strains maintain an enzyme
450 required only upon infection of capsidiol-producing plants, even if said gene is
451 completely repressed in its expression under normal circumstances. Given that *B.*
452 *cinerea* has an extremely wide host range, some strains will go for long periods
453 where capsidiol detoxification ability is not a selection pressure. One possibility is
454 that BcCPDH has functions other than capsidiol degradation. For example,
455 BcCPDH might be involved in the detoxification of antimicrobial substances
456 produced by insects, because it has been reported that spores of *B. cinerea* are
457 transmitted between plants via insects such as thrips (47). Since homologous
458 genes for *Bccpdh* are found in several insect-infecting fungi, it is expected that
459 CPDH-like enzymes in these species can metabolize insect-derived substances.
460 We, 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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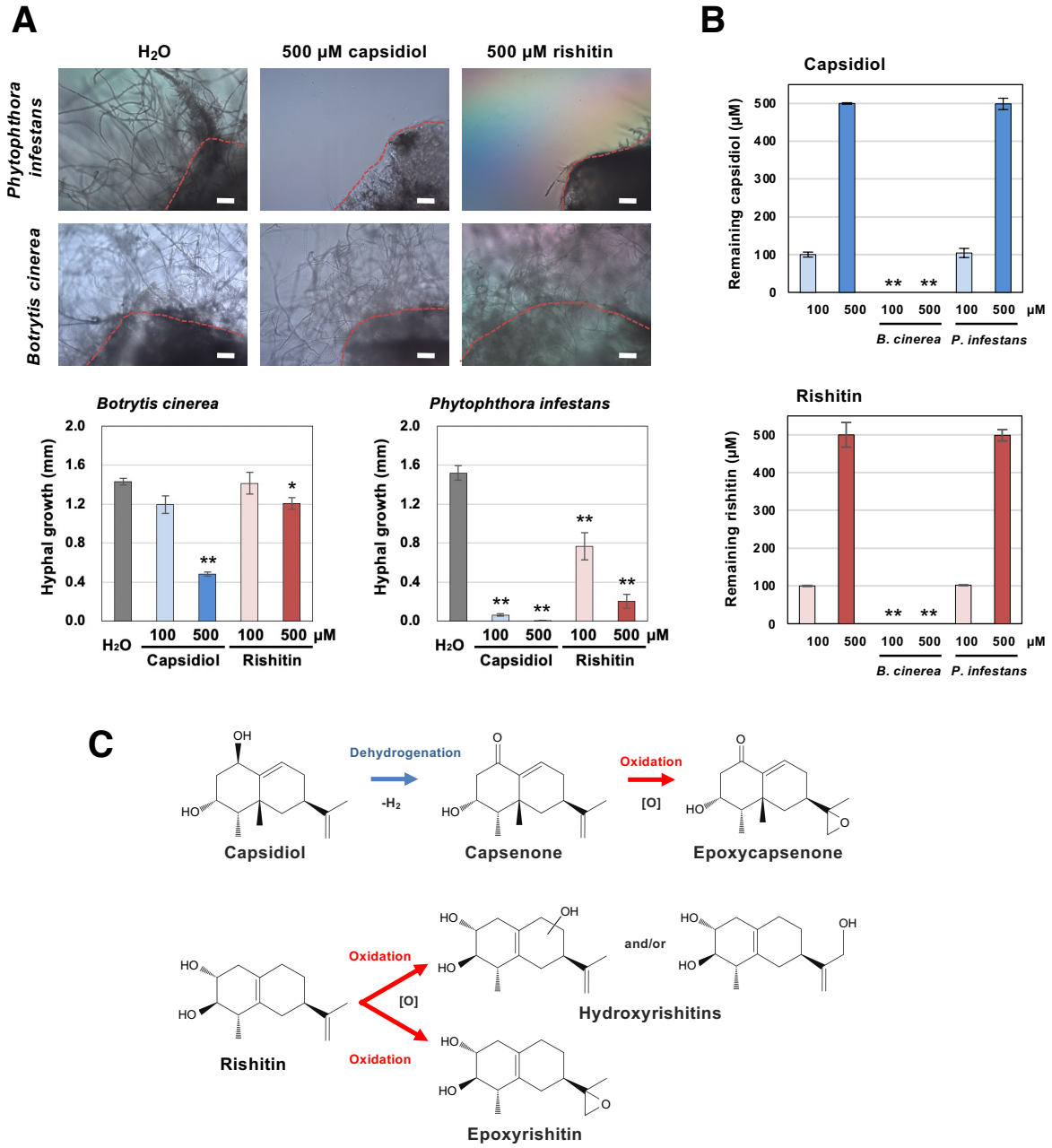
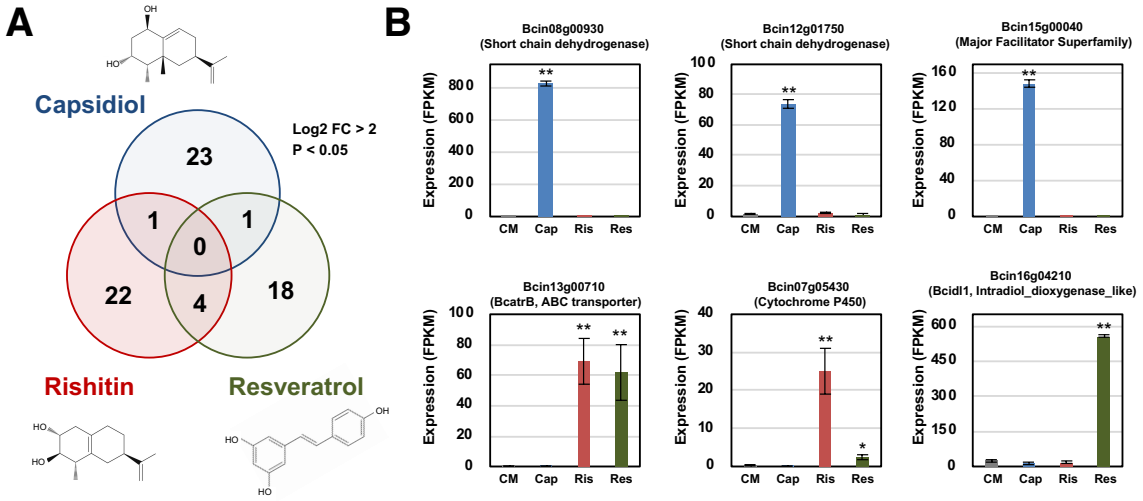
633 **Figures**



