The grey mould Botrytis cinerea employs multiple mechanisms to protect itself from antifungal plant metabolites

Jan van Kan (jan.vankan@wur.nl)
Wageningen University https://orcid.org/0000-0003-3563-1550
Yaohua You
Wageningen University
HM Suraj
Wageningen University https://orcid.org/0000-0002-5936-1420
Andrea Lorena Herrera Valderrama
Wageningen University
Paul Ruigrok
Wageningen University
Xiaoqian Shi-Kunne
Wageningen University
Frank Pieterse
Wageningen University
Henriek Beenen
Wageningen University
Edgar Chavarro-Carrero
Wageningen University
Si Qin
Wageningen University
Francel Verstappen
Wageningen University
Iris Kappers
Wageningen University
Linda Matz
Technische Universität, Institut fur Genetik
Andre Fleissner
Technische Universität, Institut fur Genetik

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The grey mould *Botrytis cinerea* employs multiple mechanisms to protect itself from antifungal plant metabolites

Yaohua You[1], HM Suraj[1,3], Linda Matz[3], A. Lorena Herrera Valderrama[1], Paul Ruigrok[1], Xiaoqian Shi-Kunne[1], Frank P.J. Pieterse[1], Henriek G. Beenen[1], Edgar A. Chavarro-Carrero[1], Si Qin[1], Francel W.A. Verstappen[2], Iris F. Kappers[2], André Fleißner[3], Jan A.L. van Kan[1]

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

[2] Wageningen University, Laboratory of Plant Physiology, Wageningen, The Netherlands

[3] Technische Universität Braunschweig, Institut für Genetik, Braunschweig, Germany

Abstract

Saponins are plant secondary metabolites comprising glycosylated triterpenoids, steroids or steroidal alkaloids with a broad spectrum of toxicity to microbial pathogens and pest organisms that contribute to basal plant defence to biotic attack. Secretion of glycosyl hydrolases that enzymatically convert saponins into less toxic products was thus far the only mechanism reported to enable fungal pathogens to colonize their saponin-containing host plant(s). We studied the mechanisms that the fungus *Botrytis cinerea* utilizes to be tolerant to well-characterized, structurally related saponins from tomato and *Digitalis purpurea.* By gene expression studies, comparative genomics, enzyme assays and testing a large panel of fungal (knockout and complemented) mutants, we unraveled four distinct cellular mechanisms that participate in mitigation of the toxic activity of these saponins. The enzymatic deglycosylation is novel and unique to this fungus-saponin combination, while the genes involved in other tolerance mechanisms have orthologs that are widely distributed in the fungal kingdom. We present a spatial and temporal model on how these mechanisms jointly confer tolerance to α- tomatine and we discuss the repercussions for other fungi and different saponins.

Keywords

Alkaloid; deglycosylation; membrane repair; plant defense; saponin.

Introduction

Plant defense reactions against microbial pathogens commonly involve the production of chemical defense compounds. This chemical armory involves constitutively produced phytoanticipins and pathogen-induced phytoalexins. A common cellular target of various defense substances from both groups is the pathogen plasma membrane, such that the presence of the plant defense compound destroys the integrity of this cellular barrier, resulting in rapid cell death. As part of the arms race between plants and pathogens, microorganisms have evolved resistance mechanisms against these plant defense compounds, which in some cases also confer resistance against (multiple) synthetic antimicrobials, such as fungicides or clinical drugs. An important category of membrane-targeting phytoanticipins are the saponins, a large group of secondary metabolites comprising glycosylated triterpenoids, steroids or steroidal alkaloids that are widely distributed in the plant kingdom (Osbourn, 1996; Piasecka et al., 2015). They are considered as basal defense compounds in plants because of their toxicity to a wide range of plant pathogens and pests including fungi, bacteria, oomycetes, nematodes and herbivorous insects (Akinpelu et al., 2014; Augustin et al., 2012; D'Addabbo et al., 2011; Hussain et al., 2019; Osbourn, 1996; Potter and Kimmerer, 1989). The importance of saponins in plant defense was illustrated by the observation that saponin-deficient (*sad*) mutants of the wild oat species *Avena strigosa* were significantly compromised in resistance against a variety of fungal pathogens (Papadopoulou et al., 1999). The growth inhibition of fungi by saponins is achieved through membrane disruption following their binding to sterols (Sandrock and VanEtten, 1998). This mode of action was demonstrated in the interactions between different saponins with natural and artificial membrane preparations (Armah et al., 1999; Claereboudt et al., 2018; Osbourn et al., 1996; Sudji et al., 2015). So far, the only known fungal resistance mechanism against saponins is their enzymatic detoxification. To detoxify saponins, fungi can secrete diverse glycosyl hydrolases (GHs) that convert saponins into less toxic products via hydrolytic deglycosylation (Osbourn, 1996; Westrick et al., 2021). The detoxification of saponins mediated by GHs is important for the capacity of fungal pathogens to infect host plants containing saponins. The ability of different isolates of *Septoria avenae* to degrade the oat leaf saponins 26-desglucoavenacosides A and B positively correlated with their pathogenicity (Wubben et al., 1996). The avenacinase activity in the fungus *Gaeumannomyces graminis* var. *avenae* that mediates hydrolysis of the oat root saponin avenacin A-1 is a ß-glucosidase (Bowyer et al., 1995) that was later assigned to the CAZyme family GH3. Avenacinase-deficient mutants of *G. graminis* var. *avenae* exhibited increased *in vitro* sensitivity to avenacin A-1 and lost the ability to infect oat while retaining pathogenicity on wheat which does not accumulate avenacin A-1 (Bowyer et al., 1995). A survey of 161 fungal isolates sampled from roots of field-grown oat and wheat plants for their sensitivity to avenacin A-1 indicated that

nearly all isolates sampled from oat where resistant to avenacin, while the isolates sampled from wheat comprised both avenacin-sensitive and avenacin-resistant fungi (Carter et al. 1999).

The importance of saponin degradation is also evident for α-tomatine, a steroidal glycoalkaloid (SGA) consisting of the steroidal aglycon tomatidine and a tetrasaccharide sugar chain (β-lycotetraose). It is the most abundant saponin in tomato vegetative tissues, green fruit as well as roots, with the highest fresh weight concentrations reported to exceed 1 mM (You and van Kan, 2021). Deglycosylation of α-tomatine by several tomato pathogens has been reported and is mediated by tomatinases from different glycosyl hydrolase (GH) families with distinct modes of action (You and van Kan, 2021). Hydrolysis of the intact tetrasaccharide and the concomitant release of the aglycon tomatidine is catalyzed by GH10 family tomatinases in fungi including *Cladosporium fulvum*, *Fusarium oxysporum* f. sp. *lycopersici*, *F. graminearum* and *F. solani* (Carere et al., 2017; Lairini and Ruiz-Rubio, 1998; Ökmen et al., 2013). Secondly, cleavage of the terminal glucose resulting in the generation of β_2-tomatine is mediated by tomatinases belonging to the GH3 family in *Septoria lycopersici*, *Verticillium albo-atrum* and *Colletotrichum coccodes* (Woodward and Pegg, 1986; Sandrock and VanEtten, 2001; Sandrock et al., 1995). Thirdly, the generation of β_1-tomatine by hydrolytic removal of the terminal xyloside was reported to occur exclusively in *Botrytis cinerea* (Quidde et al., 1998) though the β-xylosidase protein was not identified. The GH10 tomatinases in *C. fulvum* and *F. oxysporum* are not essential for pathogenicity but rather contribute to full virulence on tomato, as manifested by less severe symptoms caused by the tomatinase-deficient mutants (Ökmen et al., 2013; Pareja-Jaime et al., 2008). Remarkably, the contribution of saponin-degrading enzymes to plant infection is not only by their role in saponin detoxification but was also reported to be related to the suppression of plant defense responses mediated by the enzymatic breakdown products, indicating another step in the arms race between pathogen and host (Bouarab et al., 2002; Ito et al., 2004).

It is important to note that all saponin tolerance mechanisms that have been reported in fungal pathogens are limited to enzymatic degradation. Studies on fungal mutants unable to degrade saponins suggest, however, that so far unknown non-hydrolytic mechanisms exist, which contribute to tolerance to saponins and virulence on saponin-containing plants. For instance, *S. lycopersici* tomatinase mutants were more sensitive to α-tomatine but their growth was not fully inhibited even at 1 mM, and the virulence on tomato was not reduced (Martin-Hernandez et al., 2000). Given the fact that saponins can disrupt fungal membrane integrity via binding to 3β-hydroxy sterols (free sterols), modification of sterols or a reduction in sterol content might lead to increased tolerance to saponins (Defago and Kerns, 1983). This can be inferred from the relatively high tolerance to α-tomatine in *Phytophthora* species, which lack the biosynthetic capacity to synthesize sterols (Steel and Drysdale, 1988). Moreover, it was proposed that

tomato and potato cells withstand high levels of their own saponins (α-tomatine and solanine, respectively) due to their capacity to glycosylate plasma membrane sterols (Walker et al., 2008; Steel and Drysdale, 1988). In addition to target modifications, active membrane repair could also counteract the membrane-disruptive action of saponins. We recently reported, that in the saprotrophic fungus *Neurospora crassa,* a mutant lacking the PEF1 protein shows increased sensitivity to tomatine. PEF-1 very likely mediates a membrane repair mechanism and readily accumulates at the plasma membrane in response to tomatine. *Botrytis cinerea*, the causal agent of grey mould disease, is a broad host range fungal pathogen affecting the global production of food and ornamental plants (Williamson et al., 2007). It produces a unique type of tomatinase activity that could be induced in liquid cultures by α-tomatine but not by other SGAs (Quidde et al., 1998). However, the *B. cinerea* gene encoding the tomatinase remains to be identified. The availability of *B. cinerea* genome information combined with a recently established CRISPR/Cas9 mutagenesis system render this fungus an ideal model to study tolerance mechanisms to saponins and their contribution to virulence (Leisen et al., 2020; van Kan et al., 2017).

Based on existing findings on fungal saponin tolerance, we investigated whether *B. cinerea* employs hydrolysis combined with other (so far unknown) resistance mechanisms to gain tolerance to α-tomatine. In order to obtain a comprehensive view of fungal cellular responses to saponins, we employed genomics, transcriptomics and functional genetic approaches. We identified a gene encoding a novel, secreted saponin hydrolase and present evidence for at least three additional, intracellular mechanisms contributing to saponin tolerance. These mechanisms are activated on transcriptional or post-translational levels, probably representing pre-formed and induced systems. The respective genes are widespread in the fungal kingdom suggesting broadly conserved functions in pathogenic but also saprophytic species.

Results

Isolate B05.10 converts α-tomatine into β_1-tomatine, while isolate M3a lacks tomatinase activity

Previous studies by Quidde et al. (1998) identified a *B. cinerea* isolate, designated M3a, which was unable to hydrolyze α-tomatine, in stark contrast to many other isolates including B05.10. To confirm these early observations and verify the phenotype of our M3a culture, we compared the formation of hydrolytic degradation products in liquid cultures of *B. cinerea* B05.10 and M3a amended with α-tomatine by liquid chromatography–mass spectrometry (LC-MS). After 9 h incubation with α-tomatine, supernatant of the B05.10 liquid culture contained β_1-tomatine as the only α-tomatine breakdown product indicating that tomatinase activity is indeed conferred by a β-xlyosidase, as proposed by Quidde et al. (1998). No hydrolytic conversion product of α-tomatine was identified in supernatant of the culture of M3a (**Fig. 1**).

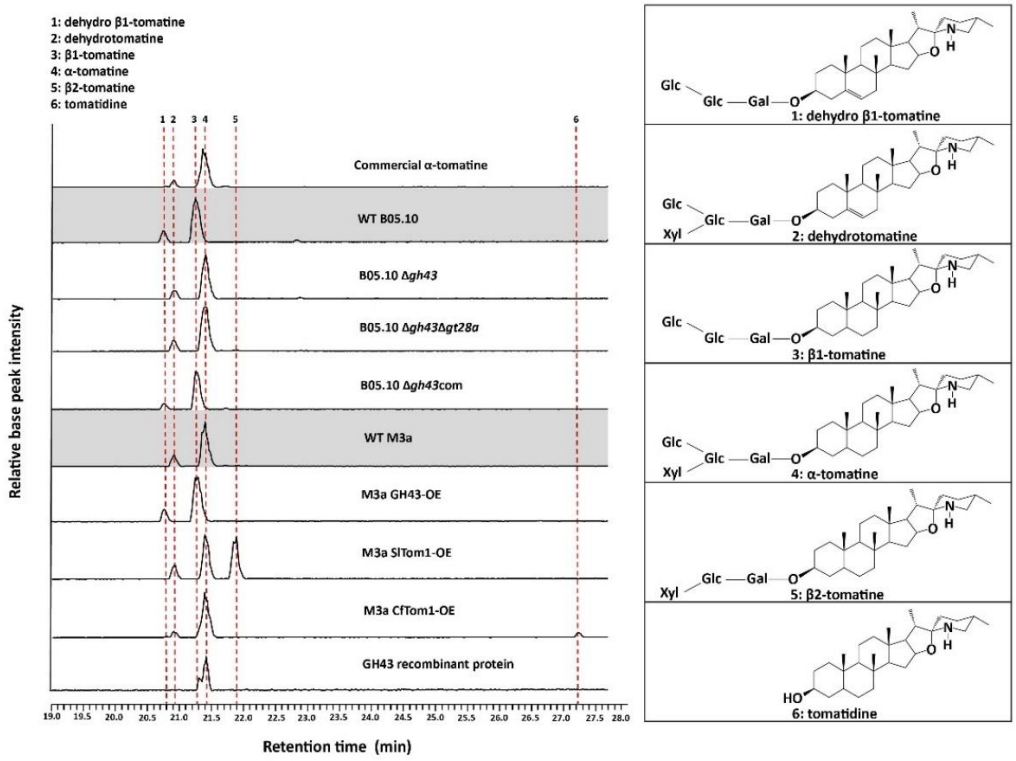


Figure 1. α-Tomatine degradation assay by LC-MS. **(A)** *B. cinerea* overnight liquid cultures were incubated with 200 µM α-tomatine for 9 h and degradation products in the supernatant were detected by LC-MS. **(B)** Structures of the metabolites 1-6. Wild type isolates B05.10 and M3a (highlighted in grey) were tested, along with several gene knockout or complemented transformants. The bottom panel shows the chromatogram of a recombinant GH43 protein incubated with α-tomatine. Note that the commercial α-tomatine used as substrate contained traces of dehydrotomatine (peak 2), which differs in the aglycone moiety, but is also a substrate for tomatinases.

