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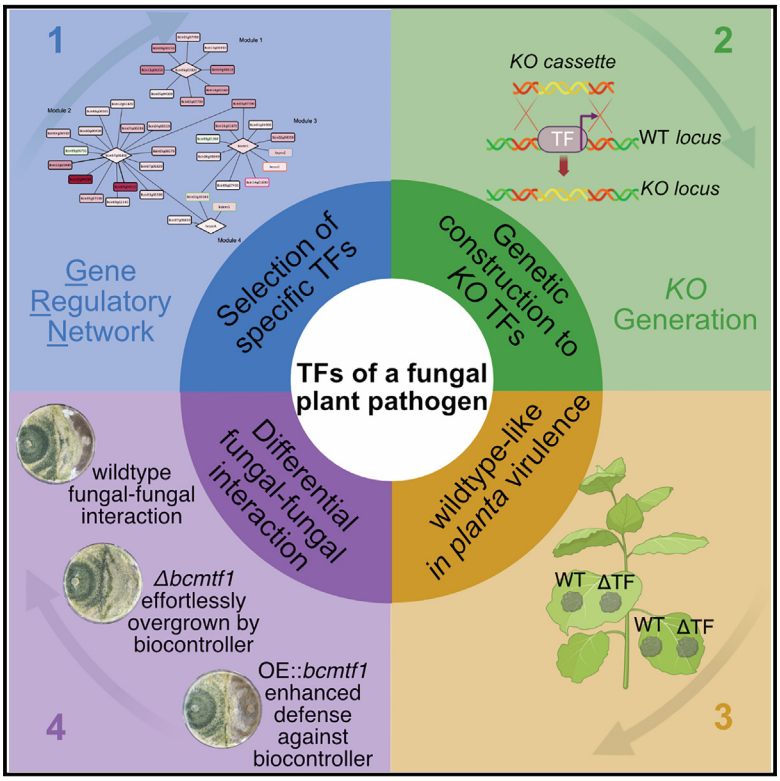
Graphical abstract



Highlights

- Gene regulatory networks identify TFs critical for fungal-fungal defense
- *B. cinerea* Δ*bcmtf1* shows increased sensitivity to biocontrol
- BcMTF1 plays a role in regulating genes essential for fungal defense
- The study of fungal-fungal defense could help in biocontrol design

Authors

Consuelo Olivares-Yañez, Nicolas Arias-Inostroza, Ruben Polanco, Paulo Canessa

Correspondence

paulo.canessa@unab.cl

In brief

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Mycoparasitic transcription factor 1 (BcMTF1) participates in the *Botrytis cinerea* response against *Trichoderma atroviride*

Consuelo Olivares-Yañez,[2,3,4,5] Nicolas Arias-Inostroza,[1,2,5] Ruben Polanco,[1] and Paulo Canessa[1,2,5,6,*]

[1]Centro de Biotecnologia Vegetal, Facultad de Ciencias de la Vida, Universidad Andres Bello, Santiago 8370186, Chile
[2]ANID–Millennium Science Initiative–Millennium Institute for Integrative Biology (iBIO), Santiago 7500565, Chile
[3]Centro de Genómica y Bioinformática, Facultad de Ciencias, Ingeniería y Tecnología, Universidad Mayor, Santiago, Chile
[4]Escuela de Biotecnología, Facultad de Ciencias, Ingeniería y Tecnología, Universidad Mayor, Santiago, Chile
[5]These authors contributed equally
[6]Lead contact
*Correspondence: paulo.canessa@unab.cl
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SUMMARY

Botrytis cinerea is a phytopathogenic fungus. Traditional control using fungicides has faced challenges, prompting the exploration of sustainable alternatives such as biocontrol. *Trichoderma atroviride*, a promising biocontroller, is well-known for its mycoparasitism. However, the molecular processes involved in this fungal-fungal interaction, particularly regarding the defense mechanisms of the pathogen, have yet to be deeply studied. Here, we investigated the transcriptional defense responses of *B. cinerea* to *T. atroviride*. We focused on four *B. cinerea* transcription factors (TFs) differentially expressed during interaction with the biocontroller. Mutants lacking these TFs exhibit increased sensitivity to *T. atroviride*, with the Bcin07g06800 (BcMTF1) loss-of-function mutant being most susceptible. Genes predicted to be regulated by mycoparasitic transcription factor 1 were differentially expressed during this interaction. BcMTF1 influences *B. cinerea*'s resistance to mycoparasitism by regulating the expression of genes potentially involved in fungal defense against *Trichoderma*. The findings provide insights into the transcriptional processes underlying fungal-fungal interactions.

INTRODUCTION

Plants represent a primary source of nutrients on Earth, providing most of the food consumed globally. Among the many dangers faced by these organisms, plant-infecting fungi pose a growing threat, representing a serious risk to global food security.[1,2] To minimize these risks, it is crucial to comprehend not just how plants get infected but also the approaches used by phytopathogens to resist the strategies utilized to limit or reduce plant infections in agricultural environments.

Among fungal phytopathogens, ten fungi have been highlighted for greater awareness[3] as they infect major food commodities, with *Botrytis cinerea* being the most prominent necrotroph. With a pathogenic toolset that includes several virulence factors (reviewed by [3–10]), the most comprehensively studied fungal necrotrophic phytopathogen is *B. cinerea*,[9] a widespread microorganism infecting over 1,000 plant species that cause massive global economic losses estimated at 10–100 billion Euros. Beyond this direct economic impact, *B. cinerea* is also associated with significant indirect costs related to the use of chemical fungicides needed for its control in productive environments.[11] The emergence of fungicide-resistant strains has granted the need to develop new fungicides or formulations, often combining two or more active compounds, which is costly and time-consuming. Moreover, the fungicide repertoire is shrinking, making it increasingly challenging to manage resistant strains effectively.[12–15] The environmental impact of fungicide use is also a concern, as chemical runoff into the soil and water bodies leads to pollution. Consequently, there is a pressing need to reduce the use of chemicals in agriculture to mitigate these adverse effects. One promising alternative or additional tool is the use of biocontroller organisms.

The biological control of plant diseases exerted by various organisms, known as phytopathogen biocontrol, has a long history in scientific research. For over 90 years,[16] *Trichoderma* species have been extensively studied to understand how they control plant disease-causing microorganisms.[17] Indeed, *Trichoderma* has recently been highlighted as a fungal genus with the most significant biocontrol potential.[18] However, most of what we know about the interspecific interaction between *Trichoderma* spp. and plant pathogens, including fungi such as *B. cinerea*, has been historically and systematically investigated to understand the strategies and mechanisms that enable phytopathogen control.[19] In contrast, the anticipated and intriguing defense mechanisms employed by phytopathogens like *B. cinerea* and others under biocontrol attack are largely

unknown, likely because plant pathogens have primarily been studied to comprehend virulence and infection mechanisms.

Mycoparasitism and biological effects exerted by *Trichoderma* spp. promote positive growth in plant seedlings and enhance plant nutrient uptake. Additionally, *Trichoderma* spp. can induce or foster plant defense responses. The biocontrol capacities have been explained, at least in part, by nutrient and space competition, lytic enzyme secretion, and the production of secondary metabolites (SMs) that act as toxins against phytopathogens. However, these fungi are also known for their ability to colonize plant roots forming symbiotic relationships that improve plant resilience to stress.[20,21] Furthermore, *Trichoderma* spp. can modulate the hormonal balance in plants, leading to enhanced growth and resistance to several pathogens.[22] This multifaceted approach makes *Trichoderma* spp. a powerful tool in sustainable agriculture.