634

635 **Fig. 1** Sensitivities and metabolic capacities of *Botrytis cinerea* and *Phytophthora*
636 *infestans* to sesquiterpenoid phytoalexins.
637 **(A)** Mycelial blocks (approx. 1 mm^3) of indicated pathogen were incubated in 50 µl
638 water, 500 µM capsidiol or 500 µM rishitin. Outgrowth of hyphae from the mycelial
639 block (outlined by dotted red lines) was measured after 24 h of incubation (n = 6).
640 Bars = 100 µm. **(B)** Residual capsidiol and rishitin was quantified by GC/MS 2 days
641 after the incubation. **(C)** Predicted metabolism of capsidiol and rishitin by *B.*
642 *cinerea*. Note that the structure of oxidized capsenone was determined in this

643 study. Data marked with asterisks are significantly different from control as
644 assessed by the two-tailed Student's *t*-test: **P < 0.01, *P < 0.05.



645

646 **Fig. 2** Unique set of genes are upregulated in *Botrytis cinerea* treated with
647 capsidiol, rishitin and resveratrol. **(A)** Venn diagram showing genes upregulated in
648 *B. cinerea* cultured in CM media containing 100 μM capsidiol, 500 μM rishitin or
649 500 μM resveratrol for 24 h. The numbers of significantly upregulated genes by
650 phytoalexin treatment with Log2 FC > 2 compared with control (CM without
651 phytoalexin) and P value < 0.05 are presented. **(B)** Expression profiles of
652 representative genes upregulated by the treatment with capsidiol, rishitin or
653 resveratrol. The gene expression (FPKM value) was determined by RNA-seq
654 analysis of *B. cinerea* cultured in CM media containing 100 μM capsidiol, 500 μM
655 rishitin or 500 μM resveratrol for 24 h. Data are mean ± SE (n = 3). Asterisks
656 indicate a significant difference from the control (CM) as assessed by two-tailed
657 Student's *t*-test, **P < 0.01, *P < 0.05.

