Characterization of the Mechanisms underlying Plant-Microbe Multipartite Interactions

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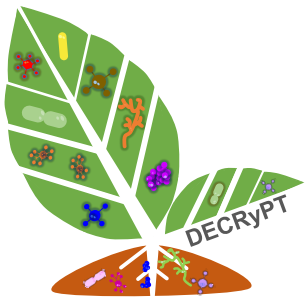

DFG Deutsche Forschungsgemeinschaft

>> In der Wissenschaft gleichen
Wir alle nur den Kindern,
die am Rande des Wissens
hie und da einen Kieselstein
aufheben, während sich der
weite Ozean des Unbekannten
vor unseren Augen erstreckt. <<

Isaac Newton
(1643 – 1727)

The Top 10 unanswered questions in MPMI

1. How do plants engage with beneficial microorganisms while at the same time restricting pathogens?
2. How does abiotic stress, such as climat change, influence plant-microbe interactions?
3. How can we translate basic research into emerging crop plants?
4. How to microbe-microbe interactions affect plant-microbe interactions?
5. Does effector-triggered immunity (ETI) potentiate and restore pathogen-associated molecular pattern-triggered immunity (PTI) - or is there really a binary distinction between ETI and PTI?
6. What is the molecular basis of nonhost resistance?
7. How do resistance proteins activate cell death?
8. Why do some pathogens need so many effectors when others need a few?
9. How do pathogens evolve novel virulence activities?
10. How do observations of binary plant-microbe interactions hold in an ecological context?

(Harris et al., 2020)

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List of Abbreviations

At	*Arabidopsis thaliana*
AQP	aquaporins
Bd	*Brachypodium distachyon*
bp	base pair
Bs	*Bipolaris sorokiniana*
CAZymes	carbohydrate active enzymes
CBM	carbohydrate binding motive
CD	carbohydrate esterase
CPP	copalyl diphosphate
CPS2	copalyl diphosphate synthase
Ct	cycle threshold
CWDE	cell wall degrading enzyme
CYP	cytochrome P450 oxygenase
Da	dalton
DAMP	damage associated molecular pattern
DAP	DNA affinity purification
DEG	differentially expressed genes
DIBOA	2,4-dihydroxy-1,4-benzoxazin-3-one
diTPS	diterpene synthases
dpi	days post inoculation
dpt	days post transfer
ECM	ectomycorrhizal
EPS	extracellular polymeric substance

ETI	effector triggered immunity
GGPP	all-*trans*-geranylgeranyl diphosphate
GH	glycosyl hydrolase
GO	gene ontology
GP	Golden Promise (barley cultivar)
HCA	hirachical clustering analysis
HR	hypersensitive response
Hv	*Hordeum vulgare*
ITS	internal transcribed spacer
ISR	induced systemic resistance
KSL4	kaurene synthase like4
M	mock
MAMP	microbe associated molecular pattern
MAPK	mitogen-activated protein kinase
MEP	methylerythritol 4-phosphate
NMR	nuclear magnetic resonance
NPRS	nonribosomal peptide synthetases
OTU	operational taxonomic unit
PGB	plant growth-promoting bacteria
PGPM	plant growth-promoting microbes
PKS	polyketide synthase
PR	pathogenisis related
PRR	pattern recognition receptor
PTI	pattern-triggered immunity
ROS	reactive oxygen species

RT-PCR	real time polymerase chain reaction
SAR	systemic acquired resistance
Si	*Serendipita indica*
Sv	*Serendipita vermifera*
SynCom	Synthetic Community
tHMGR	truncated hydroxymethylgeranyl CoA-reductase
UBI	ubiquitin
WT	wild type

Abstract

In nature, plants constantly interact with a multitude of microorganisms, collectively called the plant microbiota. Intimate associations and interdependencies between the plant and its microbiota result in a modulation of plant traits that ultimately determine plant health and yield production. Thus, unraveling the interaction networks, molecular mechanisms, and influencing factors that underlie plant-microbiota interactions is a prerequisite for developing future sustainable agricultural strategies.
Fungi of the ecologically relevant order Sebacinales feature a wide geographical distribution and host range. Root colonization by Sebacinales like *Serendipita vermifera* and *Serendipita indica* leads to beneficial effects for the host, including increased biomass and resistance to biotic and abiotic stresses. Yet little is known about factors and mechanisms that shape these widespread beneficial interactions. The studies comprised in this thesis aim to investigate the nature and relevance of the beneficial effects of Sebacinales on the host in multiple interactions.

The first study addressed the effects of the beneficial root endophyte *Serendipita vermifera* (*Sv*) and the cereal pathogen *Bipolaris sorokiniana* (*Bs*) on barley in a tripartite system. We showed that *Sv* exerts a biocontrol function by reducing pathogen colonization and host disease symptoms. Comparing the local and systemic transcriptional responses of all organisms in a gnotobiotic soil-based split-root system, we found that despite some degree of systemic protection, the major antagonistic effects were exerted upon direct contact and exhibited signs of mycoparasitism.

In the second study, the tripartite setup was extended by including a second plant host and bacterial synthetic communities (SynCom) to investigate the influence of biotic factors on the *Sv*-mediated beneficial traits. The combined presence of *Sv* and members of the core bacterial microbiota provides synergistic protection against *Bs* on both host plants. In addition, host- and microbe-dependent synergistic early growth promotion was observed. RNA-sequencing analysis revealed that these beneficial activities are not associated with extensive host transcriptional reprogramming but rather with the modulation of expression of fungal effectors and carbohydrate-active enzymes (CAZymes).

Therefore, the third study investigated the regulation of *Sv* effector and CAZyme expression in biotic interactions, including interactions with three plant hosts, a fungus or a bacterial SynCom at four time points. RNA-seq analysis identified commonalities and differences in the *Sv* transcriptome during fungal accommodation *in planta* or microbe-microbe confrontation in the absence of the host. Further, a *Sv* chitinase was identified and characterized as a determinant of fungal antagonism.

The fourth study investigated the *Bs*-induced expression of barley *HvKSL4* and *HvCPS2,* that was observed in the original tripartite setup. This led to the identification of a barley gene-cluster for barley diterpene synthesis and was a first step in decifering the role of a secreted barley diterpene during biotic interactions.

Finally, a protocol to monitor plant health and cell death via Ion leakage and Pulse Amplitude Fluorometry (PAM) was developed. This method is suitable for future multipartite setups as well as for chemical treatments and particularly useful for large-scale screenings. The studies comprised in this thesis expand our knowledge in the fields of plant-microbe interactions and biocontrol and lay the foundation for answering future research questions.

Kurzzusammenfassung

In der Natur interagieren Pflanzen kontinuierlich mit einer Vielzahl von Mikroorganismen, die zusammenfassend als Mikroflora bezeichnet werden. Die enge Assoziation und wechselseitigen Abhängigkeiten zwischen der Pflanze und ihrer Mikroflora führt zu einer Modulation von Pflanzenmerkmalen, die unmittelbar im Bezug zu Pflanzengesundheit und -ertrag stehen. Das Ergründen der Netzwerke, molekularen Mechanismen und Einflussfaktoren von Pflanzen-Mikroben-Interaktionen ist daher eine Voraussetzung für die Entwicklung zukunftsfähiger landwirtschaftlicher Strategien.

Die ökologisch bedeutende Ordnung der Wachskrustenartigen (Sebacinales) weist eine weite geographische Verbreitung und ein breites Spektrum an Wirtspflanzen auf. Die Kolonisierung der Wirtswurzeln durch Sebacinales Pilze wie *Serendipita vermifera* und *Serendipita indica* führt zu der Ausprägung positiver Pflanzenmerkmale, wie einer gesteigerten Biomasse und dem Schutz gegen biotische und abiotische Stressfaktoren. Die Faktoren und Wirkmechanismen, welche diese nützliche Interaktion beeinflussen, sind jedoch bisher noch unzureichend verstanden. Die Studien im Kontext dieser Doktorarbeit haben zum Ziel die Art und Relevanz der positiven Effekte auf die Wirtspflanze durch Sebacinales in verschiedenen Interaktionen zu untersuchen.

In der ersten Studie wurden die Effekte des Wurzelendophyten *Serendipita vermifera (Sv)* und des Getreidepathogens *Bipolaris sorokiniana* (*Bs*) in einem trilateralen System mit Gerste getestet. Wir zeigen, dass *Sv* eine Biokontroll-Funktion aufweist, da der Pilz die Kolonisierung des Pathogens sowie die Pathogen-induzierten Krankheitssymptome an der Wirtspflanze reduziert. Unter Verwendung eines gnotobiotischen, Erde-basierten Systems, dass das Wurzelsystem der Wirtspflanze in zwei separate Abschnitte teilt, haben wir die lokale und systemische transkriptionelle Reaktion der drei Organismen untersucht. Neben einem gewissen systemischen Schutz fand der Großteil des Antagonismus in direktem Kontakt beider Pilze statt und wies mykoparasitäre Züge auf.

Im Rahmen der zweiten Studie wurde der trilaterale Versuchsaufbau durch das Integrieren einer zweiten Wirtspflanze und synthetischen bakteriellen Gemeinschaften

erweitert, um die Einflüsse biotischer Faktoren auf die positiven Effekte von Sebacinales auf die Wirtspflanze zu untersuchen. Die gemeinschaftliche Anwesenheit von *Sv* und Mitgliedern der bakteriellen Kern-Mikroflora schützt beide Wirtspflanzen synergistisch vor *Bs*. Zusätzlich wurde eine Wirtspflanzen- und Mikroben-spezifische Wachstumsförderung beobachtet. RNA-Sequenzierungen haben gezeigt, dass sowohl der Schutz als auch die Wachstumsförderung nicht mit einer intensiven transkriptionellen Reprogrammierung der Wirtspflanze zusammenhängen, sondern vielmehr mit der Modulation der Expression von pilzlichen Effektoren und Kohlenhydrat-aktiven Enzymen.

In der dritten Studie wurde daher die Regulierung der Geneexpression von *Sv* Effektoren in verschiedenen biotischen Interaktionen wie der Interaktion mit drei verschiendenen Wirtspflanzen, einem Pilz oder einer synthetischen bakteriellen Gemeinschaft über vier Zeitpunkte hinweg untersucht. RNA-Sequenzierungen offenbarte Gemeinsamkeiten und Unterschiede in der transkriptionellen Regulierung während der Kolonisierung von Wirtspflanzen im Vergleich mit der Interaktion mit Mikroorganismen. Weiter wurde eine *Sv* Chitinase als relevant in der Interaktion mit *Bs* identifiziert.

Im Rahmen der vierten Studie wurde die *Bs*-induzierte Expression der Gerstegene *HvKSL4* und *HvCPS2* untersucht, die wir im ursprünglichen trilateralen System beobachtet hatten. Dies führte zu der Identifikation des ersten Gen-Clusters, dass in die Produktion von Gerste Diterpenen involviert ist. Ergänzend haben wir damit begonnen die Funktion eines sekretierten Gerste Diterpens in biotischen Interaktionen zu entschlüsseln.

Zuletzt wurde ein Protokoll zur Messung pflanzlicher Gesundheit und pflanzlichen Zelltods mittels Ion Leakage und Puls Amplituden Fluorometrie (PAM) etabliert. Die Methode kann in der Zukunft sowohl für biotische als auch für chemische Anwendungen an Pflanzen verwendet werden und ist besonders für groß angelegte Versuchsaufbaue geeignet.

Die Studien, die in dieser Arbeit enthalten sind, erweitern unser Wissen in den Bereichen der Pflanzen-Mikroben Interaktion und der Biokontrolle und legen das Fundament für die Beantwortung zukünftiger Fragestellungen.

Introduction

The Spectrum of Plant-associated Microbes

Plants constantly interact with a multitude of bacteria, oomycetes, and fungi, with whom they form parasitic, commensal, or mutualistic relationships. Parasitic microorganisms exploit their host and cause harm, while mutualistic relationships benefit both partners. Either way, the interaction with microorganisms has a massive impact on plant health and productivity (Kumar et al., 2002; Perreault & Laforest-Lapointe, 2022; Trivedi et al., 2020). From an evolutionary perspective, facultative and obligate interacting microorganisms evolved from free-living ancestors and continue to evolve. The nature of an interaction between the same organisms can change with environmental or ecological alterations within the interacting species or its surrounding communities. In this regard, microbial lifestyles are not rigid but rather move along a parasite-mutualist continuum (Drew et al., 2021; Fesel & Zuccaro, 2016; Newton et al., 2010; Xu et al., 2015).

Microbe Recognition and Plant Immunity

Plants recognize surrounding microbes via specialized cell surface pattern recognition receptors (PRRs). PRRs detect microbial structures called microbe-associated molecular patterns (MAMPs) or endogenous signals that are induced upon wounding called damage-associated molecular patterns (DAMPs). This recognition activates a first layer of immunity called pattern-triggered immunity (PTI) (Jones and Dangl, 2006). A second layer of recognition occurs in interaction with adapted microorganisms that overcome PTI via the secretion of effectors. Effectors are small, secreted molecules that affect the host or surrounding microbes (Todd et al., 2022). The direct or indirect detection of these effectors by the host via specialized, intracellular receptors leads to an enhanced immune response called effector-triggered immunity (ETI) (Jones and Dangl, 2006). The plant immune system comprising PTI and ETI was previously described in a “zig-zag”-model that depicts PTI and ETI as independent immune pathways (Jones and Dangl, 2006). However, recent research suggests that the two immunity pathways are interconnected and potentiate each other (Chang et al., 2022; Ngou et al., 2021; Pruitt et al., 2021; Tian et al., 2021; Jiang, et al., 2021; Ngou, et al., 2021). The activation of plant immunity (PTI and ETI) induces various defense responses, including the production of reactive oxygen species (ROS), mitogen-

activated protein kinase (MAPK) cascades, calcium influx, the production and secretion of defense-related proteins such as pathogenesis-related (PR) proteins and antimicrobial compounds including phenylpropanoids, oxylipids, carotenoids and terpenoids (Peng et al., 2018; Tsuda and Katagiri, 2010).

As MAMPs are a common feature of microbes across all lifestyles, successful colonization of pathogenic as well as beneficial microbes often relies on strategies to suppress or evade the plant immune responses (Jacobs et al., 2011; Pel and Pieterse, 2013; Yu et al., 2019). Till today, little is known about how plants induce immunity to restrict pathogen growth and proliferation while at the same time actively engaging with beneficial microorganisms (Dudenhöffer et al., 2016; Santoyo, 2021).

Beneficial Plant-Microbe Interactions

Interactions between plants and beneficial microorganisms are highly diverse. Colonization by certain microorganisms, such as arbuscular mycorrhizal fungi or nitrogen-fixing rhizobia, is tightly regulated by a conserved reciprocal signaling cascade between host and microbe and is often associated with the formation of specialized structures (Chaulagain and Frugoli, 2021; Oldroyd, 2013). Other beneficial microbes colonize the host plant endophytically but do not form specialized structures (Brotman et al., 2010; Hiruma et al., 2016; Weiß et al., 2016; Yuan et al., 2010). Further, certain epiphytic microbes that do not enter the plant but reside on its surface exhibit symbiotic beneficial effects (Bruisson et al., 2019; Butani et al., 2021). Interaction with beneficial microbes can improve plant nutrition, increase plant biomass, or protect plants against biotic as well as abiotic stresses (Almario et al., 2017; de Lamo and Takken, 2020; Franken, 2012; Harbort et al., 2020; Hermosa et al., 2012; Hiruma et al., 2016; Lugtenberg and Kamilova, 2009; Pieterse et al., 2014; Spaepen et al., 2007; Vlot et al., 2021; Vorholt, 2012).

Microbe-mediated plant growth promotion is often associated with enhanced nutrient availability. Plant growth-promoting microbes (PGPMs) increase the supply of essential nutrients such as nitrogen, phosphate, potassium, iron, and sulfur via a variety of mechanisms, including nitrogen fixation, inorganic phosphate solubilization, iron chelation, and the production of volatile compounds (Lopes et al., 2021; Souza et al., 2015; Varma et al., 2019). Certain endophytic fungi recruit inorganic phosphate (Pi)-solubilizing bacteria for Pi mobilization that they then take up and translocate further to the plant host (Ezawa and Saito, 2018; Meena et al., 2010; Singh et al.,

2009). Besides this, certain PGPMs function through the modulation of plant hormone status by increasing concentrations of auxin, gibberellin, and cytokinin and mitigating stress through the production and supply of exopolysaccharides, antioxidants, and osmoregulants (Lopes et al., 2021).

Microbe-mediated biocontrol activities can occur directly via microbe-microbe interactions or indirectly via the plant host. Indirect biocontrol activities comprise induced systemic resistance (ISR) and systemic acquired resistance (SAR). Direct biocontrol activities comprise, among others, antibiosis and mycoparasitism (Latz et al., 2018). Various studies reveal the relevance of secreted antimicrobial compounds or volatiles as well as microbial effectors in direct microbe-microbe antagonism (Eitzen et al., 2021; Hassani et al., 2018; Horner et al., 2012; Laur et al., 2018; Mgbeahuruike et al., 2011; Tilocca et al., 2020).

The Plant Microbiota

While the mechanisms underlying microbe-mediated host beneficial traits used to be primarily investigated in bipartite or tripartite interactions, recent research is expanding this perspective, and the plant microbiota moved into focus as a major contributor to plant health and protection (Berendsen et al., 2012; Schlaeppi and Bulgarelli, 2015). The plant microbiota comprises all microbes that live inside or in close proximity to a plant host and can be divided into the aboveground phyllosphere microbiota and the belowground rhizosphere microbiota (Berendsen et al., 2012; Raaijmakers et al., 2009; Vorholt, 2012). The plant rhizosphere comprises the soil adjacent to the roots and belowground organs and constitutes an essential source for plants to acquire water and nutrients (Liu et al., 2022). It is a hotspot for plant-microbe interactions and features a huge microbial diversity (Dlamini et al., 2022; Ling et al., 2022; Pathan et al., 2020). This microbial diversity gradually decreases from rhizosphere to the endosphere compartment, whose colonization relies on a more intimate plant microbe association (Lemanceau et al., 2017). Past studies employed environmental sampling or sampling in controlled conditions in the field and laboratory to determine members of different microbiota (Bai et al., 2015; Bulgarelli et al., 2015; Durán et al., 2018; Edwards et al., 2015; Fitzpatrick et al., 2018; Lundberg et al., 2012; Runge et al., 2022; Thiergart et al., 2020). Research identified hub and keystone species that strongly shape the microbiome community structure via direct or indirect biotic interactions (Agler et al., 2016; Zheng et al., 2021) as well as core microbiota strains that have

repeatedly been identified as endophytes across different plant species in different soil types and environmental conditions (Lemanceau et al., 2017).

Due to their strong association, some studies describe the host and its surrounding microbiota as an inseparable functional unit - the plant holobiont (Hassani et al., 2018; Lyu et al., 2021; Rosenberg and Zilber-Rosenberg, 2016; Vandenkoornhuyse et al., 2015). On the contrary, others disagree with the assumption of a high partner fidelity within the whole community (Douglas and Werren, 2016; Moran and Sloan, 2015). Indeed, plant-microbiota associations form an entangled interaction network that is shaped by abiotic factors and host factors as well as by microbe-microbe cooperation and competition (Berendsen et al., 2012; Raaijmakers et al., 2009; Snelders et al., 2018; Vannier et al., 2019). While the complexity of microbiota networks and microbial interaction patterns can be visualized using bioinformatical networks and co-occurrence analysis; understanding the mechanisms underlying these associations requires a complex scientific interface between ecological and molecular approaches with a focus on all key players, plant physiology and plant immunology, microbial responses, and environmental factors. Recent experimental approaches reconstruct plant microbiota compositions with the help of microbial synthetic communities (SynComs), and thereby attempt to bridge large-scale ecological and mechanistic studies.

Fungal and plant models to study host-microbe interactions

Sebacinales

The fungal order Sebacinales (Agaricomycetes, Basidiomycota) comprises ectomycorrhizal, endophytic, and saprotrophic species that all derived from a saprotrophic ancestor (Garnica et al., 2016; Weiß et al., 2016). Sebacinoid fungi display a wide geographical distribution and a broad host range, rendering them potential key players for terrestrial ecosystems (Oberwinkler et al., 2013; Riess et al., 2014; Verbruggen et al., 2014; Weiß et al., 2011). Among them, *Serendipita indica* and *Serendipita vermifera* have been intensively studied studies as model species for Sebacinales and root endophytes (Deshmukh et al., 2006; Dunken et al., 2022; Ghimire et al., 2009; Ghimire and Craven, 2011; Lahrmann et al., 2015; Nizam et al., 2019; Ray and Craven, 2016; Waller et al., 2008; Wawra et al., 2019).

Serendipita vermifera was originally isolated from the Australian orchid *Cyrostylis*

reniformis (Warcup, 1988) and *Serendipita indica* from the woody shrubs *Prosopis juliflora* and *Ziziphus nummularia* in the Indian dessert (Verma et al., 1998). However, they both show little host specificity and further grow saprotrophically in absence of a living host (Franken, 2012; Ray and Craven, 2016). Colonization of these species is typically associated with diverse beneficial effects including host growth promotion, nutrient acquisition, and protection (Barazani et al., 2007; Franken, 2012; Ghimire and Craven, 2011; Kumar et al., 2012; Lahrmann et al., 2015; Nautiyal et al., 2010; Qiang et al., 2012a; Shahollari et al., 2005; Waller et al., 2008). Moreover, their ability to grow axenically and their amenability to genetic modification render them excellent models to study plant-endophyte interactions.

S. vermifera and *S. indica* colonize plant roots extra- and intracellularly. Fungal growth is restricted to the epidermal cells and cortex cell layers. The fungi do not enter the endodermis, nor the vascular tissue and colonization does not extend systemically into the shoots. Intracellular colonization is further not associated with specialized structures. Colonization increases with root maturation and is most abundant in the elongation and differentiation zone (Deshmukh et al., 2006; Jacobs et al., 2011). Following an initial biotrophic growth phase, *S. vermifera* and *S. indica* undergo a switch to a cell death-associated phase that is restricted to colonized cells and does neither cause root browning nor necrosis. This localized cell death is required for successful fungal accommodation and to establish long-lasting beneficial interactions with plant hosts (Deshmukh et al., 2006; Jacobs et al., 2011; Lahrmann et al., 2013; Schäfer et al., 2009). Several fungal effectors were previously described to contribute to host colonization (Dunken et al., 2022; Nizam et al., 2019; Nostadt et al., 2020a, 2020b; Wawra et al., 2019). However, fungal effectors that contribute to the conferred host beneficial traits remain to be elucidated.

Bipolaris sorokiniana

The ascomycete *Bipolaris sorokiniana* (teleomorph: *Cochiobolus sativus*) causes black point, common root rot and leaf blotch disease in a variety of economically relevant crops including barley and wheat (Parmelee et al., 1990). Diseases caused by *B. sorokiniana* result in severe, worldwide yield losses (Al-Sadi, 2021; Krishnendu Acharya et al., 2011; Kumar et al., 2002; Sharma & Duveiller, 2006). Consequently, the hemi-biotrophic fungus was declared a major threat to wheat production in warmer

regions (Duveiller, 1994; Manamgoda et al., 2014 Ghazvini & Tekauz, 2012; Kang et al., 2020; Karov et al., 2009; Murray et al., 1998; Sharma & Duveiller, 2010).

Root rot often originates from inoculum carried on the seeds or from infections by soilborne conidia near the seedling, but the fungus can infect plants at any growth stage. The conidia attach to the plant surface, where they germinate and form germ tubes with multiple branches. From these branches appressoria are formed that penetrate the host cell surface (Krishnendu Acharya et al., 2011). *B. sorokiniana* produces a variety of sesquiterpenoid toxins that are the key factors determining fungal pathogenicity (Apoga et al., 2002; Jahani et al., 2014; Kumar et al., 2001, 2002; Nilsson et al., 1993). Furthermore, *B. sorokiniana* carries the *ToxA* gene, encoding a host-selective toxin that exhibits effector functions (Navathe et al., 2020). Besides toxins, hydrolytic enzymes such as an endo-1,4-β-xylanase and putative secreted effector genes were hypothesized to be required for host colonization and pathogenicity (Condon et al., 2013; Geimba et al., 1999; Zhang et al., 2022).

Barley

Barley (*Hordeum vulgare*) is among the oldest cultivated crops that spread from the fertile crescent in the Middle East to the rest of the World from near desert areas to arctic or subarctic regions (Nevo, 1992). Barley gained relevance as a model species from its close relatedness to wheat and its diploid genome. However, with the use of barley in alcohol production, animal feed and human food, it has significant economic relevance (Newton et al., 2011). Indeed, barley ranks fourth among all grain crops with an annual production of over 140 million tons from about 55 million ha (Zhou, 2009). Despite agricultural measures, infection with pathogens including *Bipolaris sorokiniana* cause severe barley yield losses (Dutbayev et al., 2022; Kumar et al., 2002; Manamgoda et al., 2014) underlying the need for the development of new strategies to prevent pathogen infection.

Aim of Studies

The aim of these studies was to unravel the mechanisms underlying the interactions between the beneficial root endophyte *Serendipita vermifera* (*Sv*), the hemibiotrophic pathogen *Bipolaris sorokiniana* (*Bs*) and barley as a plant host. Accounting for the complexity of plant-microbiota interactions, this system was analyzed from different perspectives including endophytic and pathogenic colonization, endophyte-mediated

host beneficial effects, the influence of biotic factors on these interactions and interaction relevant host factors.

A gnotobiotic soil-based split root system allowed the investigation of local and systemic effects of both fungi on the host plant and each other (Sarkar et al., 2019). *Sv* antagonizes *Bs* resulting in host protection. Transcriptomics further gave first insights into the underlying molecular mechanisms. Among the differentially expressed genes, a *Sv* chitinase (*Sv*CHIT-CBM5) was upregulated in response to *Bs* and hypothesized to be relevant for fungal antagonism. Barley immunity and the expression of genes associated with terpenoid biosynthesis were induced in response *Bs* but only minor in response to *Sv*.

The influence of biotic factors on the observed fungal effects was investigated by including *Arabidopsis thaliana* as a second plant host as well as two bacterial SynComs, each isolated from one of the respective host plants. Effects of non-native bacterial communities were determined using a SynCom swap approach (Mahdi et al., 2022). *Sv* and bacterial SynComs synergistically mediate host protection and growth promotion. These beneficial traits were not associated with large host transcriptional reprogramming but rather a modulated expression of fungal effectors and carbohydrate active enzymes.

To gain insight into the regulatory mechanisms that underlie microbe-plant and microbe-microbe interactions, the transcriptional responses of *Sv* to different plant hosts and microorganisms was investigated in a large time course experiment (Mahdi et al., n.d.). The data set revealed commonalities and differences in the transcriptional response of *Sv* to the different organisms and confirmed the specific upregulation of *Sv*CHIT-CBM5 in the presence of *Bs*. *Sv*CHIT-CBM5 and the *Serendipita indica* homolog *Si*CHIT-CBM5 inhibit *Bs* spore germination, revealing a direct antagonistic function.

Following up on the barley transcriptomic response to *Bs*, the pathogen-induced expression of genes associated with terpenoid synthesis was further investigated. This led to the characterization of a barley gene cluster involved in the production of labdane-related diterpenoids. Exogenous application of a major secreted barley diterpene does not inhibit spore germination and growth of *Bs* but of other fungi, suggesting an effect on the plant microbiota (Liu et al., 2021).

Finally, the increased complexity of multipartite experimental setups prompted the establishment of a screening protocol for a high throughput quantification of plant health and cell death in *Arabidopsis* (Dunken et al., 2021). The research performed in these studies gives important insights into plant-microbe and microbe-microbe interactions and supplies several large data sets that will serve as a basis for future research to understand their underlying molecular mechanisms.

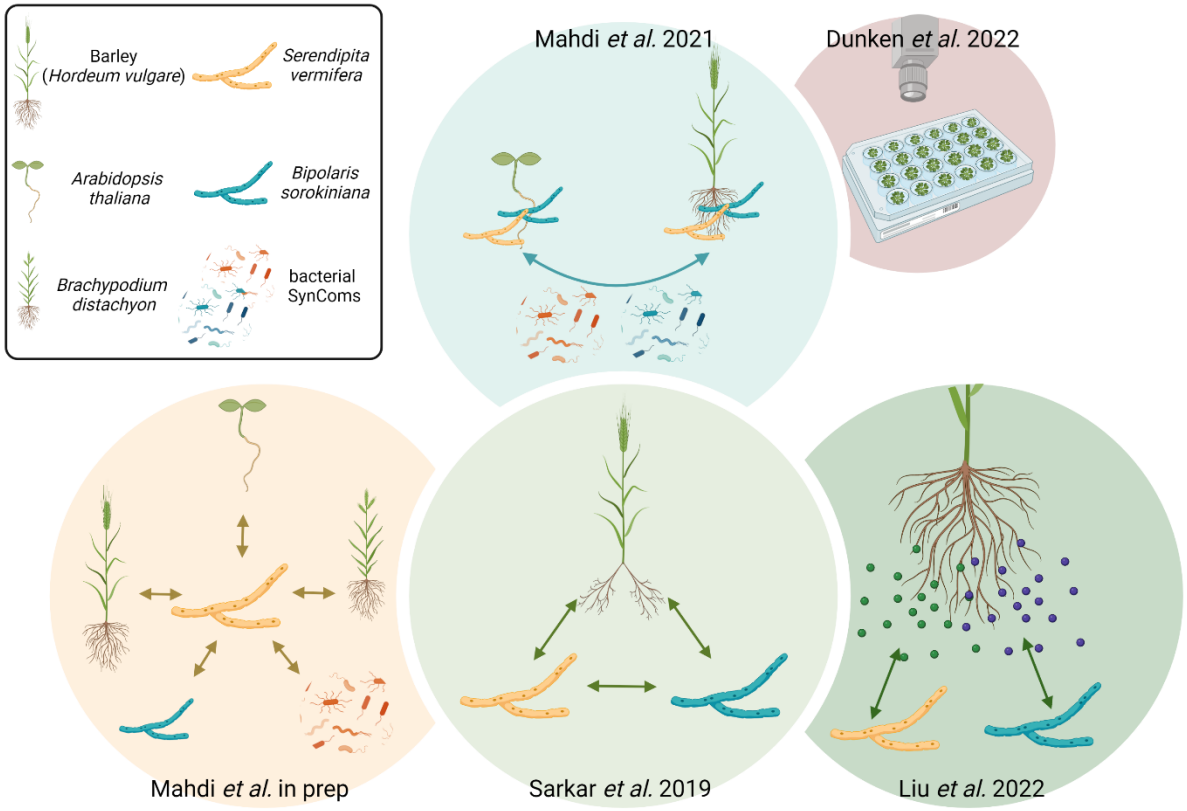

Figure 1: Schematic overview of the studies included in this thesis. 1) A tripartite system to analyze the interaction between Barley, *S. vermifera* (*Sv*), and *B. sorokiniana* (*Bs*) (Sarkar *et al.* 2019); 2) The extension of the tripartite system to analyze the stability of the tripartite system in response to changes in the biotic environment that include a second plant host as well as host- and non-host specific bacterial synthetic communities (Mahdi *et al.* 2021); 3) The analysis of *Sv* transcriptional responses to different plant hosts and microorganisms that reflect different interaction strategies; 4) The investigation of host diterpene secretion in response to *Sv* or *Bs* (Liu *et al.* 2022); 5) The development of a large-scale screening method for plant health and cell death using ion leakage and PAM fluorometry (Dunken *et al.* 2022)

Publications included in this thesis

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 doi: 10.1111/nph.15904

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Chapter 1

The inconspicuous gatekeeper: endophytic *Serendipita vermifera* acts as extended plant protection barrier in the rhizosphere.

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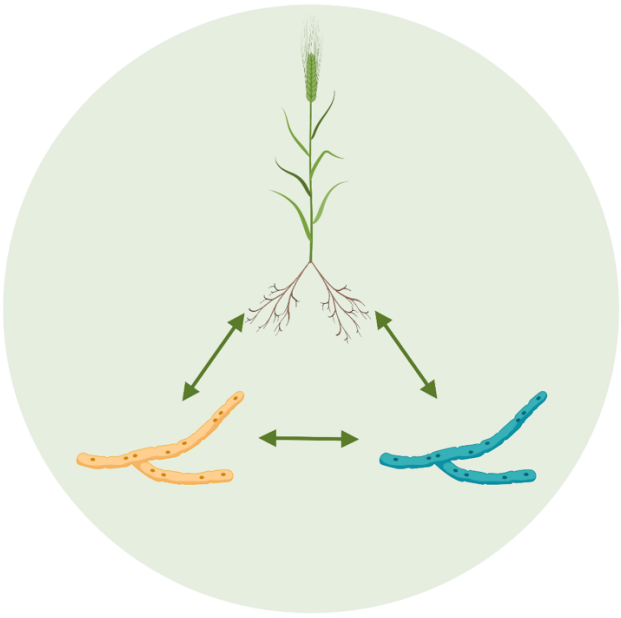
Authors contributions:

AZ, GL, MB, and DS conceived the project and planned the experiments. DS, **LM**, and HR carried out inoculations, RNA extractions, RT-PCRs and confocal microscopy. DS, GJ, HR, SN, AT, GUB and GL analyzed the data. HR, DS and AZ wrote the manuscript. DS, HR and GJ contributed equally to this work.



Research

New Phytologist

The inconspicuous gatekeeper: endophytic *Serendipita vermifera* acts as extended plant protection barrier in the rhizosphere

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Summary

- In nature, beneficial and pathogenic fungi often simultaneously colonise plants. Despite substantial efforts to understand the composition of natural plant–microbe communities, the mechanisms driving such multipartite interactions remain largely unknown.
- Here we address how the interaction between the beneficial root endophyte *Serendipita vermifera* and the pathogen *Bipolaris sorokiniana* affects fungal behaviour and determines barley host responses using a gnotobiotic soil-based split-root system.
- Fungal confrontation in soil resulted in induction of *B. sorokiniana* genes involved in secondary metabolism and a significant repression of genes encoding putative effectors. In *S. vermifera*, genes encoding hydrolytic enzymes were strongly induced. This antagonistic response was not activated during the tripartite interaction in barley roots. Instead, we observed a specific induction of *S. vermifera* genes involved in detoxification and redox homeostasis. Pathogen infection but not endophyte colonisation resulted in substantial host transcriptional reprogramming and activation of defence. In the presence of *S. vermifera*, pathogen infection and disease symptoms were significantly reduced despite no marked alterations of the plant transcriptional response.
- The activation of stress response genes and concomitant repression of putative effector gene expression in *B. sorokiniana* during confrontation with the endophyte suggest a reduction of the pathogen's virulence potential before host plant infection.

Introduction

Many plant-colonising microbes originate from the space immediately surrounding plant roots, the rhizosphere, which is a hot spot for microbial life. The fierce competition for space and nutrients in the rhizosphere has driven the evolution of plant-colonising microbes whose lifestyles range from mutualistic to pathogenic (Card *et al.*, 2016; Snelders *et al.*, 2018). To establish intimate, long-lasting relationships with beneficial microbes while fending off infections by putatively harmful invaders, plants rely on closely regulated, complex signalling cascades. Irrespective of the invader's lifestyle, early plant responses often include the activation of plant immune responses (Cook *et al.*, 2015; Couto & Zipfel, 2016; Zipfel & Oldroyd, 2017). These are counteracted by microbial secreted effector molecules, which act to facilitate niche colonisation (Rovenich *et al.*, 2014).

To date, the mechanisms underlying plant–microbe interactions have mostly been investigated in isolated plant–microbe systems. While this has revealed important insights into plant immunity and microbial infection strategies (Zipfel & Oldroyd, 2017), it is now clear that these plant–microbe interactions are more complex in nature and are largely determined by multipartite interactions (Agler *et al.*, 2016). This has led to the hypothesis that microbe–microbe competition and co-operation are crucial processes affecting plant–microbe interactions in the rhizosphere, and are possibly driven by microbial effector proteins (Snelders *et al.*, 2018). Recently, the *Zymoseptoria tritici* ribonuclease effector Zt6 was shown to display both phytotoxic as well as antimicrobial activity (Kettles *et al.*, 2018), providing first experimental evidence for a class of effector proteins required for plant colonisation as well as microbe–microbe interactions. Similarly, the gene *tvhydii1* of *Trichoderma virens*, encoding a class II hydrophobin, is expressed during *Arabidopsis thaliana* root colonisation as well as confrontation with the phytopathogen *Rhizoctonia solani* (Guzman-Guzman *et al.*, 2017). Moreover, putative effector genes of the mycoparasite *Pseudozyma flocculosa* are specifically expressed during colonisation of the phytopathogen *Blumeria graminis* (Laur *et al.*, 2018). However, the

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molecular functions of effector proteins that specifically manipulate microbe–microbe interactions remain to be elucidated.

Fungi of the order Sebacinales (Agaricomycotina, Basidiomycota) are ubiquitously present in roots of wild and cultivated plant species (Weiss *et al.*, 2011; Oberwinkler *et al.*, 2013) and were recently reported to grow in association with liverworts from the division Marchantiophyta, considered to be the most ancient nonvascular land plants (Nelson & Shaw, 2019). Depending on the plant host they can establish endophytic associations without forming any peculiar interaction structure, as well as symbioses ranging from ectomycorrhizal through ericoid to orchid mycorrhizal interactions (Weiss *et al.*, 2016). Despite low levels of colonisation observed in several environmental studies, Sebacinales have been identified as the most abundant endophyte order with no apparent host specificity (Garnica *et al.*, 2013; Riess *et al.*, 2014; Wehner *et al.*, 2014). Plant colonisation by endophytic fungi can result in profound, beneficial effects on host plants at diverse levels under laboratory and field conditions (Franken, 2012; Lahrmann *et al.*, 2015; Hiruma *et al.*, 2016; Almario *et al.*, 2017). Beneficial effects include enhanced biomass production (Deshmukh *et al.*, 2006; Ghimire *et al.*, 2009; Fakhro *et al.*, 2010; Ghimire & Craven, 2011; Banhara *et al.*, 2015) and increased resistance against biotic and abiotic stresses in barley, *Arabidopsis thaliana* and switch grass (Waller *et al.*, 2008; Ghimire & Craven, 2011). However, the molecular mechanisms governing the processes associated with such beneficial effects remain largely unknown. Closely related *Serendipita vermifera* and *S. indica* have been used in molecular studies as model species for this fungal order (Deshmukh *et al.*, 2006; Waller *et al.*, 2008; Ghimire *et al.*, 2009; Ghimire & Craven, 2011; Lahrmann *et al.*, 2015; Ray & Craven, 2016). Following an initial biotrophic growth phase, *S. vermifera*, like *S. indica*, undergoes a switch to a host cell death-associated phase during later stages of plant colonisation. Its ability to grow saprotrophically suggests that *S. vermifera* spends part of its life cycle outside the plant host and is, therefore, likely to have evolved strategies to combat microbial competitors in the rhizosphere.