Trichoderma spp. is a natural antagonist of *B. cinerea* and other phytopathogens. To survive, *B. cinerea* must protect itself against other organisms rather than taking the offensive stance observed during plant infection. Besides generalized biological strategies that include dormancy, genetic variation, as well as space and nutrient competition, some additional, though limited specific scenarios have been described. For instance, *B. cinerea* is well known for its ample SM production, many of which have antimicrobial properties. For example, in the SM dialogue between *Trichoderma arundinaceum* and *B. cinerea*, the sesquiterpene botrydial (BOT) reduces the *T. arundinaceum* response to the phytopathogen.[23] Similarly, *Trichoderma afroharzianum* displays oxalic acid degradation capacities. This organic acid promotes *B. cinerea* plant colonization by lowering the environment's pH (indeed, it is generally recognized as a virulence-related SM), also creating unfavorable conditions for the growth of competing microorganisms.[24] Interestingly, the KP4 family of toxic proteins in the fungal plant pathogen *Fusarium graminearum* also seems to have a dual role, modulating plant infection and interspecific fungal-fungal interactions against *Trichoderma gamsii,* facilitating antifungal compounds delivery into fungal cells.[25] As recently highlighted when employing *B. cinerea*, different fungal mechanisms allow diverse strategies for tolerating toxic molecules in fungi, though the transcriptional mechanisms behind toxicity tolerance are far from being completely understood.[26]

In our recent study,[19] we used a systems biology approach to understand the complex molecular dialogue between the phytopathogen *B. cinerea* and the biocontroller *Trichoderma atroviride*, two fungal models of agronomic relevance. In this approach, we thoroughly annotated the transcription factor (TF) repertoires of both fungal species. This served as the foundation for the construction of two reference Gene Regulatory Networks (GRNs): one for each fungus. GRNs are powerful tools that provide comprehensive graphical representations of regulatory interconnections between TFs and target genes.[27] The reference GRNs for *B. cinerea* and *T. atroviride* were created by combining predicted *cis*-regulatory elements in the promoter regions scanned across the genomes of both fungal species and genome-wide transcriptomic data obtained from single and dual RNA-sequencing studies, including confrontation experiments between both fungi. This resource, which we have made freely available to both scientific communities[19] has broadened our understanding of *Trichoderma* mycoparasitism and paved the way for the formulation of (multiple) testable hypotheses and an improved understanding of gene regulation, including the putative transcriptional defense mechanisms of *B. cinerea* when acting as prey during *Trichoderma* mycoparasitism.

Here, to test some of these GRN-rooted hypotheses, we generated four *B. cinerea* TF loss-of-function mutants. The data presented here demonstrate that the absence of these four TFs renders *B. cinerea* more vulnerable to mycoparasitism. This heightened susceptibility can be partly due to its reduced capacity to withstand toxic molecules. Moreover, we identified a specific TF, Bcin07g06800, which significantly influence *B. cinerea*'s ability to resist mycoparasitism upon interaction with *Trichoderma*. We have named this regulatory protein the mycoparasitic transcription factor 1 (BcMTF1).

RESULTS

B. cinerea transcription factor BcMTF1 predominantly governs the interaction with the mycoparasitic fungus and biocontroller *T. atroviride*

Employing a systems biology strategy based on the earlier construction of a reference GRN,[19] we resolved to experimentally evaluate the roles of four differentially expressed TFs in *B. cinerea* during its early (72 hpi) physical interaction with *T. atroviride*. A schematic representation of all TF proteins is depicted in Figure 1. It shows their respective DNA-binding domains, fungal-specific TF domains, and nuclear localization signal (NLS), as predicted using InterProScan[28] and DeepLoc.[29] According to the mentioned GRN (Figure S1), and judging by the predicted function of most target genes,[19] *B. cinerea* TF loss-of-function mutants are expected to have a reduced ability to withstand *T. atroviride* attack so that the outcome of this fungal-fungal interaction should favor mycoparasitism. Therefore, to test this hypothesis, the loss-of-function mutants of Bcin01g10810 (BcCPCA), Bcin02g08720 (BcSMR1), Bcin06g03820, and Bcin07g06800 (termed herein BcMTF1) were generated as indicated in the methods. Succinctly, each TF open reading frame (ORF) was replaced by homologous recombination using the hygromycin (*hph*) gene. The complete loss of each TF-encoding gene was assessed using PCR (Figures S2–S5) and qPCR (see STAR Methods).

First, we evaluated the growth rate of each ΔTF mutant. Figure S6 shows that no apparent macroscopic alterations were observed during fungal development. The growth determined among the four ΔTF mutants was highly similar, showing an equivalent growth rate as that of B05.10. Notably, only the absence of *bccpcA* leads to a slightly slower growth compared with the *B. cinerea wild-type* strain or the remaining ΔTF mutants (Figure S6).

Furthermore, homokaryotic derivatives were encountered with *wild-type T. atroviride* in confrontation assays to evaluate the behavior of each ΔTF mutant when exposed to the mycoparasitic fungus. We acquired photographs at 4-, 7-, 10-, and 14-day post-inoculation (dpi) to evaluate the fungal-fungal interaction and determined the growth of both fungal species and *T. atroviride* overgrowth. Figure S7 shows that all strains displayed similar behavior and growth phenotypes at 4 dpi, and

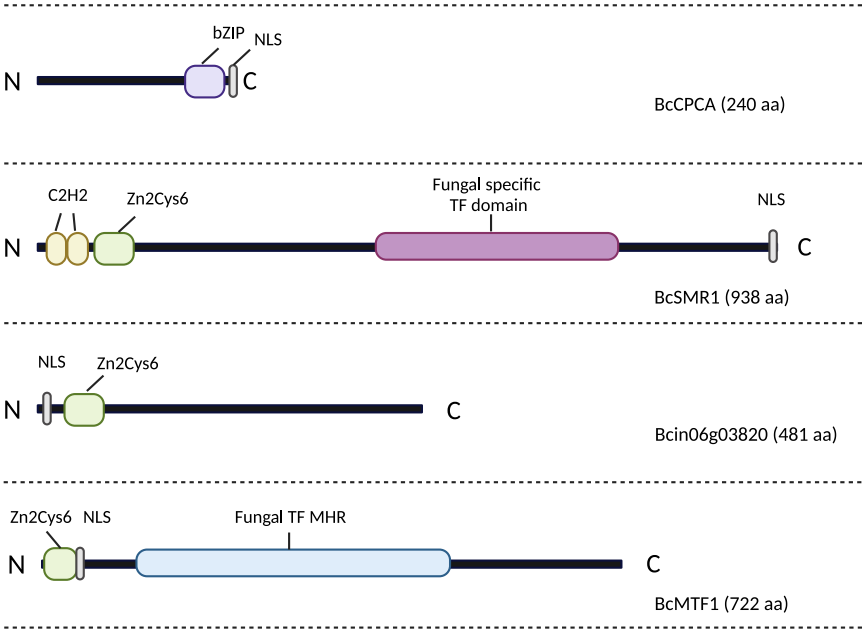

Figure 1. Schematic representation of the main domains identified in each TF under analysis

DNA-binding domains (bZIP, C2H2, and Zn2Cys6), as well as fungal-specific TF domains and nuclear localization signal (NLS, in gray), are indicated. BcCPCA contains one bZIP domain at the C-terminal half of the protein (196-230aa), while BcSMR1 contains two C2H2 domains between aa 5–32 and 33–61, followed by a Zn2Cys6 domain (74-119aa). Bcin06g03820 contains a Zn2Cys6 domain (43-87aa). BcMTF1 also presents a Zn2Cys6 domain (1-47aa). InterPro IDs, bZIP: IPR004827 (purple); Zn2Cys6: IPR001138 (green); Fungal specific TF domain: IPR007219 (lavender); Fungal specific TF MHR domain: CD12148 (light blue). TF domains and NLS were determined as indicated in methods.

no significant differences in growth or overgrowth were observed. However, on subsequent days, a distinct scenario was noted. Figure 2 shows that all the ΔTF mutant strains were more sensitive to the attack of *T. atroviride* from day 7 onward compared with B05.10. Among all ΔTF mutants, the Δ*bcmtf1* showed the highest sensitivity to *T. atroviride*, which was significantly noticeable as early as 7 dpi (Figure 2B), a period after which Δ*bcmtf1* was almost entirely overgrown by the biocontroller (the white mantle of the mycelium covered almost the entire *B. cinerea* colony). Notably, this observation was comparable with the overgrowth observed in the other three mutants by day 10. Furthermore, by 14 dpi, Δ*bcmtf1* appeared even more susceptible to *T. atroviride* than B05.10. Conversely, Δ*bccpcA*, Δ*bcsmr1*, and ΔBcin06g03820 exhibited similar behaviors at 7 and 10 dpi, displaying greater susceptibility to *T. atroviride* attack than B05.10. After 14 dpi, all ΔTF mutants were overgrown by *T. atroviride*, whereas the B05.10 strain was not entirely mycoparasitized.