M3a displays increased sensitivity to saponins and compromised virulence on tomato

To test whether the ability or inability to degrade α-tomatine correlates with differences in saponin sensitivity and virulence, tolerance and infection assays were conducted with isolates B05.10 and M3a. The sensitivity to saponins of *B. cinerea* isolates B05.10 and M3a was tested on agar plates containing different concentrations of α-tomatine or digitonin, a saponin from *Digitalis purpurea* that is structurally related to α-tomatine, especially in its glycosyl moiety. It contains a pentasaccharide that has one extra glucose moiety at the β_2-position but shares with α-tomatine the terminal xylose at the β_1-position. M3a was more sensitive to both α-tomatine and digitonin than B05.10 (**Fig. 2A**). Virulence of M3a on tomato leaves was severely compromised (**Fig. 2B**). Under conditions where B05.10 produced expanding lesions on tomato leaves, M3a displayed a disease incidence of ~20%, meaning that 80% of the inoculation droplets resulted in infection that was confined to pinpoint necrotic spots that did not expand, even upon longer incubation (**Fig. 2B**). Inoculation of M3a on *D. purpurea* was performed with agar plugs as the leaf surface of this plant contains many trichomes that interfere with inoculations using spore suspensions. On *D. purpurea*, lesions of M3a were 20% smaller than those of B05.10 (**Fig. 2C**). On *Nicotiana benthamiana*, which is not known to produce saponins, M3a displayed a disease incidence of >90% though it produced lesions that were ~20% smaller than B05.10 (**Fig. 2D**).

Genome comparison of M3a with B05.10 reveals candidate genes for α-tomatine tolerance

We reasoned that the ability and inability of B05.10 and M3a to tolerate α-tomatine should be reflected in genomic differences between the strains and that comparison of their genome sequences might reveal the molecular basis of α-tomatine tolerance in B05.10. The genome sequence of the tomatinase-producing isolate B05.10 was previously published and serves as reference for the species *B. cinerea* (van Kan et al., 2017). The genome of isolate M3a was sequenced using a combination of Illumina and Nanopore technology, and the annotated M3a genome was examined for polymorphisms with isolate B05.10. Specifically, the M3a genome was inspected for the absence of genes or for polymorphisms that would render a B05.10 gene dysfunctional. The most striking difference that was observed was the absence in M3a of a region of ~12 kb from B05.10 Chromosome 8 (**Fig. 2E**). This region contains two genes: *GH43* (Bcin08g00060) and *GT28a* (Bcin08g00070), encoding a glycosyl hydrolase containing a signal peptide and a glycosyl transferase lacking a signal peptide, respectively. The M3a genome contains in this region two different (predicted) genes and a transposable element (**Fig. 2E**). M3a displayed across the genome a total of ~92500 single nucleotide polymorphisms and ~130 gene deletions as compared with B05.10, which collectively affected the function of 166 proteins in M3a (**Supplementary Information 1**).

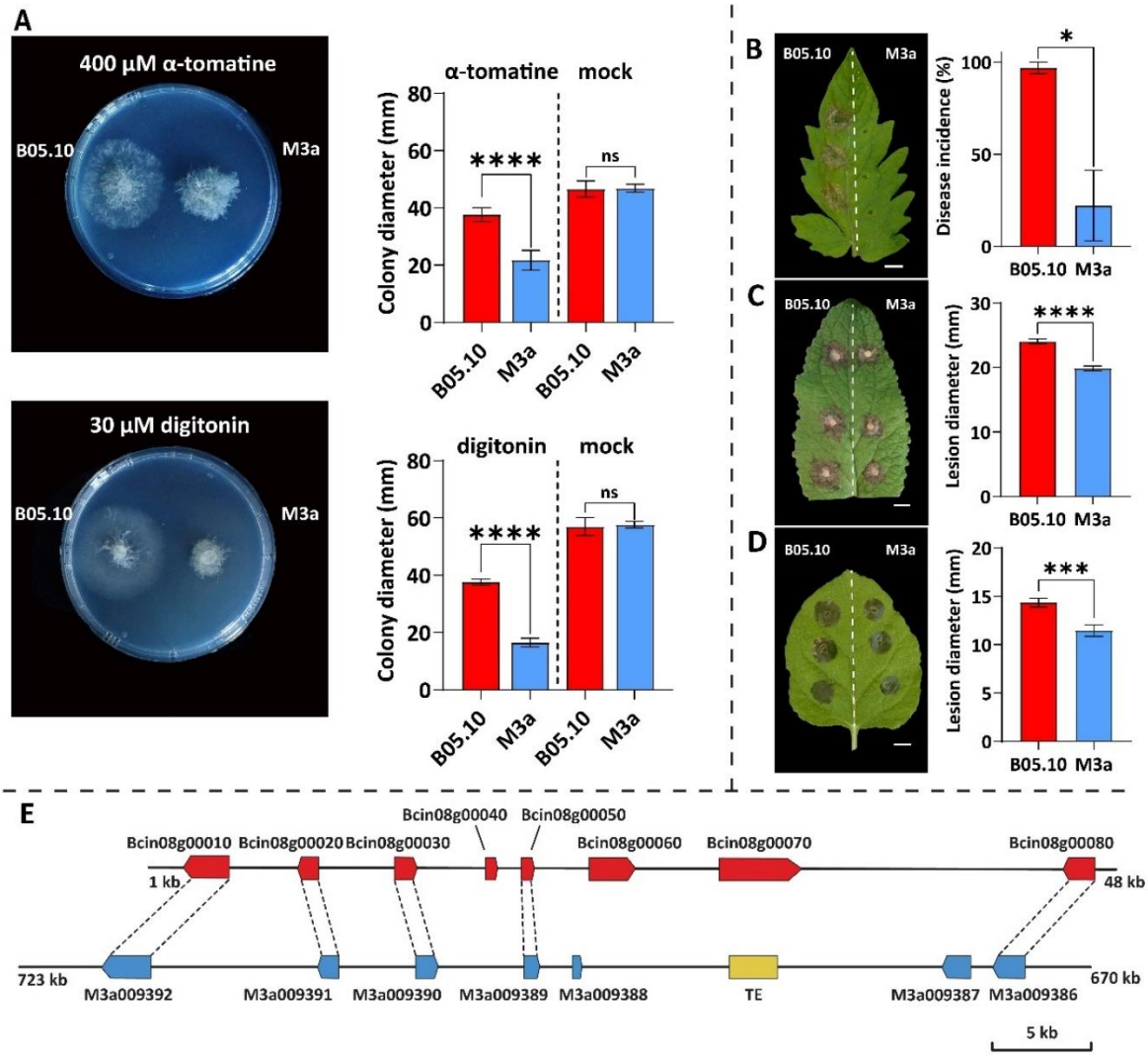


Figure 2. Differences between *B. cinerea* isolates B05.10 and M3a in sensitivity to saponins (A), in virulence on plant leaves (B-D) and in genomic composition (E). **A.** Mycelium growth on plates containing 400 µM α-tomatine and 30 µM digitonin, colony diameters were measured after 3 days. Error bars are standard error of mean (SEM) of all biological replates. **B-D.** Infection of B05.10 and M3a on tomato (B), *Digitalis purpurea* (C) or *Nicotiana benthamiana* (D). Disease incidence represents the proportion of inoculation droplets that resulted in expanding lesions. Disease incidence and lesion diameter data were based on three independent inoculation assays. Asterisks represent statistically significant differences determined by a student's t-test (* p-value <0.05; *** p-value <0.005; **** p-value <0.0001). ns indicates no significant difference. **E.** Illustration of the absence of genes Bcin08g00060 and Bcin08g00070 in isolate M3a. Red and blue boxes represent gene models in B05.10 and M3a, respectively, with the gene ID's plotted above or below the box. Dotted lines join orthologous genes. TE, transposable element in M3a. Start and end coordinates are plotted at the extremities of the contigs.

Identification of α-tomatine-responsive genes in *B. cinerea*

After identifying two candidate genes in isolate B05.10 that could either perform enzymatic glycoside hydrolysis or glycosylation, we analyzed the transcriptional response to α-tomatine. Exposure of fungi to growth inhibiting metabolites often results in the transcriptional upregulation of genes contributing to tolerance to these metabolites. This principle was exploited to identify *B. cinerea* genes that are induced in the presence of α-tomatine. An overnight culture of isolate B05.10 was divided in two parts. One half was supplemented with medium containing 200 µM α-tomatine, while the other half only received fresh medium. Samples were taken at 3 h and 6 h, and RNA was extracted for sequencing. RNA-seq analysis revealed that α-tomatine treatment in B05.10 strongly induced the transcript levels of multiple genes. Among the strongest upregulated genes **(Extended Data 1)** are the *GH43* (Bcin08g00060) and *GT28a* (Bcin08g00070) genes which are absent in M3a (**Fig. 2E**), three additional genes from the GT28 family, four genes encoding RTA1-like proteins, and a gene encoding an ABC transporter designated BcatrT. In order to obtain a detailed view of the temporal dynamics of transcript induction after α-tomatine addition, we used reverse transcription quantitative polymerase chain reaction (RT-qPCR) to study the expression profiles of a subset of α-tomatine-responsive genes as well as *PEF1* (**Extended Data 2A**). In accordance with RNA-seq results, all genes showed low, stable transcript levels in the absence of α-tomatine and were induced in response to α-tomatine treatment with comparable kinetics and induction levels (fold-changes), except for *PEF1,* whose mRNA level remained stable throughout all timepoints. Induction of α-tomatine-responsive genes was observed at 30 min and peaked between 3 and 9h. Eventually, the transcript levels decreased at 24 h after supplementing the culture with α-tomatine. Transcript levels of the same genes were also analyzed in M3a. Remarkably, induction of α-tomatine-responsive genes was compromised in M3a, except for *RTA1a* which still displayed high induction (**Extended Data 2B**), suggesting that the absence of *GH43* and *GT28a* genes in M3a might not be the sole reason for its sensitivity to α-tomatine. To test whether increased transcript levels of the above genes in B05.10 were specific to α-tomatine or resulted from a general response to membrane damage, we analyzed their expression upon treatment with the structurally related saponin digitonin or treatment with nystatin, a chemically distinct polyene antibiotic known to disrupt fungal membranes. Transcript levels of α-tomatine-responsive genes strongly increased upon digitonin treatment but not by nystatin treatment (**Extended Data 2C,D**), suggesting that transcript upregulation was in response to a saponin, rather than a response to membrane damage.

Expression levels of α-tomatine-responsive genes during plant infection

To test if α-tomatine-responsive genes are also induced during infection on the host plant tomato, we analyzed their expression during infection on tomato leaves by RT-qPCR (**Extended Data 3A**). Most of the

genes displayed low transcript levels in early infection stages that strongly increased at later time points. The increase was prominent at 24 hours post inoculation (hpi) and coincided with initiation of lesion expansion. *PEF1* transcript levels did not increase during tomato infection. The α-tomatine-responsive genes were also induced during infection on *D. purpurea* leaves from 12 hpi onwards (**Extended Data 3B**), but not induced during infection on *N. benthamiana* or the common French bean *Phaseolus vulgaris*, which do not produce α-tomatine or related compounds (**Extended Data 3C,D**).

Enzymatic detoxification of α-tomatine in *B. cinerea* is catalyzed by a GH43 protein

The hydrolytic conversion of α-tomatine by *B. cinerea* into an inactive compound was reported to be mediated by a β-xylosidase (Quidde et al., 1998; Lombard et al., 2014). According to the carbohydrate-active enzymes (CAZymes) database (Drula et al., 2022), proteins from the GH43 family have potential β-xylosidase activity and we thus presumed that the α-tomatine-responsive GH43 gene encodes an enzyme with tomatinase activity. To test this hypothesis, we produced GH43 recombinant protein in *Escherichia coli* and tested it for α-tomatine-degrading activity. After 1 h of incubation with α-tomatine, LC-MS analysis confirmed the appearance of β_1-tomatine (**Fig. 1**), the product obtained by hydrolytic removal of xylose from α-tomatine. It should be noted that a large amount of α-tomatine remained, which overlapped with β_1-tomatine in the chromatogram (**Fig. 1**). To confirm its function, the GH43 gene was deleted in B05.10, and was overexpressed in M3a through CRISPR/Cas9-mediated genome editing. Consistent with the proposed function, the B05.10 GH43 knockout (KO) mutant lost the ability to degrade α-tomatine, whereas overexpressing the GH43 gene conferred tomatinase activity on *B. cinerea* isolate M3a, as manifested by detection of β_1-tomatine by LC-MS (**Fig. 1**). These results confirm the exclusive role of GH43 in degrading α-tomatine into β_1-tomatine in *B. cinerea.* The GH43 gene will be designated as *BcTom*1 hereafter.