The ascomycete *Bipolaris sorokiniana* (teleomorph *Cochliobolus sativus*) is a serious pathogen of cereals, including barley and wheat (Kumar *et al.*, 2002). It causes common root rot and leaf spot blotch disease and was declared a major threat to wheat production in warmer regions worldwide (Duveiller & Gilchrist, 1994; Manamgoda *et al.*, 2014). Even in newly released, partially resistant wheat cultivars grain yield losses of up to 43% were reported (Sharma & Duveiller, 2006). A comparative analysis on the genetic structure of three *B. sorokiniana* populations suggested host- and tissue-specific differentiation (Gyawali *et al.*, 2012). Genome analysis identified putative effector genes encoding small-secreted proteins (SSPs) indicating that *B. sorokiniana*, like *S. vermifera*, utilises effectors to colonise its hosts (Ohm *et al.*, 2012; Condon *et al.*, 2013; Lahrmann *et al.*, 2015). However, no molecular function has been ascribed to these effector candidates and the molecular mechanisms underlying the detrimental effects of *B. sorokiniana* cereal infections, particularly of host root tissues, remain to be elucidated.

In a preliminary confrontational screen *S. vermifera* (MAFF 305830) displayed strong antagonistic effects against a series of fast-growing root pathogens, including *B. sorokiniana* ND90Pr (data not shown). Here we investigated the putative protective activity of *S. vermifera* during *B. sorokiniana* infection of barley root tissue on defined medium and in a gnotobiotic soil-based split-root system. We performed RNA-seq analysis of mixed and single mycelial soil cultures as well as (co-)colonised barley root tissue to assess fungal and plant transcriptional differences during fungal–fungal, fungal–plant and tripartite interactions. Taking advantage of the physical separation between treatments in the split-root system, we additionally examined the role of the plant host during systemic responses to fungal colonisation. By combining transcriptional data with phenotypic analyses, we provide evidence that fungal confrontation outside the plant exerts considerable biotic stress on the well-adapted cereal pathogen, weakening its ability to colonise its plant host. Conversely, the generalist root endophyte *S. vermifera* displays resilience to biotic stresses and maintains a dynamic transcriptional response that appears crucial for successful plant colonisation in a multispecies environment.

Materials and Methods

RNA-seq experiment

Sample preparation Plant and fungal materials were prepared as described in Supporting Information Methods S1 and S2. Total RNA was extracted from 100 to 200 mg ground root samples using TRIzol (Invitrogen, Karlsruhe, Germany) according to manufacturer's instructions. Ground fungal material from mixed mycelial assays was used for total RNA extraction using the Nucleospin RNA Plant kit® (Macherey-Nagel, Düren, Germany). Contaminating gDNA was removed during a DNase I treatment (Thermo Fisher Scientific, Schwerte, Germany) according to manufacturer's instructions. RNA integrity and absence of gDNA was confirmed by gel electrophoresis, quantified using a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific) and stored at −80°C. In total 1−3 μg of RNA were dissolved in 20 μl of nuclease-free water for RNA sequencing.

Illumina RNA-seq Illumina TruSeq RNA-sequencing libraries were prepared according to the manufacturer's instructions (Illumina Inc., San Diego, CA, USA). Qualified libraries were sequenced on a HiSeq 2500 system instrument at the Genome Centre of the Max Planck Institute for Plant Breeding. Libraries constructed from the fungal confrontation samples were sequenced to generate 25 million single-end reads of 100-bp length for each of the three biological replicates. For the plant–fungi tripartite samples, libraries were sequenced to generate 6 million paired-end reads with a 250-bp read length from three biological replicates. Details on the analysis of RNA-seq data and their confirmation by quantitative RT-PCR can be found in Methods S3.

Data availability

The RNA-seq data used in this study are available from the NCBI GEO database: GEO series GSE130517, BioProject PRJNA492913.

Results

Serendipita vermifera antagonises *B. sorokiniana* on defined medium and in soil

Sebacinoid fungi have been reported to increase host plant resistance to fungal pathogens (Waller *et al.*, 2005, 2008; Deshmukh *et al.*, 2006; Deshmukh & Kogel, 2007; Stein *et al.*, 2008). Our previous observations suggested that *S. vermifera* (MAFF305830) is able to antagonise root pathogens including *B. sorokiniana* (ND90Pr) (data not shown). To confirm these findings, direct confrontation assays were carried out on defined media (for details see Methods S2). During co-cultivation *S. vermifera* restricted *B. sorokiniana* growth (*Sv*+*Bs*, Fig. S1a). This inhibition was established at the interaction zone, where hyphae were in direct contact, without the formation of a halo around the fungal colonies indicating that the growth inhibition of *B. sorokiniana* was not mediated by diffusible/volatile substances or by antibiosis. Microscopic observation of the interaction zone showed that *S. vermifera* hyphae coil around hyphae of *B. sorokiniana* (Fig. S1b, upper left panel). In some cases, *S. vermifera* even penetrated and colonised *B. sorokiniana* cells (Fig. S1b, upper right panel). *Bipolaris sorokiniana* was similarly affected by the presence of *S. vermifera* at 2 d post contact (dpc) in mixed mycelial cultures in sterilised Cologne soil compared with the control, forming fewer white aerial hyphae (Fig. S1c). These phenotypes are reminiscent of mycoparasitic *Trichoderma* spp. (Harman *et al.*, 2004; Druzhinina *et al.*, 2011), and suggest that the capability of parasitising other fungi has also evolved in the order Sebacinales. While *Trichoderma* spp. are frequently used for biocontrol of crops against pathogenic fungi and oomycetes (Elad *et al.*, 1984; Howell, 2003; Benitez *et al.*, 2004; Druzhinina *et al.*, 2011), the biocontrol potential and molecular mechanisms involved in fungal antagonism are largely unexplored in Sebacinales.

Serendipita vermifera significantly reduces barley root infection by *B. sorokiniana*

To establish whether the antagonistic behaviour of *S. vermifera* affects barley root infection by *B. sorokiniana*, we first assessed the degree of fungal colonisation and disease symptoms in barley root tissue 6 d after inoculation (dpi) with *S. vermifera*, *B. sorokiniana* or both fungi together on defined plant nutrition medium (PNM) (Fig. S2; details described in Methods S4; for primer sequences see Table S1). In accordance with their growth rates in axenic cultures, *B. sorokiniana* (Bs_{PNM}) generated more biomass than *S. vermifera* (Sv_{PNM}) when inoculated separately on barley roots as determined by quantitative RT-PCR on cDNA (Fig. S2b). *Bipolaris sorokiniana*-infected barley plants displayed prominent disease symptoms including root browning (Fig. S2c), reduced shoot length (Fig. S2d), and lower plant, shoot, and root weight compared with mock controls (Fig. S2e). However, *B. sorokiniana* biomass and disease symptoms were significantly diminished in roots co-colonised by *S. vermifera* ($Sv+Bs_{PNM}$, Fig. S2b–f), indicating that the presence of *S. vermifera* affects pathogen growth *in planta*.

We then determined whether *S. vermifera* would also protect barley roots from infection by *B. sorokiniana* in soil. Roots of 7-d-old barley seedlings were inoculated and placed into split-root chambers filled with sterilised nutrient-poor Cologne soil (Fig. 1a). In addition to assessing fungal competition locally, the split-root system allowed us to test whether *S. vermifera* could protect barley from *B. sorokiniana* infection systemically. As before, *B. sorokiniana* biomass was significantly reduced (71% on average) when roots were locally co-inoculated with *S. vermifera* in soil ($Sv_{LOC}+Bs_{LOC}$, Fig. 1a,b). Although not statistically significant, a reduction of *B. sorokiniana* biomass was also observed when *S. vermifera* was present systemically (that is added to roots in separate chamber of the split-root system) ($Sv_{SYS}+Bs_{LOC}$; Fig. 1a,b). By contrast, the biomass of *S. vermifera* remained unaffected by the presence of the pathogen ($F(2,9)=0.253$, $P=0.782$; Fig. 1b, left panel). In accordance with the reduction of *B. sorokiniana* biomass, disease symptoms were also reduced in roots of plants simultaneously colonised by *B. sorokiniana* and *S. vermifera* ($F(5,120)=2.229$, $P=0.0558$; Fig. 2c). This effect was statistically significant in larger soil experiments (Fig. S3). These results highlight the potential of *S. vermifera* for biocontrol applications in agricultural systems against aggressive soil-borne pathogens and suggest that this biocontrol is not mediated solely by increased host tolerance to fungal disease.

Different transcriptional programs are activated in the two fungal species during confrontation in soil

To further investigate the mechanisms underlying the antagonistic behaviour of *S. vermifera* towards *B. sorokiniana* (*Bs*), we analysed the fungal transcriptomes during confrontation in soil by RNA-seq. We mapped 22.7–28.1 million Illumina single-end reads from three independent biological replicates onto annotated genes of both fungal genomes. For the samples containing a single organism >99% of reads uniquely mapped to *S. vermifera* or *B. sorokiniana* genes (Fig. S1d; Table S2). In the confrontation samples (*Sv*+*Bs*) *c.* 67% of the reads matched *S. vermifera* genes, whereas *c.* 33% mapped to genes of the pathogen. At 2 dpc, 1200 genes of *S. vermifera*, equivalent to 7.84% of total genes in the endophyte genome, displayed $\log_2$-fold expression changes (logFC) $\geq |1|$ (582 upregulated and 618 downregulated) compared with the control (*Sv*) and were considered to be significantly differentially expressed (Tables 1, S3). In comparison, 2249 (18.36%) of *B. sorokiniana* genes were differentially expressed during confrontation compared to *Bs* alone of which 1275 were upregulated and 974 were downregulated (Tables 1, S3).

Hierarchical clustering analysis (HCA) of the differentially expressed fungal genes (DEGs) resulted in five main clusters (C1−C5; Figs 2,3). For both *S. vermifera* and *B. sorokiniana*,

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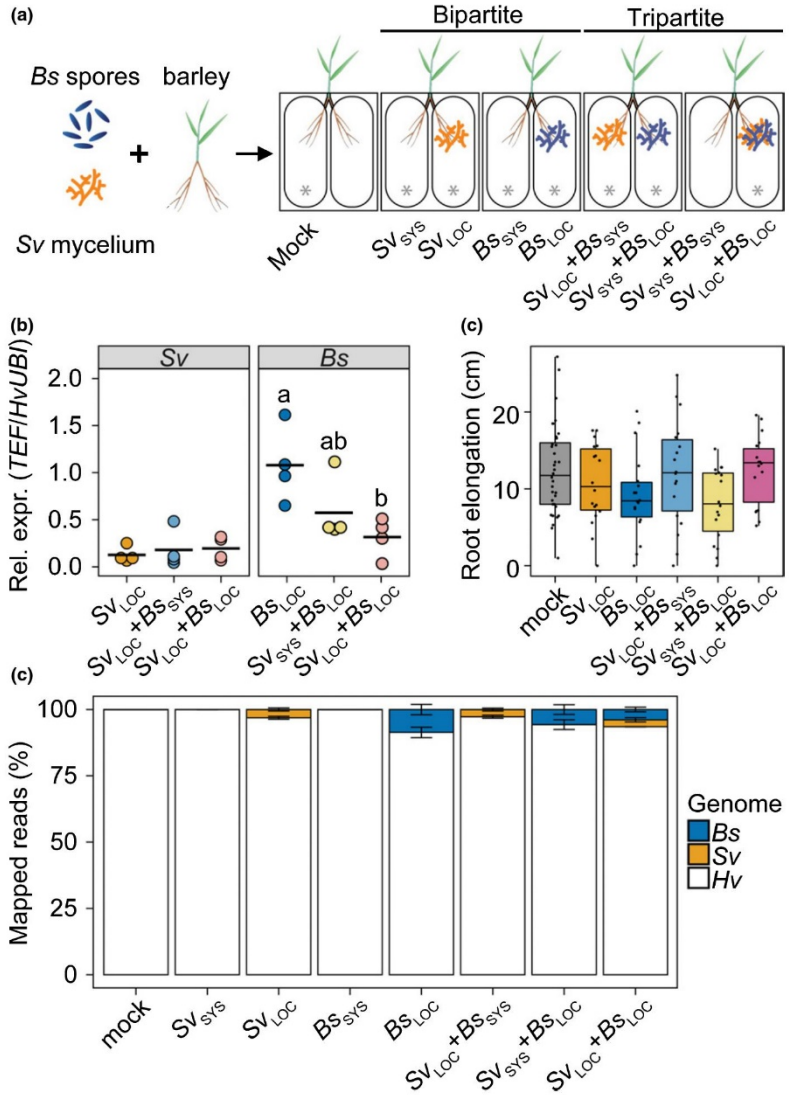

Fig. 1 Local colonisation by *Serendipita vermifera* (*Sv*) significantly reduces *Bipolaris sorokiniana* (*Bs*) biomass in barley root tissue grown in soil. (a) Schematic representation of the experimental setup of multipartite infection assays using the gnotobiotic split-root system. Root parts of 7-d-old barley seedlings to be inoculated with *GFP*-expressing *Sv* (orange hyphae) were dipped into 40 mg ml^{-1} mycelial suspensions. Following seedling transfer, each chamber was filled with 250 ml of a Cologne soil : sand mix (1 : 1), and 30 ml of a *Bs* (in blue) spore suspension with 3.4×10^4 spores ml^{-1} was added as indicated. Grey asterisks highlight samples sent for RNA-seq analysis. (b) At 6 d post inoculation, fungal colonisation in each one of four biological replicate was confirmed by quantitative RT-PCR ($n = 4$). Letters represent statistically significant differences in expression of the fungal housekeeping gene *TEF* compared with barley ubiquitin (*UBI*) according to one-way ANOVA ($F(2,9) = 5.594$, $P = 0.0264$) and Tukey's post-hoc test. The absence of letters indicates differences were not statistically significantly different. (c) To assess *Bs* disease symptoms root elongation was determined for each treatment (that is chamber in split-root system) by subtracting the root length at 0 d after inoculation (dpi) from the corresponding length at 6 dpi. Root sections were harvested for RNA extraction (for details see Supporting Information Methods S2). The boxplot shows all data points from three independent biological replicates ($n = 6$) in which the horizontal line represents the median, surrounded by the upper (25^{th}) and lower (75^{th}) percentiles. There were no statistically significant differences between means as determined by one-way ANOVA ($F(5,120) = 2.229$, $P = 0.0558$). Therefore, no letters are shown in the boxplot. (d) Relative abundance of plant and fungal transcripts during multipartite interactions in the split-root system (see (a)). Transcripts were identified by RNA-seq analysis and total reads were mapped onto annotated *Bs*, *Sv*, and barley (*Hordeum vulgare*, *Hv*) genes, respectively. Bars, mean ± SE ($n = 3$). LOC, local fungal colonisation; SYS, fungal colonisation in distant part of the root.

clusters C1 and C2 contained genes that were significantly induced or repressed during confrontation (*Sv* + *Bs*) compared with the respective control. Gene ontology (GO) enrichment analysis showed that 'transmembrane transport' and 'carbohydrate metabolism' were among the processes significantly associated with *S. vermifera* upregulated genes during confrontation

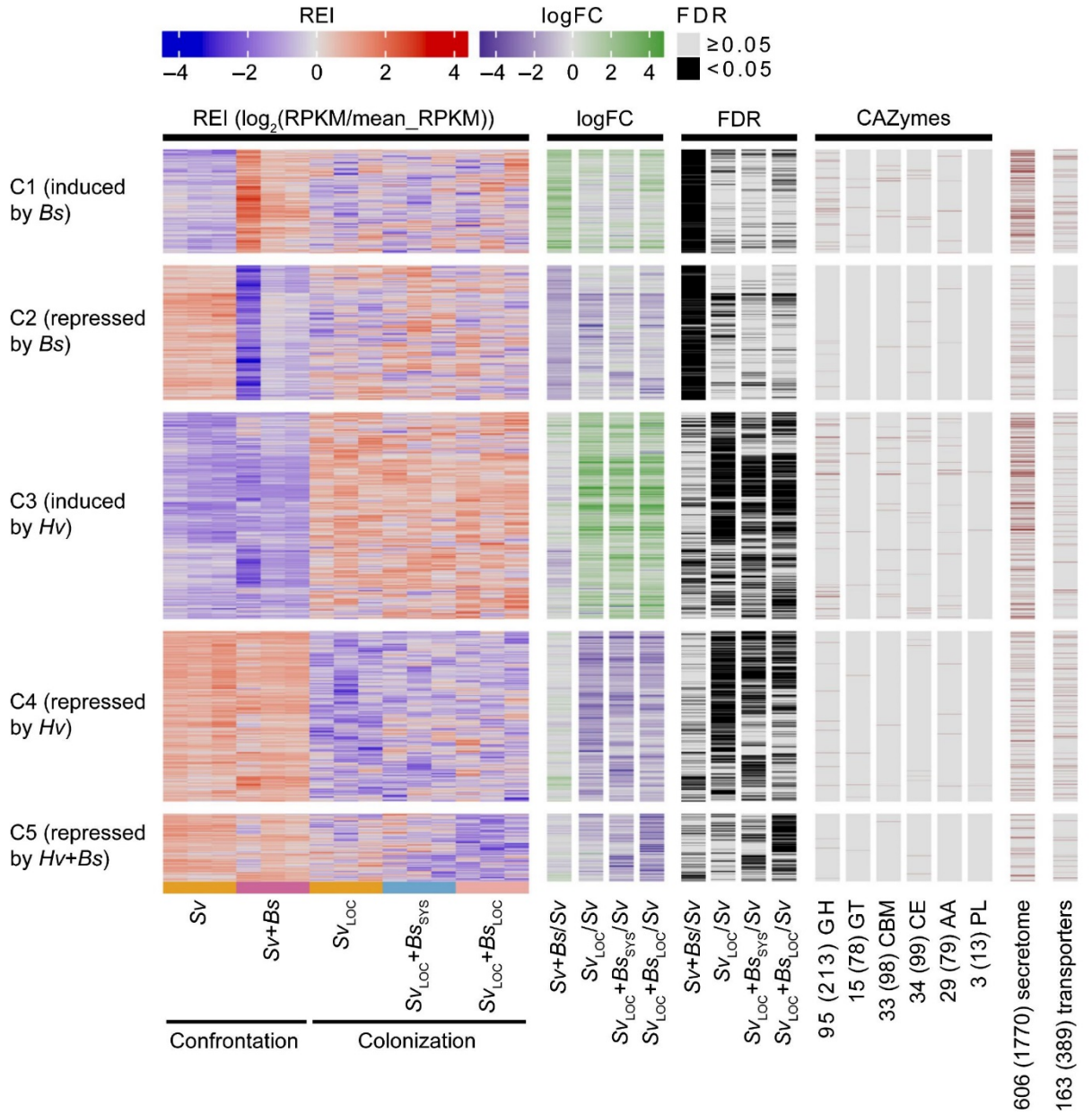

Fig. 2 *Serendipita vermifera* (*Sv*) genes encoding secreted proteins and carbohydrate-active enzymes (CAZymes) are highly induced during fungal confrontation and plant colonisation. Heatmap shows the relative expression index (REI) of *Sv* genes at 2 d post contact (dpc) with *Bipolaris sorokiniana* (*Bs*) in soil (confrontation) or 6 d after barley root inoculation in the split-root chambers (colonisation). Log_2 fold changes (logFC) indicate upregulation (green) and downregulation (blue) of gene expression under different conditions compared wih *Sv* alone. Genes were considered to be significantly differentially expressed at a false discovery rate (FDR) < 0.05 (black). Clustering patterns of genes encoding CAZymes, putatively secreted proteins (secretome) and transporters are indicated in brown. GH, glycosyl hydrolases; GT, glycosyl transferases; CBM, proteins containing chitin-binding motif; CE, carbohydrate esterases; AA, proteins with auxiliary activity; PL, polysaccharide lyases; LOC, local fungal colonisation; SYS, fungal colonisation in distant part of the root. Numbers below the last columns represent the number of differentially expressed fungal genes (DEGs) per category. The numbers of annotated genes of the same category present in the *S. vermifera* genome are shown in brackets.

with *B. sorokiniana* (Fig. S4a; Table S4). Correspondingly, an over-representation of *S. vermifera* genes encoding carbohydrate-active enzymes (CAZymes, 8.2%, χ^2 *P*-value < 0.0001) and secreted proteins (24.8%, χ^2 *P*-value < 0.0001) was detected in cluster C1 compared with 3.8% and 11.6% in the *S. vermifera* genome, respectively (Fig. 2; Table S5). Genes encoding

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Table 1 Proportion of upregulated and downregulated genes in *Serendipita vermifera* (*Sv*) and *Bipolaris sorokiniana* (*Bs*) during confrontation in soil and barley root colonisation compared to *Sv* and *Bs* controls.

Condition	Treatment	Up	%Up	Down	% Down
Genes in *S. vermifera*					
Confrontation in soil	$Sv + Bs$	582	3.8	618	4.04
Colonisation *in planta*	Sv_{LOC}	781	5.1	868	5.67
Colonisation *in planta*	$Sv_{LOC} + Bs_{SYS}$	629	4.11	667	4.36
Colonisation *in planta*	$Sv_{LOC} + Bs_{LOC}$	802	5.24	747	4.88
Genes in *B. sorokiniana*					
Confrontation in soil	$Sv + Bs$	1275	10.41	974	7.95
Colonisation *in planta*	Bs_{LOC}	1481	12.09	1079	8.81
Colonisation *in planta*	$Sv_{SYS} + Bs_{LOC}$	1301	10.62	1081	8.82
Colonisation *in planta*	$Sv_{LOC} + Bs_{LOC}$	1341	10.95	1064	8.69

Numbers of differentially expressed genes with a $\log_2$-fold change $\geq |1|$ per treatment (FDR < 0.05) are shown. LOC, local fungal root colonisation; SYS, fungal colonisation in a distant part of the root.

transporters were also overrepresented in cluster C1 with 6.2% compared with 2.5% of genes annotated as transporters in the *S. vermifera* genome (Fig. 2; Table S5; χ^2 *P*-value < 0.0001). CAZymes, particularly chitinases and β-1,6-glucanases, are fungal cell wall-degrading enzymes (CWDEs) that are important facilitators of mycoparasitism (Druzhinina *et al.*, 2011). The expression of the *S. vermifera* gene Sebve1_06375 (hereafter referred to as *SvCHIT1*), which encodes a chitinase belonging to the glycosyl hydrolase (GH) family 18 with a ChtBD3 chitin-binding domain found specifically in Agaricomycotina and chitinolytic bacteria, increased 13-fold only during direct confrontation with the pathogen in soil (Table S5). The hydrolytic domain of *Sv*CHIT1 is homologous to that of *Trichoderma* ech42, which plays an important role in mycoparasitism (Carsolio *et al.*, 1994; Woo *et al.*, 1999). Quantitative RT-PCR analysis confirmed that *SvCHIT1* was specifically induced during confrontation both on PNM and in soil (Fig. S5a, left panel), suggesting that *S. vermifera* employs specific CWDEs to hydrolyze *B. sorokiniana* cell walls during direct confrontation. *S. vermifera* genes downregulated during confrontation were associated with 14 biological processes including 'lipid metabolism' and 'proteolysis' (cluster C2 in Figs 2, S4b; Table S4).

By contrast with *S. vermifera*, the most significant biological processes associated with upregulated *B. sorokiniana* genes during direct fungal confrontation were related to secondary metabolism (Fig. S4c; Table S4). Accordingly, the expression of four polyketide synthase (PKS) genes (out of 18 encoded in the *B. sorokiniana* genome), required for the biosynthesis of polyketide secondary metabolites, was significantly elevated in the presence of the endophyte (cluster C1 in Fig. 3; Table S5). The most strongly induced *PKS* gene (*BsPKS1*, Bipso1_02104, 15-fold; Table S3) is the orthologue of *B. oryzae PKS1* and *B. maydis PKS18*, which are part of the 1,8-dihydroxynaphthalene (DHN)−melanin biosynthetic cluster in these plant pathogens and have been implicated in fungal stress responses (Henson *et al.*, 1999; Moriwaki *et al.*, 2004; Eliahu *et al.*, 2007). Quantitative RT-PCR analysis confirmed that *BsPKS1* expression was strongly induced during direct confrontation with *S. vermifera* on PNM and in soil (Fig. S5b, left panel), suggesting that the endophyte exerts strong biotic stress onto *B. sorokiniana*. Consistently, eight heat-shock protein encoding genes were significantly upregulated under this condition (Table S5). Biological processes associated with downregulated *B. sorokiniana* genes in the presence of *S. vermifera* include 'polysaccharide catabolism', 'carbohydrate metabolism', and 'isoprenoid biosynthesis' (Fig. S4d; Table S4). Interestingly, 24.1% (161) of *B. sorokiniana* genes that were significantly repressed in the presence of *S. vermifera* in soil encode putatively secreted proteins compared with 13.5% in the pathogen genome (C2 in Fig. 3; Table S6; χ^2 *P*-value < 0.0001). These include several previously identified *B. sorokiniana* effector candidates (Ohm *et al.*, 2012; Condon *et al.*, 2013) (R. Ohm, personal communication). This was reflected in the list of all deregulated effector candidate genes in which, out of 140 plant-responsive putative effector genes, 50 were specifically repressed during confrontation with *S. vermifera* in soil ('*B. sorokiniana* effectors' in Table S5). These findings highlight the different antagonistic strategies employed by *S. vermifera* and *B. sorokiniana* towards other fungi. The high stress status of *B. sorokiniana* upon challenge with the endophyte, suggests that the activation of a broad spectrum of fungal CWDEs by *S. vermifera* together with a significant reduction of gene expression for plant-responsive putative effector in *B. sorokiniana* during fungal confrontation in soil reduces the capability of the pathogen to colonise its plant host.

Fungal transcriptional changes are predominantly driven by the plant host in the tripartite interaction

Based on the consistent reduction of pathogen biomass and disease symptoms in soil, we used RNA-seq to assess fungal transcriptional responses during barley root colonisation. Barley roots were harvested at 6 dpi and thoroughly washed to remove extraradical fungal hyphae and soil particles, as shown in Fig. 1(a). At this time point, the endophyte has colonised root epidermal cells and first *B. sorokiniana* disease symptoms start to appear in this setup. To determine the composition of the Illumina reads in the three biological replicate samples obtained from the split-root system, we mapped the reads onto annotated genes of the barley (*Hv*) and fungal genomes. As expected, > 99.9% of reads matched barley genes in the mock treatment and fungal reads could only be identified in the inoculated samples (Fig. 1d; Table S2). On average, 2.5–3% of reads matched *S. vermifera* genes in all endophyte-containing samples. By contrast, the relative abundance of reads mapping to *B. sorokiniana* genes decreased from 8.6% in absence of *S. vermifera* to 5.7% and 3.9% when *S. vermifera* had been co-inoculated systemically or locally, respectively (Fig. 1d; Table S2). The reduction of *B. sorokiniana* reads in the presence of *S. vermifera* possibly

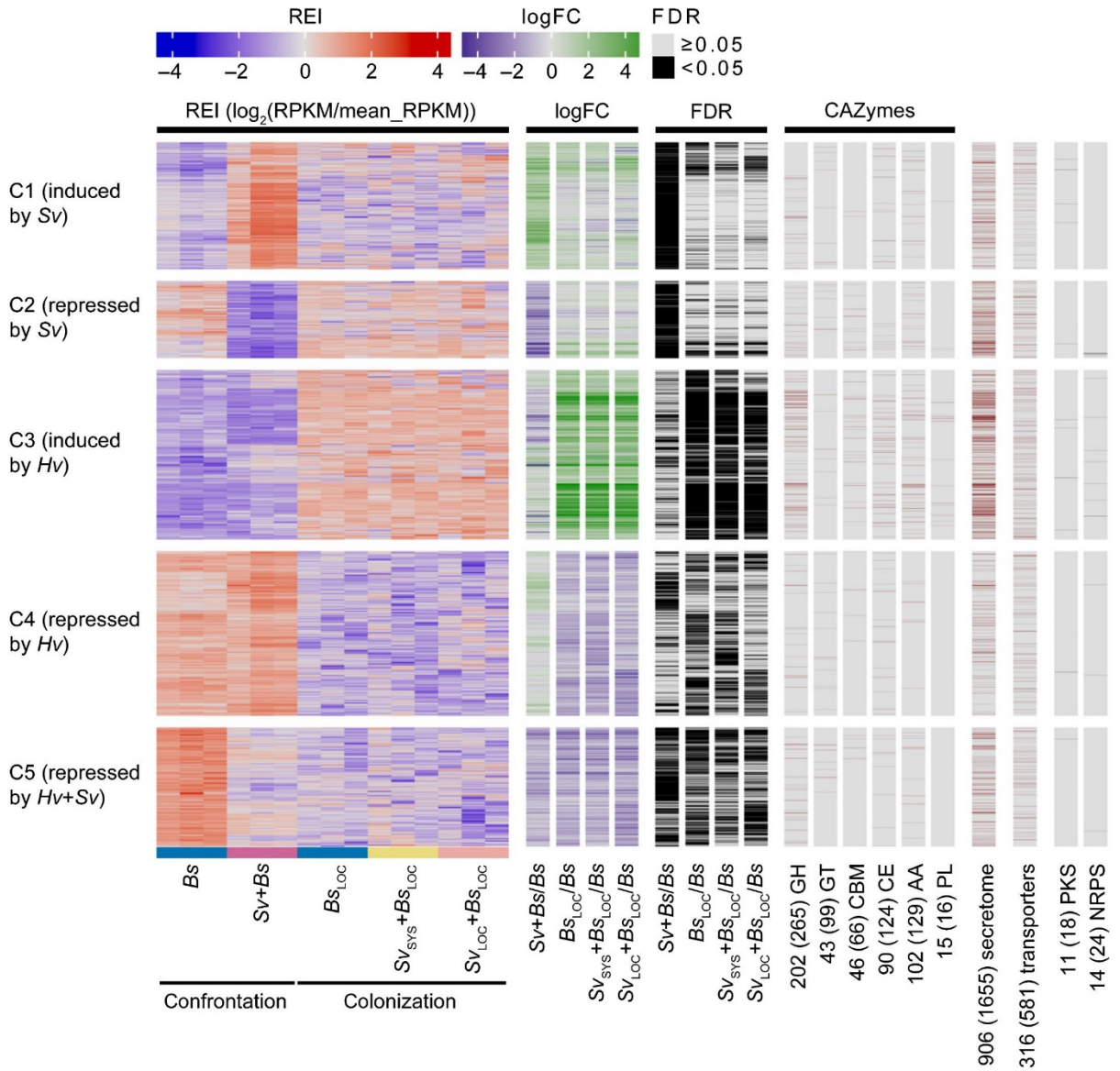

Fig. 3 *Bipolaris sorokiniana* (*Bs*) gene expression is differentially affected by direct fungal confrontation and barley root infection. Heatmap shows the relative expression index (REI) of *Bs* genes at 2 d post contact (dpc) with *Serendipita vermifera* (*Sv*) in soil (confrontation) or 6 d after barley root inoculation in the split-root chambers (colonisation). Log_2 fold changes (logFC) indicate upregulation (green) and downregulation (blue) of gene expression under different conditions compared to *Bs* alone. Genes were considered to be significantly differentially expressed at a false discovery rate (FDR) < 0.05 (black). Clustering patterns of genes encoding CAZymes, putatively secreted proteins (secretome), transporters and enzymes involved in secondary metabolism are indicated in brown. GH, glycosyl hydrolases; GT, glycosyl transferases; CBM, proteins containing chitin-binding motif; CE, carbohydrate esterases; AA, proteins with auxiliary activity; PL, polysaccharide lyases; PKS, polyketide synthases; NRPS, nonribosomal peptide synthetases; LOC, local fungal colonisation; SYS, fungal colonisation in distant part of the root. Numbers below the last columns represent the number of differentially expressed fungal genes (DEGs) per category. The numbers of annotated genes of the same category present in the *Bs* genome are shown in brackets.

reflects a lower *B. sorokiniana* biomass, which was confirmed by quantitative RT-PCR analysis displaying the ratio between constitutively expressed single copy fungal (*TEF*) and plant (*UBQ*) genes (Fig. 1b). The number of fungal DEGs did not vary much between conditions (Table 1). Moreover, the genes that were differentially regulated in the bipartite and local/systemic tripartite interactions largely overlapped (Figs 2,3; Table S5), suggesting that changes in fungal gene expression were mainly driven by the plant host, irrespective of the presence of the other fungus.

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GO enrichment analysis of upregulated *S. vermifera* genes during bipartite (Sv_{LOC}, not shown) or tripartite (Sv_{LOC} + Bs_{LOC}) interaction highlighted that, in both cases, the response to the plant host is associated with carbohydrate metabolism and sugar transport-related processes in the endophyte, indicative of an enhanced uptake of CAZyme-released and/or plant-supplied sugars via transporters (Fig. S6a; Table S7; showing the GO term analysis in the tripartite interaction). Accordingly, *S. vermifera* genes belonging to the CAZyme and secretome categories were overrepresented in cluster C3 (20.4% and 8.5% in C3 vs 11.6% and 3.8% in the genome, respectively; Fig. 3; Table S5; χ^2 *P* values < 0.0001). Notably, the groups of *S. vermifera* CWDE- and transporter-encoding genes induced during barley root colonisation differed from those activated during direct fungal confrontation (Table S5). In a previous study, microarray analysis of transcripts isolated from barley root tissue colonised by *S. indica* showed that genes encoding hexose transporters are significantly induced during later colonisation stages (Zuccaro *et al.*, 2011), including the high-affinity glucose transporter HXT5 (Rani *et al.*, 2016). Our analysis showed that the closest homologue of *SiHXT5*, Sebve1_09710 (here referred to as *SvHXT*), is among the most highly expressed barley-responsive genes at 6 dpi (27-fold; Table S8). Quantitative RT-PCR confirmed that *SvHXT* is induced during barley root colonisation on PNM and in soil and its expression is independent of the presence of *B. sorokiniana* (Fig. S5c, right panel), representing a valuable *S. vermifera* symbiosis marker gene. GO enrichment analysis of downregulated *S. vermifera* genes during the bipartite (not shown) and the tripartite interaction showed a significant enrichment in 'organonitrogen compound metabolism', 'cellular amide metabolism', and 'cellular nitrogen compound biosynthesis' processes (Fig. S6b; Table S7) indicating that, like *S. indica*, *S. vermifera* is experiencing nitrogen starvation at the onset of saprotrophy, at *c.* 6 dpi in barley (Lahrmann *et al.*, 2013).

A similar situation was found for *B. sorokiniana* genes induced during host infection (Fig. S6c; Table S7). Accordingly, we observed an enrichment of CAZyme (26.3% vs 13.5% in genome, χ^2 *P*-value < 0.0001), secreted protein (17.8% vs 4.9% in genome, χ^2 *P*-value < 0.0001) and transporter (7.4% vs 4.7% in genome, χ^2 *P*-value < 0.0001) gene categories in cluster C3 (Fig. 3; Table S5). In addition to genes encoding CAZymes and secreted proteins, seven *NPS* genes, coding for nonribosomal peptide synthetases (NRPSs), were significantly induced in *B. sorokiniana* during barley root infection (Fig. 3; Table S5). NRPSs produce small bioactive peptides that include virulence determinants and play a dominant role during plant infection by species of the *Bipolaris* genus (Finking & Marahiel, 2004; Condon *et al.*, 2013). Five of the induced *BsNPS* genes (Bipso1_11817, Bipso1_12059, Bipso1_04962, Bipso1_12079, and Bipso1_12060) have previously been described as strain-unique and three of those (Bipso1_12059, Bipso1_04962, and Bipso1_12060) were found to be specifically induced at 12 hpi in *B. sorokiniana* infecting leaves of barley cv Bowman (Condon *et al.*, 2013). We confirmed by RT-PCR that the expression of Bipso1_12059, referred to as *BsNPS1*, was significantly induced during barley root infection on PNM and in soil, and this induction was independent of the presence of *S. vermifera* (Fig. S5d, right panel), confirming *BsNPS1* as a robust virulence marker gene (Condon *et al.*, 2013). A closer inspection of barley-responsive genes of *B. sorokiniana* (upregulated in Bs_{LOC} and Sv_{LOC} + Bs_{LOC}) showed that the expression of eight genes (Bipso1_11810, Bipso1_11817, Bipso1_11820, Bipso1_11821, Bipso1_11828, Bipso1_11831, Bipso1_11837), whose products share moderate identity (35–49%) with gliotoxin biosynthetic enzymes of *Aspergillus fumigatus* (Schrettl *et al.*, 2010; Dolan *et al.*, 2015), was 16- to 2000-fold higher during infection compared with *B. sorokiniana* growing in soil (Table S8). GO term enrichment analysis identified distinct processes among downregulated *B. sorokiniana* genes during infection that are different from those found for *S. vermifera*. They include processes related to autophagy and cell division (Fig. S6d; Table S7), suggesting that the pathogen is likely to be starving in the nutrient-poor soil in the absence of the host. Together, these findings indicate that, similar to leaves (McDonald *et al.*, 2018), *B. sorokiniana* utilises secondary metabolites and potentially detoxifies phytoalexins to successfully infect host roots. Interestingly, by contrast with the gene expression changes in *B. sorokiniana* during fungal confrontation, genes involved in DHN−melanin biosynthesis were not activated during barley root colonisation, suggesting that this pathway is not required during compatible interaction with the plant host.

Barley transcriptional changes during the tripartite interaction are almost solely driven by pathogen infection

We monitored changes in barley gene expression of infected and noninfected roots using the RNA-seq data from the split-root system (Figs 1a,S7; Table S3). Principal component analysis of the dataset showed that the first two principal components (leading logFC1 and 2) explained 24.8% and 15.4% of the variation, respectively (Fig. 4a). The leading logFC1 separated roots infected with *B. sorokiniana* from non-*B. sorokiniana*-infected roots. Strikingly, samples isolated from mock-treated and *S. vermifera*-colonised roots grouped together along the first principal component, suggesting few transcriptional differences between those samples. Indeed, root colonisation by the endophyte resulted in the differential expression of 41 barley genes (29 with logFC ≥ |1|) compared with mock-treated roots (Table 2). By contrast, infection with *B. sorokiniana* resulted in the differential expression of 2741 barley genes (1643 with logFC ≥ |1|) compared with mock controls (Table 2). Most barley genes, 1257 (1090 up, 167 down), were differentially expressed in response to pathogen infection independent of the presence of *S. vermifera* locally or systemically (Figs 4b,S8; Table S9). Accordingly, GO term enrichment analysis on barley genes upregulated during *B. sorokiniana* infection in absence and presence of *S. vermifera* (Bs_{LOC} and Sv_{LOC} + Bs_{LOC}, respectively) yielded the same most significantly associated biological processes, including 'protein phosphorylation' and 'protein modification' (Fig. S9; Table S10). The pathogen infection resulted in the induction of barley genes associated with isoprenoid biosynthesis, chitin hydrolysis, and amino acid metabolism (Table S10).