Finally, considering that Δ*bcmtf1* showed the most noticeable effect, we also tested whether this mutant is impaired in its ability to counteract the attack of other relevant fungi, including the biocontrollers *Clonostachys rosea*[30] and *Chaetomium globosum*.[31] As observed for *T. atroviride,* Figure S8 shows that Δ*bcmtf1* is more sensitive to the *C. rosea* and *C. globosum* attack.

Plant infection appears unaffected by the absence of all transcription factors under analysis

If the *B. cinerea* TFs identified in response to *T. atroviride* mycoparasitism are specific to this response (fungal-fungal interaction), we do not expect an alteration in plant virulence, the most studied *B. cinerea* interorganismal interaction. *P. vulgaris* primary leaves were inoculated with loss-of-function mutants lacking each TF. Among these regulatory molecules, BcCPCA is particularly intriguing because *Aspergillus fumigatus* strains lacking CpcA have shown reduced virulence in a murine model.[32] Figure 3 shows that after 72 hpi, neither Δ*bccpcA* nor the remaining ΔTF mutants displayed reduced virulence, showing no significant differences with the lesion caused by B05.10. Therefore, this observation indicates that these TFs do not significantly affect fungal virulence, as measured in bean leaves, reinforcing the notion of their participation in regulating relevant biological processes associated with fungal-fungal interactions.

Finally, since both fungal-fungal and fungal-plant interactions involve a stressful environment (e.g., reactive oxygen species), we also decided to test whether *Δbcmtf1* has a disruption in its ability to cope with common fungal stressor agents. We selected *Δbcmtf1* since it displays the strongest phenotype in the fungal-fungal interaction. As shown in Figure S9, none of the chosen stressors —NaCl, sorbitol, and the cell wall-perturbing agents calcofluor white and Congo red— had a significant detrimental effect on *Δbcmtf1* compared to B05.10. This result suggests that BcMTF1 is not involved in regulating a basal stress response.

Bcin01g10810 (*bccpcA*) loss-of-function mutant in *B. cinerea* is slightly more sensitive to fungicides with diverse modes of action

The ORF of Bcin01g10810 (*bccpcA*) comprises 723 base pairs (bp). It is interrupted by a single intron of 60 bp situated near the 5′-end of the gene. *bccpcA* encodes a TF comprising 240 amino acids (aa) with a basic leucine zipper (bZIP) domain (Figure 1). The bZIP DNA-binding domain (DBD) is positioned in the C-terminal half of the protein between aa 196 and 230. Based on the DeepLoc 2.0 software,[29] the polypeptide is predicted to localize in the nucleus and possesses an NLS characteristic of eukaryotic proteins (probability of 0.8197; Figure 1). BLASTp analyses indicated that the BcCPCA protein shares an overall aa identity of 49.1% with the bZIP TF CpcA of *A. fumigatus* (gene ID AFUA_4G12470) and 50.0% with the GCN4 protein of *S. cerevisiae.* In *Aspergillus niger*, CpcA has been primarily associated with cross-pathway (control) regulation, hence the name.

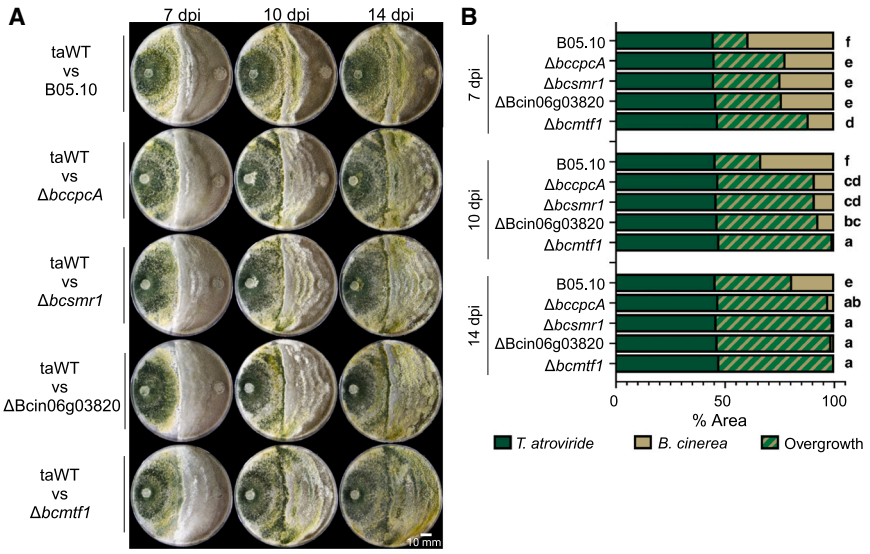

Figure 2. Time-course progression of the confrontation assay between each *B. cinerea* TF mutant and the wild-type *T. atroviride* strain

(A) Confrontation assays were conducted in 90 × 16 mm Petri dishes containing PDA as indicated in the Methods. A control *wild-type/wild-type* interaction (taWT vs. B05.10) is depicted at the top of the Figure. A representative picture was selected for each *B. cinerea* loss-of-function mutant. Each column shows the days elapsed post-inoculation (7, 10, and 14 dpi), whereas rows specify the confronted strains. Both fungi were inoculated as agar plugs, with *T. atroviride* placed on the left side of the plate and *B. cinerea* on the right. Scale bar (bottom right corner) represents 10 mm.

(B) Quantification of the growth area for the phytopathogenic fungus, the biocontroller, and the observed overgrowth of the latter as depicted in (a). Distinct letters denote statistically significant differences ($p < 0.05$).

This TF responds to aa starvation by coordinating a transcriptional response that derepresses genes involved in aa and nucleotide biosynthesis processes.[33] Its most extensively studied ortholog is the yeast GCN4 protein, which is a well-known activator of aa biosynthesis upon depletion.[34]

Notably, genome-wide expression analysis of *A. fumigatus* has unveiled that CpcA is a fungicide-induced TF-encoding gene.[32] Based on the expected secretion of toxic molecules by *T. atroviride* targeting *B. cinerea* during the fungal-fungal interactions, the Gene Ontology (GO) analysis of our published GRN for BcCPCA (Figure S1) suggests its potential role in controlling transport/export-related processes[19] which may help to counteract toxic effects. Therefore, as we have experimentally validated (Figure 4), the *ΔbccpcA* strain is slightly (but significantly) more sensitive to the fungicides iprodione, benomyl, and boscalid, all of which have diverse modes of action.

