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Authors

Eichfeld, Ruben

Mahdi, Lisa K

De Quattro, Concetta

et al.

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Research

New Phytologist

Transcriptomics reveal a mechanism of niche defense: two beneficial root endophytes deploy an antimicrobial GH18-CBM5 chitinase to protect their hosts

Ruben Eichfeld[1,2], Lisa K. Mahdi[1], Concetta De Quattro[1,2], Laura Armbruster[1,2], Asmamaw B. Endeshaw[1], Shingo Miyauchi[1,3], Margareta J. Hellmann[4], Stefan Cord-Landwehr[4], Daniel Peterson[5], Vasanth Singan[5], Kathleen Lail[5], Emily Savage[5], Vivian Ng[5], Igor V. Grigoriev[5,6], Gregor Langen[1], Bruno M. Moerschbacher[4] and Alga Zuccaro[1,2]

[1]University of Cologne, Institute for Plant Sciences, Cologne, 50674, Germany; [2]Cluster of Excellence on Plant Sciences (CEPLAS), Cologne, 50674, Germany; [3]Okinawa Institute of Science and Technology Graduate University, Onna, Okinawa, 904-0495, Japan; [4]Institute for Biology and Biotechnology of Plants, University of Münster, Münster, 48149, Germany; [5]U.S. Department of Energy Joint Genome Institute, Lawrence Berkeley National Laboratory, Berkeley, CA 94720, USA; [6]Department of Plant and Microbial Biology, University of California Berkeley, Berkeley, CA 94720, USA

Author for correspondence:
Alga Zuccaro
Email: azuccaro@uni-koeln.de

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Key words: beneficial endophytes, chitinase, fungal effectors, niche defense, root colonization, Sebacinales, time-resolved transcriptomics.

Summary

- Effector secretion is crucial for root endophytes to establish and protect their ecological niche.
- We used time-resolved transcriptomics to monitor effector gene expression dynamics in two closely related Sebacinales, *Serendipita indica* and *Serendipita vermifera*, during symbiosis with three plant species, competition with the phytopathogenic fungus *Bipolaris sorokiniana*, and cooperation with root-associated bacteria.
- We observed increased effector gene expression in response to biotic interactions, particularly with plants, indicating their importance in host colonization. Some effectors responded to both plants and microbes, suggesting dual roles in intermicrobial competition and plant–microbe interactions. A subset of putative antimicrobial effectors, including a GH18-CBM5 chitinase, was induced exclusively by microbes. Functional analyses of this chitinase revealed its antimicrobial and plant-protective properties.
- We conclude that dynamic effector gene expression underpins the ability of Sebacinales to thrive in diverse ecological niches with a single fungal chitinase contributing substantially to niche defense.

Introduction

Beneficial root-endophytic fungi are major players within the consortia of plant-associated microorganisms collectively referred to as 'plant microbiota' (Glynou *et al.*, 2016, 2018; Trivedi *et al.*, 2020; Mahdi *et al.*, 2022). While the composition of plant microbiota varies between different host species and depends on environmental factors (Tkacz *et al.*, 2015; Strullu-Derrien *et al.*, 2018), a balanced microbiota contributes to plant performance by improving host nutrient uptake and increasing resistance to biotic and abiotic stress (Raaijmakers *et al.*, 2009; Hermosa *et al.*, 2012; Finkel *et al.*, 2017; Mahdi *et al.*, 2022). Beneficial properties have been observed in plant interactions with ectomycorrhizal (ECM) and arbuscular mycorrhizal (AM) fungi, as well as fungal endophytes (Zuccaro *et al.*, 2014). These symbiotic interactions have evolved over millions of years, giving rise to fine-tuned relationships not only between fungi and their host plants but also among the various members of the microbiota (Mesny *et al.*, 2023). The health of plants is directly influenced by intermicrobial relationships. This is illustrated by microorganisms that manifest high pathogenic potential in mono-associations but are effectively restrained in a microbial community context (Sarkar *et al.*, 2019; Mesny *et al.*, 2021; Mahdi *et al.*, 2022). Host priming, along with direct intermicrobial competition and cooperation, collectively contribute to this phenomenon.

Root endophytes of the order Sebacinales, notably *Serendipita indica* (*Si*) and *Serendipita vermifera* (*Sv*), provide protection to various plant species against the aggressive phytopathogenic fungus *Bipolaris sorokiniana* (*Bs*) (Kumar *et al.*, 2002; Sarkar *et al.*, 2019; Y. Li *et al.*, 2023). This well-adapted cereal pathogen poses a significant threat, causing diseases such as common root rot and spot blotch, which lead to substantial yield losses, particularly in warmer agricultural regions (Kumar *et al.*, 2002). Recent research has shown that Sebacinales not only protect plants directly but also cooperate with other microbiota

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members. Notably, *Sv* has been shown to act synergistically with beneficial bacteria in the plant microbiota to enhance the protection of barley and Arabidopsis roots against *Bs* (Mahdi *et al.*, 2022).

Through a split-root system, we previously demonstrated that *Sv* reduces *Bs* biomass in barley roots through a combination of systemic and local effects. These effects did not depend on extensive host transcriptional reprogramming but correlated with the downregulation of the phytopathogen effector repertoire *in planta*. Direct fungal confrontation experiments in soil revealed an induction of hydrolytic enzymes and effectors in *Sv* in the presence of *Bs*. Such an antagonistic response was not activated during the tripartite interaction in barley roots. This indicates that *Sv* effectors reduce the virulence potential of *Bs* in the rhizosphere before host colonization, enabling the endophyte to act as a host-protective barrier against the phytopathogenic intruder (Sarkar *et al.*, 2019). A similar capacity for effector-induced host microbiota manipulation has been reported in pathogenic fungi. The soilborne fungus *Verticillium dahliae*, for instance, secretes the antimicrobial effector *Vd*Ave1, which suppresses antagonistic bacteria and thereby facilitates the infection of tomato plants (Snelders *et al.*, 2020, 2023). While effectors were originally described as small-secreted proteins (SSPs), which suppress plant immunity and manipulate host metabolism in order to promote microbial colonization and reproduction (De Wit *et al.*, 2009), these recent findings call for an expansion of the traditional effector concept toward supporting a role of effector secretion in shaping the niche (Veneault-Fourrey & Martin, 2011; Hemetsberger *et al.*, 2012; Win *et al.*, 2012; Lo Presti *et al.*, 2015; Snelders *et al.*, 2022).

Sv expresses distinct sets of effectors during bipartite confrontation with *Bs* in soil and tripartite interactions with barley. As of now, it remains unclear whether this differential expression of effectors is linked to diverging functions in host colonization and niche defense. The genomes of *Sv* and its close relative *Serendipita indica* (*Si*) encompass large repertoires of genes encoding for proteins involved in carbohydrate binding, plant cell wall degradation, and protein hydrolysis, as well as numerous SSPs with effector-like properties (Zuccaro *et al.*, 2011, 2014). While for some of these proteins, roles in the evasion and suppression of plant immunity have been reported, the function of most of these proteins remains elusive (Jacobs *et al.*, 2011; Lahrmann *et al.*, 2013). In this study, we conducted a time-resolved transcriptomic analysis of *Si* and *Sv*, examining their transcriptional responses when exposed to monocot and dicot host plants, the phytopathogen *Bs*, or a synthetic community of beneficial root-associated bacteria. We investigated the expression profiles of putative effector genes with a focus on SSPs and carbohydrate-active enzymes (CAZymes). Our aim was to identify Sebacinales effectors induced specifically in response to host plants and/or microbes in order to discern their roles in host colonization and niche protection. We identified a GH18-CBM5 chitinase that was specifically induced in response to *Bs* in both *Si* and *Sv* and characterized the enzyme as a novel antimicrobial effector of Sebacinales involved in plant protection through fungal antagonism in the rhizosphere.

Material and Methods

Plant, fungal, and bacterial materials

Hordeum vulgare (*Hv*, L. cv Golden Promise), *Brachypodium distachyon* (*Bd*, Bd21-3), and *Arabidopsis thaliana* (*At*, Col-0) were used as plant hosts. *Serendipita vermifera* (*Sv*; MAFF305830), *Serendipita indica* (*Si*; DSM11827), and *Bipolaris sorokiniana* (*Bs*; ND90Pr) were used as fungal models. The bacterial SynCom consists of four taxonomically diverse strains from the *At*Sphere collection (R11, R172, R189, and R935) which were described previously (Mahdi *et al.*, 2022).

Growth conditions and microbial inoculations

Hv and *At* seeds were sterilized and germinated as previously described (Mahdi *et al.*, 2022). *Bd* seeds were sterilized in 3% sodium hypochlorite and 0.1% Triton-X for 30 min under constant shaking and then washed four times with sterile water every 15 min. Seeds were stratified for 10 d in darkness at 4°C on wet filter paper and subsequently transferred to sterile glass vials containing 1/10 PNM (Plant Nutrition Medium, pH 5.7) for germination on a day–night cycle of 16 h : 8 h at 22°C : 18°C, 60% humidity, and a light intensity of 108 μmol m^{-2} s^{-1} for 8 d. *Sv* was propagated on MYP medium (Lahrmann *et al.*, 2015), *Si* on CM medium (Hilbert *et al.*, 2012), and *Bs* on modified CM medium (Sarkar *et al.*, 2019), each containing 1.5% agar, at 28°C in darkness for 21 (*Si* and *Sv*) and 14 (*Bs*) d, respectively. Mycelial suspensions of *Si and Sv* and spore suspensions of *Bs* were prepared as described previously (Hilbert *et al.*, 2012; Sarkar *et al.*, 2019). Bacteria were grown in liquid TSB medium (Sigma-Aldrich; 15 g l^{-1}) at 28°C in the dark at 220 rpm for 1–3 d depending on the growth rate. Bacterial suspensions were prepared as described previously (Mahdi *et al.*, 2022). Plant roots were inoculated on 12 × 12 cm Petri dishes (*At*) or sterile glass jars (*Hv* and *Bd*) containing 1/10 PNM with *Si* or *Sv* mycelium (0.12 g for *Hv* and *Bd* or 0.02 g for *At*), or sterile water as control. Microbe–microbe confrontation experiments were performed on Petri dishes containing 1/10 PNM. Plates were inoculated with (a) a pure suspension of *Sv* or *Si* mycelium (0.08 g), (b) a mixed suspension of *Sv* or *Si* mycelium with *Bs* spores (10.000 spores), or (c) a mixed suspension of *Si* or *Sv* with the bacterial SynCom (2 ml at $OD_{600} = 0.01$). All samples were kept on a day–night cycle of 16 h : 8 h at 22°C : 18°C, 60% humidity, and 108 μmol m^{-2} s^{-1} light intensity for 1, 3, 6, and 10 d postinoculation (dpi). Samples for microbial confrontation were collected by scraping the fungal and bacterial material from the plate surface. Plant roots of all species were washed in MilliQ water to remove extraradical fungal hyphae. All samples were snap-frozen in liquid nitrogen and used for RNA extraction. For plant protection assays in *At* or *Hv*, plants were co-inoculated with *Si* mycelium (0.02 or 0.12 g, respectively) and *Bs* spores (5.000 spores per plate or 15.000 spores per jar respectively). Plants were grown for 6 d before harvesting. Pulse amplitude modulation (PAM) fluorometry was used to assess disease symptoms in *At* (Mahdi *et al.*, 2022). Fungal colonization was

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quantified in *At* and *Hv* by reverse transcription quantitative polymerase chain reaction using the primers listed in Supporting Information Table S1. Pathogenicity assays were carried out with four to nine independent biological replicates with 4 technical replicates for *Hv* and 10 technical replicates for *At*.

RNA extraction for RNA-seq analysis

RNA was extracted as described previously (Sarkar *et al.*, 2019). RNA sequencing was performed at the US Department of Energy Joint Genome Institute (JGI) under a project proposal (ID: 505829; Zuccaro, 2020). Stranded RNA-seq libraries were generated and quantified by reverse transcription quantitative polymerase chain reaction. The sequencing was performed with Illumina technology in 151PE mode. Raw reads were filtered and trimmed using the JGI QC pipeline. BBDuk was used to filter raw reads for artifact sequences by kmer matching (kmer = 25), allowing one mismatch. Detected artifacts were trimmed at the 3′ end. RNA spike-in reads, PhiX reads, and reads containing NS were removed. Quality trimming was performed using phred trimming set at Q6. After trimming, the reads with a length below 25 bases or one third of the original read length were removed – whichever is longer. Filtered reads from each library were aligned to the *S. vermifera* MAFF 305830 v.1.0 or *S. indica* DSM 11827 reference genomes downloaded from Mycocosm (https://mycocosm.jgi.doe.gov/mycocosm/home) using HISAT2 v.2.2.0. The raw gene counts were generated using FEATURECOUNTS and the *Si* and *Sv* gff3 annotations. Only primary hits assigned to the reverse strand were included in the raw gene counts. In the principal component analysis (PCA), samples with low numbers of reads (< 100 000 for *Si* and < 20 000 for *Sv*) were not considered. Subsequently, genes with less than a total of 10 raw counts across all samples were filtered out. After the filtering, raw counts were normalized with the DESeq rlog transformations and PCA plot were drawn with the plotPCA function and customized with ggplot2. Raw counts can be accessed via the bioproject number (Table S2).

Differential gene expression analyses

The proportion of reads assigned to organisms per RNA-seq sample was examined. The consistency of normalized transcription for the biological replicates was confirmed by assessing the distribution of the number of genes and then the correlation of the biological replicates. Spearman's rank correlation was calculated using the normalized number of genes of all biological replicates. Transcript counts of genes were normalized using the R package DESEQ2 (Love *et al.*, 2014) and then $\log_2$ transformed. Significant differentially expressed genes (DEGs) specific to conditions (> 2 $\log_2$FC; FDR-adjusted $P < 0.05$) were visualized using the R package UPSETR (Conway *et al.*, 2017). Heatmaps were generated with the COMPLEXHEATMAP package in R. K-means clustering was performed with the kmeans function in R setting the number of cluster to be generated to three. Functional annotations of the *S. indica* and *S. vermifera* genomes were downloaded from Mycocosm, Joint Genome Institute (https://mycocosm.jgi.doe.gov/mycocosm/home). Gene Ontology (GO) enrichment analysis was performed using the function enricher of ClusterProfiler setting the pvalueCutoff = 1.