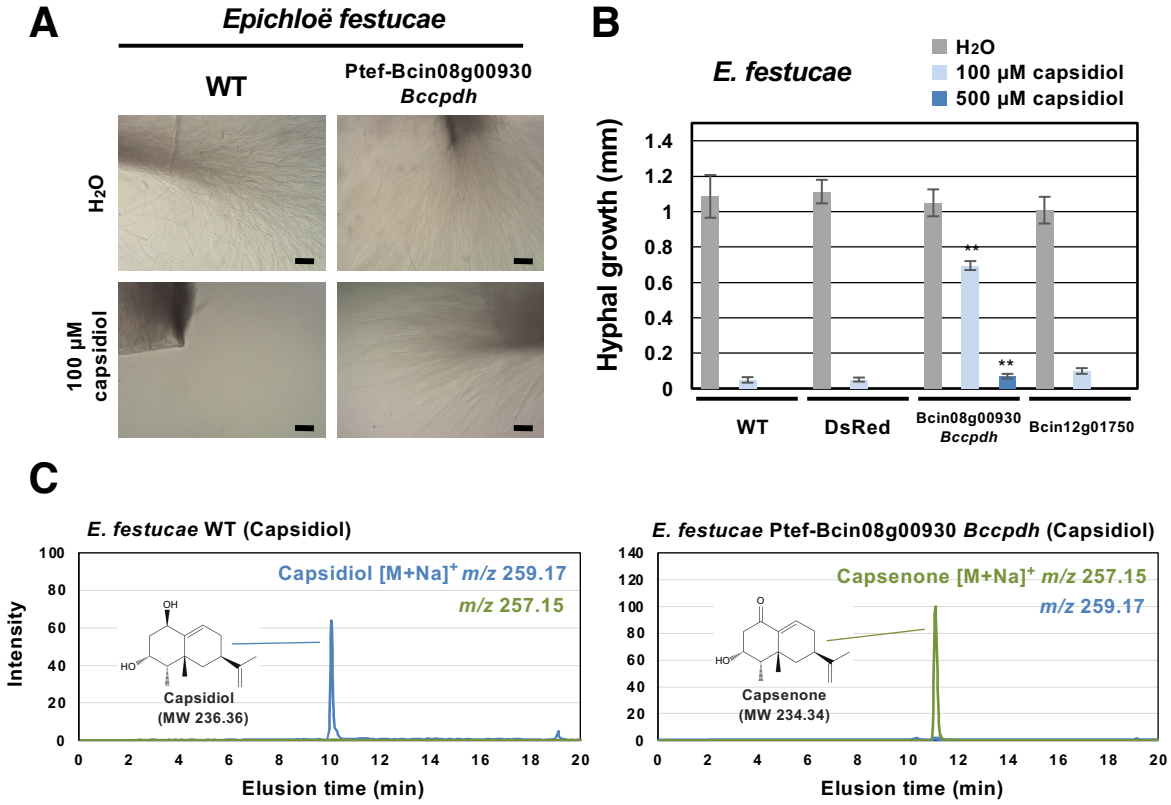