Among the *B. sorokiniana*-induced genes were several *PATHOGENESIS-RELATED* (*PR*) genes including *HvPR17a* (HORVU1Hr1G043910), *HvPR17b* (HORVU7Hr1G013170),

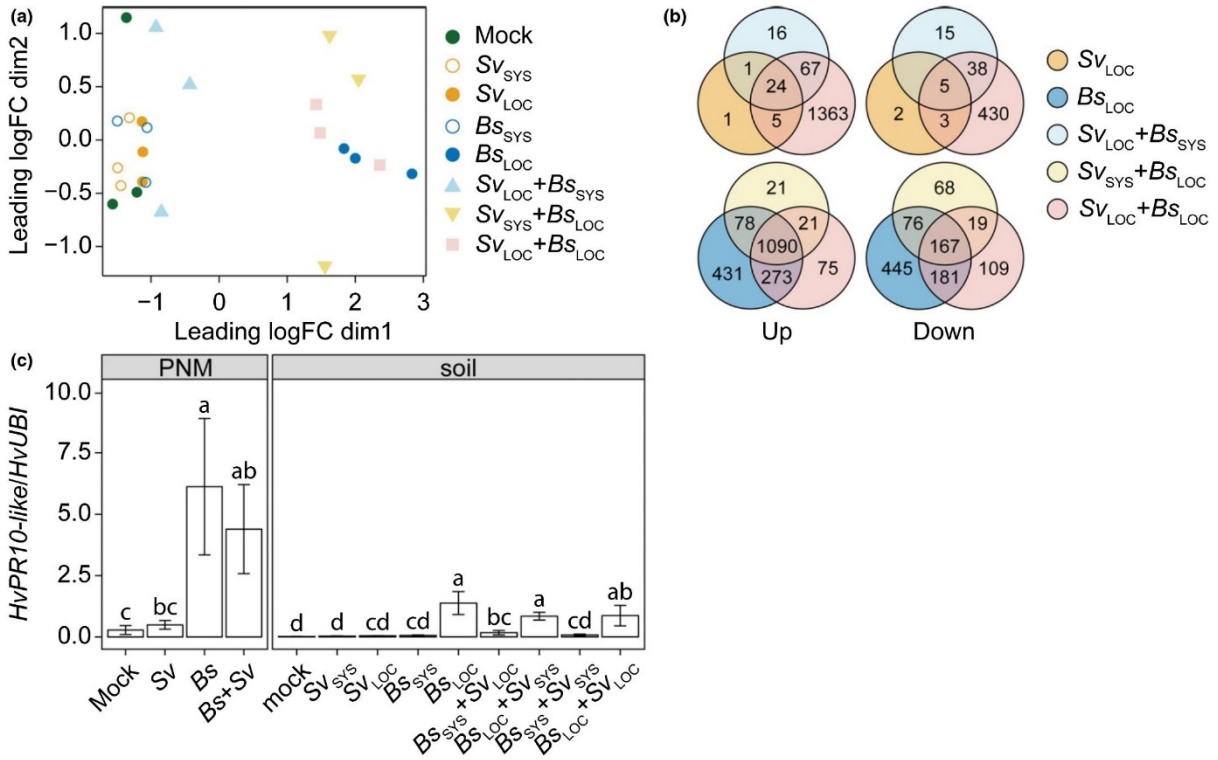

Fig. 4 Barley defence gene expression is induced during pathogen infection but not during colonisation by *Serendipita vermifera* (*Sv*). (a) Principal component analysis (PCA) of gene expression levels in barley roots during separate or simultaneous colonisation by *Sv* and *Bipolaris sorokiniana* (*Bs*) 6 d after inoculation. PCA was executed using the built-in multidimensional scaling (MDS) plots of the EDGER package in R on the regularised $\log_2$-transformed data. Plotted are the first two principal components where leading logFC dim1 accounts for 24.77% or variance and leading logFC dim2 explains 15.42% of variance. (b) Venn diagrams of differentially expressed barley genes (FDR < 0.05) during single or simultaneous fungal colonisation. (c) Changes in expression of the barley defence gene *PR10-like* in response to endophyte colonisation and/or pathogen infection on plant nutrition medium (PNM) and in soil were determined by quantitative RT-PCR. Primers targeting the barley ubiquitin gene (*HvUBI*) were used for sample calibration. Bars, mean ± SE ($n = 4$). Letters represent statistically significant differences in gene expression according to one-way ANOVA ($F(3,12) = 10.05$, $P < 0.01$ for *in vitro*, $F(8,27) = 18.61$, $P < 0.001$ for soil-grown plants) and Tukey's post-hoc test. LOC, local fungal root colonisation; SYS, fungal colonisation in distant part of the root.

and a *PR10* family gene (HORVU0Hr1G011720, hereafter referred to as *HvPR10-like*) whose product shares 94% sequence identity with HvPR10 (GenBank ID: AAP04429.1) (Table S9). Quantitative RT-PCR analysis confirmed that *HvPR10-like* expression is highly induced during infection by *B. sorokiniana* in roots of plants grown on PNM and on soil (Fig. 4c). By contrast, *HvPR10-like* expression was only weakly affected by *S. vermifera* indicating that defence responses remain at a minimum level during endophyte colonisation of barley root tissue. Similarly, our transcriptomic data showed that local and systemic root infection with *B. sorokiniana*, but not colonisation by *S. vermifera*, resulted in strong upregulation of several genes involved in the methylerythritol 4-phosphate (MEP) pathway and in the biosynthesis of terpenoids (Figs S10,S11a; Table S11). Sequence comparison of *HORVU2Hr1G004620* (from this point named *HvCPS2*) with homologues from other monocot and dicot species showed that it is most closely related to wheat (*Triticum aestivum*) copalyl diphosphate synthase 2 (TaCPS2, GenBank ID: BAH56559.1, 90% identity) (Fig. S11b). TaCPS2 was shown to catalyze the formation of normal, rather than *ent*-copalyl, diphosphate (Wu *et al.*, 2012). Given the high percentage of amino acid identity (90%), the same catalytic activity for HvCPS2 is expected. This suggests that HvCPS2 is not involved in the biosynthesis of gibberellins, which are derived from *ent*-CDP, but rather in the biosynthesis of specialised diterpenoid phytoalexins. HORVU2Hr1G004540 (hereafter referred to as *HvKSL4a*) encodes a protein that displays three amino acid differences to barley *ent*-kaurene-like synthase 4 (HvKSL4, GenBank ID: AK370792) (Li *et al.*, 2016). This difference is likely to be due to the different barley cultivars that provided these sequences. Phylogenetic analysis placed HvKSL4a in a clade with OsKSL4, TaKSL1, and TaKSL4 (Fig. S11c). Its closest homologue OsKSL4 was shown to be involved in the biosynthesis of the rice-specific diterpene phytoalexins momilactone A and B (Toyomasu *et al.*, 2008). Therefore, like HvCPS2, HvKSL4a is likely to play a role in diterpene phytoalexin biosynthesis in barley. Interestingly, *HvCPS2* and *HvKSL4a* are localised on chromosome II in close vicinity to each other and lie embedded in a cluster of nine

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Table 2 Proportions of upregulated and downregulated genes in *Hordeum vulgare* (*Hv*, barley) during root colonisation by *Bipolaris sorokiniana* (*Bs*), *Serendipita vermifera* (*Sv*) or both fungi simultaneously in the split-root system.

Treatment	DEGs	Up	%Up	Down	% Down	DEGs (logFC ≥ |1|)	Up (logFC ≥ 1)	%Up (logFC ≥ 1)	Down (logFC ≤ −1)	%Down (logFC ≤ −1)
Sv_{SYS}	8	7	0.009	1	0.001	6	5	0.006	1	0.001
Sv_{LOC}	41	31	0.038	10	0.012	29	24	0.029	5	0.006
Bs_{SYS}	149	112	0.137	37	0.045	91	68	0.083	23	0.028
Bs_{LOC}	2741	1872	2.292	869	1.064	1643	1238	1.516	405	0.496
$Sv_{LOC} + Bs_{SYS}$	166	108	0.132	58	0.071	143	95	0.116	48	0.059
$Sv_{SYS} + Bs_{LOC}$	1540	1210	1.481	330	0.404	1110	927	1.135	183	0.224
$Sv_{LOC} + Bs_{LOC}$	1935	1459	1.786	476	0.583	1294	1072	1.312	222	0.272

Numbers represent significantly differentially expressed genes (FDR < 0.05) with or without a $\log_2$ fold change (logFC) ≥ |1| per treatment. LOC, local fungal root colonisation; SYS, fungal colonisation in distant part of the root; DEGs, differentially expressed fungal genes.

genes encoding cytochrome P450s (CYPs; Table S11). Several of these genes were also strongly induced during *B. sorokiniana* infection and their products display similarity to CYPs typically involved in diterpene biosynthesis (Fig. S10; Table S11).

Despite the large differences in overall barley transcriptional responses to *S. vermifera* and *B. sorokiniana*, we identified 37 genes that were similarly deregulated during local pathogen or endophyte colonisation (Table S9). Among the upregulated genes are genes coding for peroxidases, laccases and germin-like proteins. Interestingly, a clear systemic root response was observed during fungal colonisation irrespective of the microbe's lifestyle. Seven genes were upregulated, including an aquaporin (AQP)-like protein (HORVU4Hr1G085250) and a myb-like transcription factor (HORVU7Hr1G001830) was downregulated (Table S9).

Pathogen-induced changes of the plant host environment have an effect on *S. vermifera* gene expression

Despite the overwhelming influence of the host on fungal transcriptional changes, we specifically examined whether fungal gene expression during host colonisation is affected by the presence of another fungus. To this end, we compared the sets of *S. vermifera* and *B. sorokiniana* DEGs from the mixed mycelial confrontations in soil (*Sv* + *Bs*) with samples harvested from roots colonised by the respective fungus alone (Sv_{LOC}, Bs_{LOC}) or by both fungi simultaneously ($Sv_{LOC} + Bs_{LOC}$). We found that 474 genes (177 up, 297 down) and 342 genes (83 up, 259 down) of *S. vermifera* and *B. sorokiniana*, respectively, were specifically differentially expressed (FDR < 0.05) during fungal confrontation *in planta* (Fig. S12; Table S12). No GO terms were significantly enriched among *S. vermifera* DEGs or upregulated *B. sorokiniana* genes in this condition. However, 87 GO terms of biological processes related to replication and transcription were significantly enriched among *B. sorokiniana* genes repressed during confrontation *in planta* (Table S13). A closer inspection of the top 30 induced genes of *B. sorokiniana* showed that the majority encodes proteins involved in plant carbohydrate hydrolysis, secondary metabolism, and fungal virulence including 7 effector candidates (Table S12), representing the same gene categories that are induced during *B. sorokiniana* infection alone (cluster C3; Table S5). Conversely, while most of the top 30 induced *S. vermifera* genes were not annotated, annotated genes encode a peroxidase, cytochrome P450s and a heat-shock protein with putative roles in redox homeostasis and detoxification (Table S12). Only two were predicted effector candidates (Table S12). Twelve of these top 30 *S. vermifera* genes were also significantly induced when *B. sorokiniana* colonised the roots systemically. These findings suggest that the pathogen-induced changes of the host plant environment are perceived by the endophyte and lead to tailored alterations of *S. vermifera* gene expression.

Discussion

Serendipita vermifera-mediated local and systemic barley protection from pathogen infection is not based on extensive host transcriptional reprogramming

Several beneficial fungal species with mycorrhizal or endophytic lifestyles are able to defend their plant hosts against pathogen infection (Bonfante & Genre, 2010; Druzhinina *et al.*, 2011; Zuccaro *et al.*, 2014; Weiss *et al.*, 2016; Li *et al.*, 2018). Like *S. indica*, *S. vermifera* was previously shown to induce systemic resistance to foliar infections by the obligate biotrophic pathogen *Blumeria graminis* f.sp. *hordei* (Deshmukh *et al.*, 2006; Waller *et al.*, 2008). In this study, we tested the ability of *S. vermifera* to interfere with barley root infections by *B. sorokiniana*. We show that *S. vermifera* consistently reduced barley disease symptoms on defined medium and in soil locally and systemically (Figs 1,S2c–e). Especially, systemic protection from infection was reported to be based on the ability of beneficial fungi to reprogramme host transcription (Shoresh *et al.*, 2010). Our analysis demonstrated that *S. vermifera* induces moderate transcriptional changes with 41 and 8 barley genes being significantly differentially regulated locally and systemically, respectively, 6 d after barley root inoculation in soil (Figs 4a, b, S8; Table 2). Similarly, the root endophyte *T. harzianum* has a limited impact on the transcriptome of Arabidopsis roots (Morán-Diez *et al.*, 2012). These findings support the concept that beneficial endophytic root-colonising fungi have to be separated in their mode of action from, for example, arbuscular mycorrhizal fungi and pathogens that activate extensive host transcriptional reprogramming for successful establishment of a beneficial or detrimental relationship with their plant hosts, respectively (Bonfante &

Genre, 2010; this paper). A recent study also reported a limited effect of the endophyte *Fusarium oxysporum* Fo47 on the tomato host proteome compared with the closely related pathogenic Fol007 (de Lamo *et al.*, 2018). Of the *S. vermifera*-responsive genes, 29 and 7 were also upregulated during local and systemic pathogen infection, respectively (Table S9), highlighting their role in barley–fungal interactions irrespective of the microbe's lifestyle. Locally induced genes encode several enzymes involved in lignification, a process of cell wall reinforcement forming a physical barrier against fungal infections (Zheng *et al.*, 2005; Miedes *et al.*, 2014; Fiorilli *et al.*, 2015; Bajaj *et al.*, 2018; Ma *et al.*, 2018), suggesting that barley roots establishes basal resistance to both invading fungi at sites of physical contact. During systemic fungal colonisation the putative AQP-like gene HORVU4Hr1G085250 was most strongly induced (three- to nine-fold) (Table S9). AQPs are major intrinsic proteins (MIPs) that mediate bidirectional fluxes of water and other substrates across cell membranes. In barley there are at least 42 genes that encode AQPs (Gattolin *et al.*, 2009). The closest HORVU4Hr1G085250 homologue in Arabidopsis is the tonoplast AQP isoform *At*TIP4;1 (Fig. S13), which transports small molecules and localises to the tonoplast in root epidermal cells (Gattolin *et al.*, 2009). Generally, *At*TIPs are implicated in transport of water, urea and H_2O_2 and are required for lateral root formation (Liu *et al.*, 2003; Bienert *et al.*, 2007; Reinhardt *et al.*, 2016). At the transcriptional level their regulation seems to be remarkably tissue and cell type-specific (Gattolin *et al.*, 2009). Additionally, recent data indicate that *At*TIPs are regulated by nitrogen availability in the root via the shoot (Wu *et al.*, 2019). Thus, HORVU4Hr1G085250 represents an interesting candidate for systemic communication during barley root colonisation by beneficial and pathogenic fungi.

According to our current understanding of the molecular mechanisms governing plant–fungal interactions, plants perceive the presence of microbes through the recognition of conserved microbe-derived or modified plant molecules that indicate invasion (Cook *et al.*, 2015; Sánchez-Vallet *et al.*, 2015; Fesel & Zuccaro, 2016). This recognition can occur even before plant and microbe establish physical contact and results in the quick activation of plant immune responses including the expression of hallmark plant defence genes. The fact that we did not observe extensive changes in expression of barley defence genes in response to *S. vermifera* colonisation suggests that this endophyte efficiently evades and/or suppresses plant immunity at 6 dpi possibly through the activity of effector proteins or modification of metabolites as recently shown for *S. indica* (Fig. 5a) (Wawra *et al.*, 2016; Nizam *et al.*, 2019). Moreover, as the genes deregulated during the tripartite interaction largely overlapped with those differentially expressed during pathogen infection, we propose that *S. vermifera*-mediated barley protection does not require extensive host transcriptional reprogramming.

Serendipita vermifera is resilient to biotic stress and dynamically adapts to changes in the plant host environment

By contrast with *S. vermifera*, local *B. sorokiniana* infection resulted in the deregulation of 3.4% of barley genes (Table 2). Several studies have reported a massive deregulation of host genes, ranging from 13% to 22%, during infection by foliar pathogens (Doehlemann *et al.*, 2008; Kawahara *et al.*, 2012; De Cremer *et al.*, 2013), while Arabidopsis root infection with pathogenic *Fusarium oxysporum* resulted in the deregulation of 1.6% of genes (protein-coding and noncoding) (Zhu *et al.*, 2013). This may indicate that root colonisation generally prompts fewer transcriptional changes than leaf colonisation by fungal microbes. *Bipolaris sorokiniana*-responsive genes were significantly associated with 'protein phosphorylation', and 'macromolecule modification' processes encoding important components of the plant immune system, including PR proteins, subtilisin-like proteases, peroxidases, chitinases, glucanases, and several leucine-rich repeat (LRR)-containing receptor proteins (Figs 4, S9; Tables S9, S10) (van Loon *et al.*, 2006; Figueiredo *et al.*, 2014; Sánchez-Vallet *et al.*, 2015; Fesel & Zuccaro, 2016). Moreover, genes of the MEP pathway with a putative role in diterpene phytoalexin biosynthesis were strongly induced by the pathogen locally and systemically (Figs S10, S11; Table S11). Diterpenoid phytoalexins have been identified in other *Poaceae* such as maize and rice and were shown to play a role in the defence of these plant species against various pathogens (Schmelz *et al.*, 2014). To the best of our knowledge, no diterpenoid phytoalexins have been identified in barley, urging for future studies on their characterisation and biosynthesis.

These findings indicate that barley recognises *B. sorokiniana* root infections and activates immune signalling to halt pathogen invasion (Fig. 5b). During co-colonisation, when *B. sorokiniana* disease symptoms and biomass are reduced by *S. vermifera*, barley transcriptomic changes were still largely dictated by the presence of the pathogen (Figs 4, S7, S8, S9; Table S10), again highlighting that *S. vermifera*-mediated barley protection is not based on the induction of local plant resistance at a transcriptional level (Fig. 5c). Most importantly, this also indicates that the impact of *B. sorokiniana* infection on barley is likely to result in profound physiological changes in the plant host that can be interpreted as a change of environment for the colonising endophyte. The transcriptomic data show that *S. vermifera* perceives the pathogen-induced changes during root colonisation resulting in the activation of stress-related genes that help maintain redox homeostasis and detoxify harmful compounds (Fig. 5c). Our observation that *S. vermifera* biomass levels were stable during local and systemic colonisation despite the pathogen-activated host immune system (Figs 1b, S2b) shows that this fungus is able to activate a transcriptionally dynamic response to *B. sorokiniana*-triggered stress, which is, most likely, required for *S. vermifera* successful host colonisation and biotic stress resilience.

Fungal competition in soil reduces the virulence potential of *Bipolaris sorokiniana*

In addition to plant transcriptional reprogramming, several other mechanisms contribute to fungal-mediated host protection in the rhizosphere including competition for space and nutrients, (myco)parasitism and amensalism (antibiosis) (Harman *et al.*, 2004; Philippot *et al.*, 2013). The establishment of mycoparasitic

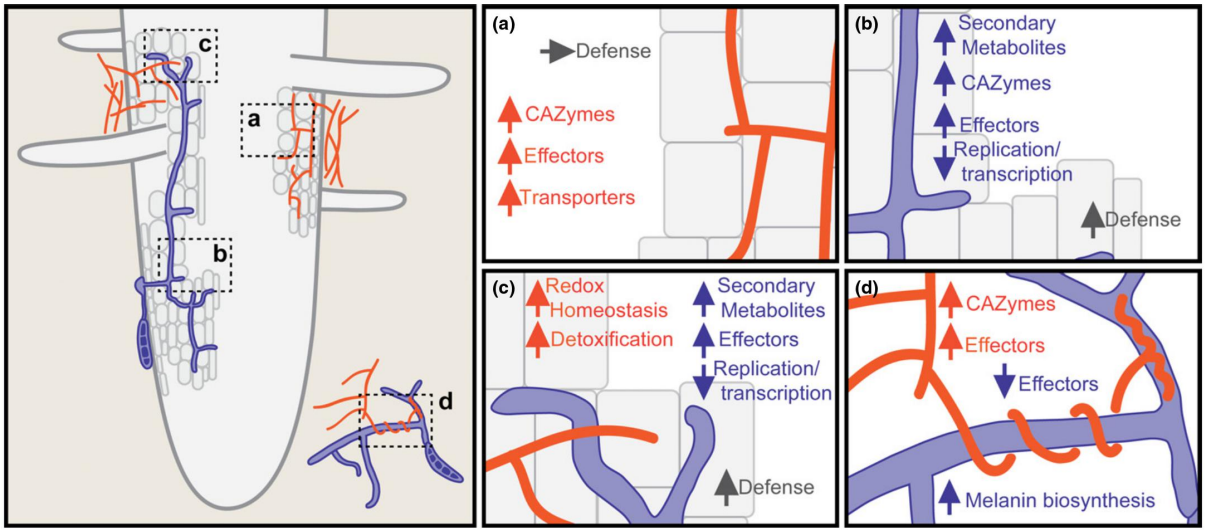

Fig. 5 Model of transcriptomic changes during bipartite and tripartite interactions. (a) Root colonisation by *Serendipita vermifera* does not lead to an extensive deregulation of plant defence gene expression. Genes encoding CAZymes, putative effectors and transporters are upregulated in *S. vermifera* towards the end of the biotrophic growth phase at 6 d after inoculation (dpi). (b) Pathogen infection causes a massive induction of plant defence gene expression. In *Bipolaris sorokiniana*, effector, CAZyme and secondary metabolite genes are upregulated during barley root colonisation. (c) Host defence gene expression is induced in response to co-colonisation by the endophyte and the pathogen, similar to *B. sorokiniana* infection alone. Fungal transcriptional reprogramming is mainly driven by the plant host. Genes encoding putative effectors , CAZymes and transporters are induced in both fungal species. However, subtle differences in fungal gene expression could be ascribed to fungal confrontation *in planta*. Especially *S. vermifera* appears to perceive the pathogen-induced changes of the host plant environment resulting in the upregulation of genes involved in redox homeostasis and detoxification. (d) Fungal confrontation in soil results in a significant induction of *S. vermifera* genes that encode CAZymes and putative effectors while the expression of genes encoding secreted proteins (including putative effectors) is suppressed in *B. sorokiniana*. The expression of secondary metabolism genes, especially those involved in DHN-melanin biosynthesis, are significantly upregulated in the pathogen during confrontation with *S. vermifera* highlighting the strong biotic stress the endophyte exerts onto the pathogen during saprotrophic growth. Organisms and categories of differentially expressed fungal genes (DEGs) of *S. vermifera*, *B. sorokiniana*, and barley are shown in orange, violet and grey, respectively.

relationships requires microbial host recognition followed by attachment to prey hyphae (Druzhinina *et al.*, 2011). Mycoparasitic *Trichoderma* spp. grow alongside and coil around the mycelium of their prey (Druzhinina *et al.*, 2011; Lace *et al.*, 2015), while *Ampelomyces* species form papilla-like structures that are analogous to appressoria of plant pathogenic fungi (Kiss *et al.*, 2010). Co-cultivation in artificial media and in soil showed that, similar to *Trichoderma* spp., *S. vermifera* coils around and occasionally colonises *B. sorokiniana* hyphae (Fig. S1), indicating that *S. vermifera* is able to attack and hydrolyze the pathogen's cell wall. Accordingly, our transcriptomic analysis revealed an overrepresentation of a specific set of CAZyme-encoding genes in *S. vermifera*, including chitinases and glucanases that were specifically induced during fungal confrontation in sterile soil (Figs 2, S4a,S5a; Tables S4,S5). Such fungal CWDEs are implicated in mycoparasitism (Howell, 2003; Harman *et al.*, 2004; Druzhinina *et al.*, 2011) and the activation of their expression indicates that *S. vermifera* utilises CWDEs to attack *B. sorokiniana*. Comparative genomic analyses have shown that gene families encoding CWDEs are expanded in the genomes of *S. vermifera* and *S. indica* (Kohler *et al.*, 2015; Lahrmann *et al.*, 2015). During Arabidopsis root colonisation, *S. vermifera* genes encoding GHs of family 3 and 28 were induced at different colonisation stages (Lahrmann *et al.*, 2015). Here, 18 *S. vermifera* genes belonging to 11 GH families were specifically induced during barley colonisation and differed from those induced during fungal confrontation (Table S5). Plant and fungal cell walls consist of different carbohydrates. It is, therefore, conceivable that *S. vermifera* requires specific sets of CWDEs for the hydrolysis of the different substrates it encounters during microbial competition in soil and during root colonisation of different plant species.

Trichoderma spp. produce secondary metabolites during interaction with their prey (Druzhinina *et al.*, 2011). However, we did not observe the activation of the few secondary metabolite genes present in the genome of *S. vermifera* specifically during confrontation with *B. sorokiniana* in soil suggesting that the antagonistic strategy of *S. vermifera* does not include toxic metabolites (Fig. 5d). By contrast, genes required for the biosynthesis of secondary metabolites were upregulated in *B. sorokiniana* during confrontation. Out of four polyketide synthase genes, *BsPKS1* was most strongly induced in the presence of *S. vermifera* (Figs S4c, S5b; Tables S4, S5). While the role of *BsPKS1* in DHN-melanin biosynthesis requires confirmation (Griffiths *et al.*, 2018), our data indicated that the presence of *S. vermifera* induces melanisation in *B. sorokiniana* hyphae (Fig. 5d). Among other functions, fungal melanin has been reported to play an important role in environmental stress responses including oxidative stress caused by reactive oxygen

species (ROS) that can be produced during fungal competition (Nosanchuk & Casadevall, 2003; Haedens *et al.*, 2005; Eisenman & Casadevall, 2012). In *T. atroviride* ROS production by fungal prey results in the induction of HSPs required for detoxification (Seidl *et al.*, 2009). Interestingly, eight *B. sorokiniana* HSP-encoding genes were significantly upregulated during confrontation with *S. vermifera* (Table S5). Taken together with the putative induction of DHN–melanin biosynthesis, the pathogen appears to experience substantial biotic stress during direct contact with the endophyte. Notably, the direct confrontation did not induce stress-related gene expression in *S. vermifera*.

In addition to CWDEs and secondary metabolites, effector molecules are thought to play a crucial role in microbial competition (Rovenich *et al.*, 2014; Snelders *et al.*, 2018). Therefore, we expected that effector gene expression would be upregulated in both fungal species during confrontation in soil. Indeed, out of 644 *S. vermifera* genes specifically induced in the presence of *B. sorokiniana* (cluster C1, Fig. 2), 36 encode putative effectors (Fig. S12; Table S5) showing significant enrichment ($\chi^2 = 0.0217$) (Lahrmann *et al.*, 2015). Surprisingly, however, the expression of several plant-responsive effector candidates was significantly repressed in the pathogen during confrontation with *S. vermifera* in soil ('*B. sorokiniana* effectors' in Table S5). While both fungi simultaneously colonise the same barley root (Figs 1b, S2b,f), *B. sorokiniana* spreads from its original site of entry through the root to the shoot tissue (Kumar *et al.*, 2002). Instead, *S. vermifera* remains localised to the epidermis and outer cortex layers (Lahrmann *et al.*, 2015). This finding indicates that the two fungi may often not be in direct contact within the root cells. However, in the rhizosphere, where the fungi are in close physical contact, *S. vermifera* exerts its antagonistic activity towards *B. sorokiniana* that, together with its ability to suppress pathogen effector gene expression, may reduce the virulence potential of the pathogen limiting its capability to colonise its plant host.

In conclusion, we show that both root-associated fungi express genes encoding microbe-targeting effectors during confrontation in soil and plant-targeting effectors during root colonisation, indicating that specific effectors are used to combat/antagonise other fungi. Additionally, a core set of few effectors was induced during plant colonisation and fungal confrontation, supporting the idea that effectors with roles in the manipulation of both the plant host and the local microbial community also exist (Snelders *et al.*, 2018). The challenge now is to functionally characterise these effectors in microbe–microbe interactions. The systemically increased resistance in barley to *B. sorokiniana* implies an endophyte-driven plant response. Nevertheless, the major effects were observed when the endophyte was in direct contact with the pathogen outside the root, suggesting that it functions as a gate keeper. Most importantly we show that the inconspicuous sebacinoid fungus, which does not overcolonise the host root, has the potential to affect multispecies interactions and is itself very resilient. This finding is in accordance with environmental studies that describe sebacinoid fungi as often of low abundance but ubiquitously and consistently present in plant roots and the rhizosphere.

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Author contributions

AZ, GL, MB, and DS conceived the project and planned the experiments. DS, LM, and HR carried out inoculations, RNA extractions, RT-PCRs and confocal microscopy. DS, GJ, HR, SN, AT, GUB and GL analyzed the data. HR, DS and AZ wrote the manuscript. DS, HR and GJ contributed equally to this work.

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Supporting Information

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

Fig. S1 *Serendipita vermifera* shows signs of mycoparasitism in direct confrontation with *B. sorokiniana*.

Fig. S2 *Serendipita vermifera* significantly reduces barley root infection by *B. sorokiniana* on defined medium.

Fig. S3 *Serendipita vermifera* counteracts the reduction of barley shoot weight caused by *B. Sorokiniana* in soil.

Fig. S4 Gene Ontology (GO) cluster representatives of differentially expressed fungal genes during direct confrontation.

Fig. S5 Fungal- and plant-responsive marker gene expression in *S. vermifera* and *B. sorokiniana*.

Fig. S6 Gene ontology (GO) cluster representatives of differentially expressed fungal genes during barley root colonisation.

Fig. S7 Pathogen infection drives barley transcriptional dynamics during tripartite interactions.

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Fig. S9 Gene ontology (GO) enrichment analysis of barley genes induced during pathogen infection and tripartite interaction.

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Chapter 2

The fungal root endophyte *Serendipita vermifera* displays inter-kingdom synergistic beneficial effects with the microbiota in *Arabidopsis thaliana* and barley

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Authors contributions:

LM and AZ designed the experiments. **LM**, CU, DB, and SRA performed the experiments, isolated the barley bacterial strains, and designed the *Hv*SynCom. **LM**, SM, GL, YN, RGO, RG, and AZ analyzed the data. **LM**, AZ, JP, CU, and SM wrote the paper with editorial input from all other authors.

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The fungal root endophyte *Serendipita vermifera* displays inter-kingdom synergistic beneficial effects with the microbiota in *Arabidopsis thaliana* and barley

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Plant root-associated bacteria can confer protection against pathogen infection. By contrast, the beneficial effects of root endophytic fungi and their synergistic interactions with bacteria remain poorly defined. We demonstrate that the combined action of a fungal root endophyte from a widespread taxon with core bacterial microbiota members provides synergistic protection against an aggressive soil-borne pathogen in *Arabidopsis thaliana* and barley. We additionally reveal early inter-kingdom growth promotion benefits which are host and microbiota composition dependent. Using RNA-sequencing, we show that these beneficial activities are not associated with extensive host transcriptional reprogramming but rather with the modulation of expression of microbial effectors and carbohydrate-active enzymes.

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INTRODUCTION

Plant pathogenic fungi limit crop productivity globally. These threats are expected to increase with global warming [1]. Decades of advances in agrochemicals and plant breeding have expanded farmers' toolkits with fungicides and resistant varieties to limit the detrimental effects of these organisms on crop yield. Yet, current tools are becoming environmentally unsustainable or ineffective against rapidly evolving pathogens [1]. A key example of this scenario is represented by the soil-borne plant pathogen *Bipolaris sorokiniana* (syn. *Cochliobolus sativus*, hereafter *Bs*), the causal agent of spot blotch and common root rot diseases that threaten cereal production in warm regions [1–3]. Root rot normally originates from inoculum carried on the seed or from soil-borne conidia, but the fungus can infect plants at any developmental stage. However, as the importance of root-inhabiting pathogenic fungi has often been underestimated, very little is known about the molecular mechanism behind the detrimental interaction of *Bs* with roots [4].

Microbial communities living at the root−soil interface, collectively referred to as the plant root microbiota, have gained centre-stage in pathogen protection [5]. Past studies across a variety of plant species employed environmental sampling or controlled conditions in the field and laboratory to characterize the root microbiota [6–10], with an overall greater focus on bacteria than on filamentous fungi [11]. Microbial diversity and abundance gradually decrease between the soil and vicinity of the root (rhizosphere), and further between the rhizosphere and root internal compartments (endosphere). Moreover, a number of bacterial taxa (e.g., Proteobacteria, Actinobacteria, Bacteroidetes, and Firmicutes) consistently occur in the root endosphere of different examined plant species [10]. This latter feature underpins the "bacterial core microbiota" concept, in which strains from specific taxa are commonly selected as endophytes across plant species, soil types, and environmental conditions [12]. By contrast, studies of geographically distinct populations of *Arabis alpina* and *Arabidopsis thaliana* (hereafter Arabidopsis) showed that few fungal taxa are prevalent in the root endosphere, and that endophytic fungal communities are strongly influenced by location and climate [9, 13].

The functions and benefits of root microbiota members in the context of abiotic or biotic stresses have been extensively investigated under laboratory conditions using single microbial strains and, more recently, synthetic bacterial communities (SynComs) [14]. Several bacterial and fungal isolates have the capacity to directly increase plant biomass via growth hormone production and/or by providing plants with limiting macro- or micro-nutrients [13, 15–19]. Although diseases caused by pathogens have been shown to be directly or indirectly reduced by the addition of single or multiple beneficial microbes [4, 8, 20–23], how fungal root microbiota members with beneficial functions influence and are influenced by bacterial colonization remains less understood.

Sebacinales fungi (Basidiomycetes) are a remarkable group of plant mutualists with worldwide occurrence in soils and as endophytes. While individual Sebacinales strains can interact with roots in the absence of differentiated structures, they can also form specialized interactions with distinctive morphological characteristics on relevant hosts, as in orchid- or ectomycorrhiza

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symbioses [24]. Root colonization by these fungi improved host growth and development, increased grain yield, and enhanced root phosphate uptake in several plant species [25–28]. The positive effects of Sebacinales on the host plant extend well beyond growth and development and cannot be explained by enhanced host nutrition alone [24, 26, 29]. Recently, it was shown that effector molecules derived from the Sebacinales root endophyte *Serendipita indica* contribute to the establishment of the fungus−host interaction [30–33]. *S. indica* effectors suppress plant defence responses and modulate plant metabolism to promote compatibility in the roots, but their contribution to beneficial outcomes is unclear. Similarly, the nature of host transcriptional programs and signalling networks that lead to a mutually beneficial fungus−plant partnership are not well understood.

In the past few years, microbe−microbe interactions have emerged as an additional important element shaping plant host−microbe interactions [4, 22, 34, 35]. Using a soil-based split-root system, we demonstrated that both local and systemic colonization by the Sebacinales endophyte *Serendipita vermifera* (syn. *Sebacina vermifera*, hereafter *Sv*) afford protection against *Bs* infection and disease symptoms in *Hordeum vulgare* (barley) [4]. Here, we explore how *Sv* and *Bs* colonization capacities in two plant species, barley, and Arabidopsis, are modulated by the presence of individual members of the core bacterial microbiota or SynComs isolated from the barley rhizosphere [36] or Arabidopsis roots [37]. The finding that *Bs* also infects and causes disease symptoms in Arabidopsis roots motivated us to develop a set of physiological measurements to characterize disease severity and plant growth in Arabidopsis under different microbe treatment regimes. These measurements include ion leakage (quantified *via* electric conductivity) and photosynthetic activity (measured using pulse amplitude modulation fluorometry) as readouts for host cell death progression and biotic stress during the host−microbe interaction. Analyses of inter-kingdom activities in barley and Arabidopsis revealed that *Sv* can functionally replace the core bacterial component of the rhizosphere by mitigating pathogen infection and disease symptoms in both hosts. Additionally, we show that cooperation between bacteria and beneficial fungi leads to inter-kingdom synergistic beneficial effects, thereby providing insights into the complex relationships of the rhizosphere. Finally, RNA-seq experiments with selected bacterial strains alone or combined with *Sv* and/or *Bs* give insights to how microbes synergistically protect plants. We conclude that plants have evolved to preferentially accommodate communities that support their health and that root-associated prokaryotic and eukaryotic microbes can act synergistically with the plant host in limiting fungal disease.

MATERIAL AND METHODS

Plant, fungal, and bacterial materials

Barley (*Hordeum vulgare* L. *cv* Golden Promise) and *Arabidopsis thaliana* Col-0 were used as plant hosts. *Serendipita vermifera* (MAFF305830) and *Bipolaris sorokiniana* (ND90Pr) were used as fungal models. The *At*SynCom consists of four bacterial strains from the *At*Sphere collection [37]. The *Hv*SynCom consists of 26 bacterial strains of an existing collection [36] as described in Fig. S1.

Growth conditions and microbe inoculations

Barley seeds were surface sterilized in 6% sodium hypochloride for 1 h under continuous shaking and subsequently washed each 30 min for 4 h with sterile water. The seeds were germinated on wet filter paper in darkness at room temperature for 4 days, transferred to 1/10 PNM (Plant Nutrition Medium, pH 5.7) in sterile glass jars for growth at a day/night cycle of 16/8 h at 22/18 °C, 60% humidity under 108 µmol/m^{2}s light intensity.

Arabidopsis seeds were surface-sterilized two times in 70 and 100% EtOH for 5 min each and sown on ½ MS (Murashige−Skoog-Medium including vitamins, pH 5.6) with 1% sucrose after ethanol removal. Following two days of stratification at 4 °C and darkness, the seeds were germinated at a day/night cycle of 8/16 h at 22/18 °C, 60% humidity, and a light intensity of 125 µmol/m^{2}s for seven days. Seedlings of similar size were transferred to 1/10 PNM medium in 12 × 12 cm square Petri dishes 1 day prior to microbe inoculation.

Single bacterial strains were grown separately in liquid TSB medium (Sigma Aldrich) (15g/l) at 28 °C in darkness with shaking at 120 rpm for 1 −3 days depending on growth rates. Final OD_{600} was adjusted to 0.01 prior to inoculation of single strains or mixtures in equal amounts for SynComs constitutions to a final OD_{600} of 0.01.

Sv was propagated on MYP medium [38] and *Bs* on modified CM [4] medium both containing 1.5% agar at 28 °C in darkness for 21 days and 14 days pre inoculation respectively. *Sv* mycelial and *Bs* conidia suspensions were prepared as described in [4].