Genetic complementation and overexpression of BcMTF1 support its involvement in fungal-fungal interactions

Notably, only one of the four TFs reported herein, sclerotia melanin regulator 1 (BcSMR1), has been previously characterized. As the

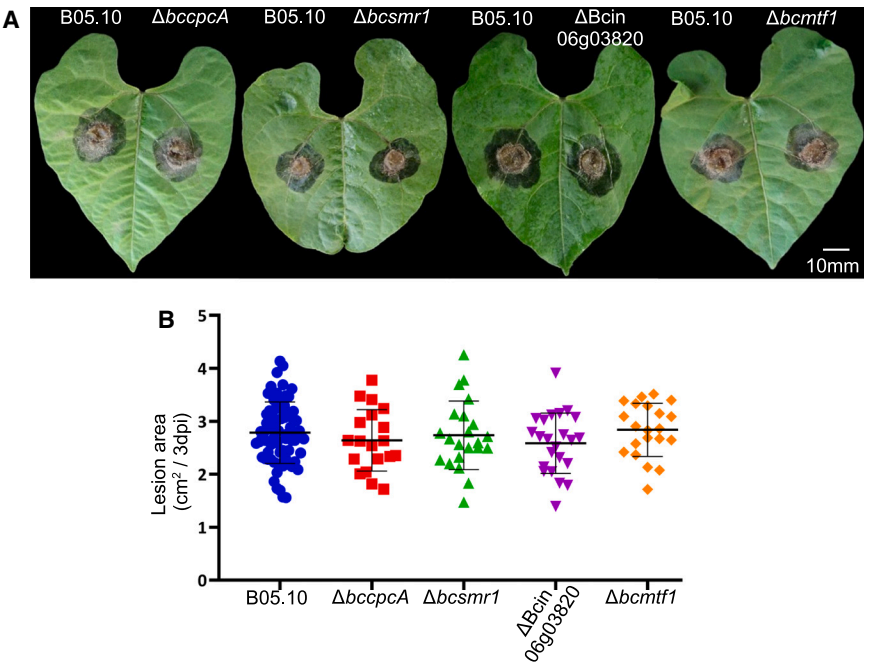

Figure 3. Loss-of-function mutants of each *B. cinerea* TF show no significant lesion variation in their ability to cause infection on *P. vulgaris* leaves

A 6 mm agar plug of each *B. cinerea* strain was inoculated on one-week-old *P. vulgaris* leaves and kept inside plastic boxes at 20°C for 72 h (3 dpi).

(A) Representative images of the virulence assay conducted on bean leaves. *B. cinerea* B05.10 was inoculated on the left section of each leaf, whereas TF mutants were placed on the right, as indicated in each case. Scale bar represents 10 mm.

(B) The necrotic lesion area (mean values ±standard error; black lines) was assessed after 3 dpi. No significant differences were observed.

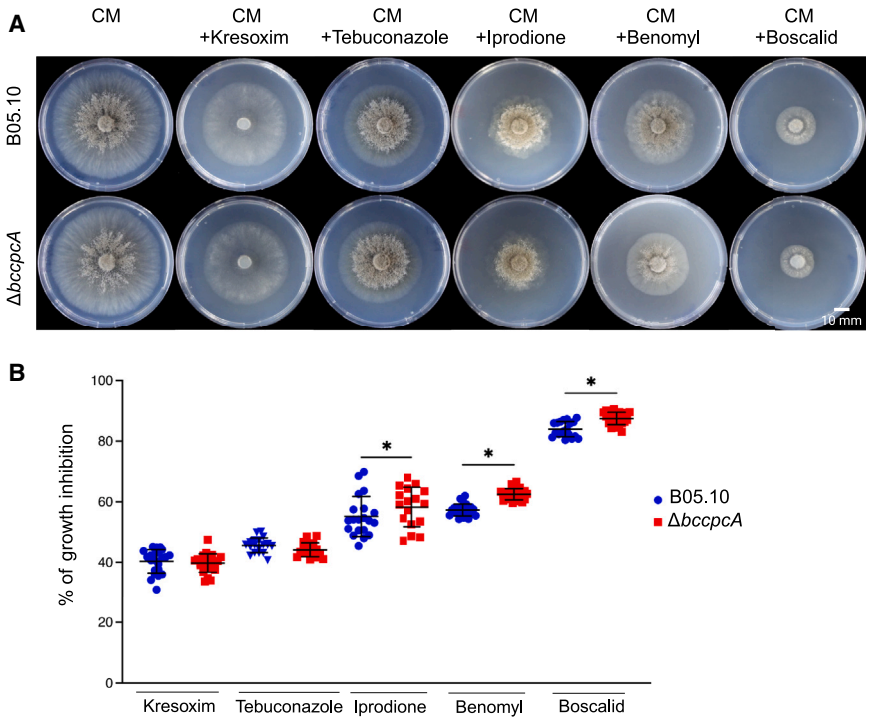

Figure 4. BcCPCA TF loss-of-function mutant results in increased susceptibility to antifungal agents

(A) The B05.10 strain and the *ΔbccpcA* mutant were inoculated in CM medium (control) and CM supplemented with different antifungals as indicated in the Methods. Kresoxim: 20 μM, Tebuconazole: 1.25 μM, Iprodione: 0.25 μM, Benomyl: 250 μM, and Boscalid: 2.5 μM. Representative images are shown. Scale bar (bottom right corner) represents 10 mm.

(B) Quantification of growth inhibition (%) of B05.10 and *ΔbccpcA* recorded after 3 dpi (mean values ±standard error). In the case of Iprodione, Benomyl, and Boscalid, the loss-of-function mutant showed significantly (*$p < 0.05$) higher growth inhibition than that observed for B05.10.

name indicates, it regulates melanin synthesis in the highly melanized sclerotia of *B. cinerea*, which serves as a resilient fungal overwintering structure.[35] According to our previously published data,[19] the target genes of BcSMR1 are functionally associated with stress and cellular detoxification (Table S1). Therefore, it is expected that the absence of BcSMR1 favors the mycoparasite in the fungal-fungal interactions (Figure 2).

However, in contrast to BcSMR1 and BcCPCA, our understanding of the Zn2Cys6 fungal-type TFs encoded by Bcin06g03820 and BcMFT1 (Figure 1) remains very limited. According to OrthoDB, both are found in several fungal species. The Bcin06g03820 gene encodes a 481 aa TF, whose DBD is located in the N-terminal half of the protein. According to DeepLoc and OrthoDB, Bcin06g03820 possesses an NLS (probability of 0.7495) with functional categories associated with energy production and conversion, aa transport and metabolism, and carbohydrate transport and metabolism. Conversely, BcMTF1 is functionally linked to inorganic ion transport and metabolism and SM biosynthesis, transport, and catabolism. With a DBD located at the N-terminus, this 722 aa regulatory protein displays an NLS with a DeepLoc probability score of 0.8559. InterPro analysis also unveiled the presence of a Fungal TF regulatory domain MHR (middle homology region; Figure 1) and that this TF belongs to the protease transcriptional activator prtT family (IPR051089).

Based on the profound sensitivity of the *Δbcmtf1* to *T. atroviride* (Figure 2) and its pivotal role as the most interconnected TF in our predictive GRN (Figure S1), we deemed it crucial to delve deeper into the involvement of this TF on *B. cinerea's* response to the biocontroller. Therefore, mutants with disrupted BcMTF1 function were subjected to genetic complementation. For this purpose, an arbitrarily selected *Δbcmtf1* mutant, exhibiting a single integration of the knockout *cassette*, was selected for genetic complementation, validating the confrontation phenotypes associated with the deletion of the TF-encoding gene (Figure 5). The *bcmtf1* gene was directed to the *wild-type locus* under the control of its endogenous promoter (Figure S10) using the standard *B. cinerea* transformation procedure. This resulted in a hygromycin-sensitive and fenhexamid-resistant complemented mutant strain, designated as *Δbcmtf1+bcmtf1*. Homologous recombination of the complemented mutant was verified using PCR (Figure S10). Figure 5 shows that the genetic complementation of BcMTF1 partially reversed the *B. cinerea*'s sensitivity to *T. atroviride* mycoparasitism. Finally, the B05.10 strain was subjected to the overexpression of *bcmtf1* to further prove the function of BcMTF1 in fungal-fungal interactions. In this case, *bcmtf1* targeted the *ku70 locus*[5] under the control of the actin promoter, showing a significant overexpression compared to the endogenous gene (Figure S11). The resulting mutant, termed OE::*bcmtf1*, leads to the expected opposite phenotype compared with *Δbcmtf1*, showing increased resistance to *T. atroviride*, which was observed as early as 7 dpi (Figure 5). Notably, OE::*bcmtf1* was more resistant to *T. atroviride* mycoparasitism compared with B05.10.