Chitinase expression and purification in *E. coli*

The coding sequences of *SiCHIT* and *SvCHIT* were amplified using the primers listed in Table S1 and cloned into an expression vector (pQE-80 l; Qiagen, Hilden, Germany). Protein production was induced with IPTG in *E. coli* Mach1 cells, and the proteins were purified by affinity chromatography via a N-terminal 6 × His-tag. For a detailed protocol, see Methods S2.

Chitin azure assay

Chitin azure (Sigma Aldrich) was adjusted to 4 mg ml^{-1} in 50 mM phosphate buffer (pH 6), and 100 µl were added to 2 ml reaction tubes. Recombinant protein was added to a final concentration of 5 µM in 200 µl. The samples were incubated at 28°C and 120 rpm overnight. Next, samples were boiled at 95°C for 5 min and centrifuged at 16 200 ***g*** for 10 min and supernatants were transferred to a 96 well plate. Absorption was measured at 560 nm.

Bs spore germination assay

Bs spores were isolated as previously described (Sarkar *et al.*, 2019) and diluted in TSB medium to a final concentration of 125 000 spores ml^{-1}. Recombinant protein was added to a final concentration of 5 µM, filled into 8 well chamber slides (VWR), and incubated for 8 h at 28°C. The germination rate of three independent replicates was quantified by noninvasive counting using an inverted microscope.

In planta protection assays

To measure protection of *At* from *Bs*, *At* seeds were sterilized and germinated as described above. After transferring 5-d-old seedlings to 1/10 PNM plates, they were inoculated with *Si* (0.02 g mycelium), *Bs* (5000 spores), or both fungi together. Four independent biological replicates with each 10 technical replicates were used. To measure the protective role of *Si*CHIT, *Bs* spores were incubated in 50 mM phosphate buffer (pH 6.0) overnight with or without (Mock) 5 µM *Si*CHIT the day before plant inoculation. Five days after inoculation with *Bs*, seedlings were transferred into 24-well plates with water and PAM fluorometry was measured over 7 d (Dunken *et al.*, 2022). Four independent biological replicates were used. For *Hv* inoculation, *Bs* spores were treated with recombinant enzyme as described above before root inoculation. After 4 d, *Hv* plants were harvested and roots were weighed after washing. Colonization of *Bs* was assessed by reverse transcription quantitative polymerase chain reaction following RNA extraction and cDNA synthesis as described previously (Sarkar *et al.*, 2019). Four to six independent biological replicates with each four technical replicates were used.

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Results

Root-associated bacteria trigger minimal transcriptional changes in Sebacinales compared with plant hosts and the phytopathogenic fungus *Bs*

To investigate the molecular mechanisms of how *Si* and *Sv* interact with a wide range of organisms from different kingdoms, we generated an RNA-seq dataset covering bipartite interactions of *Si* or *Sv* with the host plants *Hordeum vulgare* (*Hv*), *Brachypodium distachyon* (*Bd*) and *Arabidopsis thaliana* (*At*), as well as with the plant pathogen *B. sorokiniana* (*Bs*) or a bacterial synthetic community (SynCom) consisting of four taxonomically distinct bacteria derived from *At* roots and present in the *At*Sphere collection (Bai *et al.*, 2015). In co-culture with Sebacinales, these bacteria (R11, R172, R189, and R935) are tightly associated with the fungal glucan matrix (Mahdi *et al.*, 2022). The confrontation of *Si* or *Sv* with *Bs* or the bacterial SynCom was conducted in the absence of host plants, elucidating specific changes driven solely by the presence of microbial cooperation or antagonistic partners. We addressed possible temporal differences in the establishment of interaction stages by including samples collected at four different time points postinoculation (Fig. 1a). To assess the similarity between the treatments, we conducted a PCA. We found that the transcriptional profiles of *Si* and, to a lesser extent, *Sv* separated into three groups based on their interaction partners: plant hosts (green), the bacterial SynCom (red), and the phytopathogenic fungus *Bs* (orange) (Fig. 1b).

To further investigate the gene expression changes induced in *Si* and *Sv* during biotic interactions, we performed a differential gene expression analysis. When comparing transcriptional patterns between axenically cultured fungi and fungi challenged with hosts or microbes, we found a total of 4838 (*Si*) or 5606 (*Sv*) genes, which were differentially expressed (> 2 $\log_2$FC or < -2 $\log_2$FC, adjusted P-value < 0.05) in at least one of the interactions at one or more time points. These DEGs accounted for 40% of annotated *Si* and 37% of annotated *Sv* genes. Since we were interested in effector expression dynamics, we focused our analysis on upregulated genes with a $\log_2$FC > 2 (2999 *Si* and 2185 *Sv* genes). To identify commonalities and differences in gene expression during the interaction with host plants and microbes, we collapsed the significantly upregulated genes at different time points for each treatment (Fig. 1c).

While both *Si* and *Sv* responded to all host plants and to the phytopathogen *Bs* with extensive transcriptional alterations, the Sebacinales displayed only minor transcriptional changes in the presence of bacteria despite their close physical association. This observation is consistent with the notion that most fungal responses to beneficial, neutral, or antagonistic bacteria are attenuated within a few hours of initial contact (Mela *et al.*, 2011; Deveau *et al.*, 2015; Satterlee *et al.*, 2022).

Sebacinales express a core set of genes in response to monocot and dicot hosts

The responses of *Si* and *Sv* to the three plant hosts largely overlapped, with 837 (*Si*) and 393 (*Sv*) genes upregulated in the presence of all three hosts. These accounted for 31% and 19% of all 2676 (*Si*) or 2038 (*Sv*) plant-inducible genes, respectively (Table S3). This suggests that exposure of *Si* and *Sv* to different plant species triggered the expression of a set of core genes required for host colonization in both monocots and dicots. One of these genes encodes the nuclease *NucA* (Pirin1_72917; PIIN_02121 for *Si*, and Sebve1_52856 for *Sv*), which acts synergistically with the nucleotidase E5′NT (Pirin1_71782; PIIN_01005 for *Si* and Sebve1_17 804 for *Sv*) in the suppression of immunity and initiation of host cell death (Nizam *et al.*, 2019; Dunken *et al.*, 2022). Moreover, intracellular colonization of all three plant hosts was associated with upregulation of fungal proteases and CAZymes. By degrading plant cell walls and host-derived proteins, these enzymes might facilitate entry into the host cell and provide a nitrogen and carbon source for the endophytes. An organic nitrogen source is particularly relevant for *Si*, as this fungus is unable to utilize nitrate as a nitrogen source (Olivieri *et al.*, 2002; Naumann *et al.*, 2011; Zuccaro *et al.*, 2011, 2014; Lahrmann & Zuccaro, 2012; Balestrini *et al.*, 2014; Jashni *et al.*, 2015a,b; Tang *et al.*, 2021; Valadares *et al.*, 2021).

Despite commonalities between responses across all plant hosts, significant sets of genes were exclusively induced in the presence of specific plant species. Several of these host species-specific genes seemed to serve similar functions. Pirin1_74456 (PIIN_03655, upregulated specifically in response to *At*) and Pirin1_80981 (PIIN_10163, upregulated specifically in response to *Bd*) for instance both encode CE4 polysaccharide deacetylases. Deacetylases can be exploited by root-associated fungi to modulate chitin in their cell walls, aiding in evading plant immunity (Rizzi *et al.*, 2021). Moreover, we were able to pinpoint enzymes that were specifically upregulated during colonization of monocots but not *At*, potentially hydrolyzing specific substrates present in monocots but absent in dicots (Figs S1, S2).

Endophytes display partially conserved transcriptional responses to host plants and the phytopathogenic fungus *Bs*

To gain more insight into the biological functions of the Sebacinales genes induced in response to host plants or microbes, we analyzed the two sets of genes separately (Fig. S3). Employing K-means analysis, we divided both sets into three clusters, representing genes upregulated either throughout the interaction, in the early or in the late stages of interaction. A GO term analysis revealed that similar processes were induced in response to microbes and plants in both fungal endophytes. These include 'carbohydrate metabolic process' (GO:0005975), 'proteolysis' (GO:0006508), and 'transport' (GO:0006810). Genes assigned to all three terms were induced, but not necessarily significantly enriched, during all stages of colonization and might relate to nutritional processes. This indicates that *Si* and *Sv* assimilate nutrients from both plant and microbial biomass. Another GO term likely related to nutrient acquisition was 'cell wall catabolic process' (GO:0016998). Interestingly, genes related to this term were strongly induced in both Sebacinales in the early phases of the response to *Bs*, but not plants. The induction of this specific set of genes could be interpreted as a sign of mycoparasitism.

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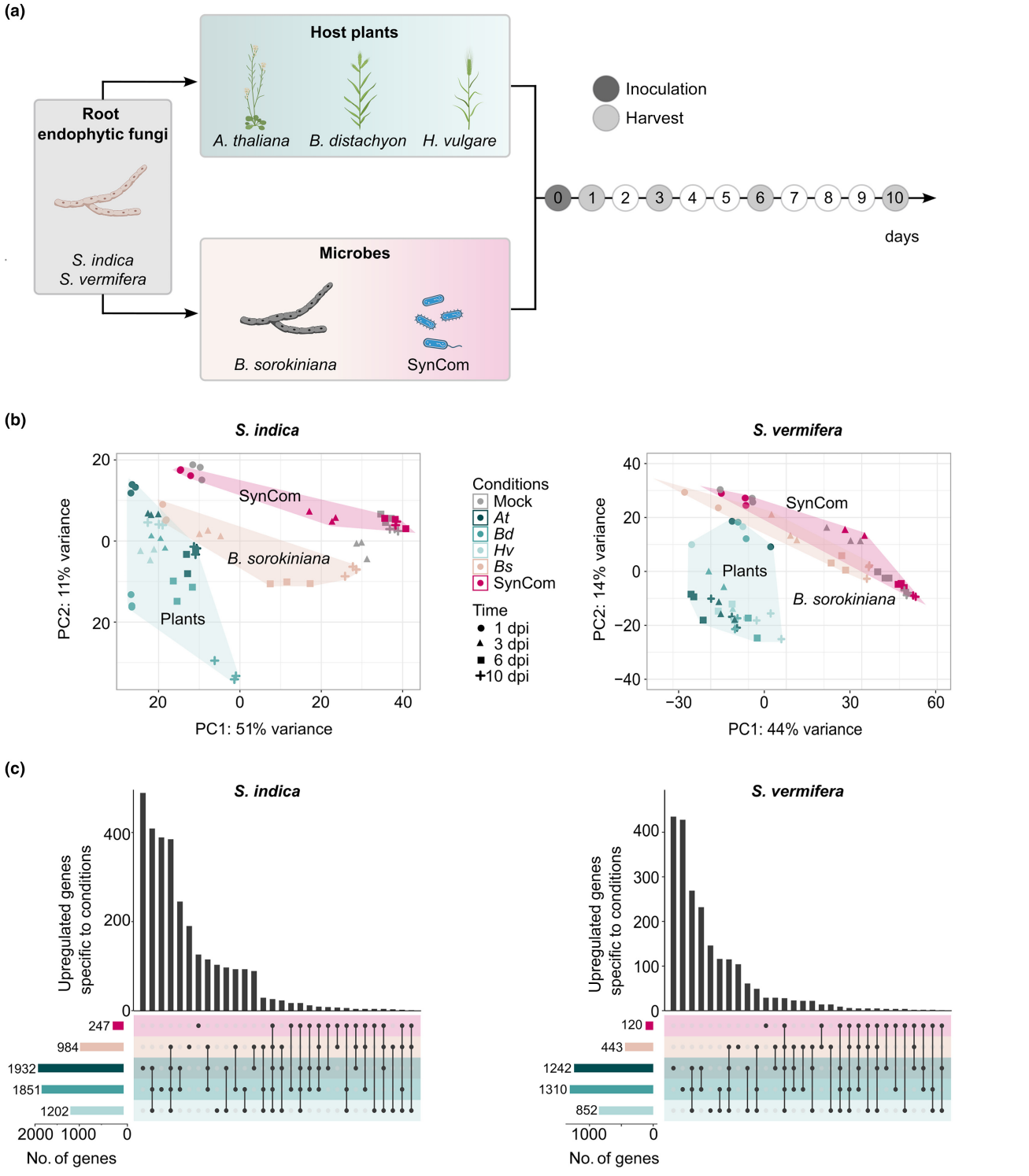

Fig. 1 Transcriptional response of *Serendipita indica* and *Serendipita vermifera* to different interaction partners. (a) Schematic overview of the experimental setup. Bipartite interactions between *S. indica* (*Si*) or *S. vermifera* (*Sv*) and the host plants *Arabidopsis thaliana* (*At*), *Brachypodium distachyon* (*Bd*), and *Hordeum vulgare* (*Hv*) or the microbes *Bipolaris sorokiniana* (*Bs*) and a synthetic bacterial community (SynCom) at different days postinoculation (dpi). Confrontation of *Si* or *Sv* with *Bs* or the SynCom was performed in absence of host plants. (b) Principal component analysis (PCA) plots comprising the top 500 most variable genes of *Si* (left) and *Sv* (right) in response to the different interaction partners across all time points. Transcriptomic responses to host plants, the SynCom, and *Bs* are highlighted with green, dark red, and orange backgrounds, respectively. (c) UpSet plot of upregulated genes (FDR-adjusted *P*-value < 0.05 and $\log_2 FC > 2$) aggregated across all time points in *Si* (left) and *Sv* (right).