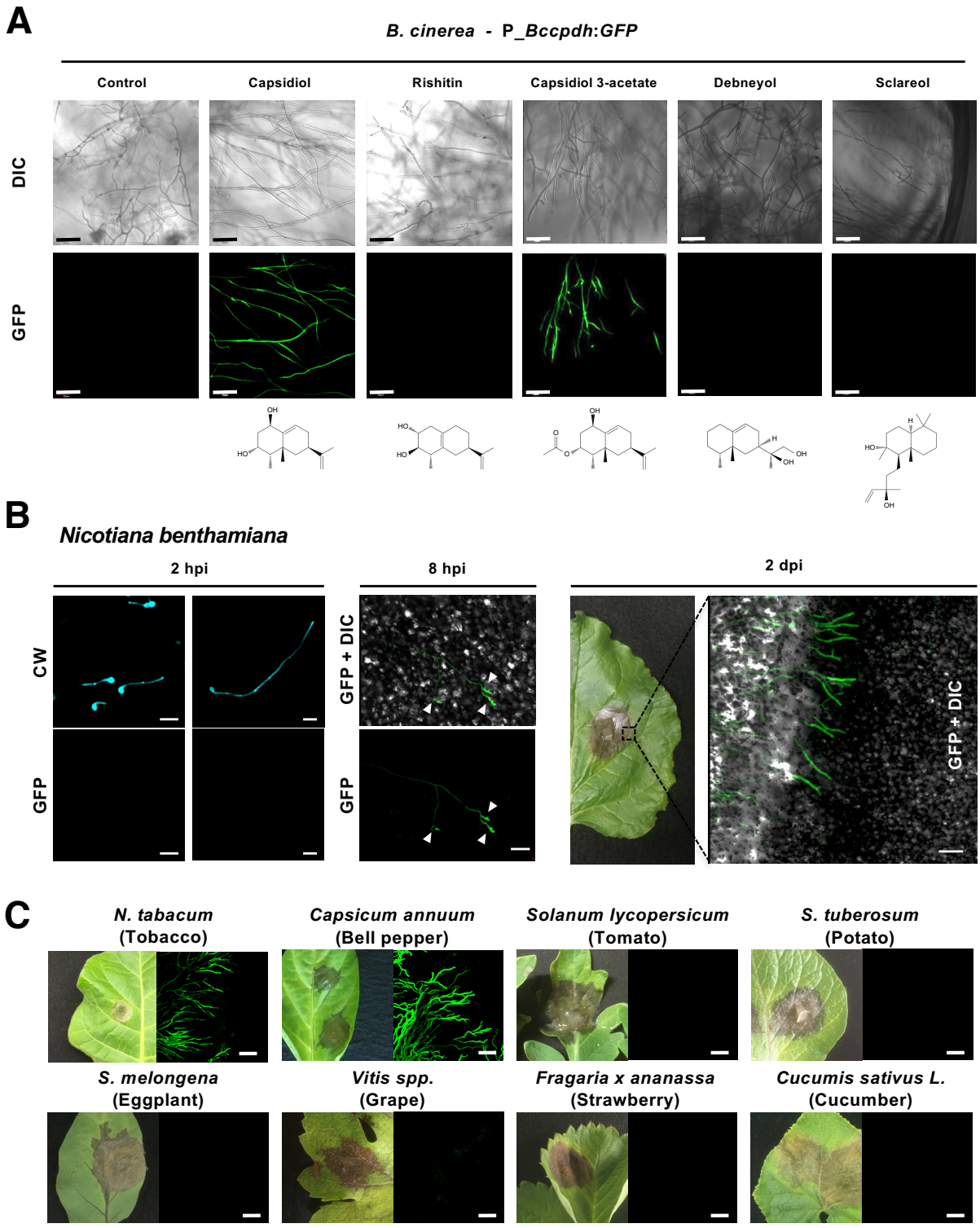