Most genes predicted to be regulated by BcMTF1 show differential expression in the TF loss-of-function mutant

According to the prediction provided by the interaction-specific GRN published previously,[19] BcMTF1 regulates the expression of 16 genes (Table S1). Published RNA-sequencing data[19] indicates that almost all these genes were upregulated in *B. cinerea* after 72 h of its interaction with *T. atroviride* (Figure S1). A GO analysis did not reveal any enriched functional categories among the putative targets of BcMTF1.[19] However, further protein sequence analysis using UniProt revealed that two of these targets (Bcin07g04510 and Bcin12g04330) likely encode peptidases. Another gene (Bcin03g00500 or *bcspl1*) has been

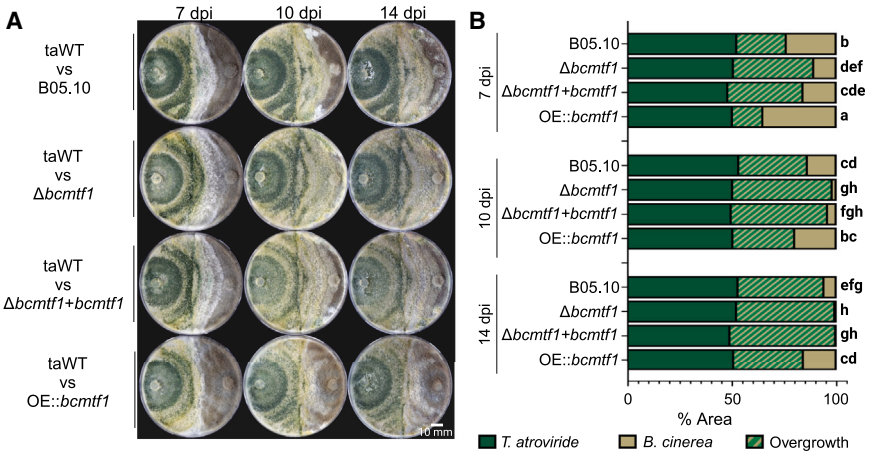

Figure 5. Overexpression of the BcMTF1 TF enhances *B. cinerea's* ability to counteract *T. atroviride* mycoparasitism

(A) Confrontation assays were conducted in 90 × 16 mm Petri dishes containing PDA as indicated in the Methods. A control *wild-type/wild-type* interaction (taWT vs. B05.10) is depicted at the top of the figure. A representative picture was selected for *B. cinerea* loss (*Δbcmtf1*) and gain-of-function mutants (complemented: *Δbcmtf1+bcmtf1,* and overexpression: OE::*bcmtf1*). Each column shows the days elapsed post-inoculation (7, 10, and 14 dpi), whereas rows specify the confronted strains. Both fungi were inoculated as agar plugs, with *T. atroviride* placed on the left side of the plate and *B. cinerea* on the right. Scale bar (bottom right corner) represents 10 mm.

(B) Quantification of the growth area for the phytopathogenic fungus (B05.10, *Δbcmtf1*, *Δbcmtf1+bcmtf1*, and OE::*bcmtf1*) and the biocontroller, and the observed overgrowth of the latter as depicted in (a). Distinct letters denote statistically significant differences ($p < 0.05$).

described as a *B. cinerea* virulence factor that induces a strong plant defense response.[36] Therefore, we decided to assess gene expression levels in the *Δbcmtf1* mutant strain during the *B. cinerea-T. atroviride* interaction using RT-qPCR to determine which of these 16 genes are indeed de-regulated in the absence of the TF. For this purpose, confrontation assays were conducted using the *wild-type* B05.10 strain and *Δbcmtf1* with *T. atroviride*, following the procedure outlined in our previous study.[19]

We successfully assessed the expression of 15 of the 16 genes potentially regulated by BcMTF1, observing three gene expression patterns (Figure 6). The expression levels of Bcin04g06500 (predicted target) were extremely low, preventing us from measuring its mRNA levels under our experimental conditions. The first group included the BcMTF1-dependent up-regulated genes, which showed the most drastic changes in expression levels. Notably, the gene IDs Bcin07g04150 and Bcin12g04330 (mentioned above) displayed three orders of magnitude increase in mRNA levels in the *B. cinerea-T.* atroviride interaction but showed no increase when the *Δbcmtf1* genetic background was analyzed (Figure 6A). Their relevance, if any, in fungal-fungal interactions has not yet been evaluated.

The second group of putative targets (Figure 6B) includes those displaying TF-dependent expression, as higher mRNA levels were observed in the *Δbcmtf1* genetic background. No evidence of the involvement of a transcriptional repressor exists; however, judging from the observed expression patterns, we cannot rule out this possibility. Finally, 6 of the 15 evaluated genes belonged to the last group: BcMTF1-independent upregulation (Figure 6C). As we have previously shown,[19] these genes display an increase in their expression levels after 3 dpi when both fungi interact. However, this up-regulation seems not to depend on the BcMTF1 since similar expression levels were determined in the presence or the absence of the TF (bcWT-taWT and *Δbcmtf1*-taWT interactions; Figure 6C, green and yellow bars, respectively). Therefore, these results demonstrate that our GRN has high predictive accuracy: only 40% of the evaluated genes were not effectively regulated by the TF analyzed in detail.

DISCUSSION

B. cinerea has been extensively studied for its ability to infect plants by attacking these organisms using diverse tools.[4] However, the strategies used by *B. cinerea* when it serves as prey in fungal-fungal interactions remain poorly explored. Notably, both scenarios have some similarities, as the production and secretion of SM and hydrolytic enzymes are crucial for attacking organisms. We can also illustrate this observation by considering the production of reactive oxygen species (ROS) during the interaction between *B. cinerea* and the plant and the relationship between *B. cinerea* and *T. atroviride*.[4,37]

Previous studies have indicated that biocontrol is a highly dynamic process that likely results from a combination of different modes of action. For instance, biocontrollers compete for physical space and nutrients, often engaging in a fierce interrelationship. Notably, the biocontrol by *T. atroviride* leads to hyphal penetration. This interaction is accompanied by the secretion of several lytic enzymes and toxic compounds. Many of these mechanisms have been extensively studied and reviewed for *T. atroviride*.[17,18,22,37]

Furthermore, to understand this process, recent studies on *Trichoderma harzianum* have focused on identifying the mycoparasitism-related genes.[38] However, these investigations only considered changes in gene expression in the biocontrol agent, with an experimental design that did not contemplate the response of the prey. In this harsh biological warfare, it is reasonable to imagine that a phytopathogen like *B. cinerea*, when subjected to the biocontrol exerted by *T. atroviride*, might employ "counter maneuvers" that, in part, reflect the diverse strategies and molecular tools to which it is exposed. In this study, using a reverse genetics approach, we provide evidence that specific TFs in *B. cinerea* significantly affect the outcome of fungal-fungal interactions, impeding *B. cinerea*'s ability to withstand *T. atroviride* mycoparasitism but, importantly, not its ability to infect plants (*P. vulgaris*).

Our research primarily delved into the roles of two of the four TFs. Specifically, those encoded by gene IDs Bcin01g10810