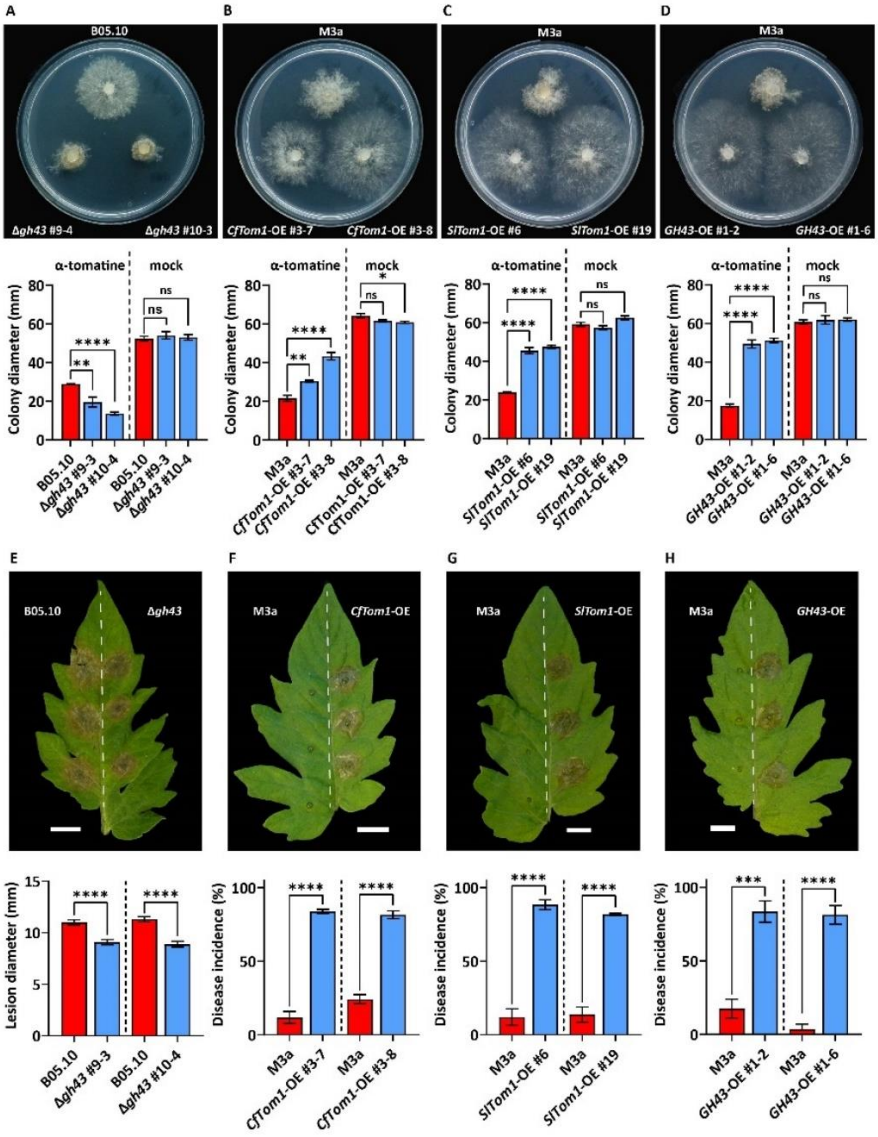
Phylogenetic analysis of BcTom1

Phylogenetic analysis of the BcTom1 protein sequence indicated that within the Sclerotiniaceae, this gene is only present in the genus *Botrytis*. Specifically, we identified orthologs in *B. aclada* (BACL_015g04100) and *B. calthae* (BCAL_0134g00010) (Valero-Jiménez et al., 2019; Valero-Jiménez., 2020), pathogens that infect onion (*Allium cepa*) and marsh-marigold (*Caltha pallustris*), respectively. Orthologs of BcTom1 were also identified in distantly related plant pathogenic fungi that can infect tomato including *Corynespora cassiicola*, *Stemphylium lycopersici*, *Fusarium solani* and *Fusarium oxysporum* f.sp. *lycopersici*, as well as in fungi that infect distinct plant species such as *Colletotrichum orbiculare, Neonectria ditissima, Macophomina phaseolina, Cadophora sp.* or *Rutstroemia sp.* (**Extended Data 4**).

Hydrolytic enzymes contribute to tolerance to plant saponins and virulence

After demonstrating the enzymatic activity of BcTom1, we tested its role in tolerance to α-tomatine and in virulence. *BcTom1*-KO mutants displayed increased sensitivity to α-tomatine on agar plates, manifested as slower radial growth than the wild type (WT) B05.10, as well as reduced virulence on tomato (**Fig. 3**). To test whether heterologous tomatinase activity can also enhance tolerance to α-tomatine and virulence of *B. cinerea* on tomato, we overexpressed tomatinase genes from a different CAZyme family, that possess a different mode of action (**Extended data 5**). To this end, we transformed *CfTom1* (GH10) from *C. fulvum* and *SlTom1* (GH3) from *S. lycopersici* in *B. cinerea* isolate M3a, which lacks the *BcTom1* gene. The overexpression of heterologous tomatinase genes conferred on M3a the corresponding ability to degrade α-tomatine, manifested by the detection of the appropriate breakdown products deduced from their catalytic activity: tomatidine and β_2-tomatine, respectively (**Fig. 1**). Expression of these heterologous tomatinases in M3a resulted in an increased tolerance to α-tomatine and a higher proportion of expanding lesions (disease incidence) upon inoculation on tomato (**Fig. 3**).

These same transformants were tested for tolerance to digitonin in plate assays and for virulence on *D. purpurea.* The *BcTom1*-KO mutants displayed increased sensitivity to digitonin on agar plates, while M3a transformants expressing CfTom1 or BcTom1 displayed increased tolerance and the M3a transformants expressing SlTom1 grew equal to the recipient **(Extended data 6**). Inoculation of these transformants on *D. purpurea* showed a reduced virulence for the B05.10 *BcTom1*-KO mutants, while M3a transformants expressing SlTom1 or BcTom1 displayed increased virulence and M3a transformants expressing CfTom1 were equally virulent as the recipient **(Extended data 6**). When inoculated on *N. benthamiana* leaves, M3a transformants expressing different tomatinase genes or B05.10 *BcTom1*-KO mutants did not exhibit differences in lesion sizes as compared with their recipients, except for M3a expressing *SlTom1*-OE, which formed slightly smaller lesions.



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258 **Figure 3.** Phenotypic characterization (sensitivity to α-tomatine and virulence on tomato) of tomatinase gene
259 transformants of isolates B05.10 and M3a. **A**. Comparison of wild type B05.10 and Δ*BcTom1* mutant. **B-D.**
260 Comparison of M3a with transformants overexpressing *C. fulvum CfTom*1 (B), *S. lycopersici SlTom*1 (C), or *BcTom*1
261 (D), respectively. Mycelium growth was on plates containing 800 µM α-tomatine or medium lacking α-tomatine
262 (mock), colony diameters were measured at 3 dpi. The asterisks represent significant differences determined by a
263 student's t-test (* p-value <0.05; ** p-value < 0.01; **** p-value <0.0001; ns indicates no significant difference).
264 **E-H.** Infection of *B. cinerea* transformants on tomato leaves. Wild type recipient was inoculated on the left side of
265 the central vein (depicted by a dashed line), while transformants were inoculated on the right side of the vein.
266 Scale bar indicates 1 cm. Error bars represent SEM of at least two independent assays. For B05.10 knockout
267 mutants, the lesion diameter (in mm) is presented. For overexpression transformants of M3a, the disease
268 incidence (proportion of inoculation spots that results in expanding lesions) is shown. Asterisks represent
269 significant differences determined by a student's t-test (**** p-value <0.0001, ns, no significant difference).

Contribution of non-hydrolytic mechanisms for tolerance to saponins

Based on the transcriptome data, which revealed significant upregulation of several genes in the presence of α-tomatine, we considered that besides enzymatic degradation by BcTom1, additional cellular mechanisms might contribute to saponin tolerance. To test this hypothesis, we generated a large set of single and multiple gene knockout mutants for the α-tomatine-responsive genes of isolate B05.10. Specifically, the attention was focused on the GT28 glycosyl transferase family and the RTA1 gene family, for which multiple members of each family were strongly upregulated by α-tomatine. Also, the PEF1 gene was studied that was earlier reported to contribute to membrane damage mitigation in response to α-tomatine in *N. crassa* (Schumann et al., 2019). The ABC transporter gene *BcAtrT* was not included in further studies, as it appeared to be a pseudogene with a single nucleotide insertion that causes a frameshift, resulting in a protein that only contains 6 (instead of 12) transmembrane domains. Combinations of mutants in distinct types of genes were also generated. The mutants were tested in growth assays for sensitivity to α-tomatine and digitonin, using both germination assays with dilution series of spores and radial growth assays with mycelium on agar plugs to distinguish the effects on colony establishment and the growth of mature hyphae, respectively. Furthermore, mutants were tested for virulence on tomato, *D. purpurea* and *N. benthamiana* (Table 1). We here only discuss the results for a subset of mutants that showed an altered phenotype (in saponin sensitivity and/or virulence). The phenotypic characterization of these mutants in radial growth assays and infection assays is presented in **Extended Data 7 and 8**.

B05.10 knockout mutants in the *BcGT28a* gene displayed increased sensitivity to α-tomatine and digitonin in a spore germination assay, but not in a mycelial growth assay. The mutant showed a reduced virulence on tomato but not on *D. purpurea*. Complementing the knockout mutant with the *BcGT28a* gene restored the phenotype to that of the wild type. The increased sensitivity was not exacerbated by deleting additional GT28 genes, however the *Δgh43Δgt28a* double mutant was significantly more sensitive to α-tomatine and digitonin than the B05.10 *Δgh43* and B05.10 *Δgt28a* single mutants, separately. These observations suggest that GH43 and GT28 function in independent pathways.

B05.10 knockout mutants in three RTA1 genes displayed an increased sensitivity only to digitonin, and this was associated with a reduced virulence on *D. purpurea*, but not on tomato or *N. benthamiana.* Analysis of RTA1 single and double mutants revealed that the phenotype was predominantly resulting from the deletion of the *BcRTA1b* gene (**Table 1**).

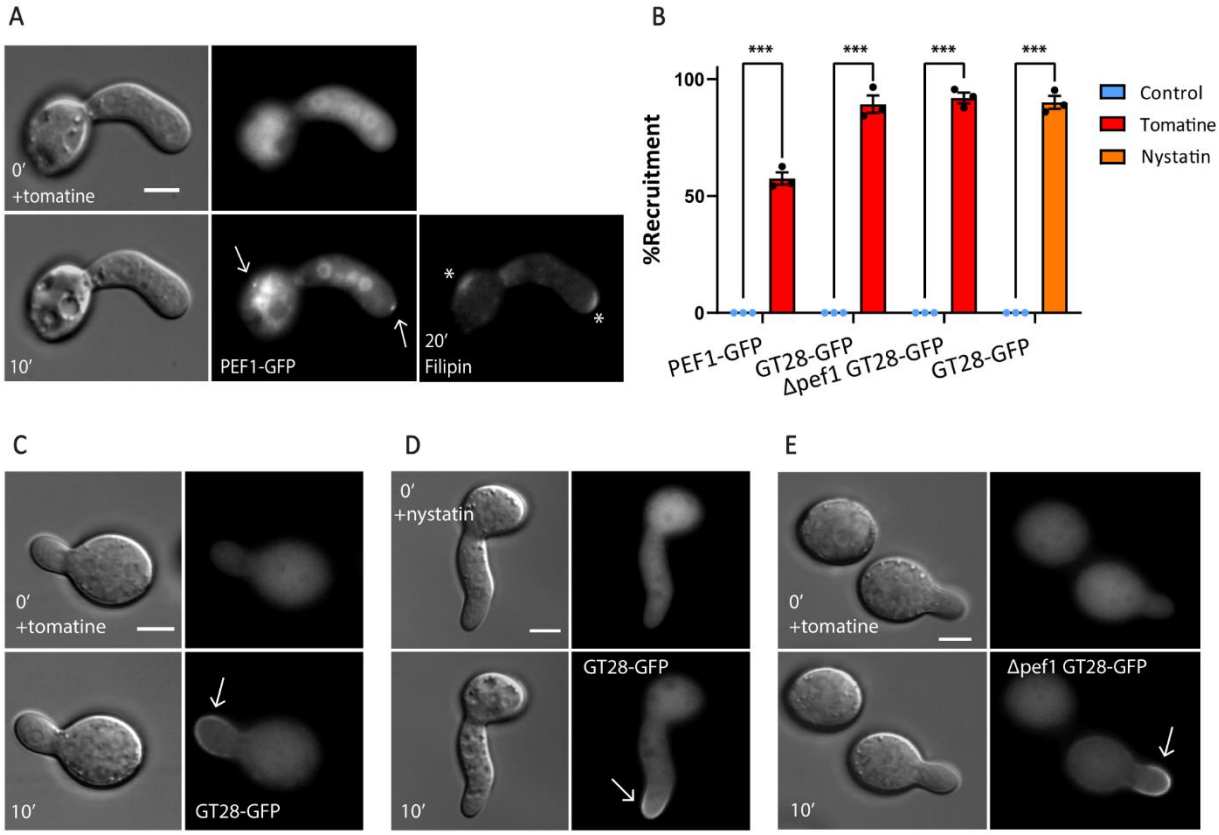
Table 1. Phenotypic analysis of *B. cinerea* mutants in tomatine-responsive genes involved in non-hydrolytic tolerance mechanisms.