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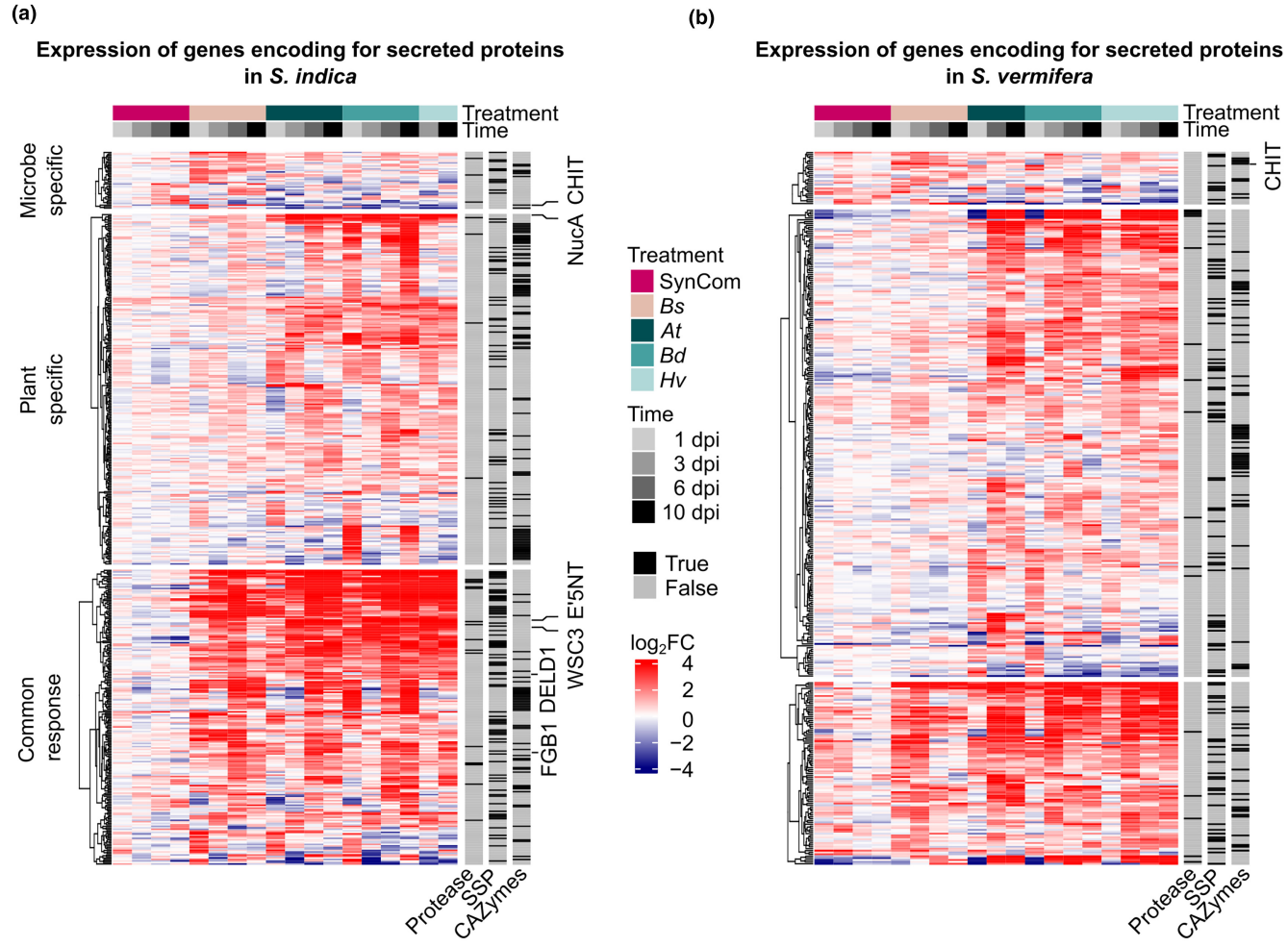

Fig. 2 Interaction partner specific expression patterns of putative effector-coding genes in (a) *Serendipita indica* (*Si*) and (b) *Serendipita vermifera* (*Sv*). Genes encoding for secreted proteins were identified through the Predector pipeline ($n = 1183$ for *Si* and 1434 for *Sv*). Of these genes, 467 (*Si*) or 373 (*Sv*) were upregulated significantly (FDR-adjusted *P*-value < 0.05 and $\log_2FC > 2$) in response to at least one biotic interaction partner at one or more time points (dpi: days postinoculation). These genes were annotated as 'proteases' or 'small secreted proteins' (< 300 amino acids) by the pipeline described by Pellegrin *et al*. (2015) or in case of Carbohydrate-active enzymes ('CAZymes') by the Predector pipeline. The clustering was performed separately for genes upregulated specifically in response to microbes (top), plants (center), or both (bottom). Effectors with characterized functions were marked on the right (NucA: Nizam *et al*., 2019; WSC3 and FGB1: Wawra *et al*., 2019; E'5NT: Dunken *et al*., 2022; DELD1: Nostadt *et al*., 2020).

Besides a large overlap of upregulated genes between all plant hosts and specific genes induced in response to *Bs*, we identified a set of commonly upregulated genes in response to both plants and *Bs* (787 genes in *Si*; 291 genes in *Sv*). These genes accounted for 80 and 66% of the total *Bs* inducible genes in *Si* and *Sv*, suggesting underlying mechanistic parallels in the interaction of Sebacinales with plants and fungi.

Sebacinales induce specific sets of effector candidates in response to host plants or microbes

To investigate whether host colonization and intermicrobial interactions require the expression of specific sets of effector genes, we identified putatively secreted proteins in *Si* and *Sv* with the Predector pipeline (Bendtsen *et al*., 2004; Sperschneider *et al*., 2016, 2018a,b; Almagro Armenteros *et al*., 2019; Kristiansingsih & MacLean, 2021; Sperschneider & Dodds, 2022; Teufel *et al*., 2022) (1183 in *Si* and 1434 in *Sv*). A substantial share of these putative effector genes (467, 40% in *Si*, and 373, 26% in *Sv*) was significantly upregulated (> 2 log2FC, adjusted *P*-value < 0.05) in response to at least one biotic interaction partner at one or more time points (Fig. 2). The vast majority of these genes were specifically upregulated in response to host plants (233, 50% in *Si*, and 248, 66% in *Sv*) or induced by both plants and microbes (196, 42% in *Si*, and 97, 26% in *Sv*). A smaller proportion (38 or 28; 8% in both, *Si* and *Sv*) of the putative effector genes were specifically induced by microbes.

GH18-CBM5 chitinases are exclusive to the Basidiomycota within the fungal kingdom

In both *Si* and *Sv*, a chitinase from the GH18 family with a CBM5 carbohydrate-binding motif emerged as one of the most strongly upregulated genes specifically in response to *Bs* (Fig. 3a).

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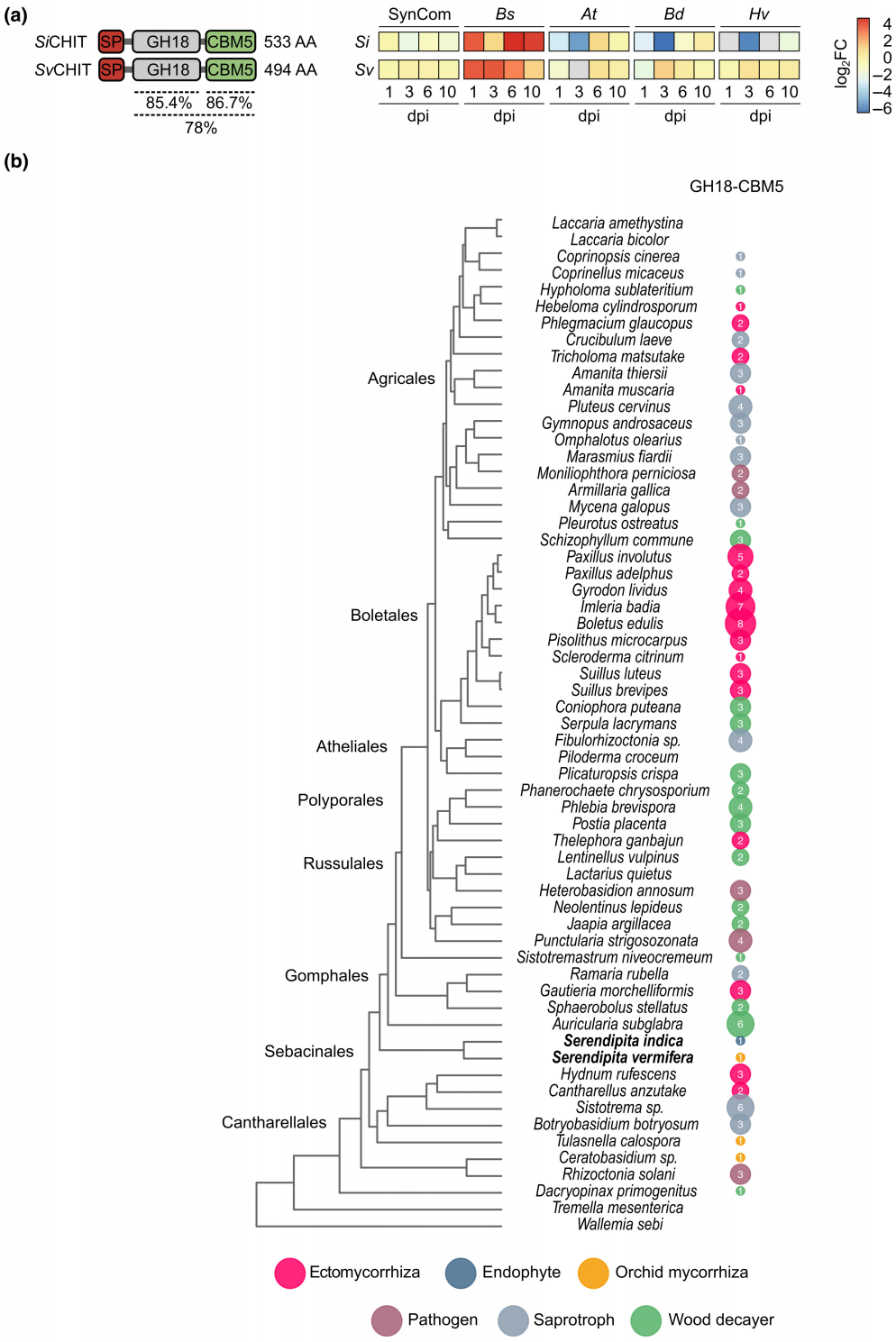

Fig. 3 GH18-CBM5 chitinases are widespread among the Basidiomycota independent of their lifestyle. (a) Domain architecture and expression pattern of *Si*CHIT and *Sv*CHIT during different biotic interactions at various time points (dpi: days postinoculation). Percentages show the sequence similarity. (b) Occurrence of GH18-CBM5 chitinases in different Basidiomycota with varying lifestyles.

Fungal nutrient acquisition heavily relies on the secretion of CAZymes, particularly in breaking down soil organic matter and the cell walls of living plants and other fungi (Auer *et al.*, 2023). Fungi across different divisions express an array of GH18 chitinases, each playing diverse roles in fungal development, nutrient uptake, and interactions with other organisms (Ihrmark *et al.*, 2010; Chen *et al.*, 2020). Here, we investigated the distribution of GH18 chitinases across 135 distantly related fungal species with different lifestyles, spanning Ascomycota, Mucoromycota, and Basidiomycota (Fig. S4; Table S4; Methods S1). Within the fungal kingdom, chitinases featuring both a GH18 and a CBM5 domain are solely present in the Basidiomycota (Fig. S4). Within the Basidiomycota, the occurrence of GH18-CBM5 chitinases is not related to fungal lifestyle, as GH18-CBM5 chitinases are found in saprotrophic as well as beneficial and phytopathogenic fungi. In addition, the GH18-CBM5 copy