Fig. 3 Bcin08g00930 encodes a capsidiol detoxification enzyme, capsidiol dehydrogenase BcCPDH.
(A) Mycelia of *Epichloë festucae* wild type (WT) or a transformant expressing Bcin08g00930 were incubated in water or 100 μM capsidiol and outgrowth of mycelia was observed 7 days after inoculation. Bars =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

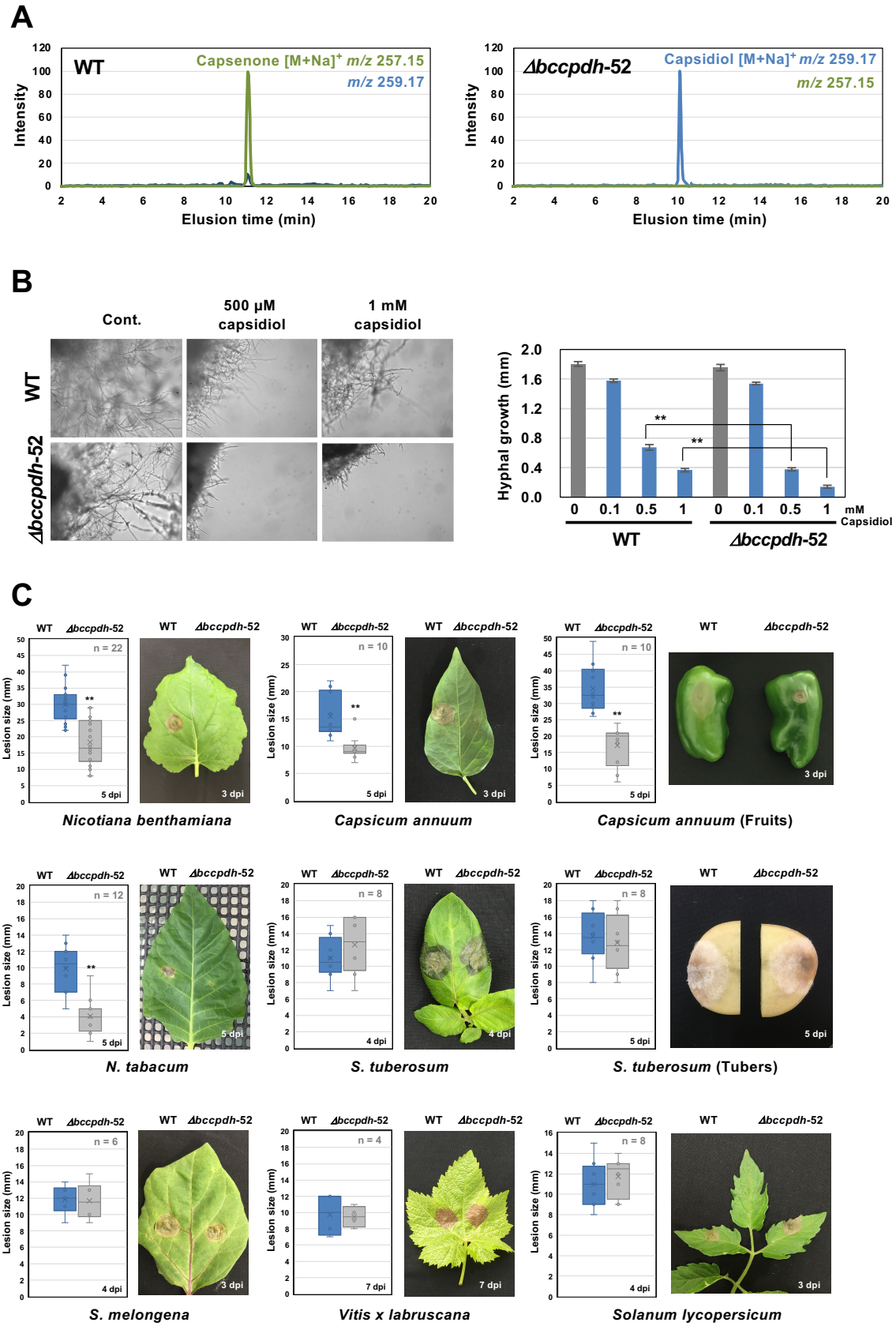

A
B. cinerea - P_Bccpdh:GFP
Control
Capsidiol
Rishitin
Capsidiol 3-acetate
Debneyol
Sclareol
DIC
GFP
B
Nicotiana benthamiana
2 hpi
8 hpi
2 dpi
CW
GFP
GFP + DIC
C
N. tabacum
(Tobacco)
Capsicum annuum
(Bell pepper)
Solanum lycopersicum
(Tomato)
S. tuberosum
(Potato)
S. melongena
(Eggplant)
Vitis spp.
(Grape)
Fragaria x ananassa
(Strawberry)
Cucumis sativus L.
(Cucumber)