Gene family	Strains compared	*In vitro* sensitivity				Virulence[c]		
		Germination assay[a]		Colony growth assay[b]				
		tomatine	digitonin	tomatine	digitonin	tomato	*N.benthamiana*	*Digitalis*
GT28	B05.10 vs B05.10 Δgt28a	↑[d]	↑	=	=	↓	=	n.t.
	B05.10 Δgt28a vs complemented	restored	restored	n.t.	n.t.	n.t.	n.t.	n.t.
	B05.10 vs B05.10 Δgt28b	=	=	n.t.	n.t.	n.t.	n.t.	n.t.
	B05.10 vs B05.10 Δgt28c	=	=	n.t.	n.t.	n.t.	n.t.	n.t.
	B05.10 vs B05.10 Δgt28aΔgt28b	↑	↑	n.t.	=	n.t.	n.t.	n.t.
	B05.10 vs B05.10 Δgt28aΔgt28bΔgt28c	↑	↑	=	=	↓	=	=
	B05.10 Δgh43 vs B05.10 Δgh43Δgt28a	↑	↑	=	=	↓	=	n.t.
	M3a vs M3a GT28a-OE	↓	↓	↓	↓	↑	=	=
RTA1	B05.10 vs B05.10 Δrta1aΔrta1bΔrta1c	=	↑	=	↑	=	=	↓
	B05.10 vs B05.10 Δrta1a	=	=	n.t.	=	n.t.	n.t.	n.t.
	B05.10 vs B05.10 Δrta1b	=	↑	n.t.	↑	n.t.	n.t.	n.t.
	B05.10 vs B05.10 Δrta1c	=	=	n.t.	=	n.t.	n.t.	n.t.
	B05.10 vs B05.10 Δrta1a Δrta1b	=	↑	n.t.	↑	n.t.	n.t.	n.t.
	B05.10 vs B05.10 Δrta1a Δrta1c	=	=	n.t.	=	n.t.	n.t.	n.t.
	B05.10 vs B05.10 Δrta1b Δrta1c	=	↑	n.t.	↑	=	=	n.t.
PEF1	B05.10 vs B05.10 Δpef1	=	=	n.t.	=	=	=	=
	M3a vs M3a Δpef1	↑	=	↑	=	↓	n.t.	=
	B05.10 Δgh43 vs B05.10 Δgh43Δpef1	↑	=	n.t.	=	n.t.	n.t.	n.t.
	B05.10 Δgh43gt28 vs. B05.10 Δgh43gt28Δpef1	↑	=	n.t.		n.t.	n.t.	n.t.

[a] germination assay by spotting serial dilutions of conidia on saponin-containing agar plates
[b] radial growth of colonies after mycelium transfer to saponin-containing agar plates
[c] necrotic lesion diameters after inoculation on leaves of the host plants indicated
[d] ↓, decreased; ↑, increased; = no difference; n.t., not tested

α-Tomatine induces membrane disruption and recruits BcPEF1 and GT28 at ergosterol-enriched domains

As α-tomatine causes membrane disruption by complexing with sterols, we examined the distribution of sterols in *B. cinerea* membranes by filipin staining and observed that ergosterol is enriched at hyphal tips and in septa (**Extended data 9**). We studied the pore-forming activity of α-tomatine on *B. cinerea*. B05.10 transformants were used that overexpress *PEF1-GFP*, whose homolog in *N. crassa* is mobilized to damaged sites at the membrane in response to Ca^{2+} influx and thereby acts as a marker for membrane damage (Schumann et al., 2019). Comparable to observations in *N. crassa*, the BcPEF1-GFP signal was mainly cytoplasmic with some accumulation at the endoplasmic reticulum around nuclei in the absence of α-tomatine (**Fig. 4A)**. Addition of α-tomatine resulted in rapid cell lysis indicated by vacuolized appearance of the fungal cells. BcPEF1-GFP accumulated in punctate structures at the cell periphery, mostly co-localizing with ergosterol-enriched sites as determined by filipin stain (**Fig. 4A**). More than 55% of the hyphae treated with α-tomatine showed recruitment of BcPEF1-GFP to the tips (**Fig. 4B**). These observations confirm the membrane-targeting action of α-tomatine and reveal that BcPEF1 was efficiently recruited to this damaged site, as was also reported in *N. crassa* (Schumann et al., 2019). To determine the subcellular localization of GT28a, a GT28a-GFP fusion protein was expressed in B05.10 under control of a constitutive promoter. In medium without α-tomatine, fluorescent signal was homogenously distributed in the hyphae, indicating GT28a is predominantly a cytoplasmic protein. After addition of α-tomatine or the chemically unrelated antibiotic nystatin to the culture, the fluorescent signal upon both treatments accumulated at hyphal tips (**Fig. 4 C,D**) and the recruitment of GT28-GFP to hyphal tips was even higher than for PEF1-GFP (**Fig. 4B**). Since PEF1 and GT28 showed similar localization patterns, we tested if PEF1 influences the dynamics of GT28. A *PEF1* deletion mutant was constructed in the background of B05.10 expressing GT28a-GFP and the dynamic of GT28 was tested. The mobilization of GT28a-GFP to the membrane of hyphal tips was still observed with high incidence in absence of PEF1 (**Fig. 4 B,E**), indicating that GT28 does not require PEF1 for translocation to the damaged sites in the membrane.



329

330 **Figure 4.** Localization of PEF1 and GT28 proteins upon membrane perforation. **A.** PEF1-GFP signal observed at 10
331 mins after application of 190 µM α-tomatine. Then the sample was stained with filipin at 20 mins to detect
332 ergosterol in the membrane. Signals are indicated by a white arrow (GFP) or a star (*) (filipin staining). The scale bar
333 indicates 5 µm. **B**. Quantification of recruitment of PEF1-GFP and GT28a-GFP to the tips of the germlings. Untreated
334 samples are indicated in blue, α-tomatine treated in red and or nystatin treated in orange. Error bars indicate SEM
335 from 3 independent experiments (n =100). Asterisks represent statistically significant differences determined by a
336 student's t-test (* p-value <0.05; ** p-value < 0.01,*** p-value <0.005). **C, D**. GT28a-GFP signal at 10 mins after
337 application of 190 µM α-tomatine (C) or 0.05 mg/mL nystatin (D); **E.** GT28a-GFP signal in *PEF1* KO mutant at 10
338 mins after treatment with 190 µM α-tomatine.

Discussion

B. cinerea employs a novel tomatinase for saponin degradation

Saponins are a common class of plant defense compounds found in many different plant genera. Fungal pathogens of these host plants must therefore have evolved efficient resistance mechanisms to cope with these membrane toxic compounds. For the last ca. 30 years, enzymatic deglycosylation and absence of the saponin target ergosterol have been the only known resistance mechanism against saponins in fungi (Defago and Kern, 1983; Westrick et al. 2021). Consistent with earlier studies, we identified the α-tomatine hydrolyzing enzyme BcTom1 as a major factor in α-tomatine resistance in *B. cinerea*. BcTom1 is the first α-tomatine degrading β-xlyosidase, thus representing a new class of α-tomatine degrading enzymes. The comparison of the host range of isolates M3a (lacking BcTom1) and B05.10 (containing BcTom1) confirmed the earlier observed correlation of the host range and the ability to chemically degrade the host defense compounds. A recent study by Mercier et al. (2021) compared various field isolates of *B. cinerea* sampled from tomato or grapevine. Their findings revealed that strains isolated from tomato consistently exhibit three copies of a 25 kbp genomic region, which includes BcTom1 and BcGT28a genes. By contrast, four strains obtained from grape lack the BcTom1 gene. The annotation employed in that study, suggested that the *BcTom1* gene participates in hemicellulose degradation, but did not allude to its role in α-tomatine detoxification (Mercier et al. 2021). The presence of multiple copies of two genes that confer tolerance to α-tomatine provide clear evidence for evolutionary adaptation in *B. cinerea* to host species. It may be interesting to explore if gene duplication events in *B. cinerea* isolates of other host plant species may also be indicative for an involvement in host preference.

Our study demonstrated that introducing tomatinases from unrelated fungi in *B. cinerea* isolate M3a enhances its tolerance to this saponin and increases disease incidence in tomatoes. This suggests that the overall degradation ability of the enzymes is more significant for the plant-fungus interaction outcome than their specific enzymatic activity in breaking down the saponin. The fact that different α-tomatine degrading enzymes are found within the fungal kingdom supports the notion that the ability to degrade α-tomatine has independently evolved in different lineages from distinct ancestral glycosyl hydrolases of the GH3, GH10 and GH43 families. Interestingly, *BcTom1* orthologs were detected in distantly related tomato pathogens, suggesting that horizontal transfer between unrelated phytopathogenic fungi may have facilitated the broadening of their host range, especially when these species share the same host or ecological niche, such as the rhizosphere or endosphere. Since mechanisms and the role of horizontal gene

transfer in fungal evolution remain enigmatic, *BcTom1* might represent a highly suitable model for further investigation of this topic, including the important question in which fungal lineage the gene originated.

Additional mechanisms contribute to α-tomatine resistance

Previous studies on gene knock-out mutants have hinted at the existence of additional saponin resistance mechanisms in fungi. These mutants displayed a reduced, yet persistent ability to infect host plants and demonstrated higher tolerance to saponin concentrations compared to non-pathogenic isolates or species, that are non-pathogenic on the respective hosts (Martin-Hernandez et al., 2000 Ökmen et al., 2013; Pareja-Jaime et al., 2008). Consistent with this notion, the *B. cinerea BcTom1* mutant tolerates higher α-tomatine concentrations than the saprotrophic fungus *N. crassa* (Schumann et al., 2019). Our comprehensive study of the response of *B. cinerea* to α-tomatine unveils for the first time novel molecular factors representing additional resistance mechanisms beyond enzymatic de-toxification or the absence of sterol. Our genetic analysis indicated that GT28a, RTA1b and PEF-1 all contribute independently from each other to α-tomatine resistance. While BcTOM1, GT28a and RTA1 are transcriptionally upregulated in the presence of α-tomatine, PEF1 represents a post-translationally activated response mechanism.

The *B. cinerea* GT28 enzymes show sequence and structural similarity to ergosterol glycosyl transferases of baker's yeast and plants (**Extended Data 10).** In the saponin producing plant species potato and tomato, membrane sterols are glycosylated by enzymes of the GT28 family to protect them from toxic interaction with their own defense compound (Ramirez-Estrada et al., 2017). The discovery of the conserved steroid binding domain in GT28a strongly supports the hypothesis that also in *B. cinerea*, this enzyme functions in modifying the α-tomatine target ergosterol in the plasma membrane, thereby providing resistance against this saponin. Upon exposure to α-tomatine or other membrane disturbing drugs, the protein is rapidly translocated from the cytoplasm to distinct regions in the plasma membrane, including the ergosterol-rich tip of actively growing germlings. These dynamics imply that GT28a undergoes posttranslational regulation and that its activity is restricted to wound sites rather than targeting sterols on a broader cellular scale. The mechanisms controlling GT28a recruitment to the membrane remain to be resolved but do not include PEF-1, which exhibits similar subcellular dynamics. GT28 homologs are widely found in filamentous fungi, suggesting a broader role than just conferring protection against a single specific plant defense compound. Given that membrane sterols serve as targets for numerous plant defense compounds, fungicides and clinical drugs, further investigation into the role of GT28 enzymes in drug resistance holds potential.

RTA1 proteins are broadly conserved and were first identified in *S. cerevisiae,* where they confer resistance to the membrane-disrupting compound 7-aminocholesterol (Soustre et al., 1996). RTA1 is proposed to operate as a fungal lipid-translocating exporter that participates in restoring membrane integrity upon exposure to membrane-disrupting compounds. It has thus far only been studied in other yeasts, such as *Candida* and *Cryptococcus* (Smith-Peavler et al., 2022) and functional studies on RTA1 genes in filamentous fungi are lacking. Our data indicate for the first time that RTA1 proteins contribute in mitigating membrane damage caused by saponins, rendering them potential virulence factors. Investigating their role in other phytopathogenic fungi and studying their ability to confer tolerance to various membrane-targeting antifungal compounds, such as agricultural fungicides and clinical drugs, is of broad interest.