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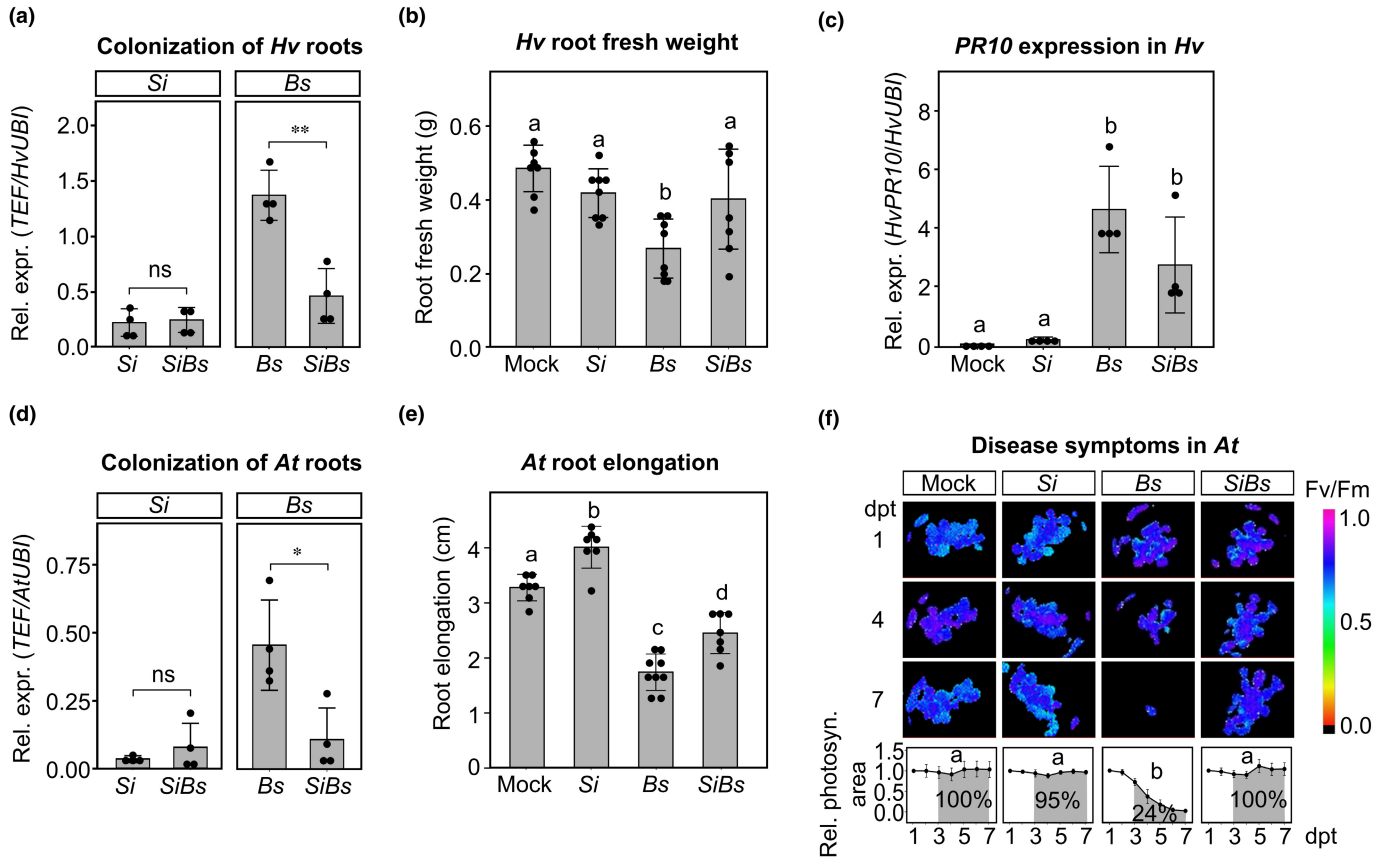

Fig. 4 Plant-protective ability of *Serendipita indica* (*Si*) in barley (*Hv*) (a–c) and *Arabidopsis thaliana* (*At*) (d–f). (a) *Si* and *Bipolaris sorokiniana* (*Bs*) colonization at 6 d postinoculation (dpi) in barley roots inferred from the relative expression of the fungal housekeeping gene *TEF* compared with the barley ubiquitin (*HvUBI*) gene. For each replicate ($n = 4$), four plants were pooled. (b) Barley root fresh weight after inoculation with *Si*, *Bs*, or both fungi at 6 dpi. For each replicate ($n > 7$), four plants were pooled. (c) *HvPR10* expression during mono- and co-inoculation of *Hv* with *Si* and *Bs* at 6 dpi. For each replicate ($n = 4$), four plants were pooled. (d) *Si* and *Bs* colonization at six dpi in *At* inferred from the relative expression of the fungal housekeeping gene *TEF* compared with the Arabidopsis ubiquitin (*AtUBI*) gene. For each replicate ($n = 4$), 10 plants were pooled. (e) *At* root elongation at 6 dpi with *Si* and *Bs*. For each replicate ($n > 7$), 10 plants were pooled. (f) Top: *At* photosynthetic activity (F_V/F_M) at 1, 4, and 7 d post transfer (dpt) corresponding to 7, 10, and 13 d postinoculation (dpi) with *Si*, *Bs*, or both fungi together. Bottom: Quantification of the photosynthetic area. Values were internally normalized to the first day of measurement. The percentages represent the remaining photosynthetic activity after the onset of disease symptoms normalized to the Mock control (area shown in gray). For each replicate ($n = 4$), 10 plants were pooled. Statistical analysis: Student's *t*-test (*P*-value < 0.01, ns: not significant) for (a, d); one-way ANOVA followed by Tukey's honest significant difference test (adjusted *P*-value < 0.05) for (b, c, e, f). Different letters indicate significant differences. Expression data were inferred from reverse transcription quantitative polymerase chain reaction and depicted values are $2^{-\Delta C_t}$. Individual biological replicates are represented as points; bars indicate averages ± SD. All replicates are independent biological replicates. Ac, acetylation; Rel. expr., relative expression.

number varies among species (Fig. 3b). Both *Si* and *Sv* carry only one copy of the GH18-CBM5 chitinase (Pirin1_74346; PIIN_03543 hereafter *Si*CHIT; Sebve1_16391, hereafter *Sv*CHIT). *Si*CHIT and *Sv*CHIT display an amino acid similarity of 78%. Taken together with the specific upregulation of both chitinases in response to *Bs*, this indicates a conserved but highly specialized function of these enzymes in both endophytes in the context of mycoparasitism (Fig. 3).

Si reduces *Bs* infection and disease symptoms *in planta*

We previously reported that *Sv* mediates protection against *Bs* in barley and *At* and hypothesized that this protective function was linked to the secretion of antimicrobial effectors (Sarkar *et al.*, 2019; Mahdi *et al.*, 2022). Similarly, *Si* has been reported to mediate the protection of wheat from *Bs* (Y. Li *et al.*, 2023). The specific induction of *SiCHIT* and *SvCHIT* in response to *Bs* indicates that both enzymes might contribute to this antagonism. To test this hypothesis, we first confirmed that *Si* displays a plant-protective phenotype against *Bs* in our host plant species (Y. Li *et al.*, 2023). To this end, we co-inoculated the roots of barley seedlings with *Si* and *Bs* spores and quantified fungal colonization by reverse transcription quantitative polymerase chain reaction at six dpi. We found that root colonization by *Bs*, but not *Si*, was drastically reduced in the co-inoculated roots compared with roots inoculated with only one fungus (Fig. 4a). To assess disease symptoms, we measured root fresh weight and found that the reduced root colonization by *Bs* in the presence of *Si* correlated with a reduction in root growth inhibition (Fig. 4b). Plant protection by *Si* was not linked to an increased

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expression of the barley defense marker gene *HvPR10* (Fig. 4c), suggesting that the host-protective capabilities of *Si* did not rely on *PR10*-mediated plant immunity. To test whether the protective ability of *Si* was host species-independent, we assessed fungal colonization (Fig. 4d) and main root elongation (Fig. 4e) of *At* seedlings upon co-inoculation with both fungi. In agreement with previous studies, we observed a growth-promoting effect of *Si* on the *At* seedlings in bipartite interactions (Del Barrio-Duque *et al.*, 2019; Scholz *et al.*, 2023). In addition, the colonization of *At* by *Bs* and *Bs*-dependent reduction in root elongation were decreased in the presence of *Si*. We further monitored the progression of disease symptoms via PAM fluorometry and demonstrated that co-inoculation with *Si* abolished the detrimental effects of *Bs* on the photosynthetic activity of *At* leaves (Fig. 4f). In summary, our findings demonstrate that *Si* has plant-protective abilities against the aggressive root rot pathogen *Bs* in barley and *At*.

The GH18-CBM5 chitinases have chitinolytic activity and inhibit *Bs* growth

For a characterization of the molecular functions of the GH18-CBM5 chitinases, we modelled the 3D structures of both enzymes using AlphaFold and docked a chitin octamer into the catalytic cleft that contains a conserved D×D×E motif required for catalysis (Figs 5a, S5A). In both cases, the substrate docked in

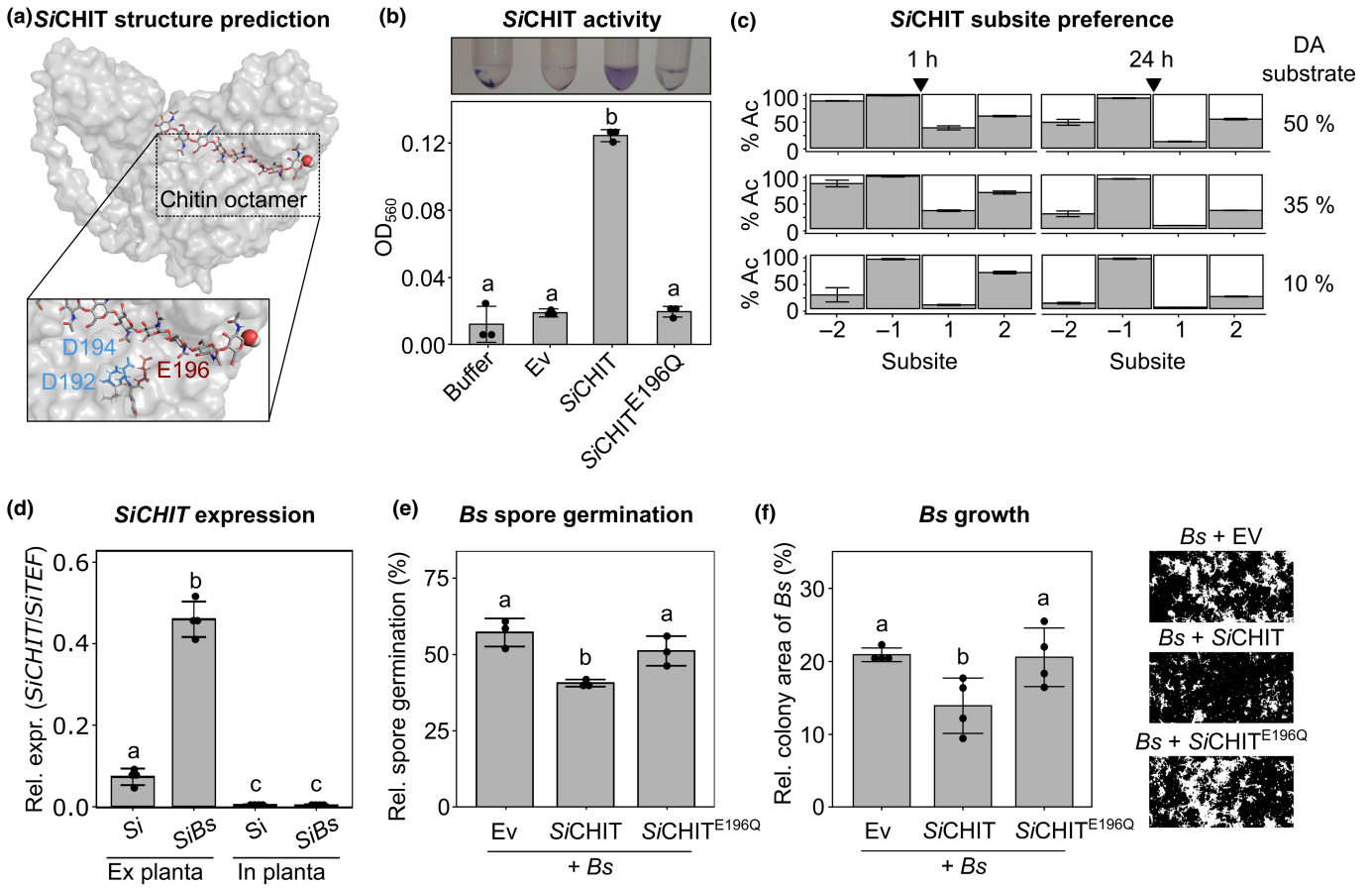

Fig. 5 Recombinantly expressed *Si*CHIT is active and inhibits spore germination and growth of the plant pathogen *Bipolaris sorokiniana* (*Bs*). (a) 3D structure of *Si*CHIT modelled using AlphaFold and visualized with PyMOL. The zoom-in shows the DIDYE motif, with aspartate (d) marked in blue and glutamate (e) marked in red. (b) Chitinolytic activity of *Si*CHIT or the catalytically inactive *Si*CHIT^{E196Q}. Chitin azure was incubated with 5 μM recombinant protein or the empty vector (Ev) control in 50 mM phosphate buffer (pH 6.0) for 24 h. Samples were spun down and the absorbance of the supernatant was measured at 560 nm (mean ± SD, $n = 3$). (c) Subsite specificity of *Si*CHIT as inferred by mass spectrometry. Chitosans of three degrees of acetylation (DA) were hydrolyzed for 1 h or 24 h and based on the sequenced products, the frequency of acetylated units at the −2 to +2 subsites of *Si*CHIT was determined. The black arrow indicates the glycosidic bond between the −1 and + 1 subsite that is cleaved by the enzyme (mean ± SD, $n = 3$). (d) *SiCHIT* expression inferred by reverse transcription quantitative polymerase chain reaction during confrontation of *Si* and *Bs ex planta* and during co-inoculation of *Hv* with *Si* and *Bs* at 6 d postinoculation (dpi). For each replicate ($n = 4$), four plants were pooled. Depicted values are $2^{-\Delta C_t}$. (e) Relative *Bs* spore germination. Germinated and nongerminated *Bs* spores were counted 8 h after incubation with the recombinant chitinase, catalytically inactive chitinase, or the empty vector (Ev) control. Different letters indicate significant differences according to a one-way ANOVA followed by Tukey's honest significant difference (HSD) test (adjusted *P*-value < 0.05, mean ± SD, $n = 3$). (f) Colony area of *Si*CHIT or *Si*CHIT^{E196Q}-treated *Bs* 6 d after plating out on PNM medium. Left: quantification of *Bs* colony area. Different letters indicate significant differences (adjusted *P*-value < 0.05) according to one-way ANOVA followed by Tukey's honest significant difference (HSD) test (mean ± SD, $n = 4$). Right: Exemplary pictures of *Bs* colonies treated with *Si*CHIT or *Si*CHIT^{E196Q}. All replicates are independent biological replicates. Ac, acetylation; Rel. expr., relative expression.