673

674 **Fig. 4** Specific activation of the *B. cinerea Bccpdh* promoter by capsidiol and its
675 derivative.
676 **(A)** Mycelia of *B. cinerea* transformant containing *GFP* gene under the control of 1
677 kb *Bccpdh* promoter (P_*Bccpdh:GFP*) was incubated in CM media containing 500
678 μM of anti-microbial terpenoids. Bars = 50 μm. **(B)** (left and middle) Leaves of *N.*
679 *benthamiana* were inoculated with conidia of *B. cinerea* P_*Bccpdh:GFP*
680 transformant. Expression of GFP in germinating conidia on the leaf surface was
681 observed by confocal laser microscopy 2 or 8 h after inoculation (hpi). CW, stained
682 with calcofluor white. Arrowheads indicate the appressoria of *B. cinerea.* Bars =
683 20 μm. (right) Leaves of *N. benthamiana* were inoculated with mycelia of *B. cinerea*
684 P_*Bccpdh:GFP* transformant and the edge of the lesion was observed by confocal
685 laser microscopy 2 d after the inoculation (2 dpi). Bar = 100 μm. **(C)** Leaves of
686 indicated plant were inoculated with mycelia of *B. cinerea* P_*Bccpdh:GFP*
687 transformant and the edge of the lesion was observed by confocal laser
688 microscopy 2 d after the inoculation. Bars = 100 μm.

689

690


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

691 **Fig. 5** *B. cinerea* BcCPDH is essential for the detoxification of capsidiol and
692 virulence in the plant species producing capsidiol.
693 **(A)** Mycelial block (approx. 1 mm^3) of *B. cinerea* wild type (WT) or *Bccpdh* KO
694 mutant strain (*⊿bccpdh*-52) was incubated in 100 μM capsidiol for 4 d. Capsenone
695 and capsidiol was detected by LC/MS. **(B)** Mycelial blocks of *B. cinerea* WT or
696 *⊿bccpdh*-52 were incubated in capsidiol and outgrowth of hyphae was measured
697 after 36 h of incubation. Data are mean ± SE (n = 10). Asterisks indicate a
698 significant difference from WT as assessed by two-tailed Student's *t*-test, **P <
699 0.01. **(C)** Leaves, tubers or fruits of indicated plant were inoculated with mycelial
700 block (5 mm^3) of *B. cinerea* WT or *⊿bccpdh*-52 and lesion size was measured
701 between 4 and 7 days after inoculation (dpi). Asterisks indicate a significant
702 difference from WT as assessed by two-tailed Student's *t*-test. **P < 0.01. Lines
703 and crosses (x) in the columns indicate the median and mean values, respectively.