Arabidopsis roots were inoculated either with *Sv* mycelium (1g/50ml), *Bs* conidia (5×10^3 spores/ml), bacteria (OD_{600} = 0.01) or a mixture of organisms contained in 0.5ml sterile MilliQ water equally spread across individual plates. Barley roots were inoculated with 3ml of *Sv* mycelium (2g/50ml), *Bs* conidia (5×10^3 spores/ml), bacteria (OD_{600} = 0.01) or a respective mixture of organisms per jar. Sterile MilliQ water was used as a control treatment. Arabidopsis and barley roots were harvested at 6 days post inoculation (dpi). Per biological replicate of each experiment and treatment, roots from 60 Arabidopsis plants or four barley plants were pooled. For RNA extraction roots of both plant species were washed thoroughly to remove extraradical fungal hyphae and epiphytic bacteria and snap-frozen in liquid nitrogen.

Pulse-amplitude-modulation (PAM) fluorometry and ion leakage measurement

For PAM fluorometry and ion leakage assays, Arabidopsis plants were picked from the plate at 6 dpi. The plant roots were washed carefully and thoroughly to remove extraradical fungal hyphae and epiphytic bacteria and subsequently transferred to a 24 well plate containing 2 ml sterile MilliQ water per well. Five plants of the same treatment were transferred to each well. PAM fluorometry and ion leakage were measured every 24 h from 1 to 7 days post transfer (dpt) as described in [39] and as indicated in the figure legends.

RNA isolation for RNA-seq and RT-PCR

RNA extraction for quantification of fungal endophytic colonization and RNA-seq, cDNA generation, and RT-PCR were performed as described previously [4]. The primers used are listed in Table S1.

Statistical analyses

For fungal colonization and plant phenotypic analyses, as well as for quantification of disease symptoms and cell death, statistical evaluation was performed using either a one-way ANOVA and Tukey's post hoc test ($p < 0.05$) or a non-parametric Kruskal−Wallis test, followed by pairwise Mann–Whitney U-tests for multiple comparisons (FDR adjusted p-value < 0.05) depending on the experiment and readout as indicated in the figure legends.

Genomic and transcriptomic data analysis

Stranded mRNA-seq Libraries were prepared according to the manufacturer's instructions (Vazyme Biotech Co., Nanjing, China). Qualified libraries were sequenced on a HiSeq 3000 system instrument at Genomics & Transcriptomics Laboratory, Heinrich-Heine University, Germany (https://www.gtl.hhu.de/en.html) to generate 50 million reads with a 150 bp read length from two to three biological replicates. Reads with Illumina adaptors and sequence quality scores lower than 15 were removed using fastp [40]. High-quality sequencing reads were then aligned to the annotated reference genomes of the three organisms (barley: IBSC Morex v2, *Bipolaris sorokiniana*: Cocsa1, *Serendipita vermifera*: *Sebacina vermifera* MAFF 305830 v1.0, Table S2) using kallisto (v.0.46.1) [41]. Read count per transcript was converted into read count per gene using an in-house custom pipeline and R package tximport [42]. Potential batch effects were excluded with Combat-seq function in SVA package [43]. We selected 25,172 of 39,734, 10,178 of 12,250, and 13,376 of 15,312 genes having more than averaged five reads per condition for *H. vulgare*, *B. sorokiniana*, and *S. vermifera* respectively for the analysis (Tables S3, S4, and S5). The log2 fold difference of the gene expression between conditions was calculated with R package DESeq2 [44]. Genes with

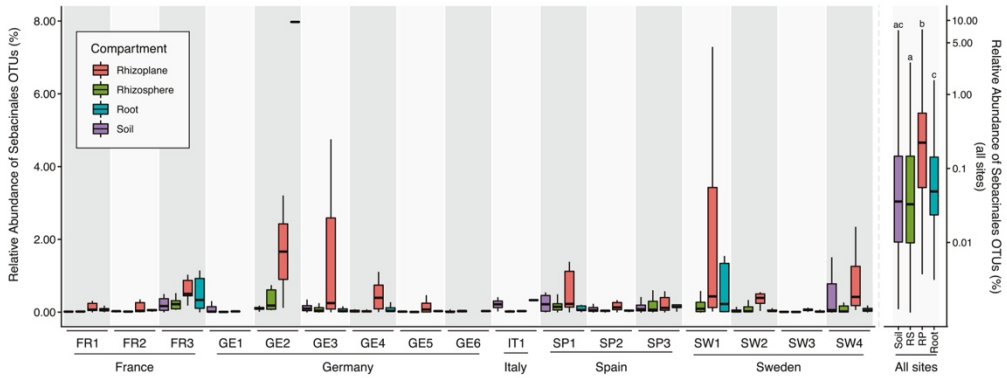

Fig. 1 Abundance of Sebacinales in Arabidopsis roots of different European locations. Abundance and location of Sebacinales OTUs in nature suggest functional association of these fungi with *Arabidopsis thaliana*. Analysis of fungal (ITS1) OTUs belonging to the Sebacinales order from sequencing data obtained from samples of soil and root-associated microbial communities across 3 years and 17 European sites where naturally occurring *A. thaliana* populations were found [9]. A non-parametric Kruskal−Wallis test with a Dunn's test for multiple comparisons on relative abundances of Sebacinales OTUs in different compartments, aggregated for all sites, shows that this fungal taxon is enriched in the rhizoplane compartment of *A. thaliana* roots compared to the other compartments. *Y*-axes have different scales for all sites (right scale) and single sites (left scale).

statistical significance were selected (FDR adjusted p value < 0.05). The consistency of normalized transcription from two to three biological replicates was confirmed by visualizing the distribution of read counts. Normalized read counts of the genes were also produced with DESeq2, which were subsequently log2 transformed. Functional annotation sets were combined using the Carbohydrate Active Enzyme database CAZy [45], the Gene Ontology GO Consortium [46], Kyoto Encyclopedia of Genes and Genomes KEGG [47], and EuKaryotic Orthologous Groups KOG [48], PFAM [49], Panther [50], MEROPS [51]. KOG, GO, KEGG, PFAM, Panther, MEROPS, best *O. sativa* hit homologues and best *A. thaliana* TAIR10 hit homologues were obtained from Phytozome, JGI (https://phytozome-next.jgi.doe.gov/). CAZymes, MEROPS, and GO terms were obtained based on KEGG, GO, and PFAM IDs using R packages KEGG.db, GO.db, and PFAM.db [52–54]. Fungal genomes and functional annotations were obtained from Mycocosm, Joint Genome Institute (https://mycocosm.jgi.doe.gov/mycocosm/home). The latest CAZy annotations were provided from CAZy team (www.cazy.org). Secreted proteins were predicted with Secretome pipeline described previously [55]. We identified the genes coding for CAZymes, lipases, proteases, small secreted proteins (less than 300 amino acids) as a subcategory. Fungal effectors were previously identified, which were combined with the predicted secretome information in this study [4]. We sorted significantly differentially regulated genes specific to the conditions (> 1 log2 FC; FDR adjusted p < 0.05) and visualized with R package UpSetR [56]. Such genes were grouped using K-means clustering with R package pheatmap [57]. Networks of k-means clustered genes were visualized with R package ggraph [58]. Genes expressed differently among the conditions were identified based on principal coordinates calculated with R package Vegan [59]. The first three principal coordinates were used to select high loading genes coding for glycosyl hydrolases and effectors of *B. sorokiniana*. Comparative analyses with a previous transcriptomic dataset [4] showed that 37 of the 50 top induced barley genes in response to *Bs* in soil are again detected to be significantly induced in the Barley_*Bs* vs Barley comparison in PNM (this study), indicating a large overlap of the highly responsive host genes to the pathogen in soil and PNM. Data are deposited at NCBI under the BioProject accession number: PRJNA715112.

Gene co-expression analysis

A self-organizing map (SOM) was trained with the normalized read count of the selected replicates using Rsomoclu and kohonen [60, 61]. A total of 1015 nodes (35 × 29 matrix) was used with a rectangular shape (four neighbouring nodes). The resolution of 25 genes per node was applied for clustering, which was empirically optimized [62, 63]. The epoch of 1000 times more than the map size was applied (i.e., 1,015,000 iterations of learning, being 1015 map size times 1000). The genes showing similar regulation trends were grouped based on the mean transcription of the nodes. We examined genome-wide condition-specific transcriptomic patterns in graphical outputs (i.e., Tatami maps). Mean transcription values were calculated from the grouped genes per condition in each node (i.e., node-wise transcription). Then, using the node-wise transcription values, highly-regulated genes specific to each of the conditions were determined by fulfilling either of two criteria: 1) > 12.6 log2 reads (above 95th percentile of the entire transcribed genes); or 2) over ± 2 log2 transcriptional differences between testing conditions and a control. The process above was performed in a semi-automated manner using co-gene expression pipeline (SHIN+GO; [62–65]. R was used for operating the pipeline (http://www.R-project.org).

RESULTS

Sebacinales associate with healthy Arabidopsis plants in diverse European locations

By monitoring root-associated microbial communities in natural *A. thaliana* populations, Thiergart et al. [9] showed that microbial community differentiation in the roots is explained primarily by location for filamentous eukaryotes and by soil origin for bacteria, whereas host genotype effects are marginal. We re-analyzed this dataset, including lower abundance operational taxonomic units (OTUs), and found that fungal OTUs of the order Sebacinales were significantly enriched in the rhizoplane compartment of healthy Arabidopsis plants in diverse European locations (Fig. 1). These environmental sampling data complement cytological studies which show that Sebacinales isolates colonize Arabidopsis by forming a loose hyphal mesh around roots with intracellular colonization limited to the root epidermis and cortex layer [38]. The frequent occurrence and enrichment patterns of Sebacinales OTUs in the roots of native Arabidopsis suggest a functional endophytic association with this host in nature. This finding motivated us to investigate the functional relevance and resilience of these fungi in a community context in the roots of Arabidopsis and to compare these with the beneficial effects observed in barley using bacterial synthetic communities.

Protection mediated by *S. vermifera* and bacteria is synergistic and largely independent of the host

We reported that *Sv* acts as an extended plant protection barrier in the rhizosphere, which reduces barley root infection and disease symptoms caused by the hemibiotrophic pathogen *Bs* on defined plant sugar-free minimal medium (PNM) and in natural soil [4]. Here we examined the role of *Sv* in augmenting the plant immune system in two different hosts for resilience against environmental threats in a bacterial community context by monitoring fungal

colonization, plant growth promotion, protection, and transcriptional response (Fig. 2A).

In the host barley, we confirmed the protective activity of *Sv* during *Bs* infection of root tissue (Fig. 2B, C) and additionally we observed enhanced *Sv* colonization through the presence of *Bs* at 6 dpi on PNM (Fig. 2D). In the host Arabidopsis, *Bs* infected seedlings displayed prominent disease symptoms at 6 dpi on PNM such as reduced main root length, rosette diameter, and lateral root number compared to mock-inoculated controls (Fig. S2A–C). *Bs* inoculated roots exhibited characteristic tissue browning, increased ion leakage, and a reduced photosynthetic active leaf area over time, indicative of host cell death progression (Fig. 3A–C and Figs. S2D, S3). As shown for barley and in accordance with their growth rates in axenic cultures [4], *Bs* generated more endophytic biomass than *Sv* upon separate inoculations of Arabidopsis roots, determined by a quantitative reverse transcription PCR (RT-qPCR) test displaying the ratio between constitutively expressed single copy fungal (TEF) and plant (UBI) genes (Fig. 3D, E). Notably, *Bs* endophytic biomass and disease symptoms were substantially diminished in roots that were co-colonized by *Sv* (Fig. 3A–D and Figs. S2A−C, S3). *Sv* endophytic colonization was enhanced by the presence of the pathogen also in this tripartite interaction (Fig. 3E). The enhanced *Sv* colonization in both hosts could be explained by the plant actively recruiting *Sv* to suppress the soil-born pathogen or *Sv* feeding on *Bs* and/or necrotic plant tissues.

Next, we determined whether bacterial strains isolated from the rhizosphere of barley (*Hv*SynCom) or the endosphere of Arabidopsis roots (*At*SynCom) can also protect barley and Arabidopsis from *Bs* infection. Both SynComs were able to reduce *Bs* colonization and partially rescue plant phenotypes caused by the pathogen in both hosts (Figs. 2B, E, 3B–D and Fig. S2B, C). Interestingly, the *Hv*SynCom alone, but not the *At*SynCom, caused increased ion leakage and reduced photosynthetic active leaf area in Arabidopsis (Fig. 3B, C and Fig. S3). This points towards an induction of host cell death in Arabidopsis by the non-native bacterial SynCom.

To clarify whether the observed host protection against *Bs* infection is a general property of root-associated bacterial strains or requires a community context, we inoculated functionally and taxonomically-paired bacterial strains from the *Hv*- and *At*SynComs (Fig. S1) individually or in combination with *Bs* on barley. We observed a strong reduction of the pathogen infection with the Proteobacteria strains bi08 (*Pseudomonas* sp.) and Root172 (*Mesorhizobium* sp.) but not with the Firmicutes strain bi80 (*Bacillus* sp.) and Root11 (*Bacillus* sp.) irrespective of the host species origin (Fig. 2B). This indicates that not all bacterial strains in the SynComs have the ability to protect the roots from *Bs* infection but the overall protection effect is maintained in a community context.

Next, we interrogated whether the observed beneficial effects on the plant hosts mediated by *Sv* or the bacterial strains are retained or altered during inter-kingdom interactions. For this, we co-inoculated barley and Arabidopsis roots with *Sv* and *Bs* in combination with a single bacterial strain or a SynCom. We found that *Sv* colonization was only marginally affected by the presence of the bacteria or positively affected in the case of the *At*SynCom in barley (Figs. 2D and 3E). The combined presence of *Sv* and bacterial strains led to a stabilized (reduced biological variation) or potentiated host protection against *Bs* infection (Figs. 2B and 3D). Potentiated protection to *Bs* infection was most evident during co-inoculation of *Sv* with Root11 in barley (Fig. 2B). These data show a robust inter-kingdom protective effect of *Sv* with bacteria against an invasive fungal root pathogen.

Finally, to measure whether the host plant contributes to the effects displayed by *Sv* and the examined bacterial strains in limiting pathogen biomass, we additionally performed direct microbe−microbe confrontation assays on PNM. In these assays, we largely recapitulated the antagonism observed against *Bs in planta* with a general reduction of *Bs* colony areas in the presence of bacteria and/or *Sv* but not with Root11 alone (Fig. S4). We, therefore, concluded that microbe−microbe interactions rather than the host plant are most important for conferring the root protective properties of *Sv* or the tested bacteria. This notion is also supported by *in planta* cytological analyses in which we observed a direct interaction between *Bs* and Root172 at the rhizoplane of Arabidopsis and extensive lysis of the fungal extracellular polysaccharide matrix surrounding *Bs* hyphae (Fig. 4).

S. vermifera confers plant growth promotion in cooperation with selected root-associated bacteria

Sv promotes plant growth in different host species at late stages of colonization [66–68]. At an early colonization time point of 6 dpi in barley, neither *Sv* alone nor any of the single bacterial strains or SynComs led to a significant change in root fresh weight (Fig. 2E). By contrast, a combination of *Sv* and bacterial strains Root11, bi08 or bi80, significantly increased barley root fresh weight at 6 dpi (Fig. 2E). This early inter-kingdom mediated root growth promotion effect was strain-specific, not restricted to bacterial strains isolated from the barley rhizosphere, and maintained in a community context (Fig. 2E). Co-inoculation with heat-inactivated bacterial SynComs failed to increase barley root fresh weight (Fig. S5A), underlining the importance of living bacteria in promoting root growth.

In Arabidopsis, we observed root growth inhibition at 6 dpi upon inoculation with *Bs* or the SynComs irrespective of the number of bacterial strains and their host origin (Fig. S2A). Co-inoculation with *Sv* largely alleviated the *Bs*-mediated root growth inhibition but did not increase root or shoot size compared to controls (Fig. S2A–C). Only the combination of Root172 with *Sv* led to a significant increase in Arabidopsis rosette diameter at 6 dpi (Fig. S2E, F). This phenotype was, however, not retained in a bacterial community context, suggesting that it is less robust and/or plant growth-promoting microbes suffer from competition by other community members.

Inter-kingdom synergistic beneficial activities are not associated with extensive host transcriptional responses

To investigate mechanisms underlying the synergistic beneficial effects displayed by a combined fungal endophyte and bacterial inoculation, we analyzed the barley root transcriptome during fungal and bacterial colonization by RNA-seq. The multipartite systems used for transcriptomics included the two fungi (*Sv* and *Bs*) and the bacterial strains Root172 and Root11, selected based on their distinctive and robust *in planta* activities with *Bs* and *Sv* at 6 dpi. Namely, Root172 conferred strong host protection against *Bs* whereas Root11 had a strong root growth promotion phenotype (Fig. 2B, E). To determine species representation in the Illumina RNA-seq reads, we mapped reads to annotated genes of the barley and fungal reference genomes. Bacterial reads were not present in the dataset due to the method used for the library preparation. On average, 7.9% of reads matched *Sv* genes in all endophyte-containing samples (Fig. 5A; Table S2). By contrast, the relative abundance of reads mapping to *Bs* genes decreased from 13.1% (*Bs* alone) to 8.6%, 12.9% or 5.7% when *Sv*, Root11 or Root172 were co-inoculated with the fungal pathogen, respectively. Co-inoculation of Root11 or Root172 with *Sv* and *Bs* reduced the relative abundance of pathogen reads, to 2.6 and 2.7%, respectively (Fig. 5A; Table S2). The reduction in *Bs* reads with *Sv* and/or bacterial strains likely reflects reduced *Bs* biomass, confirming the quantitative RT-PCR analysis (Fig. 2B). To dissect barley transcriptomic trends and identify differentially expressed genes (DEG), we examined genes that were induced or repressed under specific conditions after transcript mapping and quality assessment (Fig. S6, see "Methods"). Consistent with our previous data [4], we detected only a weak host transcriptomic response to *Sv* (184 DEG with log2FC>1, Fig. 5B, Fig. S7; Table S6). Neither

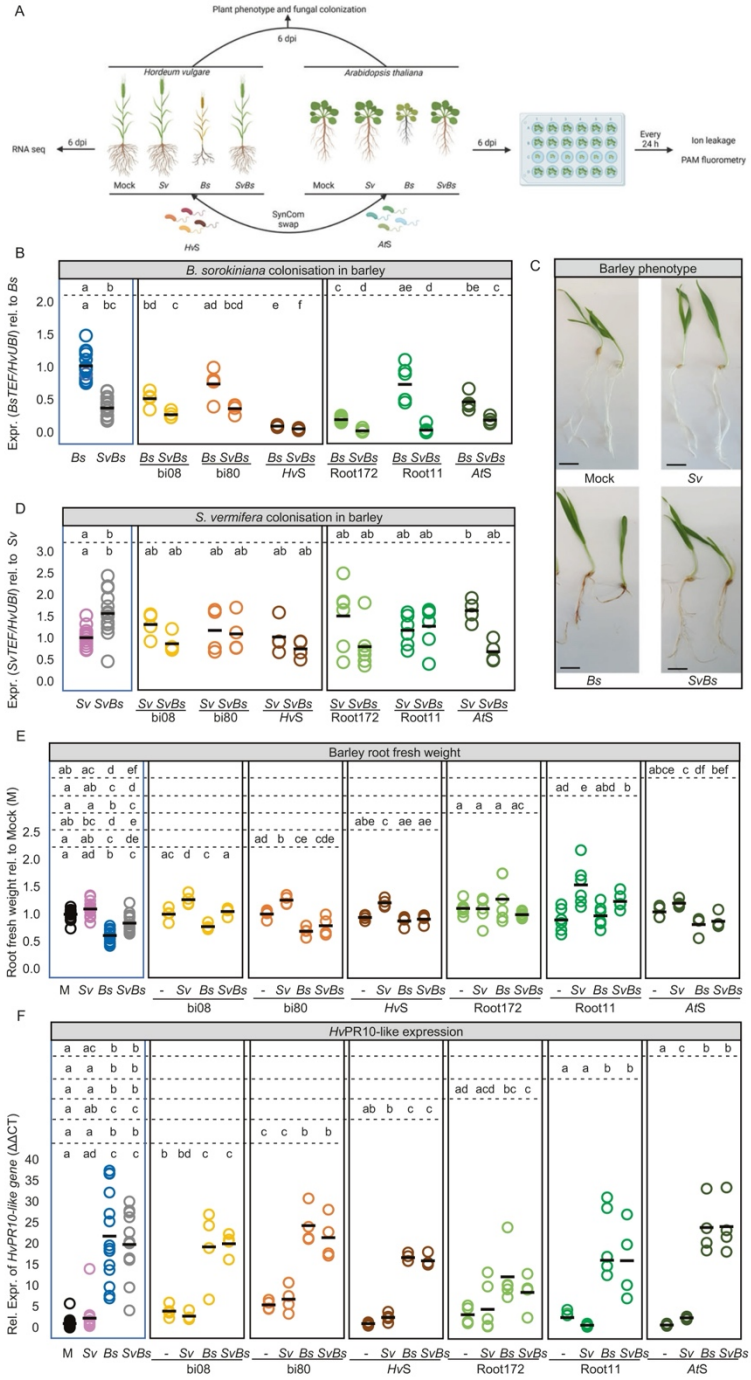

Fig. 2 Barley root colonization and responses after fungal and/or bacterial inoculation at 6 dpi. *Sv* and the bacterial SynComs display beneficial effects on barley protection and plant growth. **A** Scheme depicting the experimental setup for barley and Arabidopsis. **B** *Bs* and **D** *Sv* colonization in barley roots. Fungal colonization was inferred by expression analysis of the fungal housekeeping gene *TEF* compared to the barley ubiquitin (*UBI*) gene ($n = 4–14$). **C** Pictures showing barley inoculated with water as a control (mock), *Sv*, *Bs,* or both fungi together, scale bar = 1 cm. **E** Barley root fresh weight per biological replicate normalized to mock inoculated plants ($n = 4–14$). **F** Relative expression of *HvPr10*-like gene (HORVU0Hr1G011720) to *UBI*. Different letters in the comparison between the tripartite panel (blue frame) and combinations of any other subpanel (defined by the dashed lines) represent statistically significant differences according to non-parametric Kruskal–Wallis test followed by pairwise Mann–Whitney U-tests for multiple comparisons (FDR adjusted *p*-value < 0.05).

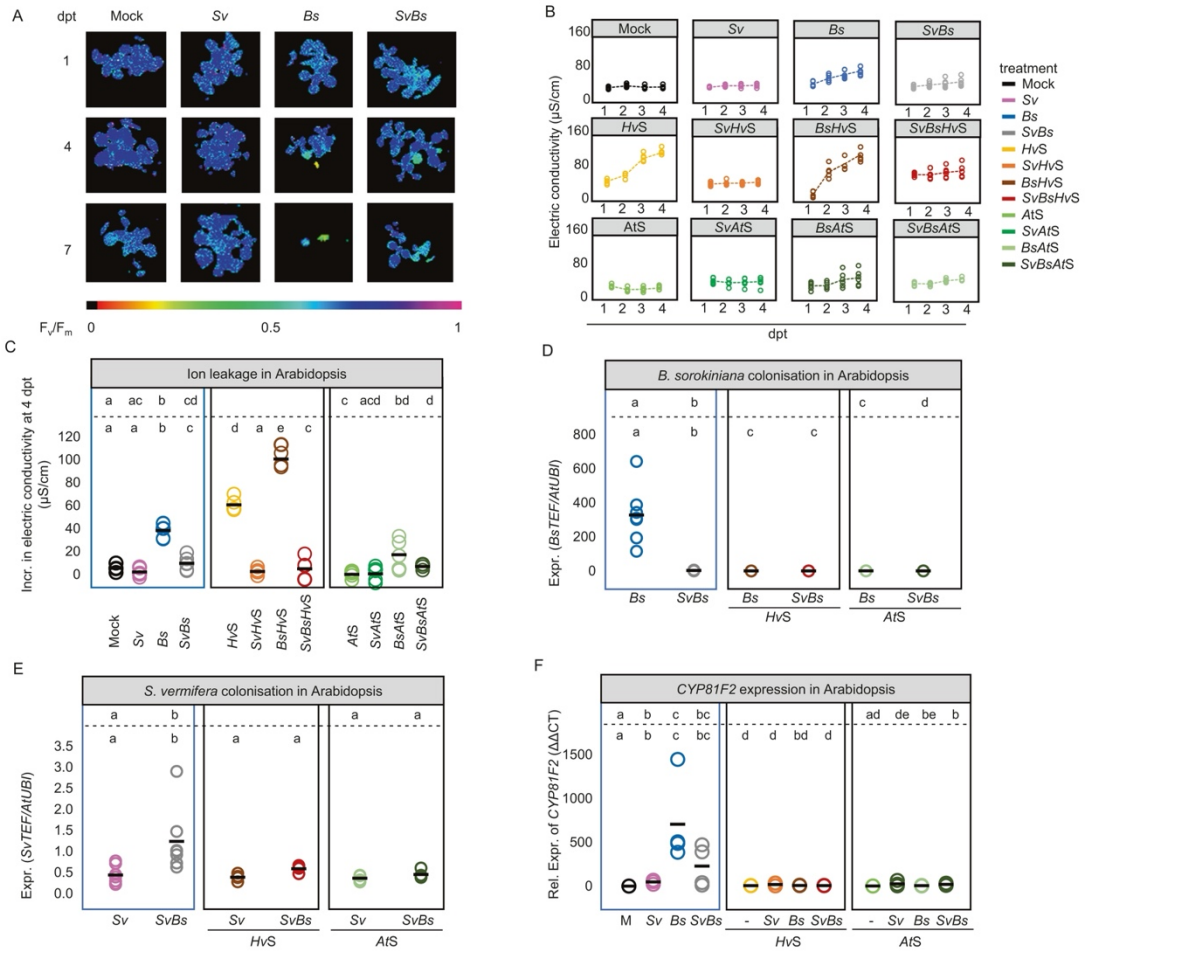

Fig. 3 Arabidopsis root colonization and responses after fungal and/or bacterial inoculation. *Sv* and the bacterial SynComs display beneficial effects on Arabidopsis protection. **A** Arabidopsis photosynthetic activity (F_V/F_M) at 1, 4, and 7 days post transfer (dpt) corresponding to 7, 10, and 13 days post inoculation (dpi), after treatments with *Bs*, *Sv*, or both fungi together. Purple/dark blue, lighter colours, and black colour indicate high, reduced, and lack of PS II activity, respectively. **B** *Bs*- and *HvS*-induced cell death measured via electric conductivity from 1 to 4 dpt ($n = 6$). **C** Total increase in electric conductivity from 1 to 4 dpt ($n = 6$). Statistical analyses were performed for each subpanel together with the tripartite panel (blue frame). **D** *Bs* and **E** *Sv* colonization in Arabidopsis inferred by expression analysis of the fungal housekeeping gene *TEF* compared with plant ubiquitin (*UBI*) ($n = 4$–7). **F** Expression of the Arabidopsis cytochrome P450 monooxygenase *CYP81F2* gene. Different letters in the comparison between the tripartite panel and combinations of any other panel (defined by the dashed lines) represent statistically significant differences according to non-parametric Kruskal–Wallis test followed by pairwise Mann–Whitney U-tests for multiple comparisons (FDR adjusted *p*-value < 0.05).

presence of the bacterial strains nor combined presence of bacteria and *Sv* led to an extensive host transcriptional response (Fig. 5B, Fig. S7; Table S6). Thus, the observed early root growth-promoting effects mediated by *Sv* with Root11 in barley were not accompanied by a strong host transcriptional response (with 14 DEG specific to this condition—3 up and 11 downregulated genes —Fig. S7B, C; Table S6).

Conversely, infection with *Bs* resulted in 2,743 barley DEG. Co-inoculation of *Bs* and Root172 reduced barley DEG to 1,517, whereas Root11 with *Bs* produced a larger number of DEG (3,528) compared to *Bs* alone (Fig. 5B and Fig. S7). Grouping DEG according to expression patterns identified 15 clusters of highly up or downregulated barley genes specific to one or more condition/s and showed that the barley response to co-inoculation with *Bs* and Root11 was most different from all other conditions (Fig. 5C; Table S7). To identify functional categories in co-regulated genes, we employed a SOM to group genes into nodes displaying similar regulation (Fig. S8; Table S3) and we performed GO enrichment analyses (Fig. S9). These analyses showed that *Bs* alone strongly induced a barley immune response and terpenoid phytoalexin production (Fig. 5D; Table S8). Root11 had no effect on immunity or terpenoid phytoalexin production, whereas Root172 slightly induced an immune response. Notably, co-inoculation of Root11 with *Bs* provoked a higher activation of immunity genes and repression of host cell wall biosynthesis and DNA modification compared to the pathogen alone (Fig. S9).

In accordance with the reduction of *Bs* biomass and disease symptoms, the presence of *Sv* reduced the number of barley DEG in response to *Bs* (Sv_Bs: 2,403; Fig. 5B). This reduction was most pronounced in combination with the bacterial strains, especially

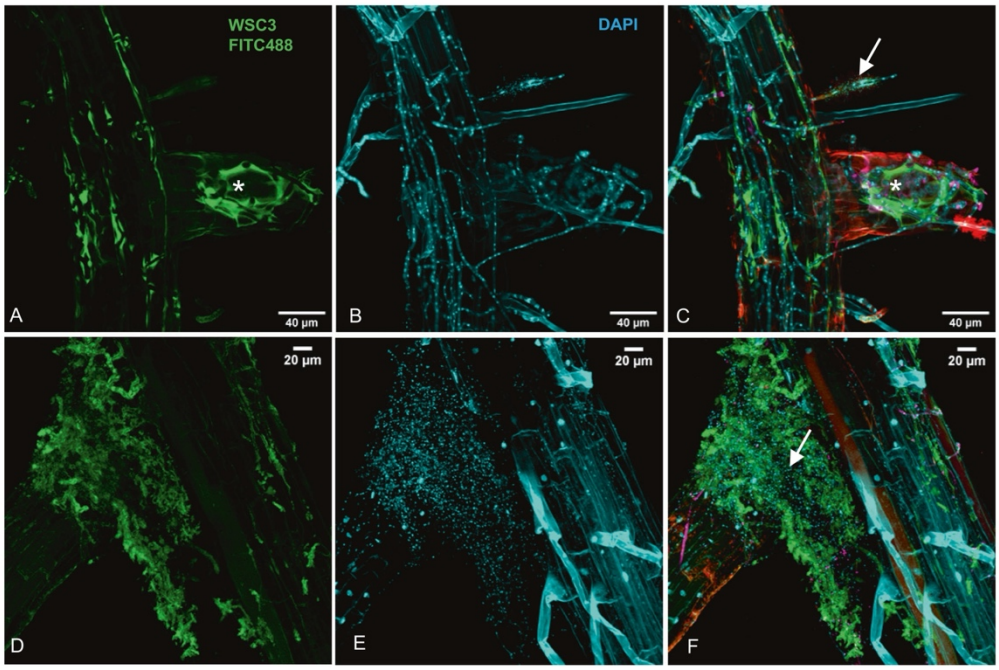

Fig. 4 ***Arabidopsis thaliana*** **Col-0 inoculated with** ***Bs*** **and Root172 at 7 dpi.** Roots were fixed with 70% EtOH and stained with the β-1,3-glucan binding lectin WSC3-FITC488, which binds to the fungal matrix (in **A** and **D**), and the fluorescent DNA stain DAPI (in **B** and **E**). Overlays (in **C** and **F**). Confocal images were recorded using a Leica TCS-SP8 confocal microscope. White arrows: lysis of *Bs* matrix in the presence of Root172. Asterisks: intact fungal matrix.

with Root172 which had the strongest effect on *Bs* colonization (Sv_Bs_Root11: 1,921; Sv_Bs_Root172: 740; Fig. 5B, Fig. S7; Table S6). Consistently, the expression of barley genes associated with terpenoid phytoalexin production was partially reduced in the multipartite interactions compared to *Bs* alone (Fig. 5D). The barley root gene expression data shows that the cooperative action of *Sv* with bacteria protects barley roots from *Bs* infection without extensive host transcriptional mobilization of immunity and defence metabolic pathways.

To test the above observation further, we investigated the immune-modulatory proprieties of the beneficial *Sv* fungal and bacterial strains in roots of Arabidopsis and barley by using specific marker genes. In Arabidopsis, we observed a reduction of the expression of the gene encoding for the cytochrome P450 monooxygenase CYP81F2 involved in indole glucosinolate biosynthesis and defence [69] in *Bs* infected roots co-inoculated with *Sv* and/or the bacteria compared to *Bs* alone (Fig. 3F) which correlates well with the reduced pathogen load. In barley, we previously identified a *PR10* family gene (HORVU0Hr1G011720, hereafter referred to as *HvPR10*-like) as a robust marker for induced immune responses to *Bs* colonization [4]. RNA-seq and quantitative RT-PCR analyses confirmed that *HvPR10*-like expression was highly induced by *Bs* infection of barley roots. By contrast, *HvPR10*-like expression was weakly induced by *Sv* and/or the bacterial strains (Fig. 2F). Despite the strong reduction in pathogen infection and disease symptoms upon co-inoculation with *Sv* and bacteria, we found that *Bs*-induced *HvPR10-like* expression was generally maintained in all combinations (Fig. 2F). This result indicates that *HvPR10-like* expression is driven principally by the pathogen and impacted less by the presence of *Sv* and bacteria. Only co-inoculation of Root172 and *Sv*, which displayed the strongest protection against *Bs* infection, significantly lowered *Bs*-induced *HvPR10-like* gene expression (Fig. 2F). Hence, in conclusion, despite the general decreased barley transcriptional response to *Bs* and the lower pathogen load, the activation of specific immune responses such as the *HvPR10-like* gene were still in place in the presence of *Sv* and/or bacteria in this host.

Synergistic actions of *S. vermifera* and bacteria reduce the virulence potential of endophytic *B. sorokiniana*

To examine mechanisms underlying the cooperative antagonistic behaviour of *Sv* and the bacteria towards *Bs*, we analyzed the fungal transcriptomes during barley root colonization at 6 dpi. We previously reported that fungal transcriptome changes are driven mainly by their interactions with the host and that *Sv* effects on the *Bs* transcriptome occur mostly in the rhizosphere [4]. Consistent with this notion, *Sv* or the bacterial treatments alone had little impact on the transcriptome of endophytic *Bs*. By contrast, the combined presence of *Sv* and Root11 had a strong impact on the *Bs* transcriptome with 65 up- and 786 down-regulated genes (Fig. 6A; Table S6). DEG of *Bs* during root infection were grouped into nine clusters (Fig. 6B; Table S7). The largest *Bs* cluster (#8) contained genes that were repressed compared to *Bs* infection of barley alone. Among the top ten repressed genes in this cluster there were 4 *Bs* genes encoding for glycoside hydrolases (Table S7). This prompted us to look into the expression of all *Bs* CAZyme and effector genes.

We observed a general transcriptional repression for genes in these categories by the combined presence of *Sv* and Root11, possibly explaining the reduced *Bs* colonization of roots (Fig. 6C, D, Figs. S10, S11; Table S9). Notably, *Bs* gene cluster #7 (with genes specifically induced in the combined presence of *Sv_Bs*_Root11) contained six upregulated genes potentially participating in the production of antibacterial compounds related to chrysoxanthone, neosartorin, and emodin (Fig. S12; Tables S10,

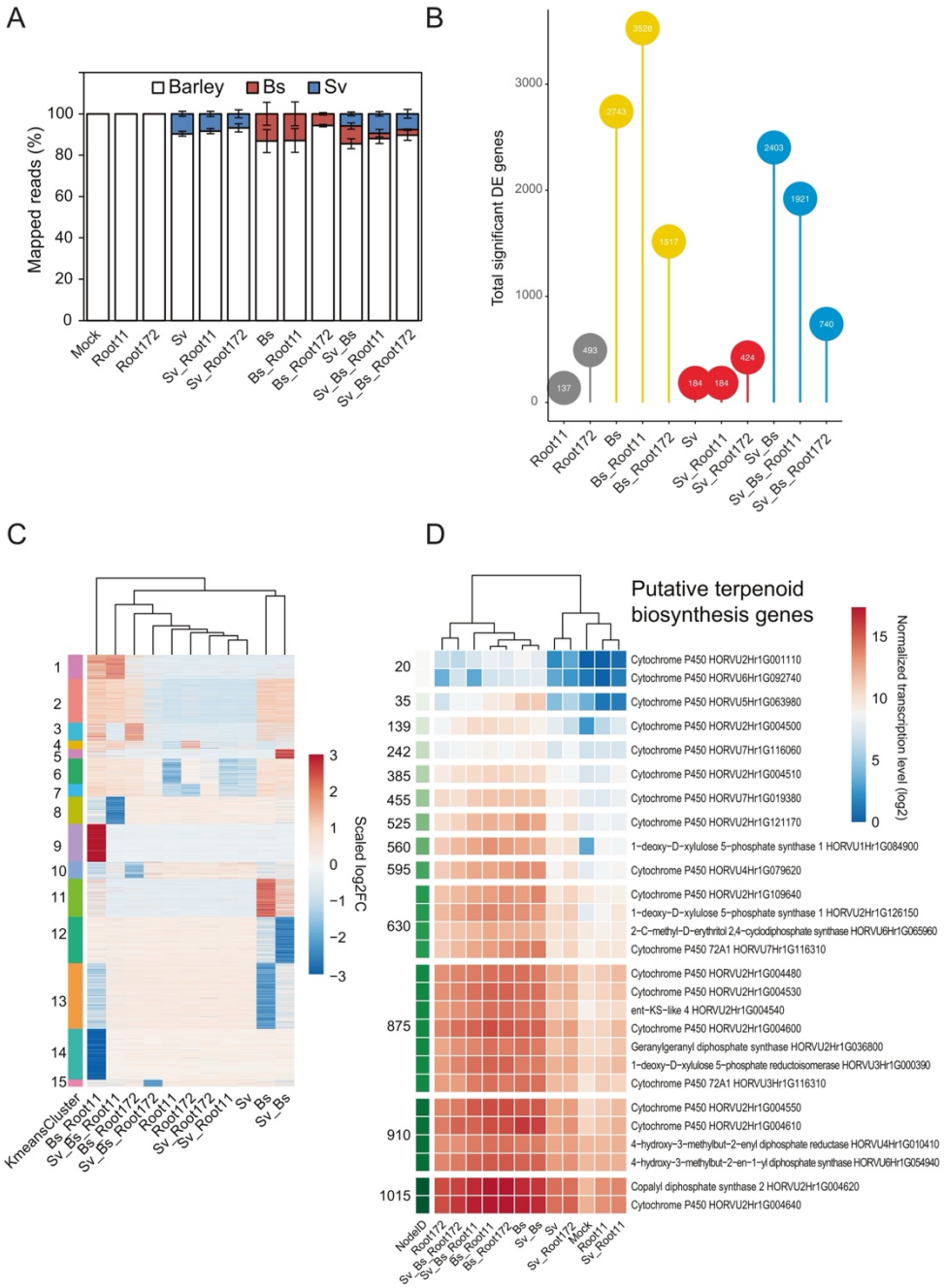

Fig. 5 Analysis of barley root transcriptional responses to fungal and bacterial colonization at 6 dpi. A Proportion of reads mapped to the organisms per sample ± SEM. A total of 34 RNA-seq samples were mapped to the corresponding organisms. Mock: *Hordeum vulgare*. See Table S2. **B** Total number of differentially expressed genes per condition (> 1 log2FC; FDR adjusted *p*-value < 0.05) in comparison to barley mock control. The numbers are shown in the circles. See Fig. S8 and Table S6. **C** K-means clustering of differentially expressed genes grouped into 15 clusters. A total of 5,539 differentially expressed genes are used for (**B** and **C**). See Tables S6 and S7. **D** Normalized transcription level of genes putatively involved in terpenoid phytoalexin synthesis. Averaged transcription in log2 is shown per condition. Terpenoid phytoalexin synthesis pathway in barley was published earlier [4]. See Table S8. *Bs: Bipolaris sorokiniana. Sv: Serendipita vermifera*. Root11 and Root172: *A. thaliana* root-associated bacterial strains Root11 and Root172 from the *At*Sphere collection.