The calcium binding protein PEF1 also contributes to saponin resistance and is part of a conserved membrane repair mechanism in filamentous fungi. Both in *B. cinerea* and *N. crassa*, the protein rapidly translocates to the plasma membrane in response to drug-induced membrane damage. Our study in *N. crassa* indicated that it also contributes to membrane repair in response to mechanical membrane rupture, for example during aberrant plasma membrane fusion, suggesting a general function in securing membrane integrity (Schumann et al., 2019). Activation of the PEF-1 response does not include gene activation but occurs post-translationally in a calcium-dependent manner, suggesting a role in an immediate cellular emergency response. Our study in *N. crassa* indicated that at least one additional PEF-1-independent repair mechanism exists, which might also participate in this response in *B. cinerea*. The future identification and comprehensive description of membrane repair mechanisms in filamentous fungi, will therefore significantly improve our understanding of the global cellular response to membrane targeting antifungal compounds.

Based on our observations, we propose a multifactorial model for saponin resistance, which likely also applies to fungal responses to other membrane targeting compounds, including fungicides and clinical drugs (**Fig. 5**). In this model, post-translational and transcriptional activation of synergistic response mechanisms occur in a stepwise and spatially distinct fashion, allowing initial survival and subsequent adaptation of the fungus upon exposure to the membrane perforating compounds. Activation of the transcriptional response requires physical contact of the cell with the compound. In the natural infection process of necrotrophic plant pathogenic fungi, this contact occurs when the fungus destroys the integrity of host cells and saponins are released in the damaged tissue. Disintegration of the plant cells likely occurs already at a distance from the fungal mycelium via secreted fungal pathogenicity factors, such as the cytotoxic sesquiterpene botrydial (Colmenares et al., 2002), proteins with cytolytic activity on plant cells

such as NLPs (Schouten et al., 2008; Cuesta Arenas et al., 2010) or other cell death-inducing compounds (Leisen et al., 2022; Bi et al., 2022). The extending fungal hyphae will come in direct contact with the plant defense compound, while the fungus colonizes the tissue. This contact induces the rapid transcriptional activation of inducible fungal resistance mechanisms but will inevitably cause immediate membrane damage. Disintegration of the membrane during this early phase would, however, result in instantaneous collapse of ion gradients across the plasma membrane and subsequent cell death. Such a life-threatening situation is counteracted by the instant activation of preformed membrane repair mechanisms, including PEF-1 (**Fig. 5**). Since such repair does not depend on gene activation, it can respond within seconds to enable fungal survival until inducible systems are activated. A common signal for membrane disintegration and the activation of adequate repair mechanisms in eukaryotic cells is a rapid increase in calcium ions in the cytoplasm. We hypothesize that saponin-induced pore formation results in such an increase and activation of PEF-1. After the short-term survival of the fungus is ensured, the inducible systems will implement its long-term adaptation. As described above, these mechanisms likely involve alteration of the cellular target (GT28a) and additional reinforcement of the membrane (RTA1). Secretion of BcTom1 will then reduce the saponin levels in surrounding plant tissue, clearing the path for hyphal extension (**Fig. 5**).

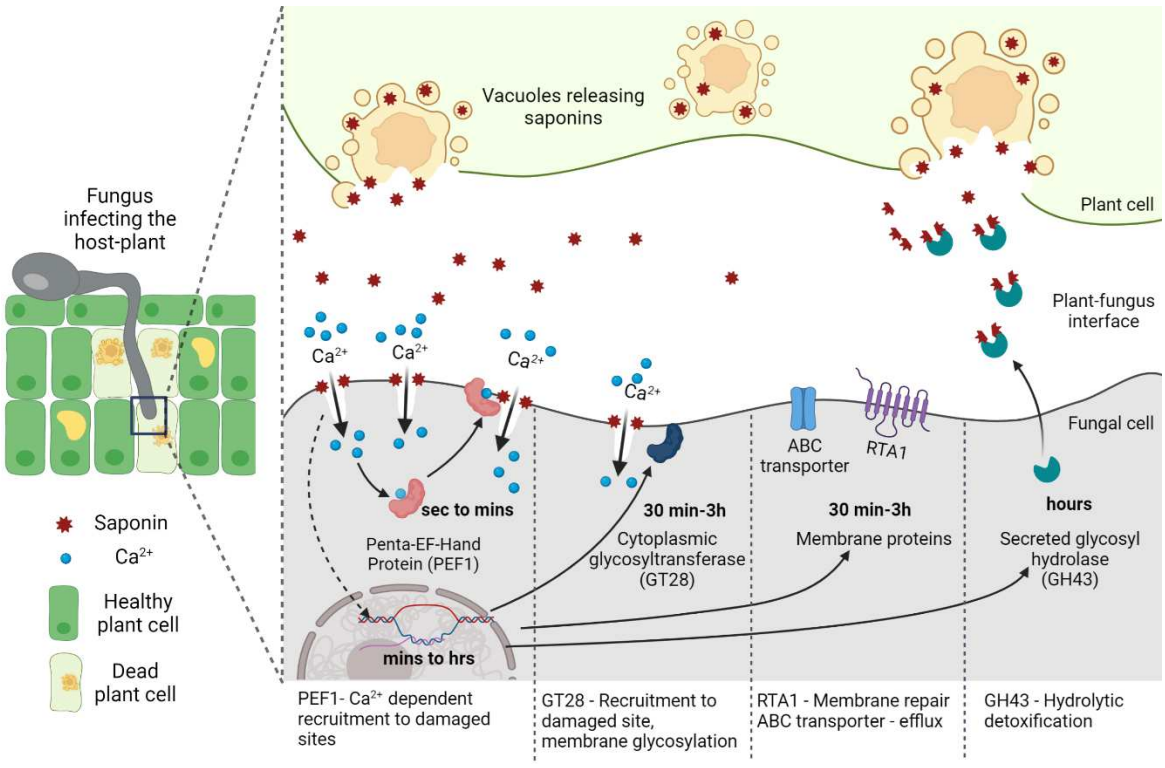


Figure 5. Model illustrating the responses of *B. cinerea* to α-tomatine, both in space and time (created with www.biorender.com). Details are discussed in the text.

For phytoalexins, this model also includes the rapid induction of efflux pumps of the MDR-MFS and MDR-ABC transporter type by their substrates. Efflux is a common cellular protection mechanism against toxic compounds and likely functions between the rapid pre-formed survival and long-term adaptation mechanisms. The combined action of phytoalexin efflux and enzymatic degradation has also been reported in *B. cinerea*, including during its interaction with tomato (Bulasag et al 2023; Schoonbeek et al., 2001; Schoonbeek et al., 2003; Stefanato et al., 2009) So far, efflux has not been described as a saponin resistance mechanism. Interestingly, our transcriptomes analysis revealed induction of an MDR-ABC transporter gene upon exposure to α-tomatine, that we designated BcAtrT. In B05.10, however, this gene contains an early stop codon and therefore does not encode a functional transporter protein. By contrast, the BcAtrT appeared to be intact in M3a. Following up the potential contribution of efflux to saponin resistance might further extend the model presented in **Fig. 5**. While induced drug resistance mechanisms are a known and common theme in fungal biology, the cellular mechanisms mediating transcriptional induction of drug-responsive genes remain cryptic. Interestingly, the observed gene induction in the α-tomatine tolerant *B. cinerea* isolate B05.10 is absent in the sensitive isolate M3a. Future comparison of the two respective genomes therefore holds much potential for approaching this important question.

The interaction between a host plant and a pathogenic fungus is intricate and encompasses numerous molecular interactions. These interactions arise from an evolutionary arms race between both partners, and the combinatorial defense response against saponins likely reflects the result of such a stepwise evolution. While obtaining an initial, single defense mechanism might not provide full resistance, it could allow limited survival in presence of the plant defense compound. This critical scenario, on the verge of death, would impose intense selection pressure for adapting to such a toxic environment. As a result, acquiring a single initial defence mechanism could rapidly lead to adaptation and an expansion of the host range of the fungus. This adaptation may also involve general non-specific defence mechanisms, such as the broad and conserved PEF-1-mediated membrane repair mechanisms. While not individually capable of providing resistance, these mechanisms can make important contributions when combined with specific factors. The evolution of broad combinatorial pathogenicity mechanisms remains mysterious, yet it presents significant potential for comprehending the emergence of new pathogens and developing effective control measures.

Materials and methods

Fungal growth and transformation

B. cinerea culturing and spore production were performed as described by Zhang et al. (2016). CRISPR/Cas9 mediated transformation of *B. cinerea* was performed according to Leisen et al. (2020). Single Guide RNAs (sgRNAs) targeting the gene of interest were synthesized prior to the transformation. For overexpression, nitrate reductase (BcniaD; Bcin07g01270) and nitrite reductase (BcniiA; Bcin01g05790) gene loci were used for targeted integration of an overexpression cassette generated in pNDH-OGG and pNAN-OGG vectors (Schumacher, 2012). The donor DNA comprising selection marker templates were amplified by PCR using primers containing 60 bp homologous recombination regions. PCR reactions using flanking primers spanning target genes were used for genotyping. At least two independent homokaryotic transformants were used for further characterization.

Plant growth and infection assays

Tomato *S. lycopersicum* cv. Moneymaker (MM), *D. purpurea, N. benthamiana* and *P. vulgaris* were grown in a greenhouse at 21/19 °C (day/night) temperatures. Detached leaves (*S. lycopersicum*, *D. purpurea*) or whole plants (*N. benthamiana* or *P. vulgaris*) were used for inoculation under lab conditions, either in Gamborg's B5 (Duchefa BV) minimal medium supplemented with 10 mM sucrose and 10 mM potassium phosphate (pH 6.0) or in potato dextrose broth (PDB) (12 g/L) at a concentration of $1x10^6$/mL. At 3 dpi, leaves where photographed and lesion sizes were measured with a digital caliper.

Gene expression analysis of α-tomatine-responsive genes in *B. cinerea*

B. cinerea spores were inoculated in liquid medium containing 3 g/L GB5 salts, 100 mM fructose, 10 mM potassium phosphate, 0.5% yeast extract and adjusted to pH 5.5 at a final concentration of $1x10^6$/mL. After overnight incubation at 20 °C 120 RPM, half of the culture was supplemented with α-tomatine (TCI Europe) and the other half was supplemented with solvent control (methanol + 0.5% formic acid). Mycelium was sampled at 0, 3 and 6 hours after α-tomatine/mock treatment and used for mRNA isolation. RNA-seq was carried out at Beijing Genomics Institute (BGI), Shenzhen, China. The reads were mapped to the B05.10 genome (van Kan et al., 2017) and expression levels were quantified as transcripts per million reads (TPM). Induction of α-tomatine-responsive genes were further validated by RT-qPCR using samples generated as described above, with more time points (B05.10: 0.5 h, 1 h, 2 h 3 h, 6 h, 9 h and 24 h; M3a: 1 h, 3 h and 9 h). Treatments with nystatin (Sigma Aldrich) or digitonin (Carl Roth) were carried out on B05.10 in a similar way and sampled at 1 h, 3h and 9 h after treatment. Expression of α-tomatine-responsive genes was

investigated during tomato and *N. benthamiana* infection at different timepoints (tomato: 0, 6, 12, 18, 24, 30, 36 and 43 hours post inoculation; *N. benthamiana*: 0, 12, 24, 36 and 48 hours post inoculation).

Sensitivity test to membrane-disrupting compounds using plate assays

Spores and mycelium were plated on 15 mL GB5 plates containing varying amounts of α-tomatine (B05.10 spores 600 µM; B05.10 mycelium 800 µM; M3A spores 200 µM; M3A mycelium 400 µM). For mycelium inoculation, agar plugs of 5 mm were taken from the growing edge of *B. cinerea* colonies on MEA plates and placed on the α-tomatine containing plates with the mycelium facing the agar. For spore inoculations, concentrations were adjusted to $1x10^6$/mL and 2 µL droplets (2000 spores) were inoculated on the plate. Each plate contains one droplet of a WT isolate (B05.10 or M3A) and two droplets with spores from two independent transformants, respectively, in five technical replicates. Plates were incubated at 20°C and colony diameters were measured at 3 dpi. For testing the sensitivity to nystatin, only mycelium plugs were inoculated on MEA plates containing 1 µg nystatin/mL. Inoculations and measurements were performed as described for the α-tomatine plate assay, and colony diameters were measured at 2 dpi.

M3a genome sequencing, assembly and comparative genomics analysis with B05.10

Genomic DNA of M3a was extracted and sequenced by Illumina and Nanopore sequencing. The Illumina and Nanopore reads were combined into an initial assembly of M3a using the MaSuRCA assembler (Zimin et al., 2013). The genomes of B05.10 and M3a were compared using four different methods. Firstly, a global alignment between the two genomes was produced using the NUCmer algorithm (Stefan et al., 2004). Secondly, an alignment-free comparison was performed using the shortest unique substrings (shustring) algorithm (Haubold et al., 2005). Thirdly, short reads obtained from Illumina sequencing on the M3a genome were aligned to the B05.10 genome using the BWA-MEM algorithm. Fourthly, long reads obtained from Nanopore sequencing on the M3a genome were aligned to the B05.10 genome using Minimap2 (Li, 2018). Based on the Illumina reads alignment, Single Nucleotide Polymorphisms (SNPs) and small insertions and deletions (indels) were mapped using the FreeBayes algorithm (Garrison and Marth, 2012). The snpEff algorithm was used to predict the effect of SNPs on proteins (Cingolani et al., 2012).