704

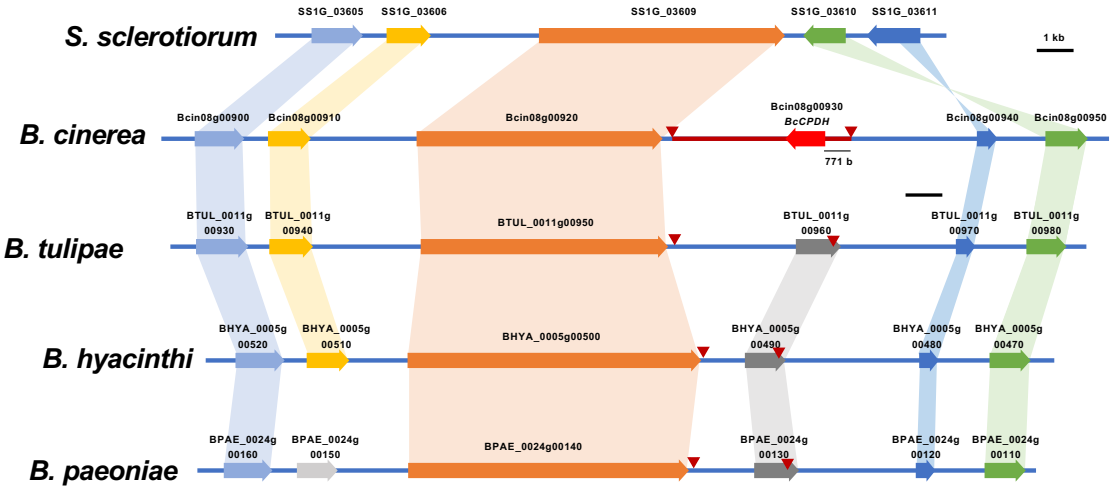


Fig. 6 Comparison of the *B. cinerea Bccpdh* gene locus with corresponding genome region of other *Botrytis* species and *Sclerotinia sclerotiorum*. Edge of conserved region among *Botrytis species* and specific region for *B. cinerea* (red line) were indicated by red arrowheads.

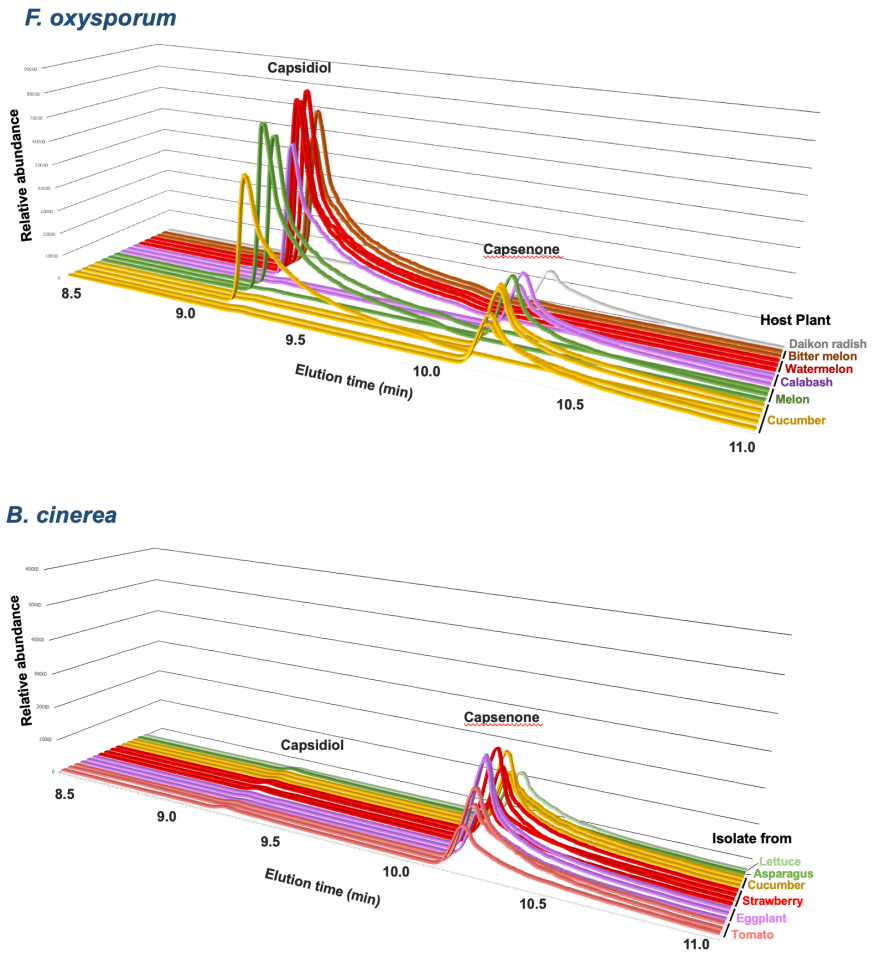


Fig. 7 CPDH activity in *F. oxysporum* and *B. cinerea* strains isolated form a variety of plants.

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.