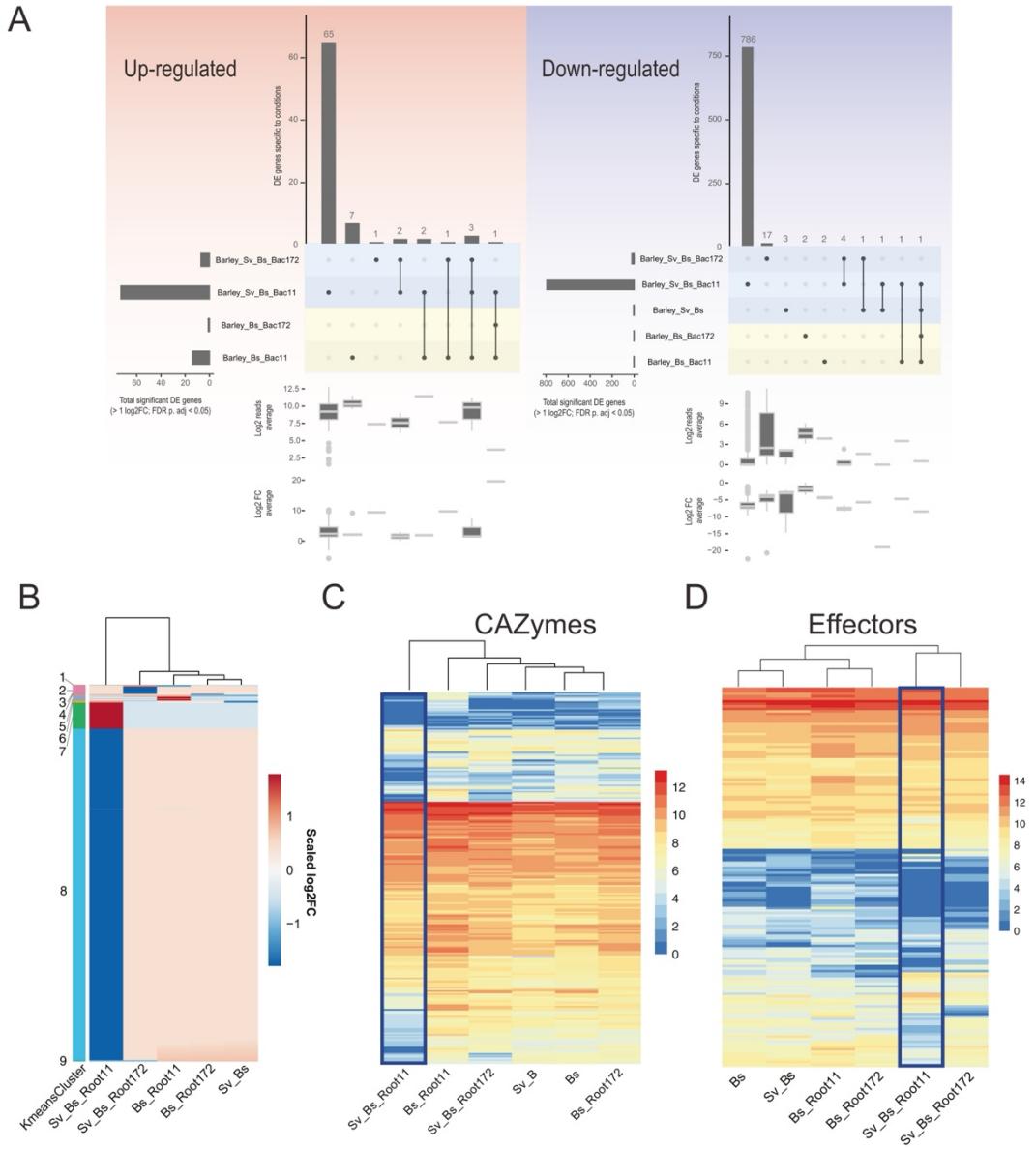

Fig. 6 ***B. sorokinana*** **transcriptional responses to** ***S. vermifera*** **and bacteria during infection of barley at 6 dpi. A** Condition-specific differentially expressed *B. sorokiniana* genes (> 1 log2FC; FDR adjusted *p*-value < 0.05) compared to barley infection alone. Horizontal bars: total number of DEG per condition. Vertical bars: number of genes unique/shared for intersections. See Table S6. **B** K-means clustering of 923 differentially expressed genes grouped into nine clusters. See Table S7. **C** Averaged log2 read counts of predicted secreted CAZyme coding genes. **D** Averaged log2 read count of predicted effector coding genes. See Table S9. *Bs*: *Bipolaris sorokiniana*. *Sv*: *Serendipita vermifera*. Root11 and Root172: *A. thaliana* root-associated bacterial strains Root11 and Root172.

S11, and S12) [70–72]. Hence, it is possible that *Bs* actively engages in antagonizing Root11 in the presence of *Sv* at 6 dpi. On the other hand, upon *Bs* co-inoculation with Root11 we observed induced expression of fungal effector and CAZyme genes (cluster 5 in Fig. 6B, C, D and Figs. S10, S11) such as several AA9, GH43, CE5, PL1, and PL3 that are known to be enriched in plant-associated fungi [38, 73]. This observation might explain the increased host immune response to the combined presence of *Bs* and Root11. Transcriptional changes in endophytic *Sv* in response to the other microbes in barley roots were generally smaller and predominantly driven by *Bs* pathogen load and the associated barley immune response (Fig. 7, Figs. S13, S14; Tables S6, S8, S9). This is in agreement with our previous data, which suggests that *Sv* transcriptional response is likely driven by the changes in the plant host environment due to the pathogen activity rather than by direct interaction with *Bs* inside the root [4].

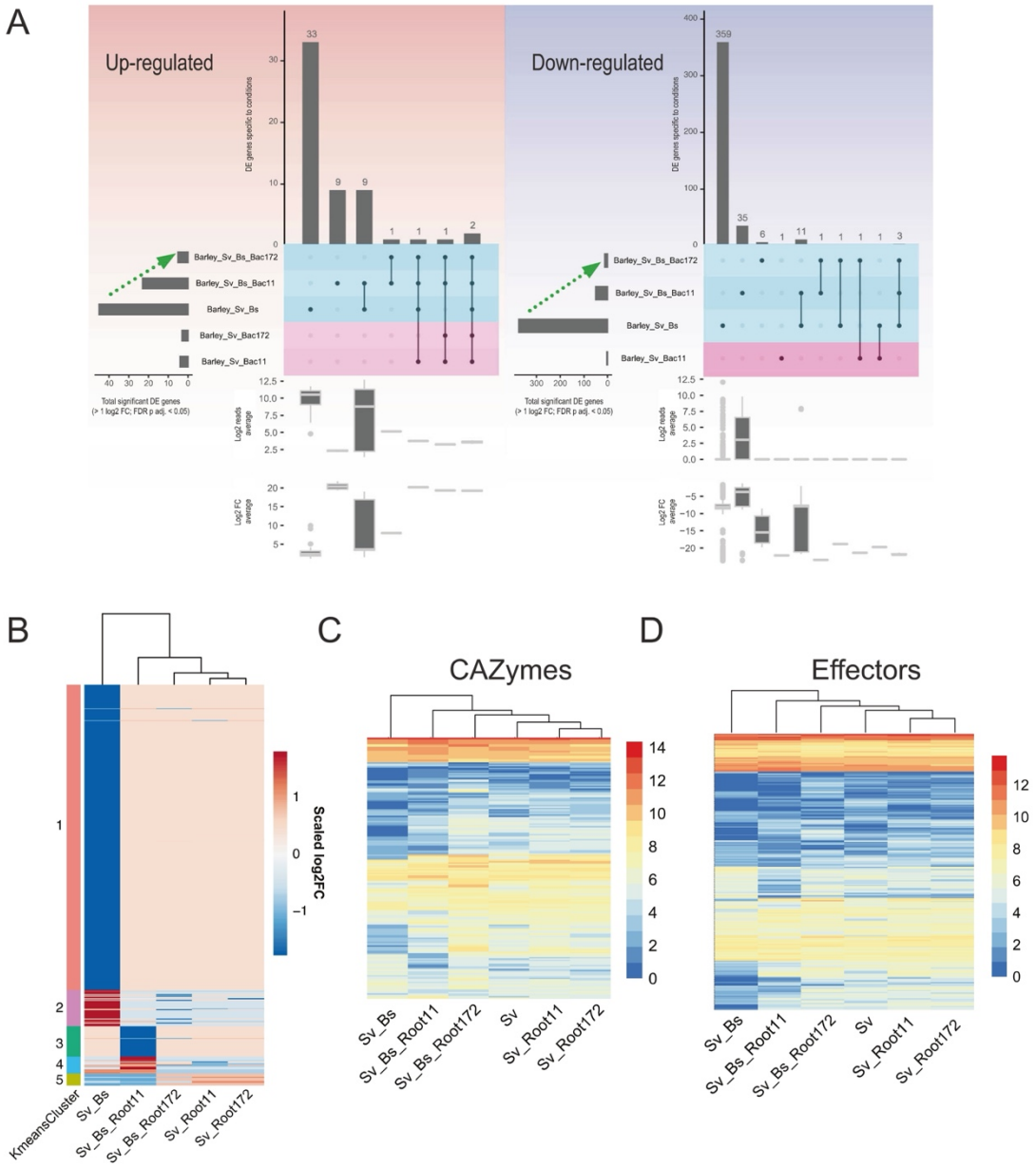

Fig. 7 *S. vermifera* transcriptional responses to *B. sorokinana* and bacteria during colonization of barley at 6 dpi. A Condition-specific of differentially expressed genes (> 1 log2FC; FDR adjusted *p*-value < 0.05) are identified by comparing to the control condition (i.e., fungus alone). Horizontal bars: total number of DEG per condition. Vertical bars: number of genes unique/shared for intersections. See Table S6. **B** K-means clustering of 520 differentially expressed genes. See Table S7. **C** Averaged log2 read count of predicted secreted CAZyme coding genes. **D** Averaged log2 read count of effector coding genes. See Table S9. *Bs*: *Bipolaris sorokiniana*. *Sv*: *Serendipita vermifera*. Root11 and Root172: *A. thaliana* root-associated bacterial strains Root11 and Root172.

DISCUSSION

In complex environments, plant−microbe interactions are not only shaped by the plant immune system [20, 74, 75] but also by microbe−microbe competition and co-operation, acting directly on or as an extension to plant immunity [76, 77]. Recent studies reveal the importance of root-associated bacteria for plant survival and protection against fungi and oomycetes [8, 78–81]. Much less attention has been paid to the role of widely distributed beneficial endophytic fungi in a multi-kingdom context. Here we show that the effects on host growth and protection that are conferred by the Sebacinales member *S. vermifera* in bipartite and tripartite interactions [4, 82] are retained in a community context. The observed robust protective function and stability of *Sv* colonization is likely due to its ability to adapt to changes in the plant host environment

[4]. The strength of its protection against an aggressive root fungal pathogen (*Bs*) is underscored by the observation that *Sv* can functionally replace core bacterial microbiota members in mitigating pathogen infection and disease symptoms in distantly related plant hosts. This finding is in accordance with Arabidopsis root microbiota samplings across European habitats which shows Sebacinales fungi to be of low abundance but consistently present in the host roots and the rhizosphere. Our data highlight the potential importance of widespread root fungal endophytes in maintaining plant host physiological fitness in nature, thereby emphasizing that low-abundance microbes can play a significant role in microbiota beneficial functions and should be considered when designing SynComs with multiple traits, such as resilience and protective activities.

Strikingly, the presence of *Sv* additionally stabilizes and potentiates the protective activities of root-associated bacteria and mitigates the negative effects caused by the non-native *Hv*SynCom in Arabidopsis (Fig. 3B–D and Fig. S3), revealing a more general protective activity of root endophytic fungi. The induction of cell death by the barley-derived SynCom in Arabidopsis could be due to the presence of specific bacterial strains that are absent in the *At*SynCom. One such bacterial group that is well represented in the *Hv*SynCom but absent in the *At*SynCom used in this study is the Pseudomonadales. Several members of this group are reported to be pathogenic whereas others with very few genome differences promote plant growth and exert biocontrol activities against different fungal pathogens [83]. However, we did not observe an increase in ion leakage upon inoculation with the *Pseudomonas* strain bi08 or other members of the *Hv*SynCom when inoculated alone (Fig. S5B–E). The pathogenicity of a single bacterial strain is likely to be suppressed in a community context, as observed for *Bs* (Figs. 2, 3). Thus, another explanation to the negative effects of the *Hv*SynCom in Arabidopsis but not in barley might be a lack of adaptation to Arabidopsis. This notion is supported by a recent analysis that detected a clear signature of host preferences among commensal bacteria from diverse taxonomic groups, including Pseudomonadales in Arabidopsis and *Lotus japonicus* [84].

Our transcriptomic analyses show that the effects of the tested bacterial strains in tripartite associations differ substantially. The general decreased barley transcriptional response to the pathogen driven by the Rhizobiales strain Root172 (Fig. 5B) and the lysis of the fungal matrix at the host rhizoplane (Fig. 4) suggest that this bacterial strain most likely acts directly on *Bs*. This is also supported by the strong antagonism of *Bs* growth in confrontation assays irrespective of the presence of a host plant (Fig. 2B and Fig. S4). Taken together, these results point to Root172 as a possible biocontrol agent against *Bs* and potentially other root-infecting pathogens. The impact of Root172 contrasted strikingly with that of the Bacillales strain Root11, which did not limit *Bs* growth but rather enhanced *Bs* pathogenicity in barley. Notably, combining these two bacterial strains with *Sv* led to a restriction of *Bs* that exceeded the protective benefits of *Sv* and the bacteria alone (Fig. 2B). These synergistic beneficial effects are decoupled from extensive host transcriptional reprogramming (Fig. 5B) and cannot be solely explained by enhanced *Sv* growth (Fig. 2D and Fig. S4B) as speculated for other fungal-bacterial synergistic beneficial effects [85, 86]. Our transcriptional and phenotypic data further suggest that *Sv*—bacterial synergism in protecting host roots have also a component that is additive because the underlying antagonistic mechanisms displayed by the fungal root endophyte and the bacterial strains are likely to be distinct and explained mainly by direct microbe−microbe interactions outside the plant. Nonetheless, we have observed a higher level of inter-kingdom mediated antagonism on *Bs* in presence of the host (Fig. 2B and Fig. S4). This suggests a minor but relevant host-dependent effect that needs to be addressed.

At the early time point of 6 dpi, growth promotion was only observed in the combined presence of *Sv* and certain bacterial strains with the strongest effect during co-inoculation with Root11 in barley and Root172 in Arabidopsis (Fig. 2E and Fig. S2E). Furthermore, growth promotion required living microbes, as co-inoculation with heat-inactivated bacteria did not increase the root fresh weight in barley. Commensal bacteria in the rhizosphere can trigger plant growth promotion and resistance to pathogen [20, 21, 87]. Among them, strains belonging to the genus *Bacillus* are often used as bioagents due to their function in eliciting ISR (induced systemic resistance) as well as growth promotion [21, 88]. However, plant growth-promoting bacteria (PGB) and Sebacinales mediated growth promotion are often reported during later stages of colonization. The early host growth enhancement observed with *Sv* and the bacteria might thus confer a competitive advantage for plants in nature. It is striking that the growth-promoting effect is not accompanied by an extensive host transcriptional response with only 14 barley DEG being specific to this condition (Table S6). Interestingly, several of these genes display differential expression across barley accessions (analyzed using Genevestigator) compared to the cultivar Golden Promise. It would therefore be informative to test growth outcomes of combined *Sv* and e.g., Root11 inoculation in different barley varieties/ecotypes. The resulting synergistic inter-kingdom benefits in plant protection against fungal disease and in plant physiology are in line with studies of the Sebacinales fungus *S. indica* with single bacterial strains on tomato [85, 89, 90], rice [91], barley [92], and chickpea [93] and underline the broad functional relevance in plant health for fungi of the order Sebacinales in multi-kingdom environments. Inter-kingdom benefits in plant–beneficial microbe interactions were reported also for native isolates of *Trichoderma* spp. and *P. fluorescens* against *Ralstonia* spp. in tomato and with *B. velezensis* against *Fusarium* in gooseberry [23, 94], suggesting that the combined application of beneficial fungi and bacteria has strong potential as biocontrol agents.

The deployment of microbiota as biocontrol agents for crop protection and enhancement is an ancient concept that is gaining increased relevance in modern agriculture [95–97]. Plant protection and growth promotion properties conferred by microbial consortia have been found to be more resilient than the use of single strains [95]. Moreover, Duran et al. 2018 showed that a complex SynCom consisting of bacteria, fungi, and Oomycetes led to the strongest beneficial effects on Arabidopsis growth and survival compared to mono-kingdom or small SynCom associations and hypothesized that selective pressures over evolutionary time favour inter-kingdom microbe−microbe interactions over interactions with single microbial strains [8]. Inter-kingom associations are frequently observed between members of the Sebacinales and bacteria. Different Sebacinales species host endobacteria of the orders *Bacillales* (genera *Paenibacillus*), *Pseudomonadales* (*Acinetobacter*) and *Actinomycetales* (*Rhodococcus*) and its close relative *S. indica* hosts an endobacteria of the order *Rhizobiales* (*Rhizobium radibacter*) [98]. Beneficial effects of these intimate inter-kingdom interactions on the plant host and the fungus itself were described between *S. indica* and *R. radibacter* [98, 99] and for interactions between arbuscular mycorrhizal fungi and bacteria belonging to different species of the orders Proteobacteria (*Rhizobiales*) and Firmicutes (*Bacillales*) [100]. Considering the pervasiveness of beneficial effects conferred by *Sebacinales* and bacteria compared to the vulnerability of *Bs* in a multipartite context, our data support the hypothesis that the establishment of beneficial inter-kingdom interactions in the plant microbiota is an evolutionary conserved and robust trait.

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AUTHOR CONTRIBUTIONS

LM and AZ designed the experiments. LM, CU, DB, and SRA performed the experiments, isolated the barley bacterial strains, and designed the *Hv*SynCom. LM, SM, GL, YN, RGO, RG, and AZ analyzed the data. LM, AZ, JP, CU, and SM wrote the paper with editorial input from all other authors.

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Chapter 3

Plant host and microbe specific gene expression in the mutualistic root endophytic fungus *Serendipita vermifera*.

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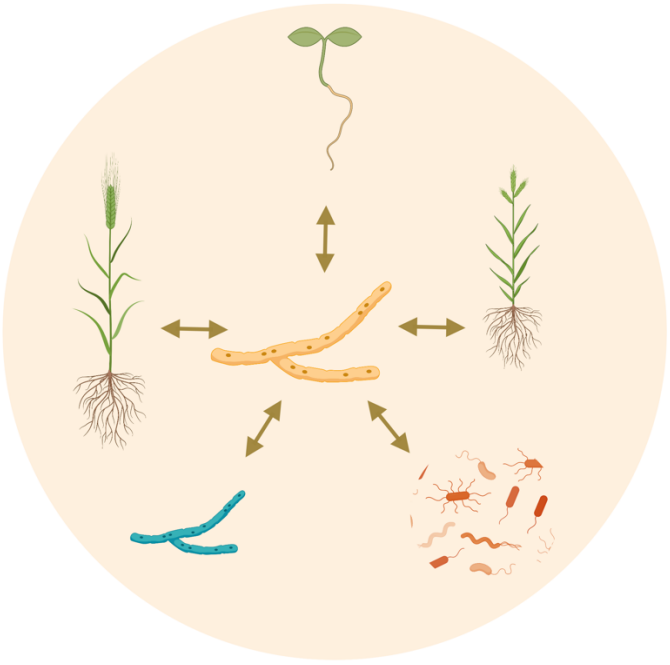
In prep

Autors contribution:

LM, GL and AZ planed the experiments. **LM** performed the inoculations and sample preparations for the RNA seq analysis. YZ, ST, DP, VN and IG organized and performed the RNA sequencing. SM and CDQ performed the bioinformatic analysis and prepared the bioinformatic figures. **LM** interpreted the data. RE performed *Si* tripartite assays, the cloning, and the chitinase-activity assay. RE and **LM** performed the fungal toxicity assay. **LM** wrote the manuscript.



Plant host and microbe specific gene expression in the mutualistic root endophytic fungus *Serendipita vermifera*

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Abstract

Associations between plants and beneficial root-associated fungi facilitate plant nutrient uptake, growth, and resistance to biotic and abiotic stresses. Endophytic generalists such as Sebacinales fungi form associations with a variety of different plant hosts and dynamically adapt to changes in the biotic environment to ensure a stable colonization. Their resilience likely relies on a large number of tightly regulated effectors and carbohydrate-active enzymes (CAZymes) that are required for both root colonization and inter-microbial interaction. However, little is known about the function, specificity, and regulation of these effectors. In this study, we examined gene expression of the Sebacinales fungus *Serendipita vermifera* in response to different plant hosts and microbes at four time points to determine interaction partner-specific expression of effector genes. Further, we identified a set of 177 DEGs that were specific to the presence of the fungal pathogen *Bipolaris sorokiniana*, including a chitinase (*Sv*CHIT-CBM5) that we identified as a determinant for fungal antagonism.

Introduction

Beneficial root-associated fungi are major players within the host microbiota, the consortia of plant-associated microorganisms (Glynou et al., 2018, 2016; Trivedi et al., 2020). They contribute to plant health and productivity by enhancing host nutrient uptake, plant growth, and resistance to biotic and abiotic stresses (Finkel et al., 2017; Hermosa et al., 2012; Mahdi et al., 2022; Raaijmakers et al., 2009). Such beneficial properties have been found in plant interactions with ectomycorrhizal fungi (ECM) and arbuscular mycorrhizal (AM) fungi, as well as non-mycorrhizal fungal endophytes (Zuccaro et al., 2014). The Sebacinales fungi *Serendipita vermifera* and *Serendipita indica* (Basidiomycota, Sebacinales) form an outer loose hyphal network around the

root and additionally colonize the epidermis and cortex cell layers of different plant hosts inter- and intracellularly (Deshmukh et al., 2006; Jacobs et al., 2011). Colonization of both fungi is associated with host beneficial traits (Deshmukh et al., 2006; Mahdi et al., 2022; Sarkar et al., 2019; Zuccaro et al., 2014). In later stages of the association, intracellular colonization leads to localized death of host cells while the beneficial effects on the plant host are maintained (Deshmukh et al., 2006; Qiang et al., 2012b). This restricted cell death is required for successful colonization and is thought to represent a nutritional strategy of Sebacinales that maintained the saprotrophic capabilities of their ancestors. The combination of their saprotrophic and biotrophic traits has likely led to the evolution of these fungi as generalists with a broad range of distantly related hosts, including mycorrhizal and nonmycorrhizal plants (Lahrmann and Zuccaro, 2012; Weiß et al., 2016, 2011). A broad host range implies that the fungus is confronted with various host-specific physiological characteristics and defense mechanisms. Indeed, *S. indica* colonization strategies are highly host-specific (Lahrmann et al., 2013; Zuccaro et al., 2014). Such adaptive capabilities are most likely associated with a large repertoire of effectors, specialized molecules that modulate the interaction between the microbe and its host. Genome analysis of *S. indica* revealed an arsenal of genes involved in plant cell wall degradation and protein hydrolysis, an expanded repertoire of proteins that function in carbohydrate binding with proteins containing LysM, WSC, and CBM1-Pfam domains, and numerous small secreted proteins (SSPs) with effector-like properties (Zuccaro et al., 2014, 2011).

Microbial effectors facilitate the evasion of host recognition, suppress the host immune response, modulate host cell physiology, or manipulate the host microbiota (Hemetsberger et al., 2012; lo Presti et al., 2015; Snelders et al., 2022; Veneault-Fourrey and Martin, 2011; Win et al., 2012). While pathogenic microorganisms use effectors to manipulate the plant microbiota and increase virulence (Snelders et al., 2022), members of the beneficial microbiota exert biocontrol activities, including antibiosis and mycoparasitism via secretion of antimicrobial compounds and volatiles or effector proteins (Eitzen et al., 2021; Hassani et al., 2018; Mgbeahuruike et al., 2011; Romero-Contreras et al., 2019; Tilocca et al., 2020). Similarly, the ability of *S. vermifera* to antagonize the necrotrophic fungus *Bipolaris sorokiniana* is associated with increased expression of putative effector proteins and carbohydrate-active enzymes (CAZymes) (Sarkar et al., 2019).

The function of fungal effectors with microbiome-manipulating capabilities may be "rooted" in the soil biome by competing with soil-dwelling bacteria. Inter-microbial competition within the plant microbiota can result in the evolution of effectors that act on both the plant host and surrounding microbes. Transcriptomic analysis of *S. vermifera* and *B. sorokiniana* during bipartite and tripartite interactions with barley revealed that while both fungi express plant host- or microbe-specific effectors, a core set of effectors is induced during both interactions, suggesting a dual function in microbe-plant and inter-microbial interactions (Sarkar et al., 2019). Examples of dual function are reported for *Trichoderma virens* tvhydii1, a class II hydrophobin, and the LysM effector Tal6 of *T. atroviride,* which are relevant for both plant root colonization and mycoparasitism against pathogenic fungi (Guzmán-Guzmán et al., 2017; Romero-Contreras et al., 2019).

This study aimed to identify the transcriptional strategies Sebacinales fungi employ to interact with different plant hosts and microbes. Therefore, we created a large RNA seq data set consisting of bipartite interactions between *S. vermifera* and different organisms, the monocot hosts barley (*Hordeum vulgare*) and *Brachypodium distachyon* (Bd21-3), as well as the dicot host *Arabidopsis thaliana*, a bacterial synthetic community (SynCom), and the fungal pathogen *B. sorokiniana*. By comparing the gene expression of *S. vermifera* in response to these interactions at four time points, we identified general determinants of host colonization as well as interaction partner-specific expression patterns. We further identified a fungal chitinase (Sebve1_16391/GH18-CBM5) as a determinant for fungal antagonism. Recombinant *Sv*CHIT-CBM5 inhibits spore germination of *B. sorokiniana*, revealing a direct antagonistic function. This study provides the first comparative data set to study fungal interaction strategies and will help to unravel other molecular mechanisms underlying fungal-plant and fungal-microbial interactions in the future.

Material and methods

Plant, fungal and bacterial materials: Barley (*Hordeum vulgare* L. cv Golden Promise), *Brachypodium distachyon* (Bd21-3), and *Arabidopsis thaliana* Col-0 were used as plant hosts. *Serendipita vermifera* (MAFF305830) and *Bipolaris sorokiniana* (ND90Pr) were

used as fungal models. The *At*SynCom consists of four bacterial strains from the *At*Sphere collection (Bai et al., 2015), which were used in (Mahdi et al., 2022).

Growth conditions and microbial inoculations: *Hordeum vulgare* and *Arabidopsis thaliana* seeds were sterilized and germinated as previously described (Mahdi et al., 2022). *Brachypodium distachyon* 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 ten days in darkness at 4 °C on wet filter paper and then transferred to sterile glass vials containing 1/10 PNM (Plant Nutrition Medium, pH 5.7) for germination on a day-night cycle of 16/8 h at 22/18 °C, 60 % humidity, and a light intensity of 108 µmol/m^2s for eight days. *Sv* was propagated on MYP medium (Lahrmann et al., 2015) and *Bs* on modified CM medium (Sarkar et al., 2019), each containing 1.5% agar, at 28 °C in darkness for 21 and 14 days, respectively. *Sv* mycelial suspensions and *Bs* spore suspensions were prepared as previously described (Sarkar et al., 2019). Bacteria were grown in liquid TSB medium (Sigma Aldrich) (15 g/l) at 28 °C in the dark at 220 rpm for 1-3 days depending on the growth rate. Bacterial suspensions were prepared as previously described (Mahdi et al., 2022). Plant roots were inoculated on a 12 x 12 cm Petri dish (*Arabidopsis thaliana*) or in a sterile glass jar (*Hordeum vulgare* and *Brachypodium distachyon*) containing 1/10 PNM with *Sv* mycelium (2g/50ml for *Hordeum vulgare* and *Brachypodium distachyon* / 1g/50ml for *Arabidopsis thaliana*) or sterile water as control. Microbe-microbe confrontation experiments on plates were performed on Petri dishes containing 1/10 PNM. Plates were inoculated with 2 ml of either a pure suspension of *Sv* mycelium (2g/50ml) or a mixed suspension of *Sv* mycelium with *Bs* spores (5000 spores/ml) or the bacterial SynCom (OD600 = 0.01). All samples were kept on a day-night cycle of 16/8 hours at 22/18 °C, 60% humidity, and 108 µmol/m^2s light intensity for 1, 3, 6, and 10 days post inoculation (dpi), respectively. 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 Milli-Q water to remove extraradical fungal hyphae. All samples were frozen in liquid nitrogen and used for RNA extraction.

RNA isolation for RNA-seq analysis: RNA extraction for RNA-seq analysis was performed as previously described (Sarkar et al., 2019). RNA sequencing was performed at JGI under a project proposal (Proposal ID: 505829) (Zuccaro and

Langen, 2020a). Stranded RNASeq libraries were generated and quantified by qPCR. The sequencing was performed with Illumina technology in 151PE mode for each sample at the U.S. Department of Energy Joint Genome Institute. On average, 18 million fragments were generated per sample. 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 the phred trimming set at Q6. After trimming, the reads with a length below 25 bases or 1/3 of the original read length were removed – whichever is longer). Filtered reads from each library were aligned to the *S. vermifera* MAFF 305830 v1.0 reference genome downloaded from Mycocosm, Joint Genome Institute (https://mycocosm.jgi.doe.gov/mycocosm/home) using HISAT2 version 2.2.0. The raw gene counts were generated using featureCounts and the *S. vermifera* gff3 annotations. Only primary hits assigned to the reverse strand were included in the raw gene counts.

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 log2 transformed. Significant DEGs specific to conditions (> 2 log2 FC or < -2 log2 FC; FDR-adjusted $p < 0.05$) were visualized using the R package UpSetR (Conway et al., 2017). Genes that were differentially expressed among conditions were identified using the principal coordinates calculated with the R package Vegan (Oksanen et al., 2020). The first five principal coordinates were used to select genes with high loadings.

Fungal gene annotations: Fungal genomes and functional annotations were obtained from Mycocosm, Joint Genome Institute (https://mycocosm.jgi.doe.gov/mycocosm/home). Secreted proteins were identified with the secretome pipeline at INRAE Nancy, France, using SignalP, WoLF PSORT, TMHMM, TargetP, and ps-scan (Pellegrin et al., 2015). Effectors were predicted using EffectorP3.0 (Sperschneider and Dodds, 2022) and selected based on the secretome

prediction above. The CAZyme annotation is from the CAZy team at the University of Aix-Marseille, France (www.cazy.org).

GO enrichment: The functional annotations of *S. vermifera* were obtained from Mycocosm, Joint Genome Institute (https://mycocosm.jgi.doe.gov/mycocosm/home). For each group of upregulated genes that are specific to a treatment or shared between treatments, Gene Ontology (GO) enrichment analysis was performed using the function enricher of ClusterProfiler. The top 10 significant GO categories were plotted for group of genes (Table S3).

Chitinase expression in *E. coli*: The coding sequences of *Sv*CHIT-CBM5 and *Si*CHIT-CBM5 were amplified and cloned into *E. coli* using the primers and protocols listed in the supplementary Method section. Protein expression was induced using 1 mM IPTG. The bacterial cultures were incubated at 16 °C and 120 rpm overnight. The pellets were collected via centrifugation at 5.000 g at 4 °C. For protein extraction, 6.5 ml lysis buffer (50mMNaH_2PO_4, 300 mM NaCl, and 10 mM Imidazole) were added per g of bacterial pellet. Pellets were 3 x sonicated for 20s on ice and centrifuged at 13500 rpm for 30 min. The supernatant was stored at 4 °C on ice. Protein concentrations were measured by Bradford assay and adjusted to equal levels. The presence and activities of both chitinases within the crude protein extracts were confirmed via SDS-Page, western blot, and chitin azure assay.

Chitin azure assay: Chitin azure was adjusted to 4 mg/mL in 20 mM phosphate buffer (pH 6), and 100 µL was added to 2 mL reaction tubes. 100 µL of 5 mg/mL crude protein extract from *E. coli* in lysis buffer was added. The samples were incubated at 28 °C and 120 rpm overnight. Next, samples were centrifuged at 13000 rpm 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. 200 uL spores were mixed with 200 uL protein extract to a final concentration of 2,8 mg/mL, filled into 8 well chamber slides (Kammerobjektträger Nunc™ Lab-Tek™ II;VWR) and incubated for 6 h at 28 °C. The germination rate was quantified by non-invasive counting using an inverted microscope.

Results

The broad host range of *S. vermifera* is associated with host-specific transcriptional responses.

To investigate how *S. vermifera* (hereafter *Sv*) colonizes a wide range of organisms while coping with plant host- and microbe-specific stresses, we created a large RNA-seq data set that includes bipartite interactions of *Sv* with the plant hosts *Hordeum vulgare*, *Brachypodium distraction,* and *Arabidopsis thaliana*, as well as with the fungal pathogen *B. sorokiniana* (hereafter *Bs*) or a bacterial synthetic community (SynCom) derived from Arabidopsis roots. Possible temporal differences in the establishment of colonization stages were addressed by examining the interactions at four time points (1, 3, 6, and 10 days post inoculation (dpi)) (Fig. 1A). In this way, we identified a total of 5606 differentially expressed genes (DEGs) compared with the fungus cultured alone, accounting for approximately 37% of all *Sv* genes.

The total number of *Sv* DEGs was highest in response to the tested plant hosts barley (*Hv*, 2781 DEGs), Brachypodium (*Bd*, 3165 DEGs), and Arabidopsis (*At,* 2908 DEGs), indicating that a large repertoire of genes is required for successful host colonization (Fig. 1B,C). Among these, 685 DEGs (12.3% of all plant-responsive DEGs) were common to all three plant hosts (Fig. 1B, Table S1). These genes are considered to be general determinants of root colonization. These common determinants include proteases and hydrolytic enzymes such as GH3 family members, which detoxify plant-derived defense compounds (Bradley et al., 2022) (Fig. 2,3b Table S2). Further, general determinants comprise genes related to nucleotide metabolism, namely a 5'-nucleotidase (Sebve1_17804; *Sv*5NT) and two nucleases (Sebve1_26106; *Sv*NucA, Sebve1_9616) (Fig. 2). Sequence homology and expression pattern of *Sv*E5NT and *Sv*NucA are reminiscent of the mechanism of cell death induction during colonization of *S. indica* by the synergistic action of *Si*E5NT and *Si*NucA (Dunken et al., 2021), indicating a similar mechanism for induction of cell death during *Sv* colonization.
Strikingly, 1043 DEGs (*At*), 945 DEGs (*Bd*), and 910 DEGs (*Hv*) were specific to each plant host, revealing large host-specific transcriptional responses (Fig. 1). Host-specific DEGs were found across all time points from initial colonization to early biotrophic and later host cell death associated colonization stages (Fig. S3). These results are consistent with host-specific transcriptional responses of fungal generalists with different lifestyles (Kusch et al., 2022; Lahrmann et al., 2013; Mela et al., 2011; Xia et

al., 2001) and highlight a versatile transcriptome as a prerequisite for fungal generalism.