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close proximity to the DxDxE motif, with the *N*-acetyl group located near the second aspartate (D194 in *Si*CHIT and D171 in *Sv*CHIT) and the β-1,4 glycosidic bond located below the catalytically indispensable glutamate (E196 in *Si*CHIT and E173 *Sv*CHIT). This predicted arrangement is in line with crystal structures of other GH18 chitinase – substrate complexes (van Aalten *et al.*, 2001). To test the catalytic activity of *Si*CHIT and *Sv*CHIT, we expressed the recombinant proteins without signal peptide in *E. coli* and purified them from the supernatant of lysed bacterial cultures by affinity chromatography (Fig. S5B; Methods S2). Thin-layer chromatography revealed that both chitinases were active on crystalline crab shell chitin (Fig. S5C). To experimentally validate the importance of the D×D×E motif, we generated catalytically inactive mutants of both enzymes by exchanging the glutamate in the D×D×E motif with glutamine (*Si*CHIT^{E196Q} or *Sv*CHIT^{E173Q}). This amino acid exchange has been shown to abolish chitinolytic activity without disrupting the chitin-binding ability in other GH18 chitinase-like effector proteins (Fiorin *et al.*, 2018). A chitinase activity assay on chitin azure verified the loss of chitinolytic activity in *Si*CHIT^{E196Q} and *Sv*CHIT^{E173Q} (Figs 5b, S5D).

GH18 chitinases exhibit a characteristic substrate specificity. They preferentially bind acetylated substrate units, but their −2, +1, and +2 subsites can also accept deacetylated substrate units, especially if the degree of acetylation (DA) of the substrate is low, as in the case of chitosan (Sørbotten *et al.*, 2005; Busswinkel *et al.*, 2018). By contrast, the −1 subsite strictly requires acetylated units for catalysis (van Aalten *et al.*, 2001). We examined the subsite specificity of *Si*CHIT by mass spectrometry of the oligomeric products generated during degradation of chitosan (Cord-Landwehr *et al.*, 2017) and found a substrate preference pattern consistent with the characteristic profile of GH18 chitinases (Fig. 5c). Collectively, the thin-layer chromatography and the chitinase activity assay provide clear evidence that *Si*CHIT and *Sv*CHIT are typical GH18 chitinases which can degrade chitin and partially deacetylated chitosan *in vitro*.

To investigate the biological role of *Si*CHIT during intermicrobial interactions and confirm the data from our transcriptomic analysis, we compared the expression of *SiCHIT* during fungal confrontation *ex planta* and during co-inoculation of barley with both *Si* and *Bs* via reverse transcription quantitative polymerase chain reaction. We observed an induction of *SiCHIT ex planta* but not *in planta* (Fig. 5d), suggesting an important role for the GH18-CBM5 chitinase in direct fungal antagonism before root colonization. To learn more about the antagonistic function of *Si*CHIT, we assessed the germination rate of *Bs* spores in the presence of both enzymes. Incubation with *Si*CHIT resulted in a significant decrease in germination of *Bs* spores (Fig. 5e). Similarly, *Sv*CHIT reduced the germination of *Bs* spores (Fig. S5E). Assessment of *Bs* fungal colony growth and morphology after treatment with *Si*CHIT, *Si*CHIT^{E196Q}, or an empty vector (Ev) control on 1/10 PNM medium showed a decrease in *Bs* growth after *Si*CHIT but not *Si*CHIT^{E196Q} pretreatment, confirming that the chitinolytic activity of the enzyme was a prerequisite for its function in fungal growth inhibition (Fig. 5f). We also compared *Bs* and *Si* growth after exposure to *Si*CHIT using spectroscopic analysis and found a significant reduction in *Bs* but not of *Si* growth, suggesting that the root endophyte is resistant to the effects of its own chitinase (Figs S5F, S5G). Our results strongly suggest that *Si*CHIT and *Sv*CHIT have antimicrobial activity against the phytopathogenic fungus *Bs*. This finding prompted us to investigate whether the exogenous application of *Si*CHIT could alleviate the disease symptoms caused by *Bs in planta*.

*Si*CHIT reduces disease symptoms of *Bs* in Arabidopsis and barley

We previously showed that Sebacinales predominantly safeguard the host plant through direct interactions among microbes occurring outside the root system (Sarkar *et al.*, 2019; Mahdi *et al.*, 2022). Therefore, to test the biocontrol ability of *Si*CHIT in barley, we inoculated barley seedlings with *Bs* spores pretreated with purified *Si*CHIT, the catalytically inactive *Si*CHIT^{E196Q}, or the Ev control. Treatment of *Bs* spores with *Si*CHIT but not *Si*CHIT^{E196Q} reduced the colonization success of the pathogen (Fig. 6a). Similarly, the reduction in root weight caused by *Bs* was significantly lower when the spores were pretreated with *Si*CHIT, but not *Si*CHIT^{E196Q} (Fig. 6b). In comparison with the Ev control, treatment with *Si*CHIT or *Si*CHIT^{E196Q} did not affect the expression of the barley defense marker gene *HvPR10* triggered by *Bs* (Fig. 6c). This implies that neither *Si*-mediated (Fig. 4c) nor the *Si*CHIT-mediated protection of barley was linked to a significant induction of *PR10*-mediated plant immunity.

Furthermore, we tested the plant-protective ability of *Si*CHIT in *At* by inoculating the seedlings with *Bs* spores pretreated with *Si*CHIT, *Si*CHIT^{E196Q}, or the Ev control. As previously observed in barley, root colonization (Fig. 6d) and root growth inhibition (Fig. 6e) by *Bs* were reduced when spores were treated with *Si*CHIT but not *Si*CHIT^{E196Q}. To assess *Bs*-induced disease symptoms, we measured the photosynthetically active plant area over 7 d via PAM fluorometry (Fig. 6f). When *At* seedlings were treated with *Bs* and the Ev control, their cumulative photosynthetically active area from the onset of the first disease symptoms to the end of the experiment was reduced to 54% of the mock control. Treatment of the *Bs* spores with *Si*CHIT resulted in a significantly less severe reduction in the photosynthetically active area to 84% of the mock control in the same time span. Similar to what we previously observed for barley, this *Si*CHIT-mediated protection from *Bs* was not accompanied by an increased transcription of *At* immune genes (Fig. S6).

These results demonstrate that the chitinolytic activity of *Si*CHIT reduces *Bs* viability, resulting in a significant decline in the pathogen's ability to establish itself in the plant niche and cause harm to its host.

Discussion

In this study, we investigated the transcriptomic landscape of two closely related beneficial root endophytes in response to different host plants and root-associated microbes. We found that *Si* and *Sv* underwent extensive transcriptional rearrangements during

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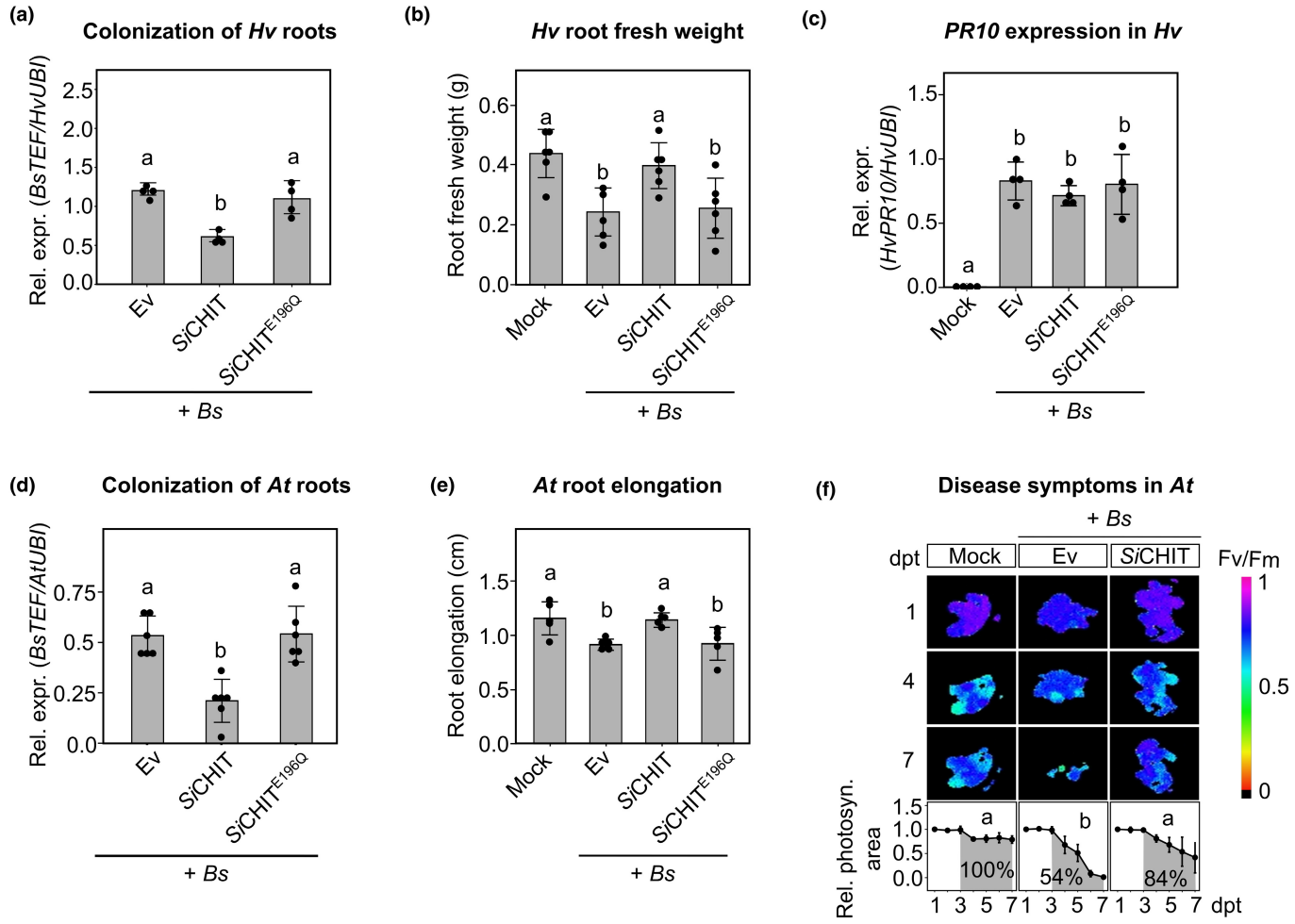

Fig. 6 Plant-protective ability of *Si*CHIT in barley (*Hv*) (a–c) and *Arabidopsis thaliana* (*At*) (d–f). (a) *Bipolaris sorokiniana* (*Bs*) root colonization at four d postinoculation (dpi) in barley roots inferred from the relative expression of the fungal housekeeping gene *TEF* compared with the barley ubiquitin (*HvUBI*) gene. For each replicate ($n = 6$), four plants were pooled. (b) Barley root fresh weight after inoculation with *Bs* spores, or *Bs* spores pretreated with *Si*CHIT or *Si*CHIT^{E196Q} at four dpi. For each replicate ($n = 6$), four plants were pooled. (c) *HvPR-10* expression in barley roots inoculated with *Bs* spores pretreated with *Si*CHIT, *Si*CHIT^{E196Q} or the empty vector (Ev) control. For each replicate ($n = 4$), four plants were pooled. (d) *Bs* colonization of *At* roots at four dpi with *Bs* spores, or *Bs* spores pretreated with *Si*CHIT or *Si*CHIT^{E196Q} inferred from the relative expression of the fungal housekeeping gene *TEF* compared with the Arabidopsis ubiquitin (*AtUBI*) gene. For each biological replicate ($n = 6$), 10 plants were pooled. (e) *At* root length at four dpi with *Bs* spores, or *Bs* spores pretreated with *Si*CHIT or *Si*CHIT^{E196Q}. For each biological replicate ($n = 5$), 10 plants were pooled. (f) *At* photosynthetic activity (F_V/F_M) at 1, 4, and 7 d post transfer (dpt) corresponding to 7, 10, and 13 d postinoculation (dpi) with *Bs*, or *Bs* pretreated with *Si*CHIT or *Si*CHIT^{E196Q}. Bottom: Quantification of the photosynthetic area. Values were internally normalized to the first day of measurement. The percentages represent the remaining photosynthetic activity after the onset of disease symptoms normalized to the Mock control (area shown in gray). For each replicate ($n = 4$), 10 plants were pooled. Expression data were inferred by reverse transcription quantitative polymerase chain reaction and values are $2^{-\Delta C_t}$. Statistical analysis: one-way ANOVA followed by Tukey's honest significant difference test (adjusted *P*-value < 0.05). Different letters indicate significant differences. Individual biological replicates are represented as points; bars indicate averages ± SD. All replicates are independent biological replicates. Ac, acetylation; Rel. expr., relative expression.

plant colonization regardless of plant species and identified a set of genes that was commonly induced in the presence of all three hosts (*At*, *Bd*, and *Hv*). These genes are likely to be general determinants of host colonization. In addition, our dataset revealed host-specific induction of genes encoding proteins, specifically tailored for the degradation of monocotyledon or dicotyledon cell walls, such as AA9, GH10, and GH11 domain-containing proteins (Lahrmann *et al.*, 2013). Host-specific transcriptional responses have also been reported for other polyspecialist fungi and are the basis for the adaptive abilities of Sebacinales to a broad host range (Cao *et al.*, 2012; Lahrmann *et al.*, 2013, 2015; Morán-Diez *et al.*, 2015).