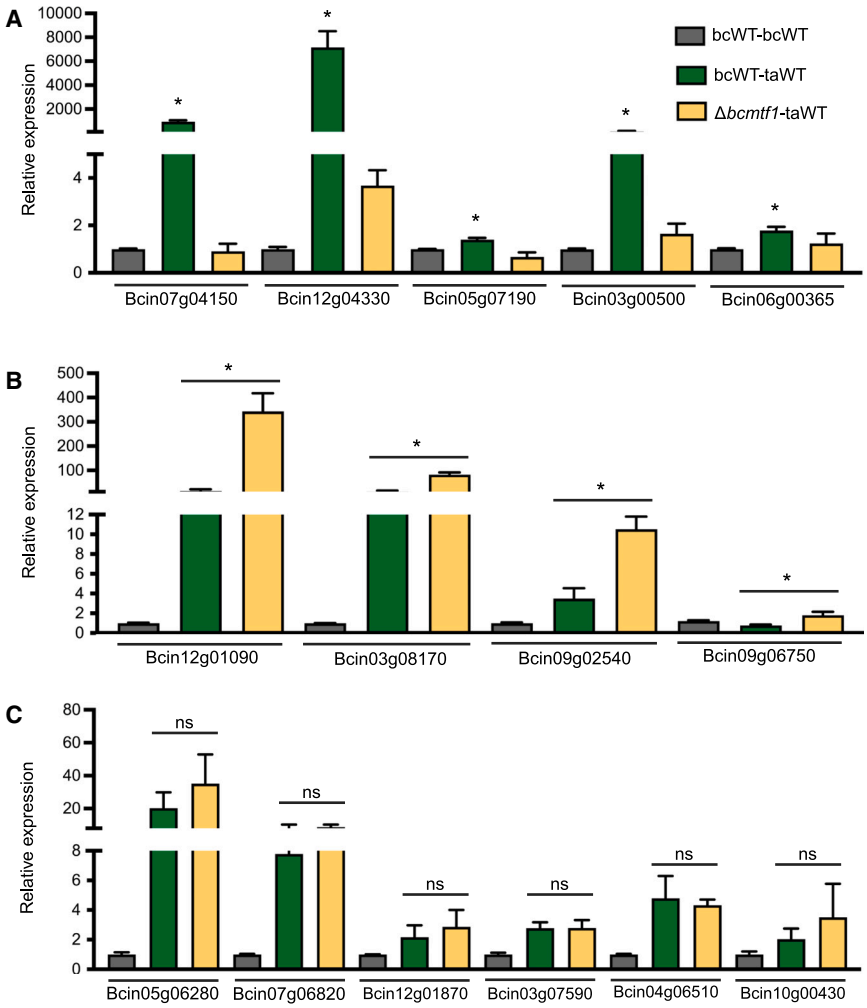

Figure 6. Regulation of gene expression by the BcMTF1

(A–C) The relative expression of each predicted target gene of BcMTF1 was assessed during the interaction of *B. cinerea* B05.10 and *Δbcmtf1* with *T. atroviride* (green and yellow bars, respectively). Control expression levels (set to 1.0) were determined for each gene in the B05.10-B05.10 interaction (gray bars). Three expression trends were observed: (A) BcMTF1-dependent up-regulation, (B) BcMTF1-dependent expression, and (C) BcMTF1-independent up-regulation. Significant differences are indicated with asterisks ($p < 0.05$; ns: not significant). Three biological replicates were performed.

(*bccpcA*) and Bcin07g06800 (*bcmtf1*). Previous studies on CpcA-like TFs in other fungi have primarily focused on the control of metabolic processes,[33,39] and information on how they may influence other functions, including defense against mycoparasitism, remains unclear. In addition, as mentioned above, the ortholog of this gene in *A. fumigatus* encodes a fungicide-induced TF,[32] and our findings indicate that the *ΔbccpcA* strain of *B. cinerea* is slightly more sensitive to different fungicides, including iprodione, benomyl, and boscalid, all of which display diverse modes of action. The target gene of iprodione is *bos1*, a regulator of the fungal osmotic signal transduction pathway.[40] Benomyl targets the tubulin-encoding gene in *B. cinerea*,[41] whereas boscalid's target gene is *sdhB,* a component of the respiration complex II.[42] Therefore, it is relevant to question why the absence of a single gene, in this case, *bccpcA* encoding a TF, has a broad effect on fungicides with such diverse molecular targets as those selected in this work. In *A. fumigatus*, differentially expressed genes upon fungicide exposure included representatives of ATP-binding *cassette* (ABC) multidrug resistance transporters and the major facilitator superfamily (MFS) of transporters. While it remains as a hypothesis, the authors have postulated that CpcA might be involved in the transcriptional regulation of genes related to detoxification, including the transporters mentioned above.[32] In *B. cinerea*, using a systems biology approach constructed on specific global gene expression data, we previously hypothesized that BcCPCA could control gene expression that allows the export of toxic molecules in fungal-fungal interactions.[19] Notably, this appears to be the case, as per our results. However, the practical implications of these findings, particularly regarding fungicide resistance, have not been reported. Consequently, these findings may also affect biocontrol effectiveness as one strategy employed by *Trichoderma* to protect plants is the secretion and utilization of toxic molecules. Therefore, the enhanced control of *T. atroviride* over *B. cinerea* observed for the *ΔbccpcA* strain can be attributed, at least in part, to a decrease in *B. cinerea's* ability to export toxic molecules produced by *Trichoderma*.

A recent report showed that among 69 fungicide-resistant *B. cinerea* isolates from grapes in China, a staggering majority (79.1%) were resistant to five chemical classes of fungicides, including iprodione and boscalid.[13] However, multidrug resistance (MDR) in *B. cinerea* isolates is not a new phenomenon. An intriguing finding, which has been documented for many years in French and German *B. cinerea* isolates, is that MDR in this fungus often appears to be associated with changes in the gene expression levels of multidrug transporters, which has also been observed (and well-documented) in fungi of clinical relevance.[14] Indeed, recent investigations have demonstrated that the Upc2A TF of *Candida glabrata* regulates the expression of a major ABC transporter,[43] explaining MDR. In *B. cinerea*, the insertion of a retroelement-derived sequence into the promoter region of the gene *bcmfsM2* (Bcin15g00270) leads to a significant increase in the mRNA levels of the encoded transporter.[12] Similarly, a gain-of-function mutation in the zinc cluster TF *Mrr1* (Bcin05g01790) results in a constant and high expression of the *atrB*-encoded efflux transporter.[15] Notably, recent

findings indicate that this transporter overexpression-associated fungicide resistance also occurs in other *Botrytis* species but in a mechanism that seems independent of the *Mrr1* TF.[44] Mutations in *bccpcA* linked to increased MDR in "natural" populations have not been documented. However, our results indicate that this TF could also drive MDR phenomena.

In this study, we experimentally evaluated the accuracy of the GRN in representing TF-target interactions during the confrontation between *B. cinerea* and *T. atroviride*. We used RT-qPCR to determine whether the expression of the potential targets of BcMTF1 depended on this regulatory protein. Notably, most predicted targets were deregulated in the absence of this TF, with the genes Bcin07g04510 and Bcin12g04330 likely encoding peptidases exhibiting the most significant changes in gene expression. As per our previous work,[19] we could infer and assign a putative DNA binding motif for *circa* 80% of the TFs encoded in the *B. cinerea* genome. This means that 1 in 5 TFs remain unanalyzed in the GRN simply because we cannot identify their putative *cis*-regulatory elements across the genome. So, we still have a partial overview of the transcriptional regulation that occurs in the fungus-fungus interaction. Furthermore, as our study primarily focused on interactions occurring at a specific time, we anticipate that different stages of interaction might reveal additional dynamics and regulatory TFs that are crucial at earlier or later stages of interaction.

B. cinerea has primarily been analyzed for its plant infection mechanisms. Similar to any microorganism within a diverse community in natural environments, *B. cinerea* must possess various regulatory mechanisms to cope with microbial antagonism,[45] which our study aims to elucidate. *Trichoderma's* mycoparasitism appears to occur in four phases: i) it uses a set of molecular tools, including chemotropism, to locate its prey; ii) it recognizes its potential host; iii) it physically interacts with the host, wrapping around the prey's hyphae; and iv) it penetrates and digests the host.[20] In our study, we did not observe any significant phenotypic or growth differences in any of the four TF mutants at early interaction times (4 dpi), which could be associated with the initial phases of biocontrol. Conversely, fungus-fungus interactions appeared to be accelerated in all TF mutants, particularly in the *Δbcmtf1*. CWDEs play a predominant role in the final phase of mycoparasitism by *Trichoderma*. Extracellular enzymes such as glucanases, proteases, and chitinases are the main enzymes secreted by this fungus to degrade the fungal cell wall of hosts.[37] Chitinases, which degrade the chitin in cell walls, are the most essential lytic enzymes secreted by this fungus. Notably, the deletion of *ech42*, which encodes an (endo)chitinase in *T. harzianum*, significantly reduces its ability to biocontrol *B. cinerea*.[46] However, despite their potential negative effects on protein activity through simple degradation, proteases are also crucial in fungal-fungal interactions, particularly in the mycoparasitic activity of *Trichoderma*. These enzymes influence the activity and stability of diverse extracellular enzymes involved in post-secretion modifications of cellulases,[47] thereby significantly contributing to the biocontrol activity of these fungal species.[48,49] Our results suggest a similar role for the proteases encoded by Bcin07g04150 and Bcin12g04330 in *B. cinerea's* defense against *Trichoderma*, opening up new avenues for further research.