α-Tomatine and digitonin degradation assay

Recombinant BcTom1 protein was produced in *E. coli* strain BL21 using a pET-15b expression vector (Abedi et al., 2012). 12.5 µg pure BcTom1 protein was incubated with 25 µM of α-tomatine for 1 h. Isolates B05.10 and M3a and their transformants were grown in liquid as described above and incubated with 200 µM α-tomatine or 100 µM digitonin for 9 h. Conversion products were analysed by LC-MS.

General microscopy and analysis

Fungal cells were observed on a Zeiss Observer 2.1 microscope using Nomarski optics with a Plan-Neofluar 100×/1.30 oil immersion objective (420493-9900) with CoolLED pE4000 as a light source for fluorescence microscopy. Images were captured with a PCO Edge 5.5 Gold (16 bit) camera and analyzed using ImageJ.

Sample preparation for microscopy and quantitative recruitment assay

To analyse GT28a-GFP recruitment in response to nystatin and α-tomatine, 5 µL of $2x10^7$ spores were incubated in 150 µL liquid mineral medium in Ibidi eight-well µ-slides (Sigma-Aldrich) for 5-6 h at 20°C. After imaging the untreated cells, 100 µL of 193 µM α-tomatine solution was added. Similarly, for nystatin 10 µL of 0.05 mg/mL was added and mixed by pipetting, and incubated for 5-10 min before analysis by Nomarski and fluorescence microscopy. For quantitative assays, 100 germlings were tested for GT28a-GFP recruitment. Each test was performed three times with multiple technical replicates.

Sterol staining

To stain membrane sterols in germlings, 100 µL of 100 µg/mL of filipin III solution in 1% (v/v) DMSO were added to the germlings grown in Ibidi eight-well µ-slides as mentioned above. Cells were incubated for 20 mins and analyzed by fluorescence microscopy using a DAPI filter setup.

Data availability statement

The sequence data discussed in this manuscript are deposited in NCBI. Sequence reads for the RNAseq experiment on α-tomatine-induced gene expression (BioProject number PRJNA955032) were deposited under SRA accession numbers are SRR24174271- SRR24174280. The genome assembly of *B. cinerea* isolate M3a is deposited under genome accession number JARWBL000000000.

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Author contributions

YY, JALvK and AF designed the study; YY, HMS, LM, ALHV, PR, HGB, SQ and FV performed the experiments; YY, XSK, FP, EACC, IK, AF, JALvK analysed the data; YY, HMS, LM, XSK made the figures and tables; YY, AF and JALvK wrote the manuscript; all authors approved the manuscript.

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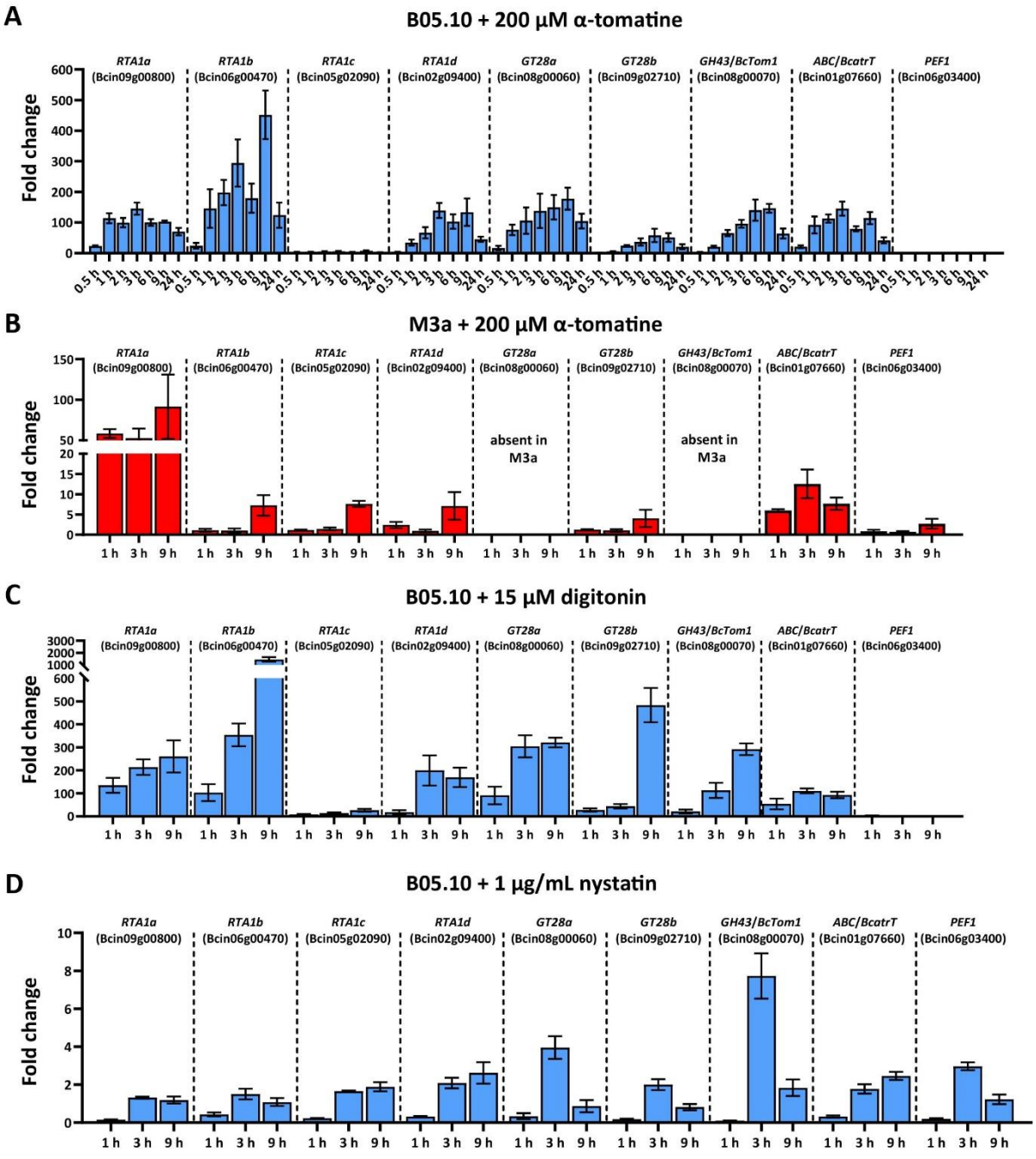
Extended Data files for the manuscript by You et al. (2023) entitled

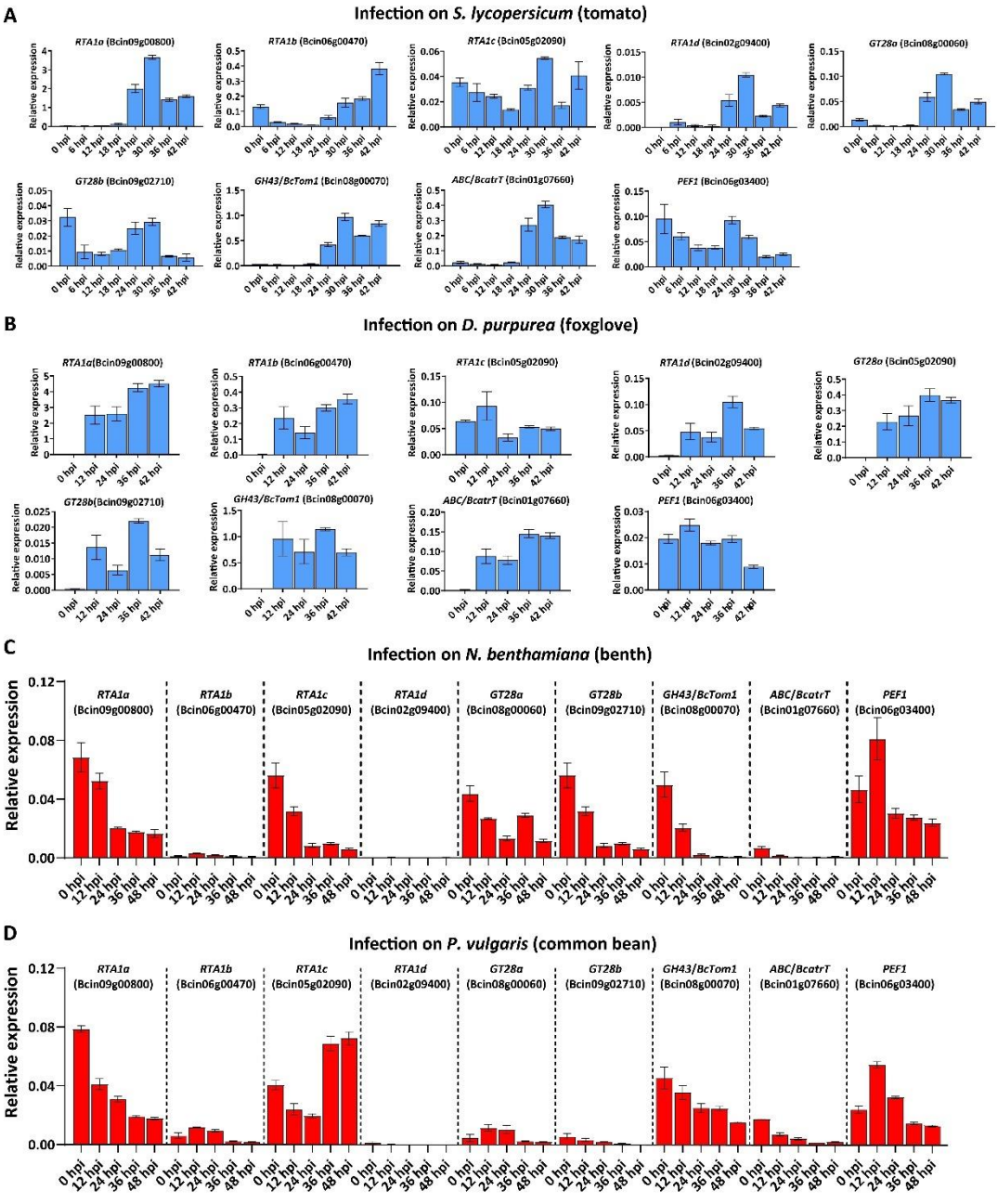
"The grey mould *Botrytis cinerea* employs multiple mechanisms to protect itself from antifungal plant metabolites"

Accession number	Pfam annotation	Fold change	
		3 h	6 h
Bcin06g00470	RTA1 like protein	569.4	765.3
Bcin09g00800	RTA1 like protein	229.0	283.7
Bcin16g00006	Berberine and berberine like protein	199.2	96.3
Bcin08g00070	Glycosyltransferase family 28	199.1	202.1
Bcin08g00060	Glycosyl hydrolases family 43	107.3	124.4
Bcin01g07660	ABC transporter	107.2	85.9
Bcin09g02710	Glycosyltransferase family 28	44.7	75.8
Bcin06g06730	hypothetical protein	41.5	37.9
Bcin09g02720	Bacterial alpha-L-rhamnosidase	36.5	151.7
Bcin02g09400	RTA1 like protein	34.2	76.0
Bcin16g00005	Glycosyltransferase family 28	19.6	13.2
Bcin06g00460	Glycosyltransferase family 28	17.3	19.6

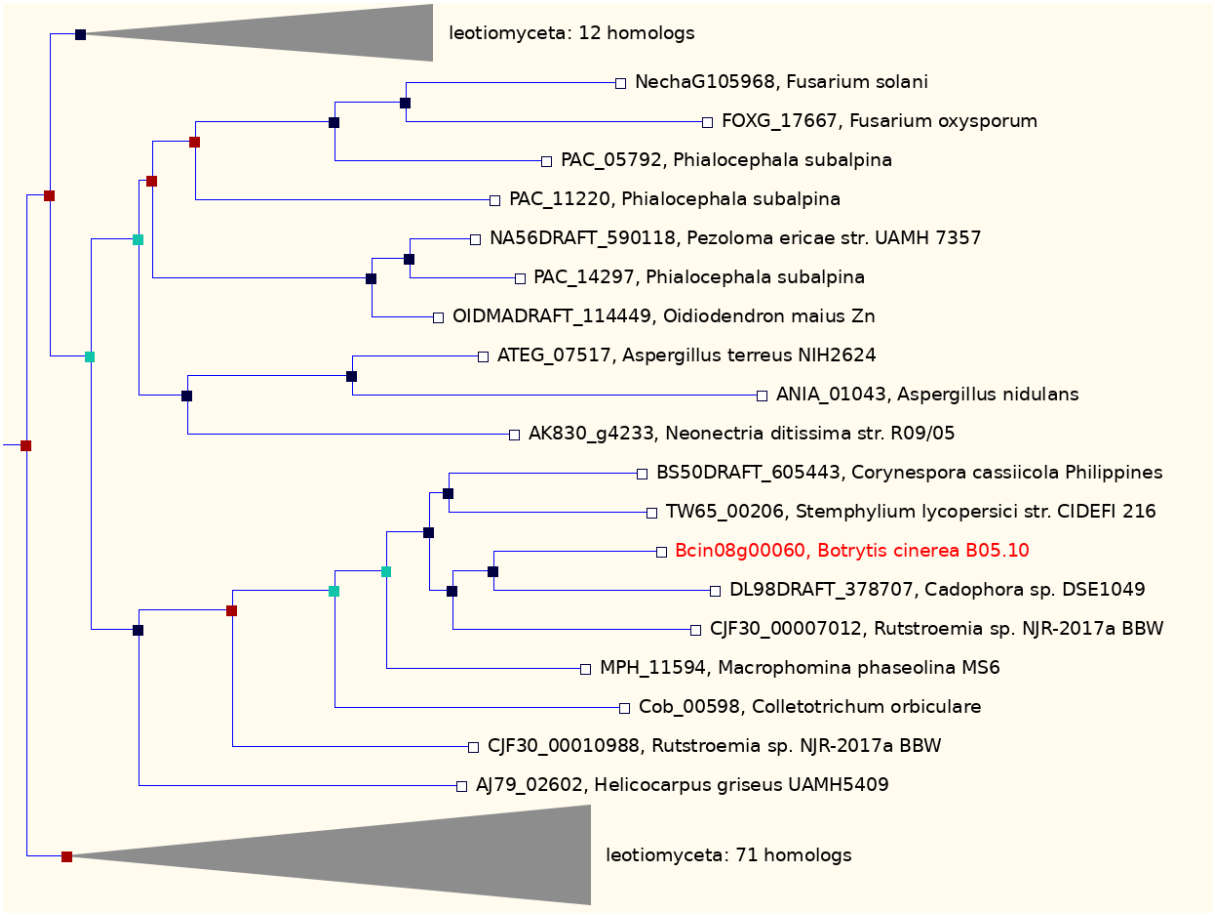
Extended Data 1. List of highly induced genes in B05.10 at 3 h and 6 h after addition of α-tomatine. Fold change represents the transcript level of *B. cinerea* genes after α-tomatine treatment as a ratio to the mock treatment. The list contains the top-10 strongest upregulated genes, supplemented with two paralogs of the *GT28* gene family and one paralog from the RTA1 family that were also significantly upregulated.