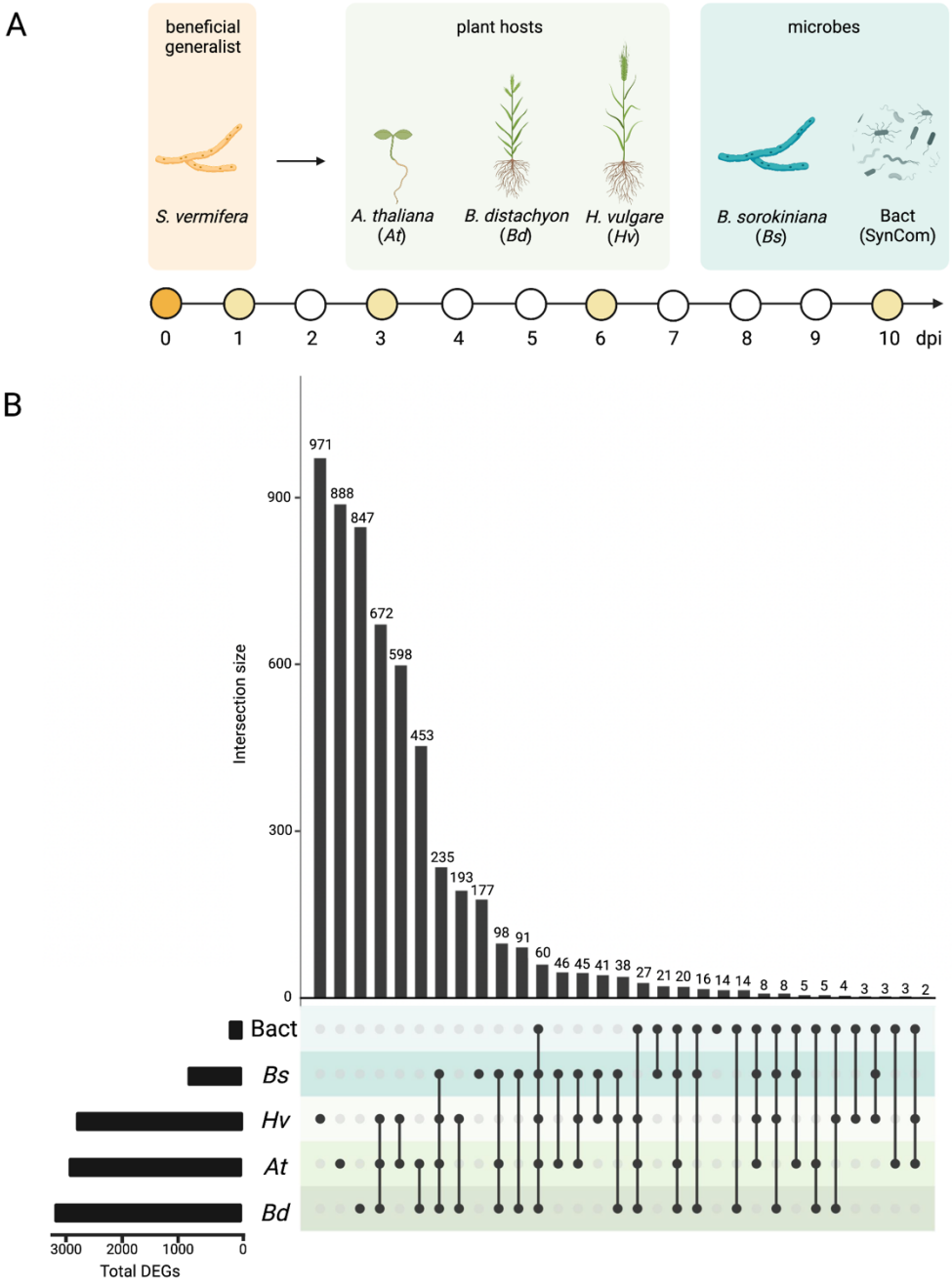

Figure 1: Differentially expressed genes (DEGs) of *Sv* in response to different interaction partners. A) Schematic overview of the experimental setup. Bipartite interactions between *Sv* and different plant hosts or microbes *At*: *Arabidopsis thaliana*. *Bd*: *Brachypodium distachyon*. *Hv*: *Hordeum vulgare*. *Sv*: *Serendipita vermifera*. *Bs*: *Bipolaris sorokiniana*. Bact: bacterial SynCom. dpi: days after inoculation. The day of inoculation is marked in orange. The analyzed time points (dpi) are marked in yellow. B) UpSetplot of significantly differentially expressed genes (DEGs) across all time points (FDR-adjusted $p < 0.05$) compared to control (fungus alone). Dotted lines indicate a combination of conditions. (Table S1 – Significant DE genes).

Interaction between *S. vermifera* and members of a core bacterial microbiota is not associated with large transcriptional responses.

Compared to the transcriptional responses to plant hosts, *Sv* responds little to the presence of the bacterial SynCom with 213 DEGs across all four time points (Fig. 1). Despite the synergistic beneficial effects on host plants mediated by Sebacinales and bacteria (del Barrio-Duque et al., 2019; Hestrin et al., 2022; Mahdi et al., 2022), little is known about the mechanisms underlying their interaction. Microscopic analyses revealed a link between *Sv* and individual bacterial strains, with the fungal glucan matrix as a major interaction zone (Mahdi et al., 2022). Accordingly, early bacterial-specific DEGs include enzymes that are associated with the modulation of carbohydrates, such as a carbohydrate esterase 4 (CD) (Sebve1_73479) and an apoplastic CBM13 effector (Sebve1_326195) (Fig. 2, Fig. 3B+S4). These proteins may alter the structure of the fungal cell wall or matrix to regulate fungal-bacterial interactions. At 10 dpi, genes associated with laccase production (Sebve1_17986) are induced, indicating fungal stress during prolonged growth in the presence of the bacteria on nutrient-poor media (Fig. 2)(Baldrian, 2004; Crowe and Olsson, 2001; Divya and Sadasivan, 2016; Freitag and Morrel, 1992). In general, the results suggest that a limited number of *Sv* genes are involved in the interaction with bacterial strains.

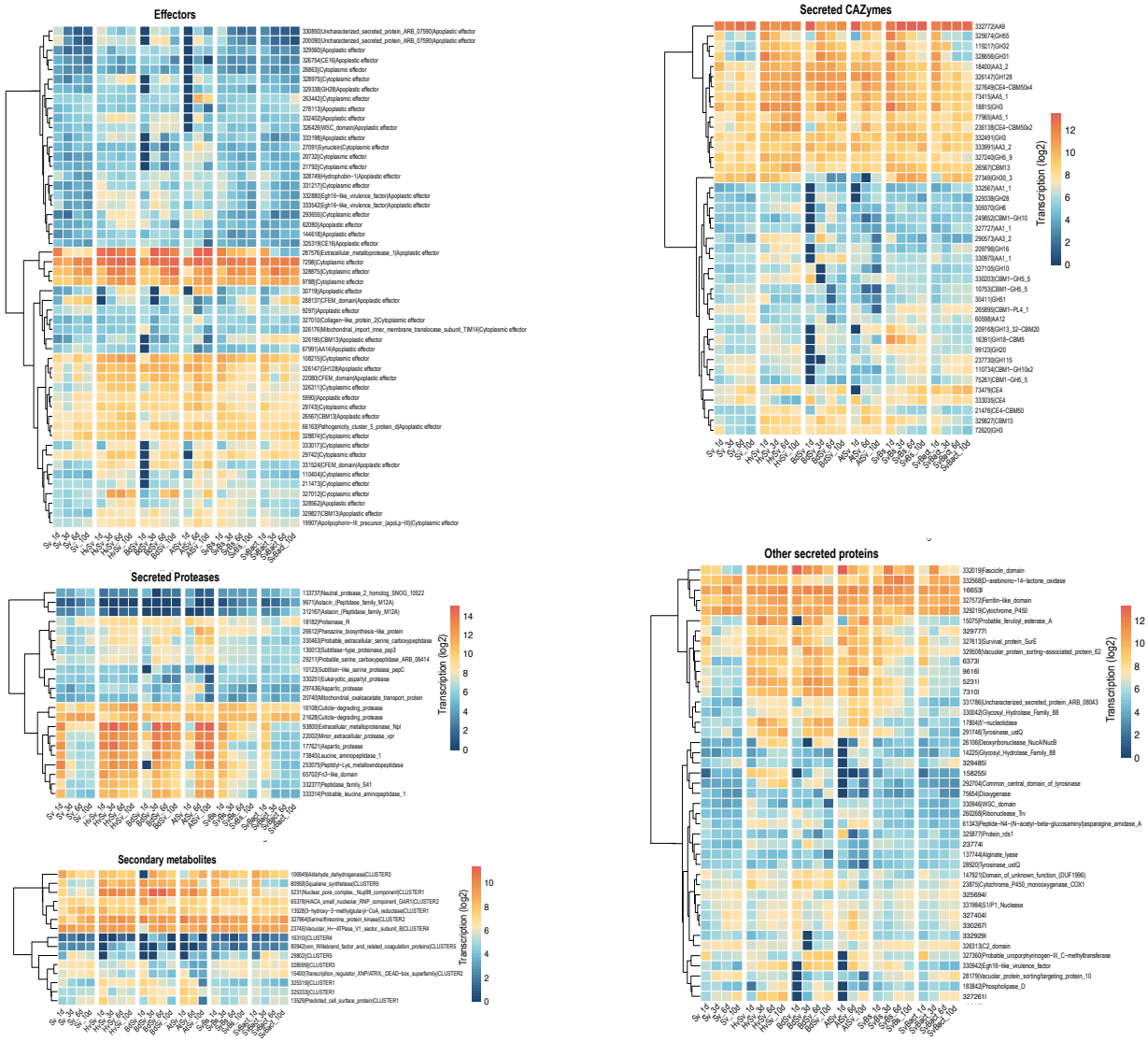

Figure 2: Overview of *Sv* DGSs encoding effectors, secreted proteins/enzymes, and secondary metabolites across different interactions and time points. Gene IDs with annotations are listed on the Y-axis, treatments, and time points on the X-axis. *At*: *Arabidopsis thaliana*. *Bd*: *Brachypodium distachyon*; *Hv*: *Hordeum vulgare*; *Sv*: *Serendipita vermifera*; *Bs*: *Bipolaris sorokiniana*; Bact: bacterial SynCom; d: days. The averaged transcription of genes combined from the replicates is indicated for each time point. Genes with more than 2 log2 different expression were selected (FDR-adjusted p < 0.05) by comparing them to the control (*Sv* alone) at the same time points (Table S2 - Selected DE genes per protein type).

S. vermifera GH18-CBM5 chitinase is a marker gene for fungal antagonism.

The transcriptional response of *Sv* to *Bs* included 912 DEGs in all treatments. In support of previous findings, we observed similarities between the transcriptomic response to *Bs* and plant hosts (Sarkar et al., 2019). 216 *Sv* DEGs (23.6% of all *Bs*-responsive genes) were shared across these treatments. GO term enrichment

identified "oxidoreductase activity," "membrane," "transport," and "transporter activity" as common processes involved in host adaptation and mycoparasitism (Fig. 3a, Table S3). Further, the shared DEGs included genes involved in carbohydrate metabolism and a secreted Egh16-like virulence factor (Sebve1_330942, hereafter *Sv*Egh16like) (Fig. 2+3b). Homologs of this Egh16like virulence factor are associated with cell wall remodeling and appressoria-mediated host penetration in plant-, insect-, and animal colonizing fungi (Cao, 2012; Grell et al., 2003; Herrera-Estrella et al., 2016; Huang et al., 2019; Shang et al., 2021; Xue et al., 2002), suggesting a role for *Sv*Egh16like during penetration of plant roots and *Bs* hyphae.

In addition to these general responses and in agreement with previous findings, several effectors and CAZymes were up-regulated explicitly in the confrontation between endophyte and pathogen. Among them, glycoside hydrolases were the most frequently induced CAZymes during interaction with *Bs* (Sarkar et al., 2019) (Fig. 2, 3b and S4, Table S1+2). The highest, uniquely upregulated gene in response to *Bs* is Sebve1_16391, which encodes a glycosyl hydrolase (GH) family 18 chitinase with a CBM5/ChtBD3 binding domain (hereafter *Sv*CHIT-CBM5). The hydrolytic GH18 domain of *Sv*CHIT-CBM5 is homologous to the Trichoderma ech42, which functions in mycoparasitism (Carsolio et al., 1994; Woo et al., 1999). The intensity and specificity of *Sv*CHIT-CBM5 expression in response to *Bs* confirm our previous findings (Sarkar et al., 2019) and suggests an important function of this gene during fungal confrontation, prompting us to select *Sv*CHIT-CBM5 for further characterization.

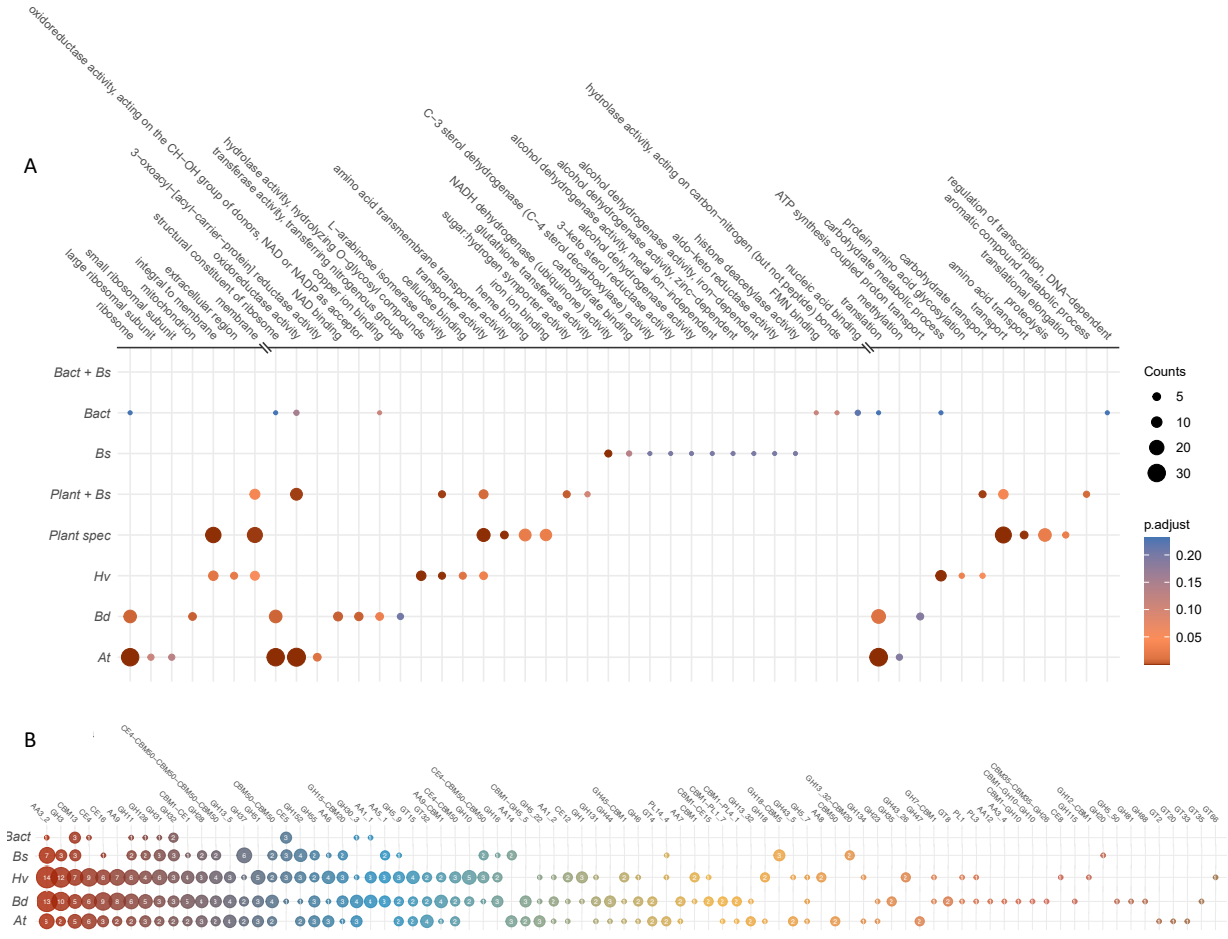

Figure 3: *Sv* GO term enrichment and CAZyme count upon different interactions. A) GO-term enrichment of *Serendipita vermifera (Sv)* genes that were either uniquely upregulated during bipartite interaction with bacteria, *Bs,* and different plant hosts or upregulated genes across different bipartite interactions. Bact: bacterial SynCom; *Bs*: *Bipolaris sorokiniana*; *Hv*: *Hordeum vulgare*; *Bd*: *Brachypodium distachyon*; *At*: *Arabidopsis thaliana*. Plant spec: all three plant hosts. The figure shows the top 10 most significantly enriched GO terms of the categories cellular component, molecular function, and biological process (from left to right as indicated by the black line) for each group of genes. The size of the dots indicated the number of genes that feature a specific GO term annotation (Table S3). B) *Sv*DEG count (>2 log2 FC) in bipartite interactions with an annotated CAZyme domain Bact: bacterial SynCom; *Bs*: *Bipolaris sorokiniana*; *Hv*: *Hordeum vulgare*; *Bd*: *Brachypodium distachyon*; *At*: *Arabidopsis thaliana*.

GH18-CBM5 chitinases are specific to Basidiomycota.

Fungi of all divisions contain a large repertoire of GH18 chitinase genes with diverse functions in development, nutrient acquisition, and biotic interactions (Chen et al., 2020; Ihrmark et al., 2010). Previously, their biological functions, domain architectures, and hydrolytic properties were thought to correlate with phylogenetic classifications (Chen et al., 2020; Seidl, 2008; Seidl et al., 2005). However, recent genome

comparisons refute the assumption that the presence of CBMs is associated with specific clades (Goughenour et al., 2021; Junges et al., 2014; Karlsson et al., 2016). Indeed, due to their patchy distribution across kingdoms, GH and CBMs are often associated with accelerated rates of gains and losses and horizontal gene transfer (HGT) (Chang and Stergiopoulos, 2015; Chase et al., 2020; Chen et al., 2020; Mayer et al., 2011; Nikolaidis et al., 2014; Sun et al., 2022). Therefore, we analyzed the distribution of genes encoding GH18-CBM5 domain-containing proteins across the fungal kingdom by performing genome comparison with 125 fungal species covering three divisions (Ascomycota, Mucoromycota, and Basidiomycota) and different lifestyles. Genes encoding GH18- and CBM-containing proteins were consistently present in all species studied (Fig. 4). However, CBM5 domains were more abundant in Basidiomycota than in Ascomycota (Zhao et al., 2013) (Fig. 4). Strikingly, genes encoding GH18-conjugated CBM5 proteins were exclusively found in Basidiomycota fungi (Fig. 4). Furthermore, within the Basidiomycota studied, genes encoding GH18-CBM5 proteins were found in more than 90% of strains (Fig. 4), indicating that GH18-CBM5 chitinases are a common feature of Basidiomycota across all lifestyles. As with other chitinases, the copy number of GH18-CBM5 varies between species (Fig. 4), suggesting a function in niche competition rather than primary metabolism (Wapinski et al., 2007). Indeed, members of the order Boletales, which have up to 8 GH18-CBM5 chitinases, exhibit not only inter- but also intra-specific competition (Hoffman et al., 2020).

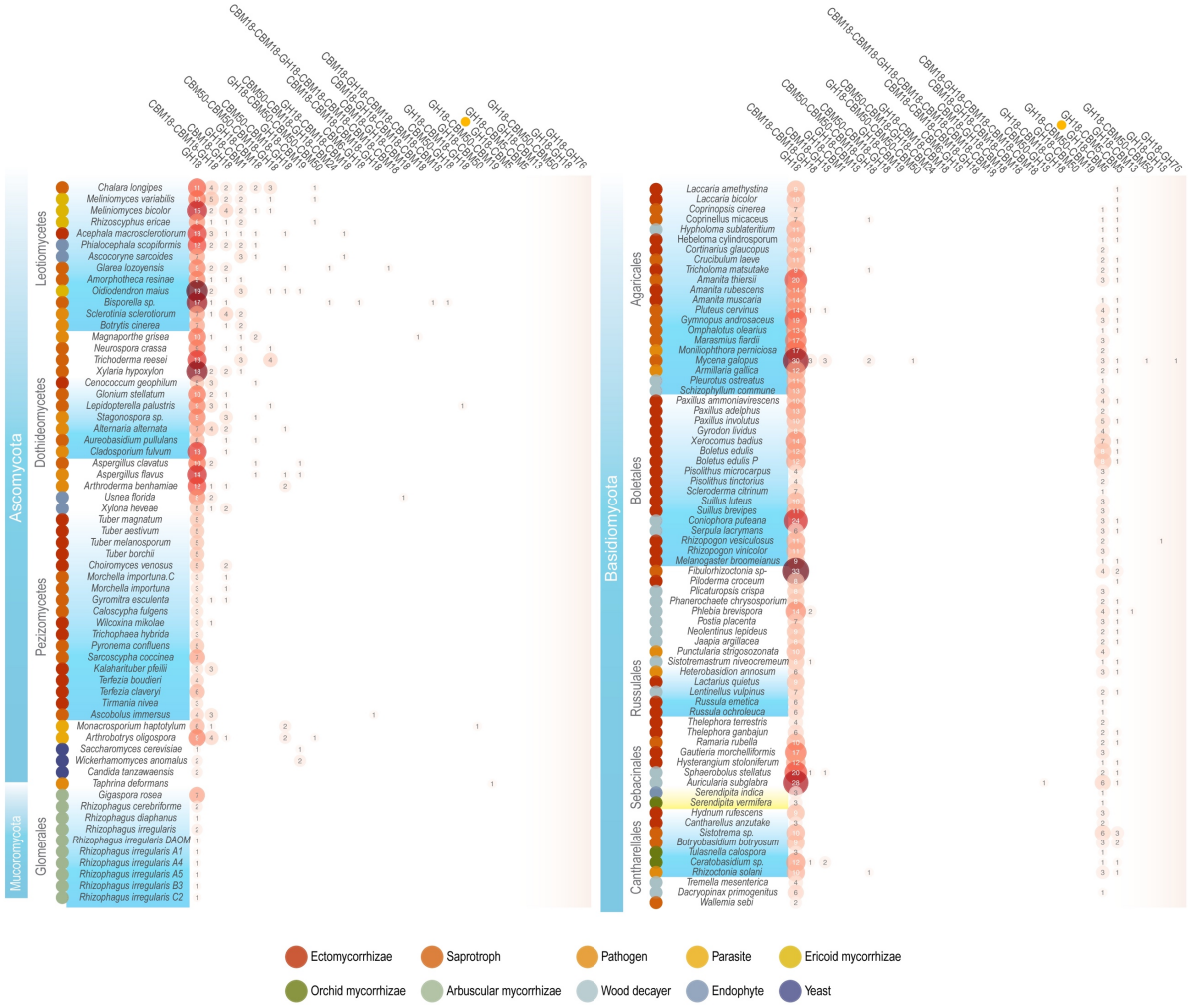

Figure 4: Overview of the different GH18-conjugated CBMs in 135 fungi across three phyla. The bubbles contain the number of genes encoding a GH18-conjugated CBM. Taxa are color-coded according to their mode of life (see bottom panel). *Serendipita vermifera* and *Serendipita indica* are colored in yellow The evolutionary order of fungi was taken from the mycocosm Ascomycota, Mucoromycota (left), and Basidiomycota (right). GH18-CBM5 is not present in Ascomycota and Mucoromycota. Both GH18-CBM5 columns are marked with a yellow dot (Table S4).

S. vermifera and *S. indica* GH18-CBM5 chitinases feature a similar structure and expression pattern and inhibit *Bs* spore germination upon exogenous application

Sv and *Si* contain only one gene encoding a GH18-CBM5 chitinase (Fig. 4) prompting us to compare the two chitinases. *Si*CHIT-CBM5 and *Sv*CHIT-CBM5 have a full-length amino acid sequence similarity of 78% and an even higher sequence similarity of 85.4% and 86.7% for the individual GH18 and CBM5 domains, respectively (Fig. 5 A). Like *Sv*CHIT-CBM5, *Si*CHIT-CBM5 expression was induced in the presence of *Bs* but not in the presence of the plant hosts or bacterial SynCom (Fig. 5 B). Given the high

structural and regulatory similarities of both chitinases, we tested the ability of *Si* to mediate host protection against *Bs* in the different hosts barley and Arabidopsis. Indeed, co-inoculation with *Si* and *Bs* in barley and Arabidopsis reduced *Bs* colonization and disease symptoms caused by *Bs* (Fig. 5C-E), resembling the protective properties of *Sv* (Mahdi et al., 2022; Sarkar et al., 2019). The results suggest that both *Sv* and *Si* exert *Bs* antagonism and host protection, and that fungal antagonism involves their respective GH18-CBM5 chitinases.

To determine the potential antifungal activity of these proteins, we expressed *Sv*CHIT-CBM5 and *Si*CHIT-CBM5 in *E. coli.* We confirmed chitinase activity of the crude extracts using chitin azure (Fig. 5F). Application of the crude protein extracts containing the Sebacinales Chitinases but not the empty vector control (Ev) inhibited *Bs* spore germination (Fig. 5G) adding further evidence for a function of *Sv*CHIT-CBM5 and *Si*CHIT-CBM5 during direct antagonism.

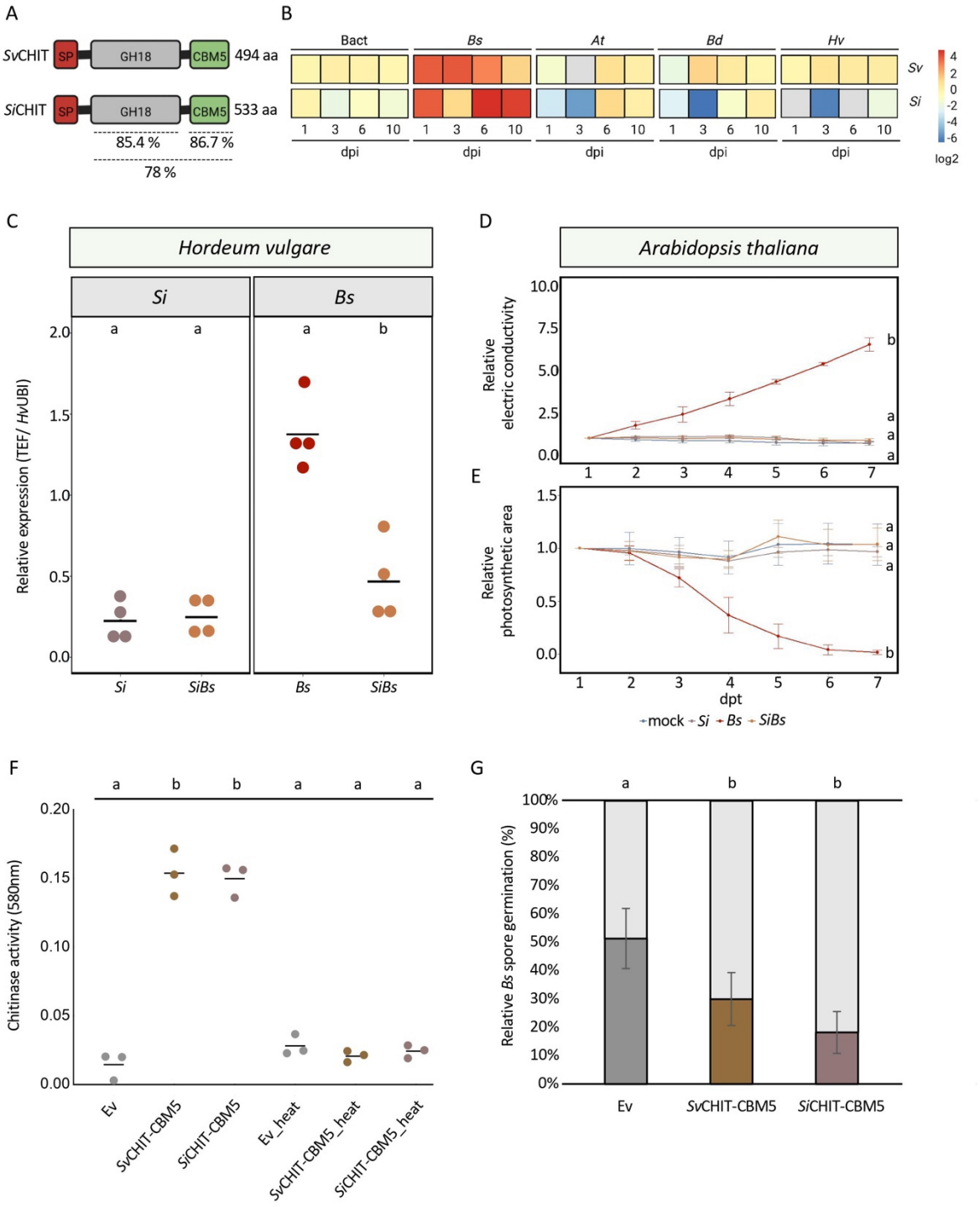

Figure 5: Domain architecture and expression comparison of *Sv*CHIT-CBM5 and *Si*CHIT-CBM5, *Si* protective abilities against *Bs* and chitinase associated inhibition of *Bs* spore germination A) Scheme showing the domain architecture of *Sv*CHIT-CBM5 and *Si*CHIT-CBM5. The percentages show the amino acid sequence similarity of each domain and the whole protein, respectively. Sequence alignment was performed using the Expasy Alignment Tool for protein sequences. (B) Interaction partner-specific expression patterns of *Sv*CHIT-CBM5 and *Si*CHIT-CBM5 at four time points (1-, 3-, 6-, and 10-days post inoculation (dpi)). Log2 differential expression is indicated according to the color scheme on the right. C) *Si* and *Bs* colonization in barley roots. Fungal colonization was derived from expression analysis of the fungal housekeeping gene TEF compared to the barley ubiquitin gene (UBI)

(n = 4). (D) Relative electrical conductance and (E) relative photosynthetic area from 1 to 7 days after transfer (dpt), corresponding to 7-14 dpi, normalized to the control treatment without fungus (mock) (n = 4). F) chitinase activity of 5mg/mL crude protein extracts from empty vector, *Sv*CHIT-CBM5 or *Si*CHIT-CBM5 was measured via chitin azure assay after overnight incubation. Heat-inactivated extracts were used as a negative control. Chitin azure degradation leads to the release of Remazol Brilliant Violet 5R, which is measured at 560 nm. G) *Bs* spore germination rate after application of crude protein extracts. Percentage of germinated spores (dark colors) and non-germinated spores (light grey) at 3 hpi (n = 3 with 200-400 spores each); Ev: empty vector control *E. coli* protein extract, *Sv: Serendipita vermifera, Si: Serendipita indica,* Heat: heat inactivated controls. Different letters represent significant differences according to one-way ANOVA and Tukey's post hoc test ($p < 0.05$). Statistical differences in D) and E) refer to the last time point (7dpi).

Discussion

Previous studies have demonstrated the importance of interaction-specific fungal gene expression for the outcome of multispecies interactions as well as for the colonization success of fungal generalists (Baetsen-Young et al., 2020; Kusch et al., 2022; Lahrmann et al., 2013; Mahdi et al., 2022; Sarkar et al., 2019; Xia et al., 2001). Conversely, multiple genes appear to be involved in several biotic interactions (Guzmán-Guzmán et al., 2017; Mahdi et al., 2022; Romero-Contreras et al., 2019; Sarkar et al., 2019). Still, little is known about the differences and similarities in fungal gene expression upon interaction with different plant hosts or microbes. The generated data set allowed us to compare interaction partner-specific transcriptomes of the Sebacinales fungus *Serendipita vermifera* across different kingdoms. We also accounted for potential temporal differences within the tested interactions by examining four time points. The resulting data provide comprehensive insights into the transcriptional regulation of *Sv* across different biotic interactions.

Sv requires greater transcriptional reprogramming to successfully colonize a plant host than to interact with surrounding microbes (Fig.1). In doing so, *Sv* largely adapts its transcriptomic response to the host species and colonization stage. Such host-specific transcriptional responses have been previously observed in other fungi with broad host ranges, indicating high adaptive abilities as a prerequisite for fungal generalism (Kusch et al., 2022; Morán-Diez et al., 2015). On the contrary, a core set of genes was classified as general determinants of host colonization. General determinants were found across all time points. They maintain putative functions during initial host colonization, fungal nutrition, and host cell death initiation. The upregulation of E5'NT facilitates colonization via the modulation of eATP levels in the closely related fungus

S. indica (Nizam et al., 2019) indicating a *Sv* mediated evasion or suppression of plant immunity to facilitate colonization. Consequently, *Sv* colonization does not result in a strong host immune response but rather a moderate induction of marker gene expression and diterpenoid secretion (Liu et al., 2021; Mahdi et al., 2022; Sarkar et al., 2019). Moreover, colonization of all plant hosts was associated with an upregulation of secreted fungal proteases (Fig. 2). Fungal proteases often target host defense proteins such as plant chitinases in plant-pathogen and plant-endophyte interactions (Balestrini et al., 2014; Jashni et al., 2015b, 2015a; Naumann et al., 2011; Olivieri et al., 2002; Tang et al., 2021; Valadares et al., 2021). In the closely related fungus *S. indica*, the secretion of extracellular proteases and metalloproteases was previously suggested to play a role in the degradation of plant tissues for nitrogen assimilation, thus providing an alternative nutritional strategy in the transition from biotrophy to the cell death-associated phase (Lahrmann and Zuccaro, 2012; Zuccaro et al., 2014, 2011). Similarly, the expression patterns of *Sv*E5'NT and *Sv*NucA resemble the expression of their homologs in *S. indica* during root colonization, where they are associated with the initiation of host cell death (Dunken et al., 2022) (Fig. 2).

In addition to direct interactions with the plant host, fungal-bacterial interactions shape the microbiome and, thus plant health (Hassani et al., 2018; Snelders et al., 2022; Vannini et al., 2016). In this context, reciprocal recognition and interaction between fungi and bacteria are associated with secreted compounds such as organic acids, sugars, toxins, and quorum-sensing molecules or direct receptor recognition (Deveau et al., 2018a; Khalid and Keller, 2021). However, research often focuses on antagonistic interactions, whereas less is known about commensal or beneficial fungal-bacterial interactions (Khalid and Keller, 2021; Salvioli et al., 2016). Here, we analyzed the interaction between *Sv* and core bacterial members of a healthy microbiota of *Arabidopsis thaliana*, a plant host commonly associated with Sebacinales in nature (Mahdi et al., 2022). When co-cultured, these bacteria are closely associated with *Sv*, especially within the fungal glucan matrix, and mediate synergistic beneficial effects on different host plants (Mahdi et al., 2022). Nevertheless, only a limited number of genes are involved in the interaction between fungi and bacteria (Fig. 1). Indeed, fungal responses to the presence of beneficial, neutral, or pathogenic bacteria are not necessarily associated with large transcriptional changes (Deveau et al., 2015; Mela et al., 2011; Satterlee et al., 2022). Acclimatization to the presence of bacteria can

result in an adaptive quenching of the fungal transcriptional response within hours after the initial interaction (Satterlee et al., 2022). Furthermore, Sebacinales, including *Sv*, harbor endobacteria (Glaeser et al., 2017). Such associations are often associated with alterations in fungal growth, signal transduction, and metabolism (Deveau et al., 2018a; Robinson et al., 2021; Salvioli et al., 2016). Accordingly, genes associated with the maintenance of endobacterial symbiosis may overlap with genes required for interaction with surrounding bacteria. The identified SynCom responsive *Sv* genes across the tested time points serve as a starting point for future genetic approaches to decipher the mechanisms underlying *Sv*-bacterial interactions in confrontation and within the plant microbiota.

The regulation of *Sv* effectors and CAZymes in the presence of *Bs* is reminiscent of their antagonistic interaction (Mahdi et al., 2022; Sarkar et al., 2019) (Fig. 2). Among all DEGs, *Sv*CHIT-CBM5 was the most strongly and uniquely regulated gene during the confrontation with *Bs* (Fig. 2; Table SX). This interaction-specific gene regulation is consistent with previous results (Sarkar et al., 2019) and highlights both the reliability of the generated data set and the relevance of *Sv*CHIT-CBM5 during fungal-fungal interactions. While the role of fungal chitinases during mycoparasitism has been extensively studied in ascomycetes, little is known about their function in basidiomycetes (Chen et al., 2020; Karlsson et al., 2016; Langner and Göhre, 2016) and no functional characterization of a basidiomycete chitinase during fungal antagonism has been described to date. In this regard, the frequent and exclusive occurrence of GH18-CBM5 chitinases in Basidiomycota increases the relevance of elucidating their function and evolution. Therefore, we compared *Sv*CHIT-CBM5 and *Si*CHIT-CBM5. We found similarities in the structure and regulation of both homologs and determined *Sv*-like host protective abilities of *Si* (Fig. 4). Importantly, exogenously applied *Sv*CHIT-CBM5 and *Si*CHIT-CBM5 inhibited the germination of *Bs* spores (Fig. 5), suggesting a direct antagonistic function.

Phylogenetically, GH18-CBM5 chitinases are distributed in the clades A (A-V) and B (B-III, B-V) that diverged prior to the separation between ascomycetes and basidiomycetes (Goughenour et al., 2021), suggesting at least two independent evolutions of this domain combination. A-V chitinases include *Sv*CHIT and *Si*CHIT, as well as ech42 from the ascomycete Trichoderma, which is active in mycoparasitism (Carsolio et al., 1994; Hermosa et al., 2012). Unlike *Sv*CHIT and *Si*CHIT, *T*ech42 lacks

a chitin-binding domain, suggesting that the presence of a CBM5 domain is not essential for the antifungal activity of *Sv*CHIT or *Si*CHIT. However, the fusion of different chitin-binding domains to *T*ech42 increased its chitinase activity and enhanced antagonism against fungal pathogens such as *V. dahliae*, *R. solani*, and several Fusarium species (Ataei et al., 2016; Kowsari et al., 2016; Limón et al., 2001). *Trichoderma harzianum* overexpressing *T*ech42 fused with the CBM5 domain of *Rhizophus oligosporus* achieved the strongest inhibitory effect against *Fusarium graminearum* among the eight different CBMs tested and the non-chimeric control (Kowsari et al., 2016). Similarly, CBMs are relevant for the efficiency of GH18 chitinases in chitinolytic bacteria, as chitinases lacking a CBM domain lose their ability to efficiently bind and hydrolyze insoluble chitin substrates (Chuang et al., 2008; Chuang and Lin, 2007; Neeraja et al., 2010; Uni et al., 2012; Watanabe et al., 1994). This suggests a functional importance of the CBM5 domain of *Sv*CHIT-CBM5 and *Si*CHIT-CBM5. In the future, knockout and complementation experiments will clarify the function of both Sebacinales chitinases and their independent domains during fungal antagonism. The establishment of gnotobiotic bipartite, tripartite, and multipartite experimental setups (Mahdi et al., 2022; Sarkar et al., 2019) and the ability to genetically modify Sebacinales fungi (Dunken et al., 2022; Lahrmann et al., 2015) are excellent prerequisites for future characterization of Sebacinales chitinases and other relevant *Sv* regulatory genes in various biotic interactions.

In this study, we report for the first time similarities and differences in the transcriptomic response of a beneficial fungal generalist to interaction partners across three kingdoms. We define determinants of host colonization and verify *Sv*CHIT-CBM5 as a marker gene for fungal antagonism against *Bs*. *Bs*-specific expression patterns and inhibitory properties of *Sv*CHIT-CBM5 and *Si*CHIT-CBM5 suggest common host protection mechanisms in different Sebacinales species. Most importantly, the generalist nature of *Sv* is associated with a highly interaction partner-specific modulation of fungal gene expression. In the future, DAP-seq analyses will help determine the transcription factors that control these highly specific transcriptional responses.

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Chapter 4

A barley gene cluster for the biosynthesis of diterpenoid phytoalexins.