Another gene induced in a BcMTF1-dependent manner is *bcspl1* (Bcin03g00500). BcSPL1 is a low-molecular-weight cerato-platanin family protein that has been described as a virulence factor that facilitates plant infection in *B. cinerea*.[36] Its phytotoxic activity has been described only in plants, triggering a hypersensitive response.[50] It belongs to a group of effector molecules known as cell-death-inducing proteins (CDIPs),[4] all of which are essential modulators of fungal virulence, but seem not active players in fungal-fungal interactions. Nevertheless, it has been described that the cerato-platanin proteins FgCPP1-2 and VdCP1 from *F. graminearum* and *Verticillium dahliae*, respectively, can protect fungal cells from chitinases shielding the cell wall from enzymatic degradation.[51,52] Further investigation is required to test whether this is also the case for BcSPL1 in *B. cinerea*.

In the aggregate, by delving into the molecular mechanisms that underpin the interaction between *B. cinerea* and *T. atroviride*, this research not only provides a framework for understanding how pathogens regulate their response to biocontrol agents but also offers a blueprint that can be extended to other fungal species and biologically meaningful interactions. This broad applicability underscores the potential of this research to reduce our reliance on chemical fungicides, thereby promoting environmentally friendly pest management practices.

Limitations of the study

Several genes controlled by BcMTF1 remain functionally uncharacterized, making it challenging to comprehensively understand their roles in fungal defense against biocontrollers. The latter implies that further unexplored pathways can be involved in the illustrated fungal-fungal interaction. Indeed, as we concentrated mainly on investigating those molecular processes occurring after 72 hpi, we still face a timeframe-restricted overview of the *B. cinerea–T. atroviride* interaction. Thus, earlier or later stages of biocontrol remain obscure, expecting other potential transcriptional dynamics under the control of a different set of TFs. This limited transcriptional network snapshot, though relevant, will be boosted as we complete the entire *B. cinerea* reference GRN; it covers today most, but not all, TF-target relationships. This scenario is further exacerbated if we consider that we have not analyzed the transcriptional response of *B. cinerea* to other biocontrollers, including bacteria. Such analyses could help us uncover potential conserved defense mechanisms as well as specific responses that might exist.

RESOURCE AVAILABILITY

Lead contact

Further requests for information and requests for resources and reagents should be addressed to Prof. Paulo Canessa (paulo.canessa@unab.cl).

Materials availability

Requests for materials should be made via the lead contact. All unique/stable reagents generated in this study are available from the lead contact without restriction.

Data and code availability

- All RNA sequencing data were available at the Sequence Read Archive (SRA) repository. Accession number is listed in the key resources table.
- This paper does not report original code.

- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

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AUTHOR CONTRIBUTIONS

P.C. and C.O.-Y. originated the idea and designed the study. N.A.-I. and C.O.-Y. carried out the research. N.A.-I., R.P., P.C., and C.O.-Y. analyzed, discussed and interpreted the data. P.C. and C.O.-Y. wrote the manuscript. P.C., R.P., and C.O.-Y. provided equipment and funding. All authors have reviewed and approved the final version of this manuscript for publication after providing comments.

DECLARATION OF INTERESTS

The authors declare no competing interests.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
Escherichia coli DH5α	Invitrogen	DH5α - 18265017
Phaseolus vulgaris cv. Venus	INIA-Chile	
Chemicals, peptides, and recombinant proteins		
Potato Dextrose Agar (PDA)	Becton Dickinson	213400
YPD	Becton Dickinson	242820
Yeast Extract	Becton Dickinson	212750
LB-Broth	Becton Dickinson	244620
Malt Extract	Becton Dickinson	218630
Casamino Acids	Becton Dickinson	223050
Polyethylene glycol (PEG) 6000	AppliChem	A1387
Sacarose	Merck	107687
Glucose	Merck	1083371000
Casein peptone	Becton Dickinson	211705
Ampicillin	Winkler	702571
Hygromycin	Invitrogen	10687010
Fenhexamid	Sigma	31713
Lysing enzyme	Sigma	L1412
VinoTaste Pro	Novozymes	VINOTASTEP
Yatalase	TaKaRa	T017
TRIzol	Invitrogen	15596018
TRIS	Merck	9210
$CaCl_2x2H_2O$.	Merck	1023821000
EDTA	Merck	16D185201
Ethyl alcohol	Merck	1085430250
RQ1-DNase	Promega	M610A
Congo Red	VWR	0379
Calcofluor white	Sigma	18909-100ML
Sorbitol	Duchefa	S0807
NaCl	Merck	106404
Deposited data		
SRA data	SRA	PRJNA756518
Critical commercial assays		
iScript cDNA Synthesis Kit	Biorad	1708891
CFX96 Touch Real Time qPCR	Biorad	
SSOAdvance Universal SYBR Green supermix	Biorad	1725272
Culture chamber	Percival	I-30BLL
Phusion HiFi DNA polymerase	ThermoScientific	F548L
Experimental models: Organisms/strains		
T. atroviride WT IMI206040	Sweden	IMI206040
B. cinerea WT B05.10	Germany	B05.10
Saccharomyces cerevisiae	EUROSCARF	BY4741
Clonostachys rosea	Chilean isolates	RP1505
Chaetomium globosum	Chilean isolates	RP2808

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Oligonucleotides		
For all oligonucleotides used for gene manipulation and RT-qPCR	See Tables S2 and S3	
Recombinant DNA		
pCSN44 (plasmid)	Fungal Genetics Stock Center	pCSN44
pRS426 (plasmid)	Fungal Genetics Stock Center	pRS426
pNDF-OCT (plasmid)	Schumacher et al.[53]	pNDF-OCT
pLaS975	This paper	pLaS975
Software and algorithms		
InterPro Scan	https://www.ebi.ac.uk/interpro/	102.0
DeepLoc	Thumuluri et al.[29]	2.1
ImageJ software	Schneider et al.[54]	1.54
R software	https://www.r-project.org/	4.1.0
GraphPad Prism	https://www.graphpad.com/	10.2.3
Cytoscape	https://cytoscape.org/	3.10.3

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Fungal strains and culture conditions

Strain IMI206040 of *T. atroviride* and B05.10 of *B. cinerea* were isolated in Sweden[55] and Germany,[56] respectively. Both fungal strains were routinely kept in Petri plates containing potato dextrose agar (PDA) at 20°C y 12:12 hours Light-Dark (LD) cycle. The same culture conditions were used to maintain *Clonostachys rosea* and *Chaetomium globosum*.

Phaseolus vulgaris culture conditions

For virulence assays, we used *Phaseolus vulgaris* cv. Venus from the Instituto Nacional de Investigaciones Agropecuarias (INIA)-Chile.

METHOD DETAILS

Cloning of gene replacement and complementation cassettes

Using the *in vivo* yeast recombinational cloning (YRC) methodology,[57] four replacement *cassettes* were constructed, one for each TF-encoding gene: *bccpcA* (gene ID Bcin01g10810), *bcsmr1* (Bcin02g08760), Bcin06g03820, and Bcin07g06800 (*bcmtf1*). The 5'- and 3'- noncoding regions of each gene were PCR amplified from the genomic DNA of *B. cinerea* B05.10 using the primers specified in Table S2. The hygromycin (*hph*) resistance *cassette* was PCR amplified from the pCSN44 plasmid.[58] The primers utilized in these PCRs contained, on average, 30-bp 5'-overhang regions to enable *in vivo* homologous YRC. To assemble the final vectors, the PCR fragments were co-transformed with the linearized pRS426 vector[59] into the uracil-auxotrophic *Saccharomyces cerevisiae* strain BY4741.[60] Following YRC, each plasmid containing the desired genetic construct (Figures S2-S5) was obtained from individual yeast colonies by *Escherichia coli* (DH5α) transformation.