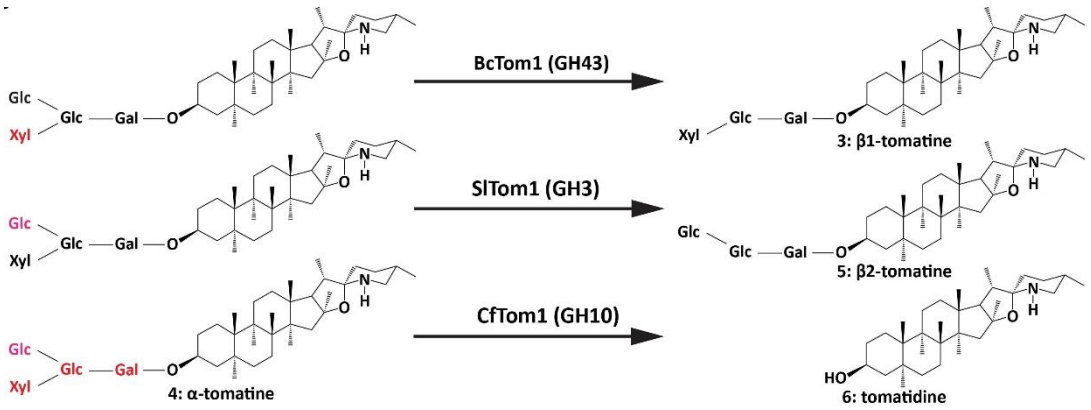
Extended Data 2. Relative expression of selected α-tomatine-responsive genes after addition of membrane-disrupting compounds in *B. cinerea* as compared to mock treatment. B05.10 treated with 200 µM α-tomatine (A); M3a treated with 200 µM α-tomatine (B); B05.10 treated with 15 µM digitonin (C); B05.10 treated with 1 µg/mL nystatin. RT-qPCR was performed using *SMT3* and *TUB* as reference genes. Error bars are SEM of three biological replicates.



Extended Data File 3. Analysis of relative expression of selected α-tomatine-responsive genes after inoculation on different plants**.** Infection on MM leaves (A), *N. benthamiana* leaves (B) or *P. vulgaris* leaves (C) by RT-qPCR using *SMT3* and *TUB* as reference genes. Error bars are SEM of three biological replicates**.**

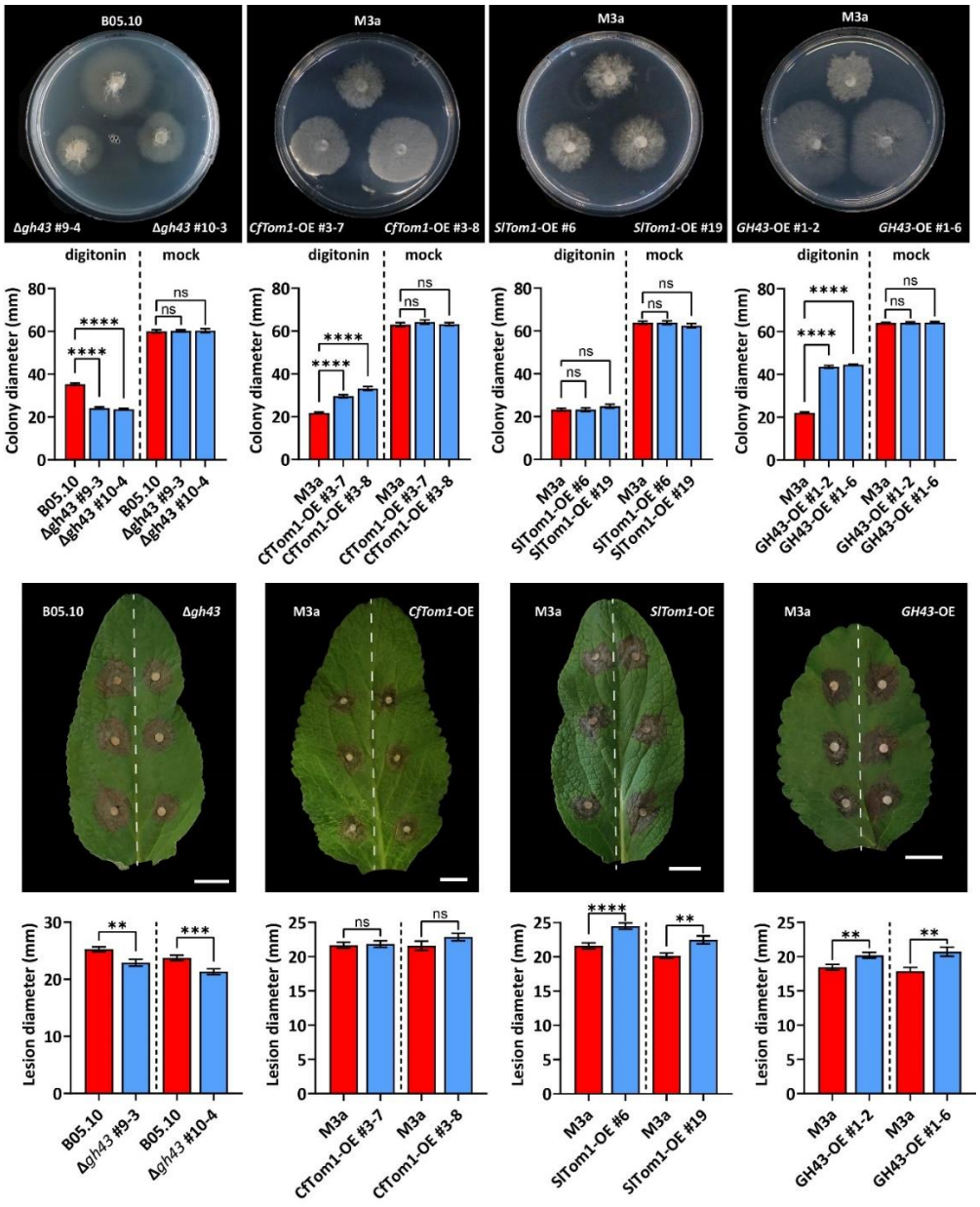


Extended Data 4. Phylogenetic tree of BcTom1 and orthologs in fungi, retrieved from Ensembl Fungi (accessed on July 28, 2023).

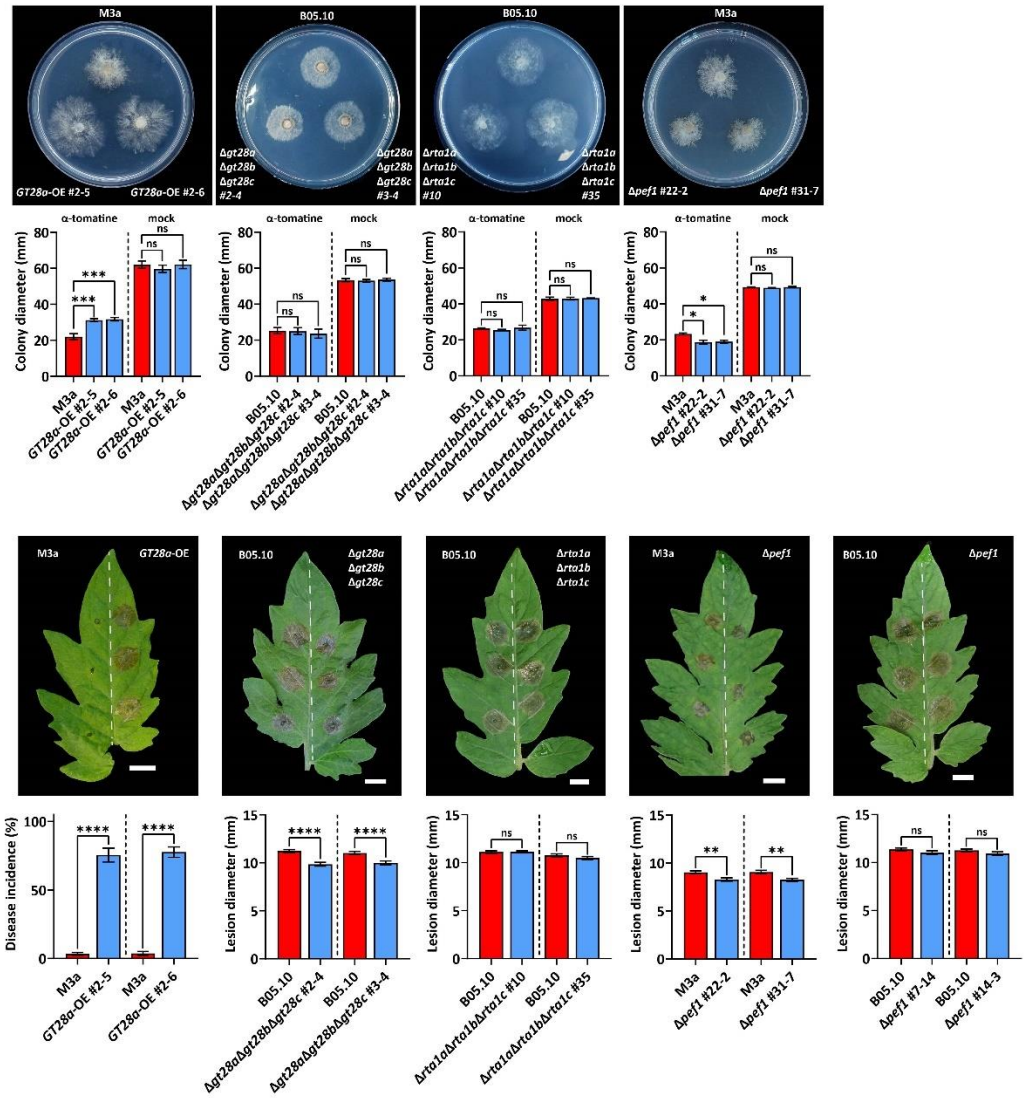


723

724 **Extended Data 5.** Modes of action on α-tomatine of three glycosyl hydrolases from different CAZyme families, that
725 were used for overexpression in *B. cinerea* isolate M3a.



Extended Data 6. Phenotypic characterization of tomatinase gene transformants.
A-D. Sensitivity to digitonin in mycelial growth assays. A. Comparison of B05.10 with Δ*BcTom*1 mutant. B-D. Comparison of M3a with transformants expressing *C. fulvum* CfTom1 (B), *S. lycopersici* SlTom1 (C), or BcTom1 (D), respectively. Mycelium growth was on plates containing 20 µM digitonin or medium lacking digitonin (mock), colony diameters were measured at 3 dpi. The asterisks represent statistically significant differences determined by a student's t-test (**** p-value <0.0001; ns indicates no significant difference).
E-H. Infection on *D. purpurea* leaves. E. Comparison of B05.10 and Δ*BcTom*1 mutant. F-H. Comparison of M3a with transformants expressing *C. fulvum* CfTom1 (F), *S. lycopersici* SlTom1 (G), or BcTom1 (H), respectively. Wild type recipient was inoculated on the left side of the central vein (depicted by a dashed line), while transformants were inoculated on the right side of the vein. Scale bar indicates 2 cm. Error bars represent SEM of at least two independent assays. The lesion diameters at 4 dpi are presented in mm. Asterisks represent statistically significant differences determined by a student's t-test (** p-value < 0.01; *** p-value <0.005; **** p-value <0.0001; ns indicates no significant difference).