The secretion of effectors allows fungi to colonize host plants and antagonize competitors through various mechanisms (Fig. 7). Recent findings shed new light on the strategies used by microbes to shape their niches, ranging from the secretion of antimicrobial effectors to the promotion of interkingdom synergies among microbes that benefit the host (Snelders *et al.*, 2020; Mahdi *et al.*, 2022; Redkar *et al.*, 2022). We found that a considerable proportion of predicted effectors (39% in *Si* and 26% in

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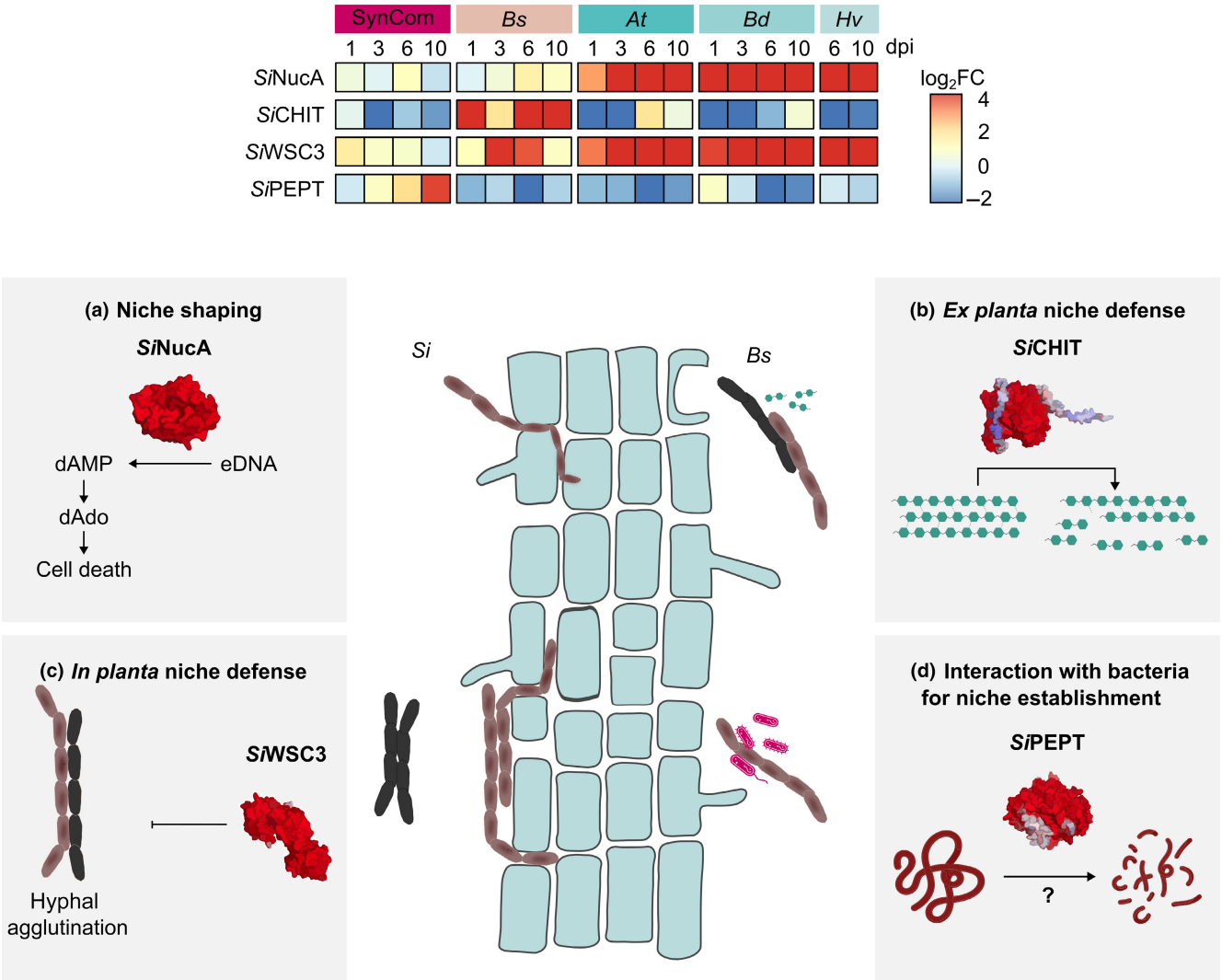

Fig. 7 Model of fungal effector functions during niche establishment and shaping. Heatmap showing the expression pattern of selected effectors in response to different biotic interaction partners. (a) The nuclease NucA (Pirin1_72917; PIIN_02121) is induced in response to host plants and suppresses host immunity by degrading extracellular DNA. Together with the nucleotidase E5′NT (Pirin1_71782; PIIN_01005), NucA produces the potent cell death initiator dAdo (Nizam *et al*., 2019; Dunken *et al*., 2022). (b) The GH18-CBM5 chitinase *Si*CHIT (Pirin1_74346; PIIN_03543) is expressed in the presence of fungal competitors and contributes to *ex planta* niche defense. (c) The lectin WSC3 (Pirin1_76632; PIIN_05825) is expressed in response to both host plants and microbial competitors. On the one hand, the lectin is involved in fungal cell wall remodeling, potentially to strengthen the *Serendipita indica* (*Si*) cell wall against external stresses occurring *in planta*. On the other hand, WSC3 mediates hyphal agglutination and could serve as biocontrol agent within the plant niche (Wawra *et al*., 2019). (d) The carboxypeptidase *Si*PEPT (Pirin1_80394; PIIN_09579) is induced in response to the presence of root-associated bacteria and could be involved in the detoxification of antifungal peptides, the modulation of host-bacteria interactions or interference with inter-bacterial communication, such as peptide-mediated signaling.

Sv) was induced strongly in response to at least one biotic interaction partner. The majority of these effectors (50% in *Si* and 66% in *Sv*) was induced specifically in the presence of host plants, but not microbes. These classical effectors most likely have important functions in evasion or suppression of plant immunity, plant cell wall degradation, and in later colonization stages, the induction of host cell death. The nuclease NucA (Pirin1_72917; PIIN_02121 for *Si* and Sebve1_52856 for *Sv*) for instance was specifically induced in response to all three host plants (Fig. 7). Together with the nucleotidase E5′NT (Pirin1_71782; PIIN_01005 for *Si* and Sebve1_ 17 804 for *Sv*), NucA is involved in the suppression of immunity and initiation of host cell death via the production of small active molecules (Nizam *et al*., 2019; Dunken *et al*., 2022).

Restricted host cell death is essential for the successful colonization of host plant by *Si* and *Sv* and is considered a nutritional strategy of Sebacinales, which have retained the saprotrophic capabilities of their ancestors (Deshmukh *et al*., 2006; Qiang *et al*., 2012). Indeed, CAZymes which are typically associated with saprotrophism are expanded in the genomes of *Si* and *Sv* and account for a substantial proportion 80/233 in *Si* and 49/248 in *Sv* of the effectors which were induced specifically in

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response to plants (Lahrmann *et al.*, 2015). Compared with pathogenic fungi, Sebacinales endophytes do not trigger massive transcriptional changes in their hosts, suggesting they have mechanisms to establish a compatible interaction without eliciting a strong immune response (Lahrmann *et al.*, 2013). In agreement with this hypothesis, our dataset showed that PIIN_08944 (Pirin1_ 79 755, no homolog in *Sv*), a known inhibitor of SA-mediated basal plant immune responses, was induced specifically in response to host plants (Akum *et al.*, 2015). The same was true for the GH18 chitinase PIIN_03542 (Pirin1_74345 in *Si*) that does not bear a CBM5. This suggests that despite its antimicrobial activity *in vitro* (X. Li *et al.*, 2023), the enzyme might be involved in the evasion of chitin-triggered plant immunity rather than direct fungal antagonism as recently demonstrated (Fiorin *et al.*, 2018; Kozome *et al.*, 2024).

Similar to plants, the fungal competitor *Bs* elicited strong transcriptomic adaptations in both *Si* and *Sv*. These responses partially overlapped, suggesting common underlying principles in the interaction of Sebacinales with plants and fungi. The secretion of effectors might be such a common principle. Indeed, a considerable fraction of the differentially expressed putative effectors (185/467 in *Si* and 87/373 in *Sv*) were induced in response to both, host plants and the phytopathogenic fungus *Bs*. We hypothesize that these effectors might be involved in intermicrobial competition *in planta* or execute moonlighting functions relevant for the interactions with both, host plants and microbial competitors. The fungal lectins WSC3 (Pirin1_76632; PIIN_05825 in *Si* and Sebve1_309621 in *Sv*) and FGB1 (Pirin1_ 74 015; PIIN_03211 in *Si*; no homolog in *Sv*) are prime examples of multi-functional effectors (Wawra *et al.*, 2016, 2019). WSC3 contains three wall stress component (WSC) domains that bind long β-1,3-linked glucans (Wawra *et al.*, 2019). These domains were first identified in the yeast *Saccharomyces cerevisiae* protein, *Sc*Wsc1, a mechanosensor for cell wall integrity anchored in the plasma membrane (Verna *et al.*, 1997; Lodder *et al.*, 1999). In *S. indica*, WSC3 is associated with the EPS matrix of *Si* (Chandrasekar *et al.*, 2022) where it potentially strengthens the matrix against external stresses by forming helical bundles with three β-1,3-linked glucan polymers (Wawra *et al.*, 2019). Additionally, *Si*WSC3 promotes adhesion between hyphal cells and efficiently agglutinates the hyphae of fungal competitors, suggesting a possible function in microbial antagonism and niche protection (Fig. 7). By contrast, *Si*FGB1, which binds to the β-1,6-linkages of various β-glucans, does not participate in hyphal agglutination. Instead, this lectin alters *Si* cell wall composition and binds soluble β-glucan fragments, thereby helping to evade plant immunity in barley and Arabidopsis (Wawra *et al.*, 2019). Further research is needed to investigate the potential role of these lectins in intermicrobial interactions.

Additionally, we found several genes encoding for proteins with conserved DELD motifs (Lahrmann *et al.*, 2013) to be upregulated *in planta* and during confrontation with *Bs*. Members of the DELD effector family, in particular *Dld1* (Pirin1_76679; PIIN_05872), have been shown to promote plant colonization by enhancing micronutrient availability to the fungus and interfering with oxidative stress and redox homeostasis (Nostadt *et al.*, 2020). The function of DELD proteins during interfungal competition, however, remains to be functionally characterized.

Only a minority of effectors (38/467 in *Si* and 28/373 in *Sv*) were induced specifically in response to root-associated microbes. A small subset of these effectors were exclusively induced in response to bacteria. For example, we identified a secreted carboxypeptidase (Pirin1_80394 and PIIN_09579) induced at late time points of *Si*-bacteria interactions. While the function of *Si*PEPT has not been characterized, carboxypeptidases in general have been discussed to be involved in fungal–bacterial antagonism since they can act on bacterial cell walls (Moretti *et al.*, 2010; Das *et al.*, 2013; Muszewska *et al.*, 2017). The small size of bacterial-responsive genes might be a result of the ubiquitous presence of microbes in the soil that calls for a constitutive rather than stimuli-dependent expression of antimicrobial effectors (Snelders *et al.*, 2018).

Among the *Bs*-induced effectors, we identified and functionally characterized an antimicrobial GH18-CBM5 chitinase (Fig. 7). In bacterial taxa, GH18-CBM5 chitinases use the CBM5 domain primarily for enhancing substrate binding and degradation of crystalline chitin (Horn *et al.*, 2006; Liu *et al.*, 2023). In fungi, GH18 chitinases have been extensively studied for their roles in mycoparasitism and biocontrol, particularly in the Ascomycota fungi *Trichoderma* spp. (Carsolio *et al.*, 1994; Woo *et al.*, 1999; Druzhinina *et al.*, 2011). While *Trichoderma* GH18 chitinases lack CBM5 domains, the fusion with other substrate-binding domains enhances substrate degradation and antagonistic activity against fungal competitors (Limón *et al.*, 2001, 2004). Thus, the presence of a naturally occurring GH18-CBM5 fusion in Basidiomycota suggests an effective strategy for combating microbial antagonists.

Pretreatment of *Bs* spores with the GH18-CBM5 chitinase reduced germination of *Bs* spores and disease symptoms in two plant hosts. This confirms that host species-independent intermicrobial interactions largely contribute to plant health in a complex tripartite system. Importantly, neither plant protection mediated by *Si* nor by application of the purified chitinase led to an increase in plant defense marker genes, demonstrating, that *Si*CHIT recapitulates the protective effect of *Si* in plants. Moreover, the sole expression of *SiCHIT* in direct confrontation with *Bs* and not in a tripartite setup with a host plant demonstrates that the local effects of plant and niche protection occur largely outside of the host. The mechanism by which *Si* and *Sv* protect themselves from the hydrolytic activity of their own GH18-CBM5 chitinases remains to be elucidated. However, it has been hypothesized that mycoparastic fungi shield their cell walls from competitor-derived CAZymes by expressing proteins that shield chitin while at the same time secreting an aggressive cocktail of enzymes designed to weaken the prey fungus (Gruber & Seidl-Seiboth, 2012). We previously demonstrated that *Si* secrets various lectins, which attach to the fungal cell wall and the surrounding soluble glucan matrix (Wawra *et al.*, 2019), and are strongly induced in response to *Bs*. This suggests that some of the numerous LysM domain-containing lectins in Sebacinales may serve a protective function. Since endophytic fungi are rich in

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lectins, chitin- and chitosan-modifying enzymes, this disease protection mechanism may be more widespread among endophytic fungi than previously thought (Govinda Rajulu *et al.*, 2010).

The secretion of a GH18-CBM5 chitinase may serve the nutritional needs of root-associated fungi through two strategies – by consuming the biomatter of the fungal competitor *Bs* and by safeguarding their ecological niche, the host plant, from the plant pathogen. This finding suggests that the effector-mediated manipulation of the microbiota by beneficial fungi extends beyond bacteria to fungal members of the plant microbiota. Fungal antimicrobial effectors emerge as pivotal players in multipartite interactions, contributing significantly to niche defense and beneficial effects of root endophytes.

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Competing interests

None declared.