In the case of the TF encoded by *bcmtf1*, gene complementation and overexpression (OE) strains were also assembled using YRC. For genetic complementation, the mutant strain of the TF previously generated was transformed with a genetic construction reestablishing the *wild-type bcmtf1 locus*. In this case, the recently described *fenR* (fenhexamid) resistance *cassette* was used as a selection marker (obtained from the pNDF-OCT vector[61]). For OE, first, we generated a backbone vector, which we refer to as pLaS975. This vector was designed to allow homologous recombination into the *ku70 locus* (*ku70* deficiencies do not disturb *in vitro* or *in planta* growth and virulence[62]), possess a *hph* resistance *cassette*, and allows the constitutive expression of a gene of interest (GOI) under the *bcactin* strong promoter and ß-tubulin terminator sequence. For both constructions, the open reading frame (ORF) of the GOI was amplified from genomic DNA using the primer pairs indicated in Table S3. The design and verification of the complementation and OE strains of *bcmtf1* can be found in Figures S10 and S11, respectively. All genetic constructs were sequenced to verify the absence of mutations before *B. cinerea* transformation.

For each TF, conserved protein domains were identified by InterPro Scan[28] (https://www.ebi.ac.uk/interpro/). Putative NLS were predicted with DeepLoc software.[29]

Generation of *B. cinerea* deletion and complemented mutants

Following the assembly of gene replacement and OE genetic constructs, the entire DNA region containing either genetic construct was PCR amplified using the external primers flanking each of them (Tables S2 and S3). For this purpose, we used the Phusion HiFi

DNA polymerase. Four 50 μl PCR products were pulled, purified, and ethanol-concentrated in 30 μl TE Buffer 1X for each *B. cinerea* protoplast transformation. Succinctly, *B. cinerea* protoplasting and transformation were conducted as described,[53] based on the first reported protocol.[63] First, *B. cinerea* conidia were incubated for 18 h at 20°C and 120 rpm in 100 ml of malt extract medium (1.5%). Second, protoplasts were generated from young mycelia using an enzymatic mixture consisting of 100 mg of lysing enzyme, 200 mg of VinoTaste Pro, and 0.5 mg of Yatalase. The resulting protoplasts were mixed with 30 μl of purified PCR products in a polyethylene glycol (PEG) solution (25% PEG 6000, 1 M $CaCl_2$, 1 M Tris-HCl, pH 7.5). Following 20 to 24 h of fungal cell-wall regeneration on SH agar, protoplasts were overlaid with SH agar containing 70 μg/ml hygromycin B or 50μg/ml fenhexamid, as appropriate for each genetic construct. Homokaryotic isolates were achieved after single-spore isolation. PCR confirmed transformants that had experienced the expected homologous recombination. The absence of *wild-type* alleles was confirmed using primers designed to amplify the substituted genomic region. Single integration of each genetic construction was determined through qPCR (see below).

Confrontation assays

To analyze mycoparasitism, confrontation assays were conducted as described.[19] In brief, each fungus was inoculated as a six mm-diameter mycelial agar plug placed on opposite sides of a PDA-containing Petri dish. Control plates were inoculated only with one fungal species. The cultures were incubated for the indicated days in each experiment (see below) at 20°C under 12h-12h light-dark cycle (LD) conditions within temperature-controlled incubators (Percival Scientific).

Growth rate, fungicide and stress assays

To determine if the deletion of each TF affects the growth of *B. cinerea*, a six-mm-diameter agar plug of each mutant strain was inoculated on PDA plates and kept in LD at 20°C for 96 h. The growth front of each strain was registered every 24 h. After incubation, a digital image was recorded, and the growth area was calculated using the ImageJ software.[54]

The effect of the different fungicides on *B. cinerea* growth was evaluated by supplementing the Complete Medium (CM) with different concentrations of each fungicide as indicated previously.[64–66] CM medium was used since it is a rich yet defined medium (1% glucose, 0.5% casein peptone, 0.25% casaminoacids, 0.25% yeast extract, 5% salt solution, 0.1% vitamin solution, 0.2% microelement solution, and 1.5% agar, pH 5.0). The final concentration of each fungicide follows: Kresoxim, 20μM; Tebuconazole, 1.25μM; Iprodione, 0.25μM; Benomyl, 250μM; and Boscalid: 2.5μM. Plates not supplemented with the different fungicides were used as controls. A six-mm-diameter agar plug of each strain was inoculated in the CM media with or without the respective fungicide (at 20°C for 72 h). After this period, images were recorded, and the area of growth was calculated using ImageJ.

To determine if the deletion of BcMTF1 implies a decrease in the tolerance to different types of stresses that could result in a more susceptible phenotype in the confrontation of this strain with *Trichoderma*, the effect of different stressors on its growth was evaluated and compared with that of B05.10. This was carried out by supplementing CM with different concentrations of the following stressor agents: Congo red (430 μM), Calcofluor white (65.4 μM), sorbitol (2M) and NaCl (0.8M). Not supplemented plates were used as controls. A 6 mm diameter agar plug of each strain was inoculated onto CM medium with or without the respective stressor (at 20°C for 72 h). After this period, images were recorded, and the area of growth was calculated using ImageJ.

Virulence assays

Virulence assays of B05.10 and ΔTF strains were conducted as described with slight modifications.[67] Briefly, primary leaves of *circa* 1-week-old *Phaseolus vulgaris* cv. Venus were inoculated with a 6 mm-diameter agar plug of each fungal strain previously grown one week on PDA medium supplemented with bean leaves. To analyze virulence, after inoculation, plants were kept inside a plastic box at room temperature for 72 h. The obtained lesions were recorded with a digital camera, and their area was calculated as described.[54]

RNA extraction, cDNA synthesis, and real-time RT-qPCR

RNA from the interacting mycelia between *T. atroviride* and *B. cinerea* B05.10 or *Δbcmtf1* was collected 3 dpi. RNA was then extracted using TRIzol as described.[19] Total RNA was treated with DNAse according to the manufacturer's specifications. Thereafter, 1μg of DNAse-treated total RNA was used to generate cDNA using the Biorad iScript cDNA Synthesis Kit. The assay efficiency of each set of primers was determined using serial dilutions of a purified qPCR product employing the qPCR Software CFX maestro (Bio-Rad). A total of 3μl of each cDNA dilution was used as a template for a standard qPCR reaction using SSOAdvance Universal SYBR Green supermix. Primer specificity was evaluated through melting curves and agarose gel analysis. Information on the qPCR primers and cycling conditions can be found in Tables S4 and S5. To assess the overexpression levels of *bcmtf1* in the OE::*bcmtf1* mutant, two sets of primers were designed to distinguish the endogenous copy of the gene from the overexpressed one (Table S4).

To determine the single integration of the *hph cassette* in the creation of loss-of-function mutants, we employed qPCR as outlined by Vasquez-Montaño et al., 2020.[67] In this process, we determined the relationship between *hph* and a single-copy gene in the *B. cinerea* genome (*bcfrq1*). We selected transformants with a *hph:bcfrq1* ratio close to 1. Details about the qPCR primers and qPCR amplicons used can be found in Table S6.

QUANTIFICATION AND STATISTICAL ANALYSIS

All statistical analyses were performed in the R environment for statistical analysis. Shapiro-Wilk normality and Levene tests were employed to assess whether normal distribution and homoscedasticity of data fulfilled statistical analysis criteria. A one-way analysis of variance (ANOVA), with Tukey's post hoc test, was used to evaluate differences in lesion area (virulence assays), strain growth, and growth inhibition assays against antifungals or fungal stressors. A Kruskall-Wallis Rank Sum test with Dunn's post hoc test was performed to evaluate differences in the relative expression of different genes (determined by RT-qPCR). As for the overgrowth assays between the strains of *B*. *cinerea* and *T*. *atroviride* under analysis, *C*. *rosea*, and *C*. *globosum*, a two-way ANOVA analysis was performed to evaluate the interaction between the different strains and days post-inoculation. All statistical analyses were assessed with a significance value of $p < 0.05$.