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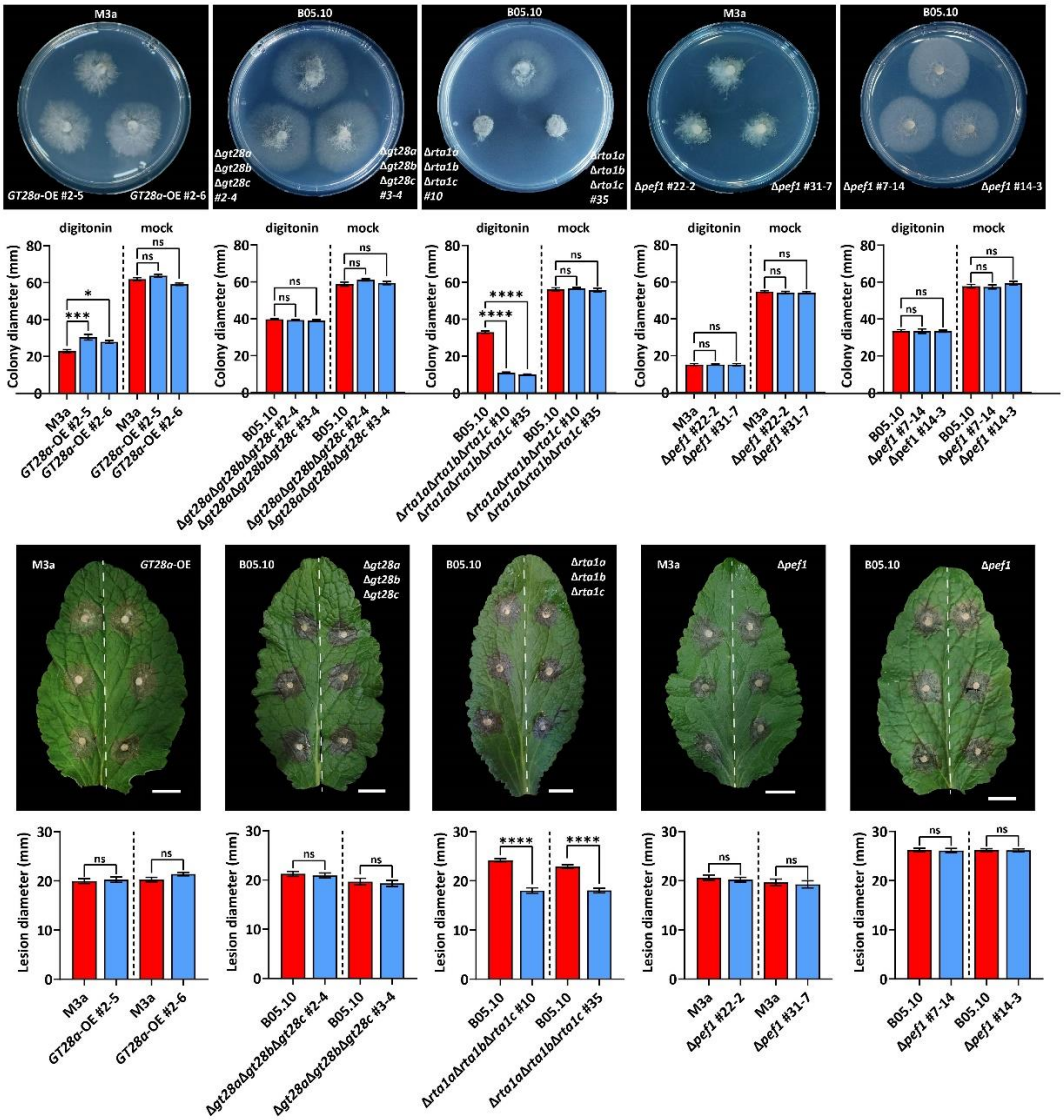
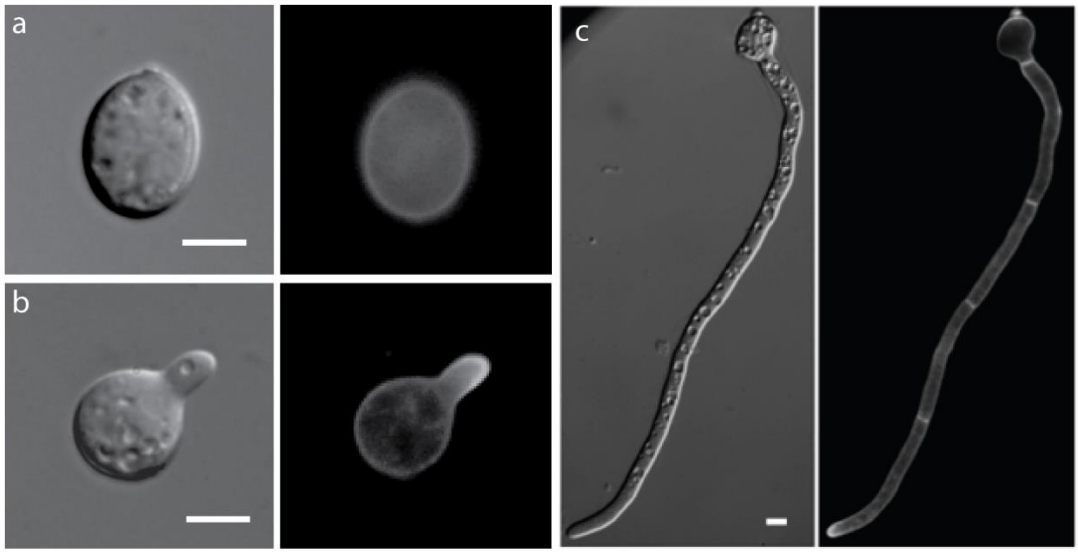
742 **Extended Data 7.** Phenotypic characterization of *B. cinerea* transformants in α-tomatine-responsive genes involved
743 in non-hydrolytic tolerance mechanisms.
744 **A-D.** Sensitivity to α-tomatine in mycelial growth assays. A. Comparison of M3a with GT28a-OE transformant. B-D.
745 Comparison of B05.10 with mutants deleted in three GT28 genes (B), three RTA1 genes (C), or in the *Bcpef*1 gene
746 (D), respectively. Mycelium growth was on plates containing 800 µM α-tomatine or medium lacking α-tomatine
747 (mock), colony diameters were measured at 3 dpi. The asterisks represent statistically significant differences
748 determined by a student's t-test (* p-value < 0.05; *** p-value <0.005; ns indicates no significant difference).
749 **E-I.** Infection on tomato leaves. WT strains were inoculated on the left side of the vein (as depicted by the dashed
750 line). Transformant strains were inoculated on the right side of the vein. E. Comparison of M3a with GT28a-OE
751 transformant. F-I. Comparison of B05.10 with mutants deleted in three GT28 genes (F), three RTA1 genes (G), or in
752 the *Bcpef*1 gene (I), or comparison of M3a deleted in the *Bcpef*1 gene (H), respectively. Scale bar indicates 1 cm.
753 Error bars represent SEM of at least two independent inoculation assays. The lesion diameters at 3 dpi are
754 presented in mm. Asterisks represent statistically significant differences determined by a student's t-test (** p-
755 value < 0.01; *** p-value <0.005; **** p-value <0.001; ns indicates no significant difference).



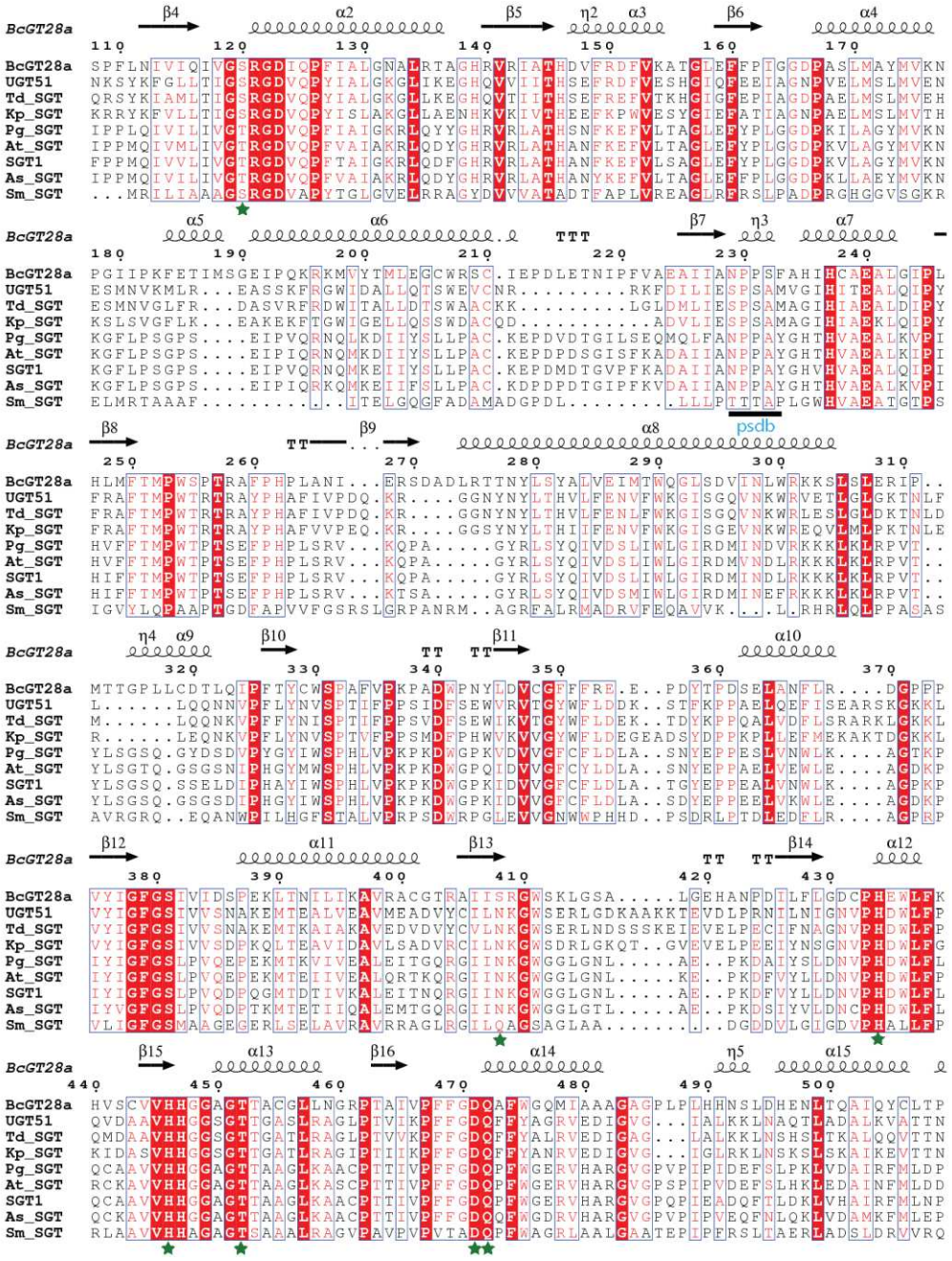
Extended Data 8. Phenotypic characterization of *B. cinerea* transformants in α-tomatine-responsive genes involved in non-hydrolytic tolerance mechanisms.

A-D. Sensitivity to digitonin in mycelial growth assays. A. Comparison of M3a with GT28a-OE transformant. B-D. Comparison of B05.10 with mutants deleted in three GT28 genes (B), three RTA1 genes (C), or in the *Bcpef*1 gene (E), or M3a mutant deleted in the *Bcpef*1 gene (D), respectively. Mycelium growth was on plates containing 20 µM digitonin or medium lacking digitonin (mock), colony diameters were measured at 3 dpi. The asterisks represent statistically significant differences determined by a student's t-test (* p-value < 0.05; *** p-value < 0.005;**** p-value <0.001; ns indicates no significant difference).

F-J. Infection on *D. purpurea* leaves. WT strains were inoculated on the left side of the vein (as depicted by the dashed line). Transformant strains were inoculated on the right side of the vein. F. Comparison of M3a with GT28a-OE transformant. G-J. Comparison of B05.10 with mutants deleted in three GT28 genes (G), three RTA1 genes (H), or in the *Bcpef*1 gene (J), or comparison of M3a deleted in the *Bcpef*1 gene (I), respectively. Scale bar indicates 2 cm. Error bars represent SEM of at least two independent inoculation assays. The lesion diameters at 4 dpi are presented in mm. Asterisks represent statistically significant differences determined by a student's t-test (**** p-value <0.001; ns indicates no significant difference).



Extended Data 9: Filipin staining of *B. cinerea* spores (a), germlings (b) and hyphae (c). Fluorescence reveals that ergosterol accumulates predominantly in the tips of germ tubes and hyphae and in the septa. The scale bar indicates a distance of 5 µm.



Extended Data 10: Sequence alignment of SGTs from different organisms. Conserved residues are shaded in red, while blue boxes indicate amino acids with similar physicochemical properties. Secondary structure elements are represented by flat arrows for β sheets and a helical arrow for α-helices. The putative steroid binding domain (psdb) and UDPG binding pockets (highlighted with green stars) are described in Chen et al. (2018). The following abbreviations and corresponding organisms were used: BcGT28a (*Botrytis cinerea B05.10*, A0A384JNQ5), UGT51 (*Saccharomyces cerevisiae* s288c, AAB67475.1), Td_SGT (*Torulaspora delbrueckii*, G8ZQN5), Kp_SGT (*Komagataella pastoris*, AAD29570.1), Pg_SGT (*Panax ginseng*, BAC22616.1), At_SGT (*Arabidopsis thaliana*, AAF27006.1), SGT1 (*Solanum lycopersicum*, A0A3Q7HJ64), As_SGT (*Avena sativa*, CAB06081.1), Sm_SGT (*Streptomyces malaysiense*, OIK24026.1).

Supplementary Files

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