Author contributions

AZ, LKM, RE and GL conceptualized the research. LKM prepared samples for RNA-seq analysis. DP, VS, KL, ES, VN and IGV performed the RNA-seq experiment and initial mapping. CDQ, SM and LA analyzed the RNA-seq data. CDQ, SM, LA and RE prepared figures for the manuscript. RE and ABE purified *Si*CHIT and characterized its antimicrobial activity against the phytopathogen *Bs* as well as its protective effects for the host plants *At* and *Hv*. RE, MJH, SC-L and BMM performed the mass-spectrometry analysis of the *Si*CHIT substrate specificity. AZ, RE, LA and LKM wrote the manuscript. All authors revised and approved the final version of the manuscript.

ORCID

Laura Armbruster https://orcid.org/0000-0002-4559-2404
Stefan Cord-Landwehr https://orcid.org/0000-0003-1915-1936
Concetta De Quattro https://orcid.org/0000-0003-2885-5468
Ruben Eichfeld https://orcid.org/0000-0001-6190-4482
Asmamaw B. Endeshaw https://orcid.org/0000-0003-4457-0366
Igor V. Grigoriev https://orcid.org/0000-0002-3136-8903
Margareta J. Hellmann https://orcid.org/0000-0002-3500-9465
Gregor Langen https://orcid.org/0000-0002-8321-1756
Lisa K. Mahdi https://orcid.org/0000-0003-1559-5492
Shingo Miyauchi https://orcid.org/0000-0002-0620-5547
Bruno M. Moerschbacher https://orcid.org/0000-0001-6067-3205
Vivian Ng https://orcid.org/0000-0001-8941-6931
Emily Savage https://orcid.org/0000-0002-7129-8989
Vasanth Singan https://orcid.org/0000-0002-9983-5707
Alga Zuccaro https://orcid.org/0000-0002-8026-0114

Data availability

The raw data of the RNA-Seq experiments were deposited in the NCBI BioProject database (https://www.ncbi.nlm.nih.gov/bioproject/) under the accession numbers listed in Table S2.

References

van Aalten DM, Komander D, Synstad B, Gaseidnes S, Peter MG, Eijsink VG. 2001. Structural insights into the catalytic mechanism of a family 18 exochitinase. *Proceedings of the National Academy of Sciences, USA* **98**: 8979–8984.

Akum FN, Steinbrenner J, Biedenkopf D, Imani J, Kogel KH. 2015. The *Piriformospora indica* effector PIIN_08944 promotes the mutualistic Sebacinalean symbiosis. *Frontiers in Plant Science* **6**: 906.

Almagro Armenteros JJ, Tsirigos KD, Sonderby CK, Petersen TN, Winther O, Brunak S, von Heijne G, Nielsen H. 2019. SignalP 5.0 improves signal peptide predictions using deep neural networks. *Nature Biotechnology* **37**: 420–423.

Auer L, Buee M, Fauchery L, Lombard V, Barry KW, Clum A, Copeland A, Daum C, Foster B, LaButti K *et al.* 2024. Metatranscriptomics sheds light on the links between the functional traits of fungal guilds and ecological processes in forest soil ecosystems. *New Phytologist* **242**: 1676–1691.

Bai Y, Müller DB, Srinivas G, Garrido-Oter R, Potthoff E, Rott M, Dombrowski N, Münch PC, Spaepen S, Remus-Emsermann M *et al.* 2015. Functional overlap of the Arabidopsis leaf and root microbiota. *Nature* **528**: 364–369.

Balestrini R, Nerva L, Sillo F, Girlanda M, Perotto S. 2014. Plant and fungal gene expression in mycorrhizal protocorms of the orchid Serapias vomeracea colonized by Tulasnella calospora. *Plant Signaling & Behavior* **9**: e977707.

Bendtsen JD, Nielsen H, von Heijne G, Brunak S. 2004. Improved prediction of signal peptides: SignalP 3.0. *Journal of Molecular Biology* **340**: 783–795.

Busswinkel F, Goni O, Cord-Landwehr S, O'Connell S, Moerschbacher BM. 2018. Endochitinase 1 (Tv-ECH1) from Trichoderma virens has high subsite specificities for acetylated units when acting on chitosans. *International Journal of Biological Macromolecules* **114**: 453–461.

Cao Y, Zhu X, Jiao R, Xia Y. 2012. The Magas1 gene is involved in pathogenesis by affecting penetration in Metarhizium acridum. *Journal of Microbiology and Biotechnology* **22**: 889–893.

Carsolio C, Gutiérrez A, Jiménez B, van Montagu M, Herrera-Estrella A. 1994. Characterization of ech-42, a *Trichoderma harzianum* endochitinase gene expressed during mycoparasitism. *Proceedings of the National Academy of Sciences, USA* **91**: 10903–10907.

Chandrasekar B, Wanke A, Wawra S, Saake P, Mahdi L, Charura N, Neidert M, Poschmann G, Malisic M, Thiele M *et al.* 2022. Fungi hijack a ubiquitous

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plant apoplastic endoglucanase to release a ROS scavenging β-glucan decasaccharide to subvert immune responses. *Plant Cell* **34**: 2765–2784.

Chen W, Jiang X, Yang Q. 2020. Glycoside hydrolase family 18 chitinases: The known and the unknown. *Biotechnology Advances* **43**: 107553.

Conway JR, Lex A, Gehlenborg N. 2017. UpSetR: an R package for the visualization of intersecting sets and their properties. *Bioinformatics* **33**: 2938–2940.

Cord-Landwehr S, Ihmor P, Niehues A, Luftmann H, Moerschbacher BM, Mormann M. 2017. Quantitative mass-spectrometric sequencing of chitosan oligomers revealing cleavage sites of chitosan hydrolases. *Analytical Chemistry* **89**: 2893–2900.

Das D, Hervé M, Elsliger M-A, Kadam Rameshwar U, Grant Joanna C, Chiu H-J, Knuth Mark W, Klock Heath E, Miller Mitchell D, Godzik A *et al.* 2013. Structure and function of a novel ld-carboxypeptidase a involved in peptidoglycan recycling. *Journal of Bacteriology* **195**: 5555–5566.

De Wit PJGM, Mehrabi R, Van den Burg HA, Stergiopoulos I. 2009. Fungal effector proteins: past, present and future. *Molecular Plant Pathology* **10**: 735–747.

Del Barrio-Duque A, Ley J, Samad A, Antonielli L, Sessitsch A, Compant S. 2019. Beneficial endophytic bacteria-*Serendipita indica* interaction for crop enhancement and resistance to phytopathogens. *Frontiers in Microbiology* **10**: 2888.

Deshmukh S, Hückelhoven R, Schäfer P, Imani J, Sharma M, Weiss M, Waller F, Kogel K-H. 2006. The root endophytic fungus Piriformospora indica requires host cell death for proliferation during mutualistic symbiosis with barley. *Proceedings of the National Academy of Sciences, USA* **103**: 18450–18457.

Deveau A, Barret M, Diedhiou AG, Leveau J, Boer W, Martin F, Sarniguet A, Frey-Klett P. 2015. Pairwise transcriptomic analysis of the interactions between the ectomycorrhizal fungus Laccaria bicolor S238N and three beneficial, neutral and antagonistic soil bacteria. *Microbial Ecology* **69**: 146–159.

Druzhinina IS, Seidl-Seiboth V, Herrera-Estrella A, Horwitz BA, Kenerley CM, Monte E, Mukherjee PK, Zeilinger S, Grigoriev IV, Kubicek CP. 2011. Trichoderma: the genomics of opportunistic success. *Nature Reviews Microbiology* **9**: 749–759.

Dunken N, Widmer H, Balcke GU, Straube H, Langen G, Charura NM, Saake P, Quattro CD, Schön J, Rövenich H *et al.* 2022. A nucleoside signal generated by a fungal endophyte regulates host cell death and promotes root colonization. *BioRxiv*. doi: 10.1101/2022.03.11.483938.

Finkel OM, Castrillo G, Herrera Paredes S, Salas González I, Dangl JL. 2017. Understanding and exploiting plant beneficial microbes. *Current Opinion in Plant Biology* **38**: 155–163.

Fiorin GL, Sanchéz-Vallet A, Thomazella DPDT, do Prado PFV, do Nascimento LC, Figueira AVDO, Thomma BPHJ, Pereira GAG, Teixeira PJPL. 2018. Suppression of plant immunity by fungal chitinase-like effectors. *Current Biology* **28**: 3023–3030.

Glynou K, Ali T, Buch A-K, Haghi Kia S, Ploch S, Xia X, Çelik A, Thines M, Maciá-Vicente JG. 2016. The local environment determines the assembly of root endophytic fungi at a continental scale. *Environmental Microbiology* **18**: 2418–2434.

Glynou K, Nam B, Thines M, Maciá-Vicente JG. 2018. Facultative root-colonizing fungi dominate endophytic assemblages in roots of nonmycorrhizal Microthlaspi species. *New Phytologist* **217**: 1190–1202.

Govinda Rajulu MB, Thirunavukkarasu N, Suryanarayanan TS, Ravishankar JP, El Gueddari NE, Moerschbacher BM. 2010. Chitinolytic enzymes from endophytic fungi. *Fungal Diversity* **47**: 43–53.

Gruber S, Seidl-Seiboth V. 2012. Self versus non-self: fungal cell wall degradation in Trichoderma. *Microbiology* **158**(Pt 1): 26–34.

Hemetsberger C, Herrberger C, Zechmann B, Hillmer M, Doehlemann G. 2012. The Ustilago maydis effector Pep1 suppresses plant immunity by inhibition of host peroxidase activity. *PLoS Pathogens* **8**: e1002684.

Hermosa R, Viterbo A, Chet I, Monte E. 2012. Plant-beneficial effects of Trichoderma and of its genes. *Microbiology* **158**(Pt 1): 17–25.

Hilbert M, Voll LM, Ding Y, Hofmann J, Sharma M, Zuccaro A. 2012. Indole derivative production by the root endophyte Piriformospora indica is not required for growth promotion but for biotrophic colonization of barley roots. *New Phytologist* **196**: 520–534.

Horn SJ, Sikorski P, Cederkvist JB, Vaaje-Kolstad G, Sørlie M, Synstad B, Vriend G, Vårum KM, Eijsink VGH. 2006. Costs and benefits of processivity in enzymatic degradation of recalcitrant polysaccharides. *Proceedings of the National Academy of Sciences, USA* **103**: 18089–18094.

Ihrmark K, Asmail N, Ubhayasekera W, Melin P, Stenlid J, Karlsson M. 2010. Comparative molecular evolution of Trichoderma chitinases in response to mycoparasitic interactions. *Evolutionary Bioinformatics* **6**: 4198.

Jacobs S, Zechmann B, Molitor A, Trujillo M, Petutschnig E, Lipka V, Kogel K-H, Schäfer P. 2011. Broad-spectrum suppression of innate immunity is required for colonization of Arabidopsis roots by the fungus Piriformospora indica. *Plant Physiology* **156**: 726–740.

Jashni MK, Dols IHM, Iida Y, Boeren S, Beenen HG, Mehrabi R, Collemare J, Wit PJG. 2015a. Synergistic action of a metalloprotease and a serine protease from *Fusarium oxysporum* f. sp. lycopersici cleaves chitin-binding tomato chitinases, reduces their antifungal activity, and enhances fungal virulence. *Molecular Plant–Microbe Interactions* **28**: 996–1008.

Jashni MK, Mehrabi R, Collemare J, Mesarich CH, Wit PJGM. 2015b. The battle in the apoplast: further insights into the roles of proteases and their inhibitors in plant-pathogen interactions. *Frontiers in Plant Science* **6**: 584.

Kozome D, Sljoka A, Laurino P. 2024. Remote loop evolution reveals a complex biological function for chitinase enzymes beyond the active site. *Nature Communications* **15**: 3227.

Kristianingsih R, MacLean D. 2021. Accurate plant pathogen effector protein classification ab initio with deepredeff: an ensemble of convolutional neural networks. *BMC Bioinformatics* **22**: 372.

Kumar J, Schäfer P, Hückelhoven R, Langen G, Baltruschat H, Stein E, Nagarajan S, Kogel K-H. 2002. *Bipolaris sorokiniana*, a cereal pathogen of global concern: cytological and molecular approaches towards better controldouble dagger. *Molecular Plant Pathology* **3**: 185–195.

Lahrmann U, Ding Y, Banhara A, Rath M, Hajirezaei MR, Döhlemann S, Wirén N, Parniske M, Zuccaro A. 2013. Host-related metabolic cues affect colonization strategies of a root endophyte. *Proceedings of the National Academy of Sciences, USA* **110**: 13965–13970.

Lahrmann U, Strehmel N, Langen G, Frerigmann H, Leson L, Ding Y, Scheel D, Herklotz S, Hilbert M, Zuccaro A. 2015. Mutualistic root endophytism is not associated with the reduction of saprotrophic traits and requires a noncompromised plant innate immunity. *New Phytologist* **207**: 841–857.

Lahrmann U, Zuccaro A. 2012. Opprimo ergo sum—Evasion and Suppression in the Root Endophytic Fungus Piriformospora indica. *Molecular Plant–Microbe Interactions* **25**: 727–737.

Li X, Wajjiha B, Zhang P, Dang Y, Prasad R, Wei Y, Zhang SH. 2023. *Serendipita indica* chitinase protects rice from the blast and bakanae diseases. *Journal of Basic Microbiology* **63**: 734–745.

Li Y, Bi M, Sun S, Li G, Wang Q, Ying M. 2023. Comparative metabolomic profiling reveals molecular mechanisms underlying growth promotion and disease resistance in wheat conferred by Piriformospora indica in the field. *Plant Signaling & Behavior* **18**: 2213934.

Limón MC, Chacón MR, Mejías R, Delgado-Jarana J, Rincón AM, Codón AC, Benítez T. 2004. Increased antifungal and chitinase specific activities of *Trichoderma harzianum* CECT 2413 by addition of a cellulose binding domain. *Applied Microbiology and Biotechnology* **64**: 675–685.