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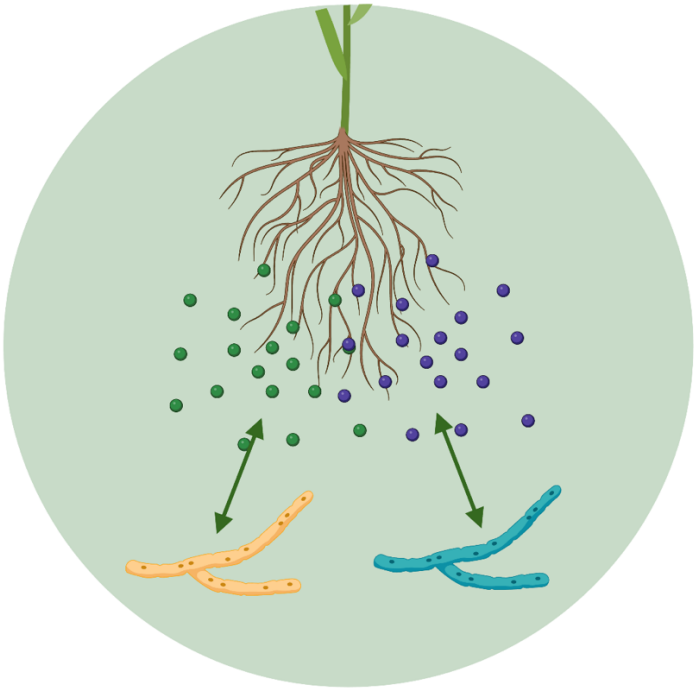
Authors contributions:

YL performed the LC-MS, GC-MS, yeast and *N. benthamiana* expression and gene expression analysis. GB provided supervision for LC-MS and data analysis. AS-H provided assistance for LC-MS sample preparation. AP performed the NMR measurements and analyses. UB supervised the yeast expression experiments. **LM** and AZ provided infected barley samples, media samples and transcriptomic data. **LM** established protocols and performed experiments to harvest fungal free media and test antifungal activity of the purified diterpene via multiple readouts. AT and GB designed the project. **LM** performed colonization experiments with barley KSL4 and CPS2 mutants. AT designed and supervised the project. AT wrote the abstract, introduction, results and discussion. YL wrote the materials and methods, except the NMR part (AP) and LC-MS (GB). AT, YL and GB prepared the figures. All authors read and approved the manuscript. The final version of the manuscript will contain the full set of the above-mentioned data.



A barley gene cluster for the biosynthesis of diterpenoid phytoalexins

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Abstract

Phytoalexins are specialized metabolites that are induced upon pathogen infection and contribute to the defense arsenal of plants. Maize and rice produce multiple diterpenoid phytoalexins and there is evidence from genomic sequences that other monocots may also produce diterpenoid phytoalexins. Here we report on the identification and characterization of a gene cluster in barley (*Hordeum vulgare* cv. Golden Promise) that is involved in the production of a set of labdane-related diterpenoids upon infection of roots by the fungal pathogen *Bipolaris sorokiniana*. The cluster is localized on chromosome 2, covers over 600 kb and comprises genes coding for a (+)-copalyl diphosphate synthase (HvCPS2), a kaurene synthase like (HvKSL4) and several cytochrome P450 oxygenases (CYPs). Expression of HvCPS2 and HvKSL4 in yeast and *Nicotiana benthamiana* resulted in the production of a single major product, whose structure was determined to be of the cleistanthane type and was named hordediene. Co-expression of HvCPS2, HvKSL4 and one of the CYPs from the cluster (CYP89E31) afforded two additional products, hordetriene and 11-hydroxy-hordetriene. Both of these compounds could be detected in extracts of barley roots infected by *B. sorokiniana*, validating the function of these genes *in planta*. Furthermore, diterpenoids with multiple oxidations and with molecular masses of 316, 318 and 332 were induced in infected barley roots and secreted in the medium, indicating that additional oxidases, possibly from the same genomic cluster are involved in the production of these phytoalexins. Our results provide the basis for further investigation of the role of this gene cluster in the defense of barley against pathogens and more generally in the interaction with the microbiome.

Introduction

Monocotyledons contribute some of the most important staple crops worldwide, including the three major ones maize (*Zea mays*), wheat (*Triticum aestivum*) and rice (*Oryza sativa*) that cover a large part of calorie intake by humans worldwide (Awika, 2011). Behind these three species, barley (*Hordeum vulgare*) is the fourth most important grain crop with an annual production of over 140 million tons and with an harvest area of almost 48 million ha worldwide in 2018 (**Table S1**). Barley is grown in temperate climates and primarily for animal feed, but also to provide substrate for the fermentation of beverages (e.g. beer), and for a range of health promoting products. It is one of the earliest cultivated crops with supporting archaeological evidence from the fertile crescent region dating to as far back as over 10,000 years before present (BP) (Badr et al., 2000). The initial use of barley was for food, but it was then later replaced by wheat (Riehl, 2019). Cultivated barley is one of 31 *Hordeum* species, with *H. vulgare* subsp. *spontaneum* believed to be the wild ancestor of cultivated barley (Badr et al., 2000). The close relatedness of barley to wheat and its diploid nature (2n = 14) make it a relevant model species for the study of temperate cereal crops. A first draft of its genome was published in 2016 and was recently updated (Beier et al., 2017; Mascher et al., 2017; Monat et al., 2019). Furthermore, genetic transformation and gene editing in barley are now well established, providing a complete toolbox for functional genetics in this species (Hensel, 2020).

Plants synthesize a complex array of secondary metabolites that contribute to the response and adaptation to a range of biotic or abiotic stresses. These metabolites can be produced constitutively, in a tissue specific manner or upon challenge by specific stresses, be they biotic or abiotic. Whereas some metabolites are common to a wide range of species, others are restricted to a species or to a taxon, thereby determining a species or taxon metabolite signature, a feature that led to the denomination “specialized metabolites” (Pichersky et al., 2006; Pichersky and Lewinsohn, 2011).

Plant pathogenic fungi impose a major burden on crop yield, and this impact is expected to increase with climate change (Miedaner and Juroszek, 2021). As the use of agrochemicals is increasingly under scrutiny by environmental agencies and while single gene-for-gene resistance may be rapidly overcome in a changing climate, there is a strong need for more durable resistance traits. The fungus *Bipolaris sorokiniana* (syn. *Cochliobolus sativus*) is the pathogenic agent of spot blotch and root rot in wheat and barley and is particularly prevalent in regions with a warmer climate (Rosyara et al., 2010) and therefore represents a typical future important threat in regions with a temperate climate in the context of global warming. *B. sorokiniana* can infect both aerial and underground parts of the plant but knowledge on how it interacts with roots is still rather limited (Sarkar et al., 2019).

There is widespread evidence that infection of plants by microbial pathogens triggers the production of a secondary metabolites (also called specialized metabolites) that exhibit antimicrobial or antioxidant activities (Ahuja et al., 2012). These compounds, called phytoalexins, are not restricted to a particular chemical class, with examples among phenylpropanoids, alkaloids or terpenoids (Ahuja et al., 2012). This is also the case in Monocotyledons, a plant clade that includes some of the most important food crops worldwide. In barley, there is a number of reports of various phytoalexins produced in response to diverse pathogens. These include phenylamides, such as the dimeric hordatine A and B, the indole-derived gramine, benzoxazinones such as 2,4-dihydroxy-1,4-benzoxazin-3-one (DIBOA), methoxychalcones as well as tyramine and related amines (Ishihara et al., 2017; Ube et al., 2017; Ube et al., 2021). However, in contrast to other monocotyledon crops such as maize and rice, no sesqui- or diterpenoid phytoalexins have been identified in barley yet. There is now extensive data available on the nature and biosynthesis of a range of terpenoid phytoalexins in these important crop species. Rice produces several classes of labdane-related diterpenoids, including momilactones (A and B) (Kato et al., 1973; Cartwright et al., 1977), phytocassanes, oryzalexins (Akatsuka et al., 1983; Kono et al., 1984; Sekido et al., 1986; Kato et al., 1993, 1994) and oryzalides (Watanabe et al., 1990; Kono et al., 1991) as well as the macrocyclic *ent*-oxodepressin (Inoue et al., 2013). Maize produces dolabralexins and kauralexins, both labdane-related diterpenoid phytoalexins (Schmelz et al., 2011), as well as zealexins, which are sesquiterpenoids (Huffaker et al., 2011).

Biosynthesis of terpenoids starts by the conversion of linear isoprenyl diphosphate chains by terpene synthases to either linear or cyclic terpenes or terpene alcohols. In the case of diterpenoids, the precursor is typically all-*trans*-geranylgeranyl diphosphate (here abbreviated as GGPP) (Bohlmann et al., 1998), although in some isolated cases it is the all-*cis*-isomer, nerylneryl diphoshate (Zi et al., 2014). Diterpene synthases (diTPS) are classified according to the mechanism underlying the initiation of the cyclization reaction. Thus class I diTPS initiate the reaction by dephosphorylation whereas class II do it by protonation of the terminal isoprenic double bond (Peters, 2010). The main products of class II diTPS are *ent*-copalyl diphosphate (*ent*-CPP), the precursor of the gibberellins, and the other stereoisomers *syn*-copalyl diphosphate (*syn*-CPP) and CPP of normal configuration (Peters, 2010). In addition, there are other products of class II diTPS with a slightly different core structure, such as clerodienyl or halimadienyl diphosphates, as well as products with an alcohol function (Nakano et al., 2005; Sallaud et al., 2012; Pelot et al., 2017). Because the products of class II diTPS still contain a diphosphate group, class I diTPS can convert them to olefinic diterpenes or diterpene alcohols. The sequential reactions catalysed by class II and class I diTPS lead to the broad group of labdane-related diterpenes, which have in common a core bicyclic decalin ring structure (Peters, 2010). Apart from *ent*-oxodepressin, which has a

119 macrocyclic structure, all diterpenoid phytoalexins from monocots identified so far belong to
120 the labdane-related group. Following cyclization by diTPS, diterpene backbones are then
121 oxidized at different positions and in a stereospecific way. Cytochrome P450 oxygenases
122 (CYPs) are the most frequently involved in these oxidations (Bathe and Tissier, 2019), but
123 other classes of enzymes such as 2-oxoglutarate dependent dioxygenases in gibberellin
124 biosynthesis (Hedden and Kamiya, 1997) or short-chain dehydrogenases/reductases as in
125 momilactone biosynthesis (Kitaoka et al., 2016) can also play a role in functionalizing
126 diterpenes. These oxidations can sometimes lead to backbone rearrangements and,
127 importantly, provide anchoring points, such as hydroxyl or carboxyl groups, for further
128 modifications by conjugating enzymes (Long et al., 2008; Rontein et al., 2008). Thus, sugar,
129 acyl, or benzoyl groups can decorate the oxidized diterpene core and provide added
130 functionalities.
131 In recent years the elucidation of the biosynthesis of rice and maize diterpenoid phytoalexins
132 has progressed significantly (Murphy and Zerbe, 2020). In rice, the labdane-related
133 diterpenoids are derived from either *ent*- or *syn*-copalyl diphosphate, whereas the maize
134 diterpenoid phytolexins derive from *ent*-CPP. In addition to the diTPS, which produce the
135 diterpene backbones of these phytoalexins, a number of CYPs are involved in the
136 functionalization of these backbones. Notably, both in maize and rice, some of the genes for
137 the biosynthesis of terpenoid phytoalexins occur are physically associated in chromosomal
138 clusters (Shimura et al., 2007; Wang et al., 2011; Ding et al., 2020; Liang et al., 2021).
139 Here we present the identification and characterization of a diterpenoid phytoalexin
140 biosynthesis cluster in barley. We show that genes in this cluster are strongly induced in
141 roots by a barley fungal pathogen, *B. sorokiniana* and we characterize the first biosynthesis
142 steps, consisting of a copalyl diphosphate synthase, a kaurene synthase like and a CYP.
143 Notably, the diterpene backbone produced by the CPS/KSL enzymes belong to the
144 cleistanthane group of labdane-related diterpenoids. To the best of our knowledge, this
145 diterpene backbone has not been detected yet in grasses. We also detected diterpenoid
146 phytoalexins in barley roots and in high amounts in the root exudate as well as the
147 intermediates produced by the first three enzymes of the pathway.
148

149 ## Results

150

151 ### Identification of a diterpenoid biosynthesis gene cluster in barley chromosome 2

152 In previous work, we generated transcriptome data of barley roots infected with either a
153 fungal pathogen, *Bipolaris sorokiniana*, or a beneficial root endophyte, *Serendipita vermifera*,
154 or both in local or systemic context (Sarkar et al., 2019). We observed that a number of
155 genes from the MEP pathway as well as a number of genes from a region in chromosome 2

encoding terpene synthases and cytochrome P450 oxygenases are strongly induced by the fungal pathogen *B. sorokiniana* and moderately by *S. vermifera* (**Fig. 1**). In the MorexV2 version of the barley genome (Monat et al., 2019), this cluster spans over 600 kb and contains one gene encoding a copalyl diphosphate synthase (CPS), one kaurene-synthase like (KSL), 8 CYP and one asparaginase (**Fig. 1**). The cluster also harbors a number of pseudogenes, including five CYPs and four CPS. As is frequently the case in regions of the genome with multiple duplications, the early versions of the genome sequence (including MorexV1 and MorexV2) still contain a number of gaps, and it is likely that the number of genes and pseudogenes in this cluster evolves as more accurate sequences become available.

Phylogenetic analysis of the CPS and KSL genes in the chromosome 2 cluster

To better characterize the genes of the cluster, we performed a phylogenetic analysis. Earlier publications reported on the identification and biochemical characterization of HvCPS1 as an *ent*-CDP synthase (Spielmeyer et al., 2004; Wu et al., 2012). The strong similarity of HvCPS1 to TaCPS3 and TaCPS4, both of which are *ent*-CDP synthases, and the exclusive presence of *ent*-CDP synthases in the same branch underscores the distinct evolutionary conservation of *ent*-CDP synthases in monocots (**Fig. 2**). The CPS in the chromosome 2 cluster has not been characterized yet and we propose to name it HvCPS2. HvCPS2 shares high similarity to TaCPS2, a (+)-CDP synthase, and belongs to the same branch as OsCPS4, a *syn*-CDP synthase, and TaCPS1, an *ent*-CDP synthase. Thus, although these data support a role of HvCPS2 in specialized diterpenoid metabolism, they do not allow us to predict its actual biochemical activity with high confidence.

The kaurene synthase-like of the chromosome 2 cluster was already identified previously and named HvKSL4 (Li et al., 2016). In the phylogenetic tree comprising other monocot KSL enzymes (**Fig. 2**), it is in the same branch as TaKSL1, TaKSL4 and OsKSL4. The substrate of TaKSL1 and TaKSL4 is (+)-CDP (Zhou et al., 2012), while that of OsKSL4 is *syn*-CDP (Otomo et al., 2004). In the neighboring branch are TaKSL2 and TaKSL3. The function of TaKSL3 is unknown but TaKSL2 also uses (+)-CDP as substrate (Zhou et al., 2012). The clear separation from KSL enzymes that use *ent*-CDP as substrate, including the barley *ent*-kaurene synthase (HvKS), indicates that HvKSL4 most likely uses either (+)-CDP or *syn*-CDP as substrate.

HvCPS2 is a (*+*)-CDP synthase and HvKSL4 produces a cleistanthane-type backbone

To determine the biochemical activity of HvCPS2, we expressed it in yeast using our Golden Gate yeast cloning system (Scheler et al., 2016) together with RoMiS, the miltiradiene synthase from rosemary (*Rosmarinus officinalis*) (Brückner et al., 2014), with CcKS, an *ent*-

kaurene synthase from coffee (*Coffea canephora*), or with HvKSL4. Co-expression with RoMiS yielded the expected diterpene product (miltiradiene), whereas no *ent*-kaurene could be detected with CcKS, demonstrating that HvCPS2 produces (+)-CDP **(Fig. 3**). Co-expression of HvCPS2 and HvKSL4 in yeast yielded a novel product with a molecular ion of 272 (**Fig. 3**), indicating that it is a diterpene olefin. Besides this major product, several much more minor products could also be detected. When expressing truncated versions of HvCPS2 and HvKSL4 together with a cytosolic geranylgeranyl diphosphate synthase (GGPPS) and a truncated version of hydroxymethylglutaryl CoA-reductase (tHMGR) in *Nicotiana benthamiana*, the same product could be detected (**Fig. S1**). Since there was no significant match in the NIST database (Mass Spectrometry Data Center, http://chemdata.nist.gov), we purified the main product and determined its structure by nuclear magnetic resonance (NMR) spectroscopy (see **Table S4**). The product was determined to have a cleistanthane backbone, with two double bonds in the C-ring at positions C8-C9 and C12-C13, a methyl group attached to C13 and an ethyl group attached to C14 in the α-configuration (**Fig. 3**). Cleistanthanes constitute a relatively small group of diterpenes that occur in some plants and fungi [see for example: (Kaufman et al., 1987; Riehl and Pinto, 2000; Shiono et al., 2010; Zheng et al., 2018)]. We could not find published reports of the exact same structure and we therefore named it hordediene (alternatively: cleistantha-8(9),12(13)-diene).

Phylogenetic analysis of the CYPs in the chromosome 2 cluster

Of the eight CYP-encoding genes in the chromosome 2 cluster, only five are expressed at significant levels and display an expression pattern similar to that of *HvCPS2* and *HvKSL4*. Furthermore in the latest annotation of the barley genome, two of these genes merged into a single one so that only four CYP-encoding genes can be considered in the current version of the barley genome (**Table 1**). A phylogenetic analysis of these sequences shows that three of them are most similar to OsCYP99A2 and to OsCYP99A3 from rice (see **Fig. S2 and Table S5)**. Although no biochemical function could be determined for OsCYP99A2 yet, OsCYP99A3 plays a role in the biosynthesis of momilactones by oxidizing *syn*-labdane related diterpenes - *syn*-pimaradiene and *syn*-stemodene - at the C19 position to generate a carboxylic acid function (Wang et al., 2011). Interestingly, OsCYP99A2 and OsCYP99A3 are located in the same tandem cluster next to OsCPS4 and OsKSL4, a situation highly reminiscent of the barley cluster presented here (Shimura et al., 2007).

The fourth CYP shares strongest similarity to enzymes of the CYP89 clan, with the closest homolog in *Arabidopsis thaliana* being CYP89A2 (AT1G64900), whose biochemical function is unknown yet. The most closely related protein sequences in the databases are homologs

from other crops, e.g. *Triticum aestivum* or *Setaria viridis*, but these are of unknown function as well. The assigned identification for this CYP is CYP89E31.

Characterization of HvCYP89E31

We first focused on the characterization of HvCYP89E31 by expressing it in yeast. We cloned a yeast optimized sequence in a vector together with HvCPS2 and HvKSL4 and in addition with the cytochrome P450 reductase from Arabidopsis. Analysis of the extracts by gas chromatography couple to electron ionization mass spectrometry (GC/EI-MS) showed the presence of two additional peaks, with a molecular *m/z* of 270 (product **2**) and 286 (product **3**), respectively (**Fig. 4**). These masses indicate one and two additional degrees of oxidation. Next, we also isolated microsomes from a yeast strain expressing CYP89Ax and performed in vitro assays with hordediene as a substrate. GC-MS analysis of the extracts from this reaction showed the same products that are produced by yeast expressing all three genes (**Fig. 4**). This conclusively demonstrates that the new products detected are directly derived from hordediene. We also expressed those three genes in *N. benthamiana* as described above for HvCPS2 and HvKSL4 and found the exact same products present in hexane extracts (**Fig. S1**). The products were purified from a large scale yeast culture and their structure determined by NMR (**Tables S6 and S7**). Compound **2** has an aromatized C-ring whereas compound **3** has a hydroxyl group on position 11 (**Fig. 4**). We propose to name these compounds hordetriene (**2**) and 11-hydroxy-hordetriene (**3**).

Hordetriene and 11-hydroxy-hordetriene are present in barley roots infected with *B. sorokiniana*

To determine if the diterpene products identified in yeast and *N. benthamiana* by metabolic engineering are also produced in barley plants, we infected barley roots with the pathogen *B. sorokiniana*, or with the beneficial fungus *Serendipita vermifera* (syn. *Sebacina vermifera*) or with both as described in Sarkar et al. (2019). Hexane extracts from the roots were then analyzed by GC-MS and compared to an extract from a yeast strain expressing HvCPS2, HvKSL4 and CYP89E31 (**Fig. 5**). Whereas hordediene (**1**) could not be detected in any of the root extracts, **2** and **3** were detected in roots infected with *B. sorokiniana* or with *B. sorokiniana* and *S. vermifera*. In mock-infected roots or in roots infected with *S. vermifera* alone, smaller amounts of **3** could be detected compared to roots infected with *B. sorokiniana*, but **2** could not be detected. These results demonstrate that the diterpenoids produced by enzymes of the chromosome 2 cluster are indeed induced during fungal pathogen infection but not significantly by beneficial endophytic colonization, an observation consistent with the transcriptome data generated previously (Sarkar et al., 2019).

Detection/identification of diterpenoid phytoalexins induced by *B. sorokiniana* infection

Given the presence of additional CYP enzymes in the barley chromosome 2 cluster, we wondered whether further oxidized diterpenoids could be detected in barley roots infected with *B. sorokiniana*. We thus performed untargeted LC-QToF-MS (negative mode) analysis of extracts from the root and from the medium. Searching for masses corresponding to additional oxidations of **3 (Fig. 4)**, i.e. 301.2, 317.2, 315.2 and 331.2, a number of peaks could be detected with a strong increase in samples from roots and medium of plants infected with *B. sorokiniana* (**Fig. 6**). By comparison, under mock control conditions this accumulation was not observed. LC-TOF-MS with negative electrospray ionization detected [M-H] adducts of six major compounds of which two products with the monoisotopic neutral mass or 316.21 Da dominated. High enrichments were also observed for three compounds with the monoisotopic formula mass 332.20 Da and for one with 318.22 Da, indicating that these main products carry 3 or 4 oxygen atoms. MS/MS spectra of these compounds imply a diterpene character based on the fragment ions 269.19, 271.21, 287.20 and 301.1 (**Fig. S3**). Neutral losses of 43.99 and 46.01 suggest the presence of lactones or carboxyl groups in all six diterpenes. As well, compound 6 has an additional carbonyl group as indicated by a neutral loss of 30.01 Da between the molecular ion and m/z 287.203.

Discussion

Evolution and diversity of diterpenoid phytoalexins in monocots

Since the discovery of diterpenoid phytoalexins in rice and the elucidation of their biosynthesis, there has been an increasing number of reports on investigations of specialized diterpenoid metabolism in monocots. This includes maize (*Zea mays*) (Schmelz et al., 2011; Ding et al., 2020), switchgrass (*Panicum virgatum*) (Pelot et al., 2018; Muchlinski et al., 2021), and wheat (*Triticum aestivum*) (Wu et al., 2012; Zhou et al., 2012). In the Triticeae, which comprise wheat, barley and rye *(Secale cereale*), despite the publication of a report describing the characterization of wheat diterpene synthases, (Wu et al., 2012; Zhou et al., 2012), to the best of our knowledge no diterpenoid phytoalexins had been identified yet. Our work shows that like maize and rice, barley is also able to produce diterpenoid phytoaelxins when challenged with a pathogen. Thus, production of diterpenoid phytoalexins appears to be a common feature of monocots. It is also noteworthy that the genes involved in the biosynthesis of these compounds are frequently localized in clusters encoding diterpene synthases (CPS and KSL) as well as CYPs or in the case of rice a short chain dehydrogenase reductase (OsMAS) (Shimura et al., 2007; Ding et al., 2020; Muchlinski et al., 2021). Clusters of genes involved in specialized metabolism are encountered in plants

and typically consist of tandem duplicated copies of one or more gene families (Nützmann et al., 2016). The molecular mechanisms driving the formation and evolution of these clusters are still enigmatic, but the physical association and duplication of genes offer a number of potential advantages. First, physical association means that possible toxic intermediates due to mutation or loss of one of the genes are less likely to accumulate. Second, the presence of multiple copies of highly similar genes provides opportunities for gene rearrangements and independent evolution, thereby opening the door to rapid chemical innovations. The capacity to evolve novel defenses rapidly constitutes a key evolutionary adaptation in the arms race against pests and pathogens.

Despite the relatedness of wheat and barley, the characterization of wheat diterpene synthases indicates that none of them can produce the cleistanthane backbone identified here (Awika, 2011). This is additional evidence that the gene clusters allow for rapid evolution of enzyme function and chemical diversification.

A search for regions in rice that are syntenic to the barley cluster on chromosome 2 using the SynFind tool (Tang et al., 2015) was unsuccessful (data not shown). This could be due to the fact that this chromosomal region is subject to frequent rearrangements. Nonetheless, in a publication investigating the synteny between rice and barley (Thiel et al., 2009), much of barley chromosome 2 was found to be syntenic with rice chromosome 4, which is where the main cluster for momilactone biosynthesis is located. Thus, it is possible that the evolution of this genomic cluster predates the divergence between Triticeae and rice.

Hordediene: an unprecendented diterpene backbone in grasses

HvKSL4 catalyzes the cyclization of (+)-CPP to hordediene, a cyclization reaction which, to the best of our knowledge, has not been reported previously, at least not in monocots. Hordediene belongs to the cleistanthane group of labdane-related diterpenes, which is characterized by the presence of an ethyl group attached to C17, instead of C13 as in pimaradiene. In the cyclization pathway we propose, after the initial dephosphorylation, the first electron migrations lead to the formation of the pimaradienyl cation (**Fig. S4**). At this point the pathway diverges from the pimaradiene cyclization via capture of a proton from C9 by the C14-C15 double bond of pimaradienyl. This results in formation of the C8-C9 double bond and the C14 carbocation. A hydride shift from C17 to C14 would then allow the migration of the ethyl group to C17 and consequently result in the presence of carbocation at C13. Resolution of this cation by proton loss from C12 would then lead to hordediene.

A survey of the literature on cleistanthane diterpenoids reveals a modest number of reports (65 in Web of Science as of May 2021), with occurrence either in plants or fungi. Many of the cleistanthanes reported are of the *ent*- configuration, such as Fimbricalyxoid A from *Strophioblachia fimbricalyx* (Cheng et al., 2016) but some of normal (+) configuration, like

hordediene, have also been described, such as zythiostromic acids (Ayer and Khan, 1996). There is little information on the biological function of these diterpenoids, although in some cases anticancer activity in the µM range could be determined (Cheng et al., 2016).

Production and potential role of diterpenoid phytoalexins in barley

We characterized three genes from the cluster (HvCPS2, HvKSL4 and HvCYP89E31) and showed that co-expression of these three genes results in the production of two major products, hordetriene and 11-hydroxy-hordetriene. The fact that we could detect both of these products in barley roots infected with *B. sorokiniana*, strongly supports a role of this cluster in the production of diterpenoid phytoalexins and in the defense reaction of barley. LC-MS analysis indicated the presence of further oxidized diterpenoids, however at this stage we were not able to isolate and purify sufficient quantity of these compounds for structural elucidation. Thus it remains to be determined whether these are also derived from the same diterpene backbone as compounds **2** and **3**. The fact that no other diterpene synthase is induced by *B. sorokinianasorokiniana* in our transcriptome data argues in favor of this hypothesis. At this stage, we can only speculate on the biological activity of these compounds. Their biosynthesis is only minimally induced by *S. vermifera*, but very strongly by the pathogenic fungus *B. .sorokiniana* (Sarkar et al., 2019). Whether the induction is specific to *B. sorokiniana* or is a general response to infection by pathogens remains to be determined. Interestingly, in a recent report on the metabolic profiling of barley plants infected with *Fusarium graminearum*, which causes head blight, two diterpenoids with masses of 302.2 and 318.2 were the most strongly induced compounds (respectively 32 and 22 fold change) (Karre et al., 2017). They were annotated as neoabietic and 7-hydroxykaurenoic acid respectively, although there was no formal identification of these compounds. We suspect that these compounds also belong to the diterpenoid group that we identified, which would suggest that they are not induced specifically by *B. sorokiniana*. Availability of larger amounts of pure compounds in the future will allow us to perform bioassays and determine their potential antimicrobial activity. Of relevance to their biological function is the fact that these compounds are for the most part secreted into the medium by the roots. Since they are present at a low basal level in non-infected plants, it is likely that their presence in the rhizosphere could influence the composition of the root microbiome. There is already evidence of specialized metabolites secreted by the roots that impact the composition of the microbiome (Massalha et al., 2017; Huang et al., 2019; Murphy et al., 2021). Conversely, specific members of the microbial community can induce secretion of specialized metabolites by the roots (Massalha et al., 2017), suggesting a complex network of signals and effectors between the microbial community and the plant in the rhizosphere.

376 Generating barley plants mutated in HvCPS2 or HvKSL4 by CRISPR-Cas gene editing will
377 allow us to address these questions in this important crop species in the future.
378
379 ## Materials and Methods
380
381 **Plant growth and fungal inoculations**
382 Barley seeds (*Hordeum vulgare* L. cv Golden Promise) were sterilized in 70% ethanol for 1
383 min, followed by washing with sterile distilled water and 1.5 h incubation in 12% sodium
384 hypochloride under continuous shaking. After 3 times 30 min washing, the seeds were
385 placed on wet filter paper in darkness and at room temperature for 4 days for germination.
386 Four seedlings were transferred to 1/10 PNM (Plant Nutrition Medium, pH 5.7) (Wawra et al.,
387 2016) in sterile glass jars and grown in a day/night cycle of 16/8 h at 22/18 °C, 60 % humidity
388 under 108 µmol m^{-2} s^{-1} light intensity.
389 *Bipolaris sorokiniana* (ND90Pr) and *Serendipita vermifera* (MAFF305830) were used in this
390 study. *Bs* was propagated on modified CM medium with 1.5% agar and *Sv* on MYP medium
391 with 1.5% agar in the dark at 28 °C for 21 days and 14 days before inoculation respectively.
392 *Bs* conidia and *Sv* mycelial were collected according to the procedures which were described
393 in (Sarkar et al., 2019). Barley roots were inoculated with 3 ml of either *Sv* mycelium
394 (2g/50ml), *Bs* conidia (5000 spores/ml) or a 1:1 mixture of the two fungi per jar for 6 days.
395 Sterile water was used as a mock treatment. Roots washed thoroughly and the
396 corresponding medium were collected and snap-frozen in liquid nitrogen for extraction of
397 metabolites.
398
399 **qRT-PCR**
400 RNA isolation from roots was performed using the Spectrum Plant Total RNA kit (Sigma-
401 Aldrich). The complementary DNA (cDNA) was synthesized using ProtoScript II First Strand
402 cDNA Synthesis Kit (New England Biolabs) following the manufacturer's instructions with
403 primer $d(T)_{23}$ VN. Quantitative real-time PCR was performed in triplicates using 10-20 ng
404 cDNA as template and gene specific primer pairs shown in **Table S8** in CFX Connect Real-
405 Time PCR System (Bio-Rad). The PCR conditions were 95□°C for 15□min; 40 cycles of 95
406 °C for 15 s, 56□°C for 30□s; 95 °C for 10 s. The melting curve was measured from 65 °C to
407 95 °C with a step of 0.1□°C per second. Relative expression of targeted genes was
408 calculated using delta Ct method (Livak and Schmittgen, 2001) and barley ubiquitin genes as
409 references (Deshmukh et al., 2006).
410
411 **Phylogenetic analysis**

Amino acid sequences (**Tables S2, S3 and S5**) were aligned and phylogenetic trees were generated using MEGA X (Kumar et al., 2018). ClustalW was used for the alignment and the maximum likelihood method with a bootstrap of 1,000 for the phylogenetic tree. For the other parameters, default settings were used.

Heterologous expression of diterpenes in yeast

Plasmids containing *GGPPS* and *ATR1* were kindly provided by colleagues in the group and have been described previously (Scheler et al., 2016). Codon-optimized DNA sequences of *HvCPS2, HvKSL4* and *HvCYP89E31* were synthesized by Thermo Fisher Scientific Inc. for yeast expression. Each gene was further cloned into Golden Gate compatible yeast expression level 1 vector, together with a synthetic galactose-inducible promoter and a terminator. Different gene combinations were finally assembled into one yeast expression level M vector by a 50 cycle restriction–ligation reaction with BpiI and T4-Ligase.

Constructs were then transformed into *S. cerevisiae* strain INVSc1 (Thermo Fisher Scientific Inc.) and plated out onto uracil-free (Ura-) selection medium (1 g l^{-1} Yeast Synthetic Drop-out Medium Supplements without uracil (Sigma-Aldrich), 6.7 g l^{-1} Yeast Nitrogen Base with Amino Acids (Sigma-Aldrich) and 20 g l^{-1} Micro Agar (Duchefa Biochemie)). Three positive colonies were picked and inoculated into 5 ml yeast extract-peptone-dextrose (YPD) medium (20 g l^{-1} tryptone and 10 g l^{-1} yeast extract) containing 2% of glucose and grown for 24 h with shaking at 30 °C. To induce protein expression, the cell pellet was resuspended in fresh YPD medium containing 2% galactose. After another 24 h of growth, the whole culture was extracted with 2 ml *n*-hexane.

Transient expression in *Nicotiana benthamiana*

Transit peptides of protein HvCPS2 and HvKSL4 were predicted by two online tools, ChloroP 1.1 (http://www.cbs.dtu.dk/services/ChloroP/) and LOCALIZER (http://localizer.csiro.au/). Truncated sequences without the predicted transit peptides of these two genes were generated by PCR reactions using designed primers and subsequently sequenced. The cDNAs of *HMG reductase*, *GGPPS* in plasmids have been described previously (Scheler et al., 2016; Yadav et al., 2019). The *HMG reductase*, *GGPPS*, *trHvCPS2*, *trHvKSL4* and *HvCYP89E31* were cloned into T-DNA vectors (binary vector pL1F-1) driven by the 35S promoter and flanked by the Ocs terminator (Weber et al., 2011). The resulting T-DNA plasmids were transformed into *Agrobacterium tumefaciens* strain GV3101::pMP90 and plated out onto LB agar plates with appropriate antibiotics. Bacteria were harvested and resuspended in infiltration medium (10 mM $MgCl_2$, 10 mM MES, 20 µM acetosyringone, pH=5.6) after 48 h inoculation at 28 °C. To co-infiltrate several genes, each bacteria suspension was diluted to a final OD_{600} of 0.4, then all strains were mixed equally to an

appropriate volume for infiltration. The suspension was infiltrated into the abaxial side of several leaves in three individual 4-week-old *N. benthamiana* plants using a syringe without needle. After treatment, the plants were cultivated in a climate controlled phytochamber for 4 days. Three leaf discs (9 mm diameter) per infiltrated spot were harvested and extracted by 2 ml *n*-hexane, followed by drying down under nitrogen flow and GC-MS analysis.

Microsome isolation and *in vitro* enzyme assay

A protocol from the literature with slight modification was used for microsome isolation (Urban et al., 1997; Scheler et al., 2016). The construct carrying *HvCYP89E31* and *ATR1* were transformed into yeast strain INVSc1. A single positive colony was picked to inoculate 5 ml of Ura-medium with 2% glucose and grown for 24 h at 30 °C with shaking. The culture was then used to inoculate 100 ml of Ura-medium with 2 % glucose in a 500 ml flask at 30 °C for 24 h. The cells were then collected by centrifugation, resuspended in 100 ml fresh YPD medium with 2 % galactose to induce protein expression and inoculated under shaking for another 24 h at 30 °C. All the following steps were carried out at 4 °C. The cells were harvested by centrifugation and resuspended in 30 ml of pre-chilled TEK buffer (50 mM Tris-HCl pH 7.5, 1 mM EDTA, 100 mM KCl), centrifuged again and resuspended in 2 ml TES buffer (50 mM Tris-HCl pH 7.5, 600 mM sorbitol, 10 g l^{-1} BSA, 1.5 mM β-mercaptoethanol) and transferred to a 50 ml tube. Acid-washed autoclaved 450–600 µm diameter glass beads were added into the tube until the surface of the cell suspension are reached. The suspension was shaken vigorously by hand for 1 min and returned back to ice for 1 min. This step was repeated four times. The glass beads were washed by 5 ml TES buffer three times, and the supernatant was collected and combined to a new tube, followed by centrifugation at 7,500 g for 10 min. The supernatant was transferred to ultracentrifugation tubes and centrifuged for 2 h at 100 000 g. The pellet was gently washed successively with 5 ml TES and 2.5 ml TEG buffer (50 mM Tris-HCl pH 7.5, 1 mM EDTA and 30% glycerol) after the supernatant was removed, then transferred to a Potter homogenizer with a spatula. 2 ml TEG buffer was added to the homogenizer and the pellet was carefully homogenized. 100 µl aliquots were transferred to 1.5 ml microtubes and stored at –80 °C until used.

In vitro CYP enzyme assays were performed in a 600 µl reaction volume, containing 40 µl of microsome preparation, 100 µM substrate, 1 mM NADPH, 50 mM sodium phosphate pH 7.4. The solution was incubated at 30 °C for 2 h with gentle shaking. Products were extracted with 1 ml *n*-hexane under strong agitation (vortex). After centrifugation, the organic phase was collected, then dried under a N_2 stream and resuspended in 100 µl *n*-hexane for GC-MS analysis.

Purification of diterpenes by silica gel column chromatography or SPE

For the purification of hordediene, 1 l of yeast culture was grown and extracted with 1 l *n*-hexane. The raw extracts were dried in a rotary evaporator and resuspended in 4 ml n-hexane, then loaded into two properly conditioned SiOH SPE cartridges (500 mg, MACHEREY-NAGEL). The cartridges were then washed with 2 ml n-hexane. The breakthrough and washing fraction were collected and combined. After drying down under nitrogen stream, an aliquot was measured by GC-MS to check the purity of the product and the rest, with an amount of around 2 mg was used for NMR structure elucidation.

Hordetriene and 11-hydroxy-hordetriene were first extracted from three liters of yeast culture. After concentration, the raw extracts were dissolved in 4 ml *n*-hexane and loaded into a pre-conditioned self-packed silica gel column (5 g, 15 mm x 100 mm). The column was then eluted by *n*-hexane and successively by 99:1, 98:2, 97:3, 96:4, 96:5 n-hexane: ethyl acetate solutions. The volume of each elution solution was 10 ml but the elution was separately collected in five 2 ml microtubes. An aliquot of each fraction was measured by GC-MS and the fractions with the same product were combined and then used for NMR structure elucidation. The yield of hordetriene and 11-hydroxyhordetriene was around 0.5 mg.

Nuclear magnetic resonance (NMR) conditions

H, ^{13}C, 2D (^{1}H,^{1}H gDQCOSY; ^{1}H,^{1}H zTOCSY;^{1}H,^{1}H ROESYAD; ^{1}H,^{13}C gHSQCAD; ^{1}H,^{13}C gHMBCAD), selective (^{1}H,^{1}H zTOCSY1D; ^{1}H,^{1}H ROESY1D), and band selective (^{1}H,^{13}C bsHMBC) NMR spectra were measured with an Agilent VNMRS 600 instrument at 599.83 MHz (^{1}H) and 150.84 MHz (^{13}C) using standard CHEMPACK 8.1 pulse sequences implemented in the VNMRJ 4.2A spectrometer software. TOCSY mixing time: 80 ms; ROESY mixing time: 300 ms; HSQC optimized for $^{1}J_{CH}$ = 146 Hz; HMBC optimized for $^{n}J_{CH}$ = 8 Hz. All spectra were obtained with C_6D_6 + 0.03% TMS as solvent at +25°C. Chemical shifts were referenced to internal TMS (δ ^{1}H = 0 ppm) and internal C_6D_6 (δ ^{13}C = 128.0 ppm).