Limón MC, Margolles-Clark E, Benítez T, Penttilä M. 2001. Addition of substrate-binding domains increases substrate-binding capacity and specific activity of a chitinase from *Trichoderma harzianum*. *FEMS Microbiology Letters* **198**: 57–63.

Liu J, Xu Q, Wu Y, Sun D, Zhu J, Liu C, Liu W. 2023. Carbohydrate-binding modules of ChiB and ChiC promote the chitinolytic system of Serratia marcescens BWL1001. *Enzyme and Microbial Technology* **162**: 110118.

Lo Presti L, Lanver D, Schweizer G, Tanaka S, Liang L, Tollot M, Zuccaro A, Reissmann S, Kahmann R. 2015. Fungal effectors and plant susceptibility. *Annual Review of Plant Biology* **66**: 513–545.

Lodder AL, Lee TK, Ballester R. 1999. Characterization of the Wsc1 protein, a putative receptor in the stress response of Saccharomyces cerevisiae. *Genetics* **152**: 1487–1499.

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Love MI, Huber W, Anders S. 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biology* **15**: 550.

Mahdi LK, Miyauchi S, Uhlmann C, Garrido-Oter R, Langen G, Wawra S, Niu Y, Guan R, Robertson-Albertyn S, Bulgarelli D *et al.* 2022. The fungal root endophyte *Serendipita vermifera* displays inter-kingdom synergistic beneficial effects with the microbiota in *Arabidopsis thaliana* and barley. *The ISME Journal* **16**: 876–889.

Mela F, Fritsche K, Boer W, van Veen JA, Graaff LH, van den Berg M, Leveau JHJ. 2011. Dual transcriptional profiling of a bacterial/fungal confrontation: *Collimonas fungivorans* versus *Aspergillus niger*. *The ISME Journal* **5**: 1494–1504.

Mesny F, Hacquard S, Thomma BP. 2023. Co-evolution within the plant holobiont drives host performance. *EMBO Reports* **24**: e57455.

Mesny F, Miyauchi S, Thiergart T, Pickel B, Atanasova L, Karlsson M, Hüttel B, Barry KW, Haridas S, Chen C *et al.* 2021. Genetic determinants of endophytism in the Arabidopsis root mycobiome. *Nature Communications* **12**: 7227.

Morán-Diez ME, Trushina N, Lamdan NL, Rosenfelder L, Mukherjee PK, Kenerley CM, Horwitz BA. 2015. Host-specific transcriptomic pattern of Trichoderma virens during interaction with maize or tomato roots. *BMC Genomics* **16**: 8.

Moretti M, Grunau A, Minerdi D, Gehrig P, Roschitzki B, Eberl L, Garibaldi A, Gullino ML, Riedel K. 2010. A proteomics approach to study synergistic and antagonistic interactions of the fungal–bacterial consortium Fusarium oxysporum wild-type MSA 35. *Proteomics* **10**: 3292–3320.

Muszewska A, Stepniewska-Dziubinska MM, Steczkiewicz K, Pawlowska J, Dziedzic A, Ginalski K. 2017. Fungal lifestyle reflected in serine protease repertoire. *Scientific Reports* **7**: 9147.

Naumann TA, Wicklow DT, Price NPJ. 2011. Identification of a Chitinase-modifying Protein from Fusarium verticillioides: TRUNCATION OF A HOST RESISTANCE PROTEIN BY A FUNGALYSIN METALLOPROTEASE. *Journal of Biological Chemistry* **286**: 35358–35366.

Nizam S, Qiang X, Wawra S, Nostadt R, Getzke F, Schwanke F, Dreyer I, Langen G, Zuccaro A. 2019. *Serendipita indica* E5′NT modulates extracellular nucleotide levels in the plant apoplast and affects fungal colonization. *EMBO Reports* **20**: e47430.

Nostadt R, Hilbert M, Nizam S, Rovenich H, Wawra S, Martin J, Kupper H, Mijovilovich A, Ursinus A, Langen G *et al.* 2020. A secreted fungal histidine- and alanine-rich protein regulates metal ion homeostasis and oxidative stress. *New Phytologist* **227**: 1174–1188.

Olivieri F, Eugenia Zanetti M, Oliva CR, Covarrubias AA, Casalongué CA. 2002. Characterization of an extracellular serine protease of *Fusarium eumartii* and its action on pathogenesis related proteins. *European Journal of Plant Pathology* **108**: 63–72.

Pellegrin C, Morin E, Martin FM, Veneault-Fourrey C. 2015. Comparative analysis of secretomes from ectomycorrhizal fungi with an emphasis on small-secreted proteins. *Frontiers in Microbiology* **6**: 1278.

Qiang X, Zechmann B, Reitz MU, Kogel K-H, Schäfer P. 2012. The mutualistic fungus *Piriformospora indica* colonizes Arabidopsis roots by inducing an endoplasmic reticulum stress–triggered caspase-dependent cell death. *Plant Cell* **24**: 794–809.

Raaijmakers JM, Paulitz TC, Steinberg C, Alabouvette C, Moënne-Loccoz Y. 2009. The rhizosphere: a playground and battlefield for soilborne pathogens and beneficial microorganisms. *Plant and Soil* **321**: 341–361.

Redkar A, Sabale M, Zuccaro A, Di Pietro A. 2022. Determinants of endophytic and pathogenic lifestyle in root colonizing fungi. *Current Opinion in Plant Biology* **67**: 102226.

Rizzi YS, Happel P, Lenz S, Urs MJ, Bonin M, Cord-Landwehr S, Singh R, Moerschbacher BM, Kahmann R. 2021. Chitosan and chitin deacetylase activity are necessary for development and virulence of *Ustilago maydis*. *MBio* **12**: e03419.

Sarkar D, Rovenich H, Jeena G, Nizam S, Tissier A, Balcke GU, Mahdi LK, Bonkowski M, Langen G, Zuccaro A. 2019. The inconspicuous gatekeeper: endophytic *Serendipita vermifera* acts as extended plant protection barrier in the rhizosphere. *New Phytologist* **224**: 886–901.

Satterlee TR, Williams FN, Nadal M, Glenn AE, Lofton LW, Duke MV, Scheffler BE, Gold SE. 2022. Transcriptomic response of *Fusarium verticillioides* to variably inhibitory environmental isolates of streptomyces. *Frontiers in Fungal Biology* **3**: 590.

Scholz SS, Barth E, Clément G, Marmagne A, Ludwig-Müller J, Sakakibara H, Kiba T, Vicente-Carbajosa J, Pollmann S, Krapp A *et al.* 2023. The root-colonizing endophyte *Piriformospora indica* supports nitrogen-starved *Arabidopsis thaliana* seedlings with nitrogen metabolites. *International Journal of Molecular Sciences* **24**: 15372.

Snelders NC, Boshoven JC, Song Y, Schmitz N, Fiorin GL, Rovenich H, van den Berg GCM, Torres DE, Petti GC, Prockl Z *et al.* 2023. A highly polymorphic effector protein promotes fungal virulence through suppression of plant-associated Actinobacteria. *New Phytologist* **237**: 944–958.

Snelders NC, Kettles GJ, Rudd JJ, Thomma BPHJ. 2018. Plant pathogen effector proteins as manipulators of host microbiomes? *Molecular Plant Pathology* **19**: 257–259.

Snelders NC, Rovenich H, Petti GC, Rocafort M, van den Berg GCM, Vorholt JA, Mesters JR, Seidl MF, Nijland R, Thomma BPHJ. 2020. Microbiome manipulation by a soil-borne fungal plant pathogen using effector proteins. *Nature Plants* **6**: 1365–1374.

Snelders NC, Rovenich H, Thomma BPHJ. 2022. Microbiota manipulation through the secretion of effector proteins is fundamental to the wealth of lifestyles in the fungal kingdom. *FEMS Microbiology Reviews* **46**: fuac022.

Sørbotten A, Horn SJ, Eijsink VGH, Vårum KM. 2005. Degradation of chitosans with chitinase B from Serratia marcescens. *The FEBS Journal* **272**: 538–549.

Sperschneider J, Dodds PN. 2022. EffectorP 3.0: prediction of apoplastic and cytoplasmic effectors in fungi and oomycetes. *Molecular Plant–Microbe Interactions* **35**: 146–156.

Sperschneider J, Dodds PN, Gardiner DM, Singh KB, Taylor JM. 2018a. Improved prediction of fungal effector proteins from secretomes with EffectorP 2.0. *Molecular Plant Pathology* **19**: 2094–2110.

Sperschneider J, Dodds PN, Singh KB, Taylor JM. 2018b. ApoplastP: prediction of effectors and plant proteins in the apoplast using machine learning. *New Phytologist* **217**: 1764–1778.

Sperschneider J, Gardiner DM, Dodds PN, Tini F, Covarelli L, Singh KB, Manners JM, Taylor JM. 2016. EffectorP: predicting fungal effector proteins from secretomes using machine learning. *New Phytologist* **210**: 743–761.

Strullu-Derrien C, Selosse M-A, Kenrick P, Martin FM. 2018. The origin and evolution of mycorrhizal symbioses: from palaeomycology to phylogenomics. *New Phytologist* **220**: 1012–1030.

Tang N, Lebreton A, Xu W, Dai Y, Yu F, Martin FM. 2021. Transcriptome profiling reveals differential gene expression of secreted proteases and highly specific gene repertoires involved in *Lactarius-pinus* symbioses. *Frontiers in Plant Science* **12**: 714393.

Teufel F, Almagro Armenteros JJ, Johansen AR, Gislason MH, Pihl SI, Tsirigos KD, Winther O, Brunak S, von Heijne G, Nielsen H. 2022. SignalP 6.0 predicts all five types of signal peptides using protein language models. *Nature Biotechnology* **40**: 1023–1025.

Tkacz A, Cheema J, Chandra G, Grant A, Poole PS. 2015. Stability and succession of the rhizosphere microbiota depends upon plant type and soil composition. *The ISME Journal* **9**: 2349–2359.

Trivedi P, Leach JE, Tringe SG, Sa T, Singh BK. 2020. Plant–microbiome interactions: from community assembly to plant health. *Nature Reviews Microbiology* **18**: 607–621.

Valadares RBS, Marroni F, Sillo F, Oliveira RRM, Balestrini R, Perotto S. 2021. A transcriptomic approach provides insights on the mycorrhizal symbiosis of the mediterranean orchid *Limodorum abortivum* in nature. *Plants* **10**: 251.

Veneault-Fourrey C, Martin F. 2011. Mutualistic interactions on a knife-edge between saprotrophy and pathogenesis. *Current Opinion in Plant Biology* **14**: 444–450.

Verna J, Lodder A, Lee K, Vagts A, Ballester R. 1997. A family of genes required for maintenance of cell wall integrity and for the stress response in *Saccharomyces cerevisiae*. *Proceedings of the National Academy of Sciences, USA* **94**: 13804–13809.

Wawra S, Fesel P, Widmer H, Neumann U, Lahrmann U, Becker S, Hehemann J-H, Langen G, Zuccaro A. 2019. FGB1 and WSC3 are *in planta*-induced β-

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glucan-binding fungal lectins with different functions. *New Phytologist* **222**: 1493–1506.

Wawra S, Fesel P, Widmer H, Timm M, Seibel J, Leson L, Kesseler L, Nostadt R, Hilbert M, Langen G *et al.* 2016. The fungal-specific β-glucan-binding lectin FGB1 alters cell-wall composition and suppresses glucan-triggered immunity in plants. *Nature Communications* **7**: 13188.

Win J, Chaparro-Garcia A, Belhaj K, Saunders DGO, Yoshida K, Dong S, Schornack S, Zipfel C, Robatzek S, Hogenhout SA *et al.* 2012. Effector biology of plant-associated organisms: concepts and perspectives. *Cold Spring Harbor Symposia on Quantitative Biology* **77**: 235–247.

Woo SL, Donzelli B, Scala F, Mach R, Harman GE, Kubicek CP, Del Sorbo G, Lorito M. 1999. Disruption of the ech42 (Endochitinase-Encoding) gene affects biocontrol activity in *Trichoderma harzianum* P1. *Molecular Plant–Microbe Interactions* **12**: 419–429.

Zuccaro A, Lahrmann U, Güldener U, Langen G, Pfiffi S, Biedenkopf D, Wong P, Samans B, Grimm C, Basiewicz M *et al.* 2011. Endophytic life strategies decoded by genome and transcriptome analyses of the mutualistic root symbiont *Piriformospora indica*. *PLoS Pathogens* **7**: e1002290.

Zuccaro A, Lahrmann U, Langen G. 2014. Broad compatibility in fungal root symbioses. *Current Opinion in Plant Biology* **20**: 135–145.

Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Upregulated *Serendipita indica* CAZymes in response to host plants and microbes.

Fig. S2 Upregulated *Serendipita vermifera* CAZymes in response to host plants and microbes.

Fig. S3 K-means clustering and Gene Ontology (GO) term annotation of microbe- and plant-induced genes in *Serendipita indica* and *Serendipita vermifera*.

Fig. S4 Overview of the different GH18-conjugated carbohydrate-binding modules (CBMs) in 135 fungi in three phyla.

Fig. S5 Purification of *Si*CHIT and *Sv*CHIT, *Sv*CHIT activity and effect of *Si*CHIT on *Serendipita indica* growth.

Fig. S6 Defense gene expression after treatment of *Arabidopsis thaliana* seedlings with *Bipolaris sorokiniana* pretreated with *Si*CHIT.

Methods S1 Biological materials, growth conditions, and microbial inoculations.

Methods S2 Purification and characterization of recombinant chitinases.

Table S1 List of primers used in this study.

Table S2 BioProject accessions for RNA-seq raw count data.

Table S3 Significantly upregulated *Serendipita indica* and *Serendipita vermifera* genes in response to different host plants or microbes.

Table S4 Genome references for GH18-CBM5 comparison.

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