Metabolites extraction from barley roots and PNM medium

100 mg (fresh weight) of frozen and cryo-ground root matter was extracted using 900 µL dichloromethane/ethanol (2:1, v/v) and 100 µl hydrochloric acid solution (pH 1.4). Extraction and duplicate removal of hydrophilic metabolites was achieved by 1 min FastPrep bead milling (FastPrep24, MP Biomedicals) followed by phase separation during centrifugation. For extraction 1.6 mL wall-reinforced cryo-tubes (Biozyme) each containing steel and glass beads were used. The upper aqueous phase was discarded and replaced for a second round of bead mill extraction/ centrifugation. Thereafter, the aqueous phase was removed and the

lower organic phase was collected. Subsequently 600 uL tetrahydrofuran (THF) was used for exhaustive extraction (FastPrep). After centrifugation the organic THF extract was combined with the first extract and dried under a stream of N_2.

Root exudates were extracted from 60 mL of gelrite media. For this, the gel was distributed into two 50 ml Falcon tubes. To each tube 4 g of NaCl and 3 mL ethyl acetate were added. The tubes were thoroughly shaken by hand and centrifuged. The upper phase (organic extract) was collected before fresh ethyl acetate was added for another two consecutive extractions. The combined extracts of three extraction rounds were combined and dried in a stream of N_2.

GC-MS

Dried extracts were suspended in 200 µl n-hexane. The analysis of yeast and plant extracts was carried out using a Trace GC Ultra gas chromatograph (Thermo Scientific) coupled to an ATAS Optic 3 injector and an ISQ single quadrupole mass spectrometer (Thermo Scientific) with electron impact ionization. Chromatographic separation was performed on a ZB-5ms capillary column (30□m × 0.32□mm, Phenomenex) using splitless injection and an injection volume of 1□µl. The injection temperature rose from 60□°C to 250□°C with 10□°C□s^{-1} and the flow rate of helium was 2□ml□min^{-1}. The GC oven temperature ramp was as follows: 50□°C for 1□min, 50 to 300□°C with 7□°C□min^{-1}, 300–330□°C with 20□°C□min^{-1} and 330□°C for 5□min. Mass spectrometry was performed at 70□eV, in a full scan mode with *m/z* from 50 to 450. Data analysis was done with the device specific software Xcalibur (Thermo Scientific).

RP-UPLC-ESI-MS/MS

For UPLC-MS analysis dried extracts were suspended in 100 uL 80% methanol/ 20% water. Separation of medium polar metabolites was performed on a Nucleoshell RP18 (2.1 x 150 mm, particle size 2.1 µm, Macherey & Nagel, GmbH, Düren, Germany) using a Waters ACQUITY UPLC System, equipped with a Binary Solvent Manager and Sample Manager (20 µl sample loop, partial loop injection mode, 5 µl injection volume, Waters GmbH Eschborn, Germany). Eluents A and B were aqueous 0.3 mmol/L NH_4HCOO (adjusted to pH 3.5 with formic acid) and acetonitrile, respectively. Elution was performed isocratically for 2 min at 5% eluent B, from 2 to 19 min with a linear gradient to 95% B, from 19-21 min isocratically at 95% B, and from 21.01 min to 24 min at 5% B. The flow rate was set to 400 µl min^{-1} and the column temperature was maintained at 40 °C. Metabolites were detected by positive and negative electrospray ionization and mass spectrometry.

Mass spectrometric analysis of small molecules was performed by MS-TOF-SWATH-MS/MS (TripleToF 5600, AB Sciex GmbH, Darmstadt, Germany) operating in negative or positive ion

mode and controlled by Analyst 1.7.1 software (AB Sciex GmbH, Darmstadt, Germany). The source operation parameters were as follows: ion spray voltage, -4500 V / +5500 V; nebulizing gas, 60 psi; source temperature 600°C; drying gas, 70 psi; curtain gas, 35 psi. TripleToF instrument tuning and internal mass calibration were performed every 5 samples with the calibrant delivery system applying APCI negative or positive tuning solution, respectively (AB Sciex GmbH, Darmstadt, Germany).

TripleToF data acquisition was performed in MS1-ToF mode and MS2-SWATH mode. For MS1 measurements, ToF masses were scanned between 65 and 1250 Dalton with an accumulation time of 50 ms and a collision energy of 10V (-10V). MS2-SWATH-experiments were divided into 26 Dalton segments of 20 ms accumulation time. Together the SWATH experiments covered the entire mass range from 65 to 1250 Dalton in 48 separate scan experiments, which allowed a cycle time of 1.1 s. Throughout all MS/MS scans a declustering potential of 35 (or -35 V) was applied. Collision energies for all SWATH-MS/MS were set to 35 V (-35) and a collision energy spread of ±25V, maximum sensitivity scanning, and elsewise default settings.

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Conflict of interest

The authors have no conflict of interest to declare.

Author Contributions

YL performed the LC-MS, GC-MS, yeast and *N. benthamiana* expression and gene expression analysis. GB provided supervision for LC-MS and data analysis. AS-H provided assistance for LC-MS sample preparation. AP performed the NMR measurements and analyses. UB supervised the yeast expression experiments. LM and AZ provided infected barley samples and transcriptomic data. AT and GB designed the project. AT designed and supervised the project. AT wrote the abstract, introduction, results and discussion. YL wrote the materials and methods, except the NMR part (AP) and LC-MS (GB). AT, YL and GB prepared the figures. All authors read and approved the manuscript.

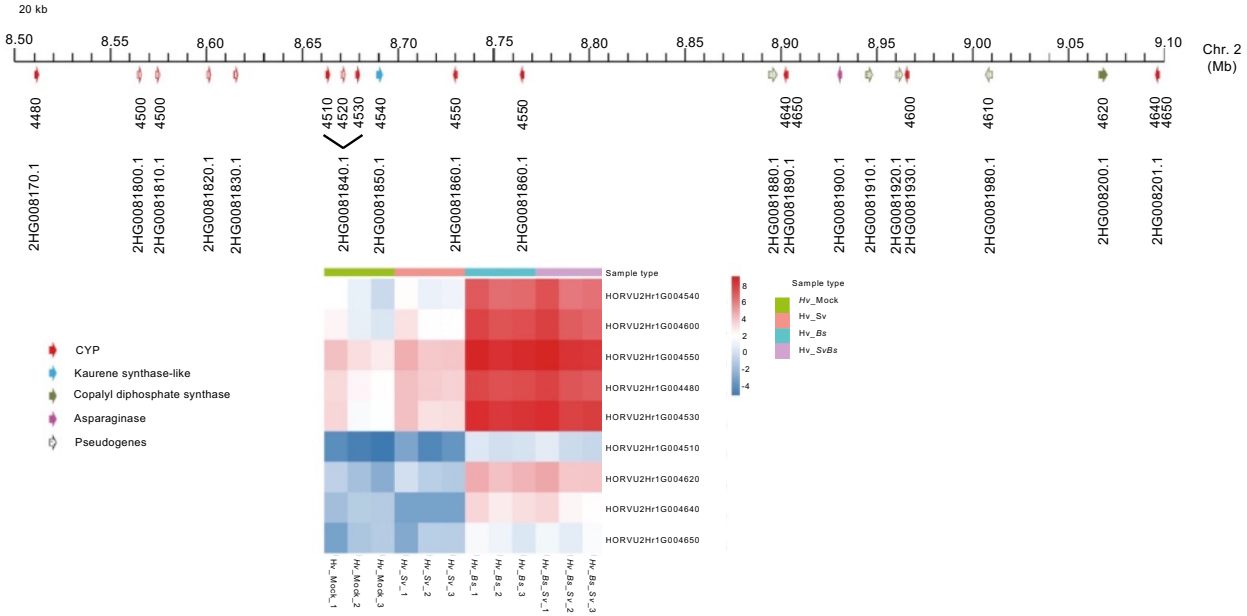
Tables

Table 1 List of genes from the chromosome 2 diterpenoid phytoalexin cluster.

Genome version			Gene status	Transcript ID	Protein ID	Name
MorexV1 (2017)	**MorexV2 (2019)**	**MorexV3 (2021)**				
2Hr1G004480	2HG0081780	2HG0099280	FL	AK252527.1		CYP99A66
2Hr1G004510 2Hr1G004520	2HG0081840	2HG0099340	Pseudo			
2Hr1G004530	2HG0081840	2HG0099350	FL		KAE8771735	CYP99A67
2Hr1G004540	2HG0081850	2HG0099360	FL	AK370792		HvKSL4
2Hr1G004550	2HG0081860 UnG0627890 UnG0631950	2HG0099370[a] 2HG0099420[a] 2HG0099430[a]	FL	AK369243		CYP89E31
NP	2HG0081880	2HG0099470	Pseudo			ψCPS1
2Hr1G004600	2HG0081930	2HG0099550	FL			CYP99A68[b]
2Hr1G004610	2HG0081980	2HG0099550	FL			CYP99A68[b]
2Hr1G004640 2Hr1G004650	2HG0081890 2HG0082010 2HG0081930 UnG0636400	2HG0099480	FL			CYP99A68[b]
	2HG0081920	2HG0099500	Pseudo			ψCPS2
2Hr1G004620	2HG0082000	2HG0099570	FL	AK364238		HvCPS2

Notes: (a) the amino acid sequences of these two genes are identical. The sequences only differ in the 5'-UTR. (b) the amino acid sequences are identical except for one amino acid change in 2Hr1G004600

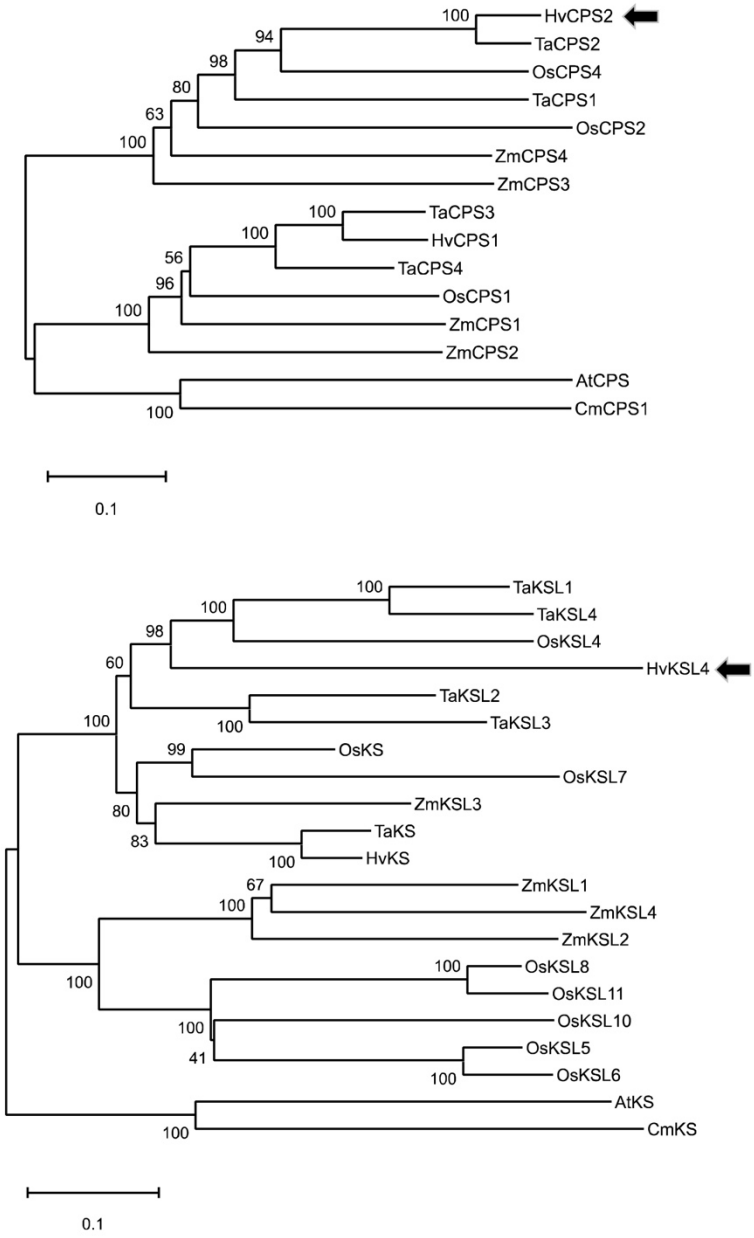
Figures



602

603 **Figure 1**. **Overview of the barley chromosome 2 cluster for diterpenoid biosynthesis**. The scale on top indicates the position on chromosome
604 2 based on the latest annotation of the barley genome (Monat et al., 2019). The genes are represented by arrows underneath the scale. The color
605 code indicates to which gene family they belong and the dotted pattern the presence of a pseudogene. The first row of numbers below the scale
606 indicates the last 4 digits of the gene ID according to MorexV1 gene models and the second row gives the gene identification in the MorexV2
607 version of the barley genome. The colored bars in the lower part represent gene expression values as log2-transformed FKPM values for genes
608 that show differential gene expression (|fold change| > 2, data from Sarkar et al., 2019). Each square for a sample type represents data from a
609 biological replicate. The samples types are indicated by the following color code: purple: barley root co-inoculated with *B. sorokiniana* and *S.*
610 *vermifera*; turquoise blue: barley roots inoculated with *B. sorokiniana*; pink: barley roots inoculated with S. vermifera; green: barley root mock
611 inoculated.

19



612

613 **Figure 2. Phylogenetic analysis of HvCPS2 and HvKSL4.** The sequences indicated were
614 aligned and processed with the MEGA X software (Kumar et al., 2018) with the maximum
615 likelihood method and 1000 bootstrap replications. The consensus trees are shown with the
616 bootstrap values for the individual branches shown. The HvCPS2 and HvKSL4 sequences
617 are indicated by grey arrows. The list of sequences used is provided in **Tables S2 and S3**.

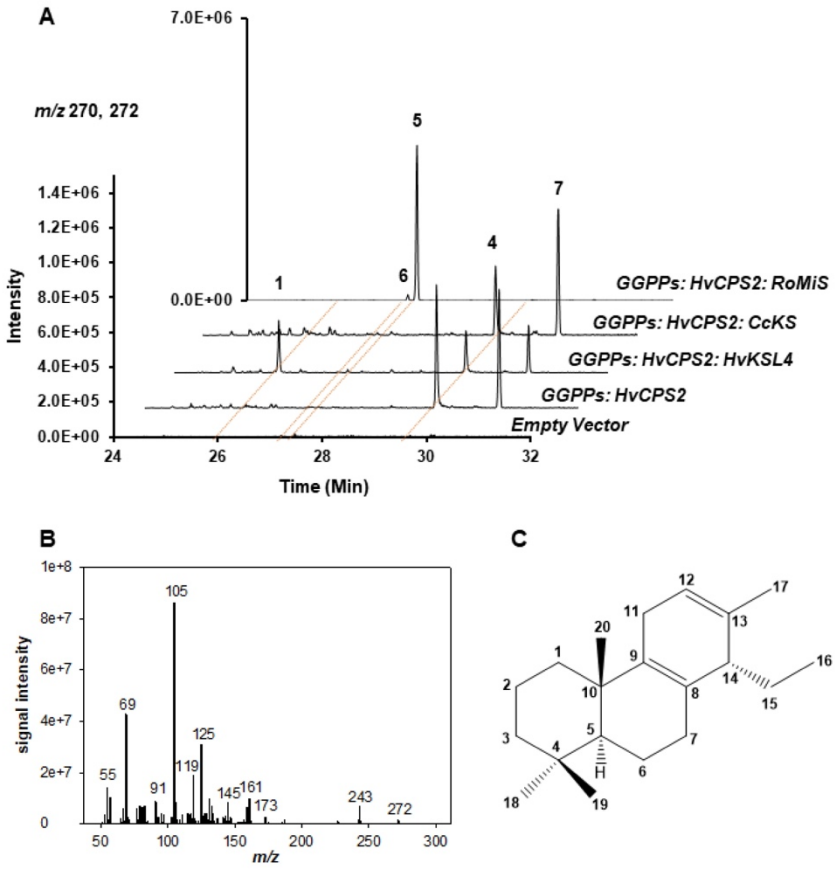
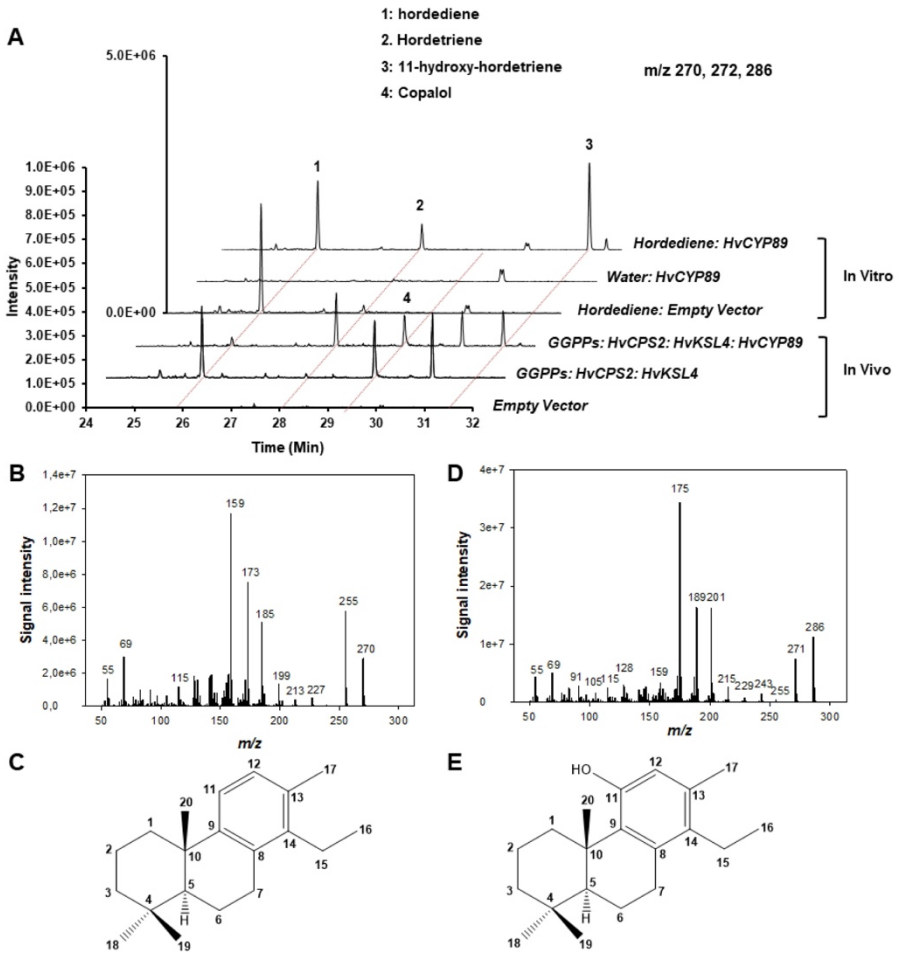


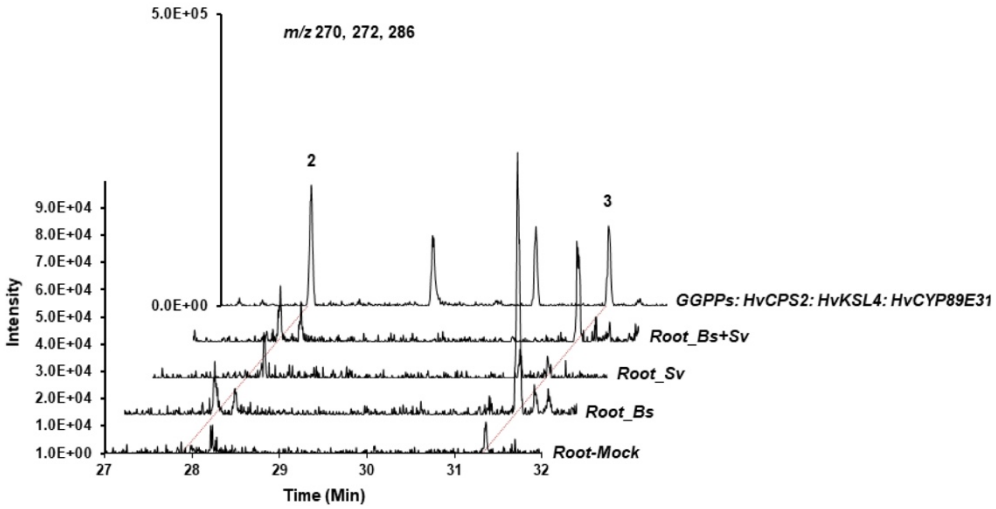
Figure 3. **Expression of HvCPS2 and HvKSL4 in yeast.** A. Selected ion (*m/z* 270 and 272) GC-MS chromatograms of *n*-hexane extracts of yeast strains expressing the gene combinations indicated on the right. CcKS: *ent*-kaurene synthase from coffee (*Coffea canephora*); RoMiS, miltiradiene synthase from rosemary (*Rosmarinus officinalis*). **1**: hordediene; **4:** (+)-copalol; **5**: miltiradiene; **6**: abietatriene; **7**: unknown copalol derivative. B. EI mass spectrum of hordediene **(1)**. Structure of hordediene as determined by NMR.



627

628 **Figure 4. Characterization of HvCYP89E31**. A. GC-MS chromatograms (single ion
629 monitoring for *m/z* 270, 272 and 286). The in vivo chromatograms show analysis of extracts
630 from transient assays in *N. benthamiana*. The in vitro chromatograms show analysis of in
631 vitro assays with microsome fractions from yeast expressing HvCYP89E31. The identity of
632 peaks is indicated by numbers. B and C. Respectively EI mass spectrum and structure of
633 compound **2**, hordetriene. D and E. Respectively EI mass spectrum and structure of
634 compound **3**, 11-hydroxy-hordetriene.

635



636

637

638 **Figure 5. GC-MS chromatograms of extracts from barley roots infected with *B.***
639 ***sorokiniana* and/or *S. vermifera*.** Single ion monitoring (m/z 270, 272 and 286) of extracts
640 from roots infected with *B. sorokiniana* (Root_Bs), *S. vermifera* (Root_Sv) or both
641 (Root_Bs+Sv) or mock inoculated (Root-Mock). An extract of yeast strain expressing
642 HvCPS2, HvKSL4 and HvCYP89E31 is shown as a reference. **2**: hordetriene; **3:** 11-hydroxy-
643 hordetriene.

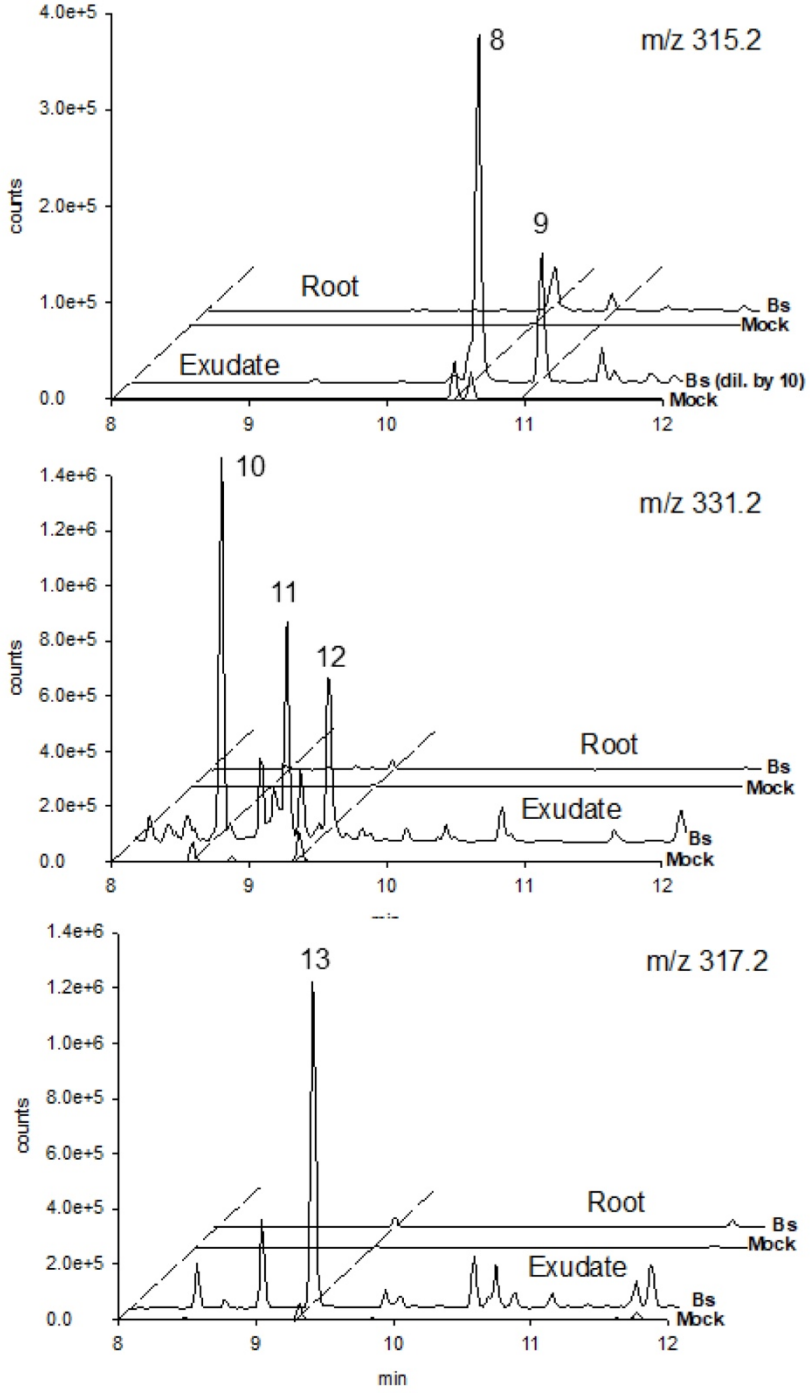


Figure 6. LC-(-)ESI-QToF-MS of major diterpene metabolites in roots and root exudates of barley 6 dpi with *B. sorokiniana* (Bs) and in respective mock infected controls. HR-MS/MS spectra of compounds 8-13 are presented in **Fig. S3**.

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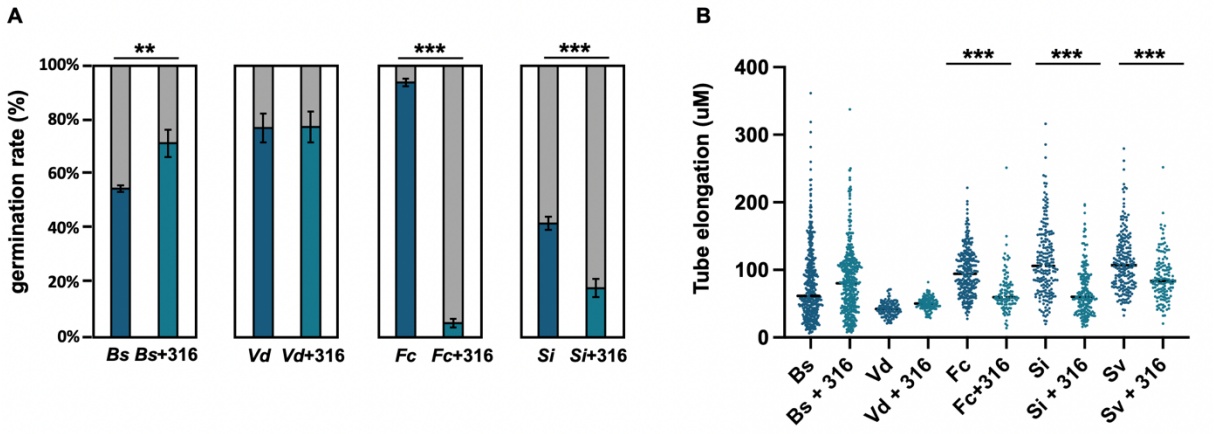
Supplementary information

The online version contains supplementary material available at: https://www.biorxiv.org/content/10.1101/2021.05.21.445084v1.supplementary-material



Additional Figure 1: Effect of barley diterpene 316 application on different fungi. The heterologous expression of barley KSL4 (HORVU2Hr1G004540), CPS2 (HORVU2HR1G004620), CYP89E31 (HORVU2Hr1G004550), CYP99A66 (HORVU2Hr1G004480) and CYP99A67 (HORVU2Hr1G004530) led to the production of a diterpene with the molecular weight of 316 Da. This diterpene is the major secreted barley diterpene upon *Bs* colonization. The diterpene was purified from yeast (kindly provided by the Group of Alain Tissier) and applied to different fungi to investigate potential effects on fungal spore germination and tube elongation A) Percentage of germinated (blue) and not germinated (grey) spores of *Bipolaris sorokiniana* (*Bs*, 3 hpi), *Verticilium dahlia* (*Vd*, 9 hpi), *Fusarium culmorum* (*Fc*, 9 hpi) and *Serendipita indica* (*Si*, 12 hpi) after application of 1% DMSO as a control (dark blue) or 1% DMSO + 500 uM of the barley diterpene 316 (turquoise). The incubation time depended on the speed of fungal spore germination. B) Tube elongation/hyphal length of *Bipolaris sorokiniana* (*Bs*, 3 hpi), *Verticilium dahlia* (*Vd*, 9 hpi), *Fusarium culmorum* (*Fc*, 9 hpi), *Serendipita indica* (*Si*, 12 hpi) or *Serendipita vermifera* (*Sv*, 12 hpi) after application of 1% DMSO as a control (dark blue) or 1% DMSO + 500 uM of the barley diterpene 316 (turquoise). Asterisks indicate significant differences between control and diterpene-treated samples (Student's *t*-test; * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$)

Chapter 5

Monitoring Cell Death via Ion leakage and PAM Fuorometry

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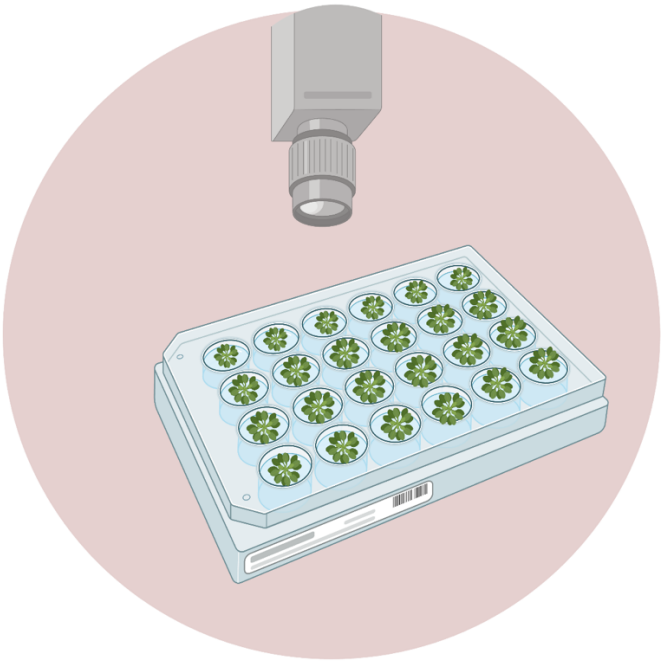
* These authors contributed equally to this work.

Authors contributions:

ND, **LM,** and AZ developed the protocol and wrote the manuscript, RH corrected the manuscript.



Chapter 14

Monitoring Cell Death Via Ion Leakage and PAM Fluorometry

Nick Dunken, Lisa Mahdi, Rainer E. Häusler, and Alga Zuccaro

Abstract

Cell death in plants plays a major role during development as well as in response to certain biotic and abiotic stresses. For example, plant cell death can be triggered in a tightly regulated way during the hypersensitive response (HR) in defense against pathogens or be elicited by pathogenic toxin deployment. Monitoring cell death and its impact on plant health can aid in the quantification of plant disease symptoms and help to identify the underlying molecular pathways. Here, we describe our current protocol for monitoring plant cell death via ion leakage and Pulse-Amplitude-Modulation (PAM) fluorometry. We further provide a detailed protocol for the sample preparation, the measurement, and the data evaluation and discuss the complementary nature of ion leakage and PAM fluorometry as well as the potential of PAM fluorometry for high-throughput screenings.

Key words Plant cell death, ION leakage, Pulse-Amplitude-Modulation (PAM) fluorometry, Pathogen treatment, Chemical treatment, Large-scale screening

1 Introduction

Plant cell death is essential for plant development [1] as well as in response to biotic and abiotic stresses [2, 3]. Forms of regulated cell death (RCD) such as apoptosis-like cell death, necroptosis, and the hypersensitive response (HR) are induced in response to different stimuli and exhibit distinct cell death characteristics. As a drastic and irreversible transition, RCD is tightly regulated by the plant [4]. However, RCD can also be induced or suppressed by plant-associated microorganisms and pathogens can trigger host cell death independently of the plant cell death machinery via the secretion of toxins and cell-wall degrading enzymes.

Monitoring cell death allows to quantify disease as well as stress symptoms of the plant and helps to unravel the underlying molecular mechanisms.

Plant cell death results in a loss of cell membrane integrity that ultimately leads to the leakage of cellular electrolytes into the surrounding medium. Measuring the corresponding increase in

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electric conductivity in the surrounding liquid is a commonly used, direct proxy to quantify plant cell death in response to abiotic and biotic stresses [5]. However, ion leakage measurements are time-consuming as each sample has to be measured individually. Thus, they are less suitable for large-scale screenings.

The photosynthetic activity reflects the health status of a plant [6, 7]. The maximum potential quantum yield of photosystem II electron transport is defined as the variable fluorescence (F_V) divided by the maximum fluorescence (F_M) (F_V/F_M), where $F_V = F_M\text{-}F_0$ (F_0. ground fluorescence). Healthy and non-stressed plants obtain a F_V/F_M value of approximately 0.8 [8, 9] whereas biotic and abiotic stresses lead to a decrease in the F_V/F_M ratio up to a total loss of photosynthetic activity upon cell death. Thus, while PAM fluorometry is an indirect proxy for cell death, it provides additional information about the overall health status of the plant. Due to its simple implementation and time-effectiveness, PAM measurements are especially useful for large-scale screenings. Furthermore, PAM measurements do not require a liquid-based system and can also be used for plants on solid medium or soil. Both ion leakage and PAM fluorometry are noninvasive and thus suitable to measure cell death progression over time on the same samples.

Here, we show the function and specificities of cell death monitoring via a combination of ion leakage and PAM fluorometry, exemplified by a chemically induced, developmental cell death as well as a biotic stress induced cell death.

Representing a chemical induced cell death, we treated *Arabidopsis thaliana* seedlings with methyl jasmonate (MeJA), a bioactive derivate of jasmonic acid (JA) that induces leaf senescence [10]. Treatment with MeJA leads to increased electric conductivity as well as to a constant, uniform decrease in the F_V/F_M ratio (Fig. 1).

Representative of a biotic interaction inducing cell death, we took advantage of a previously described tripartite system consisting of the detrimental fungal pathogen *Bipolaris sorokiniana* and the beneficial fungal root endophyte *Serendipita vermifera* in barley [11]. While *B. sorokiniana* colonization results in host cell death, co-colonization with *S. vermifera* counteracts these detrimental effects [11]. We here applied this tripartite system in *Arabidopsis thaliana* (Fig. 1). We inoculated Arabidopsis roots with *S. vermifera*, *B. sorokiniana,* or a combination of both fungi (Fig. 3). As observed in barley, *B. sorokiniana* was able to colonize Arabidopsis roots. Upon colonization, *B. sorokiniana* caused typical disease symptoms on Arabidopsis roots and shoots including root browning. Consistently, *B. sorokiniana* colonization results in a constant increase in electric conductivity. This increase is diminished in plants when the roots are additionally inoculated with *S. vermifera*, reflecting the protective function of *S. vermifera*.

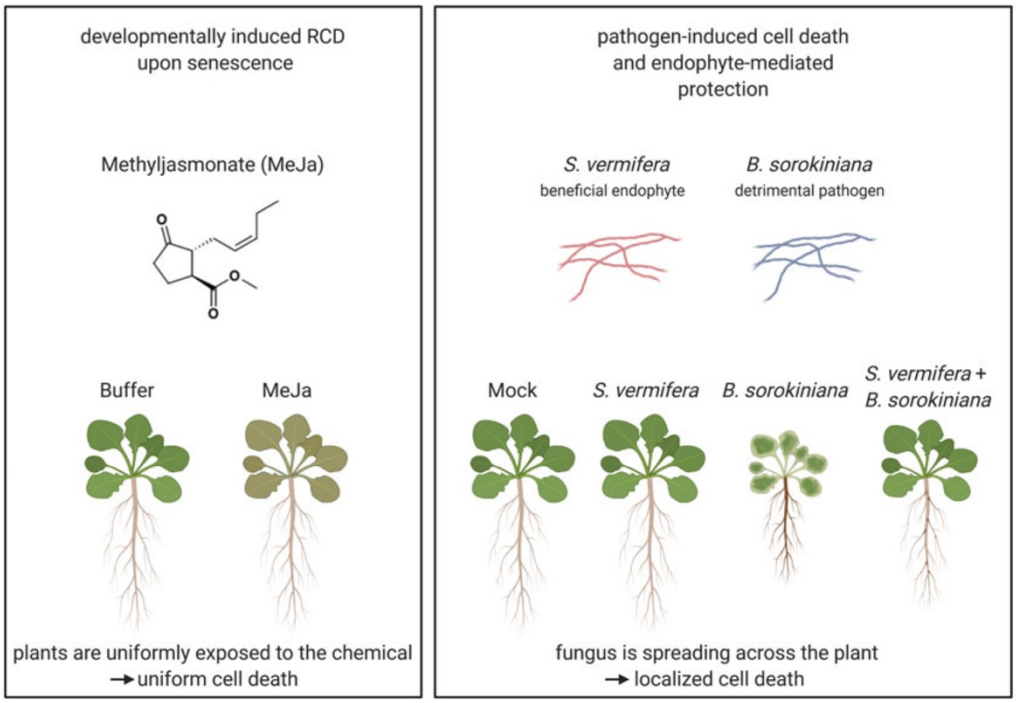

Fig. 1 Experimental setup depicting a chemically induced developmental cell death via methyl jasmonate (MeJA) (left) and a pathogen-induced cell death via *B. sorokiniana* colonization in a tripartite system with the beneficial endophyte *S. vermifera* (right)

PAM analysis revealed consistent results. Interestingly, *B. sorokiniana* colonized plants do not display a uniformly decreased F_V/F_M ratio (Fig. 2b). Instead, there is a local reduction of photosynthetic activity and cell death that slowly spreads across the whole shoot. This in turn leads to a constant reduction in the photosynthetic active leaf area over time reflecting the proceeding cell death.

2 Materials

2.1 Equipment

1. 24-Well culture plates.
2. Square plates.
3. Forceps.
4. Conductivity meter.
5. Imaging PAM fluorometer (e.g., Imaging-PAM M-Series Chlorophyll Fluorescence System (Walz)) able to measure culture plates (e.g., 24-well plates).

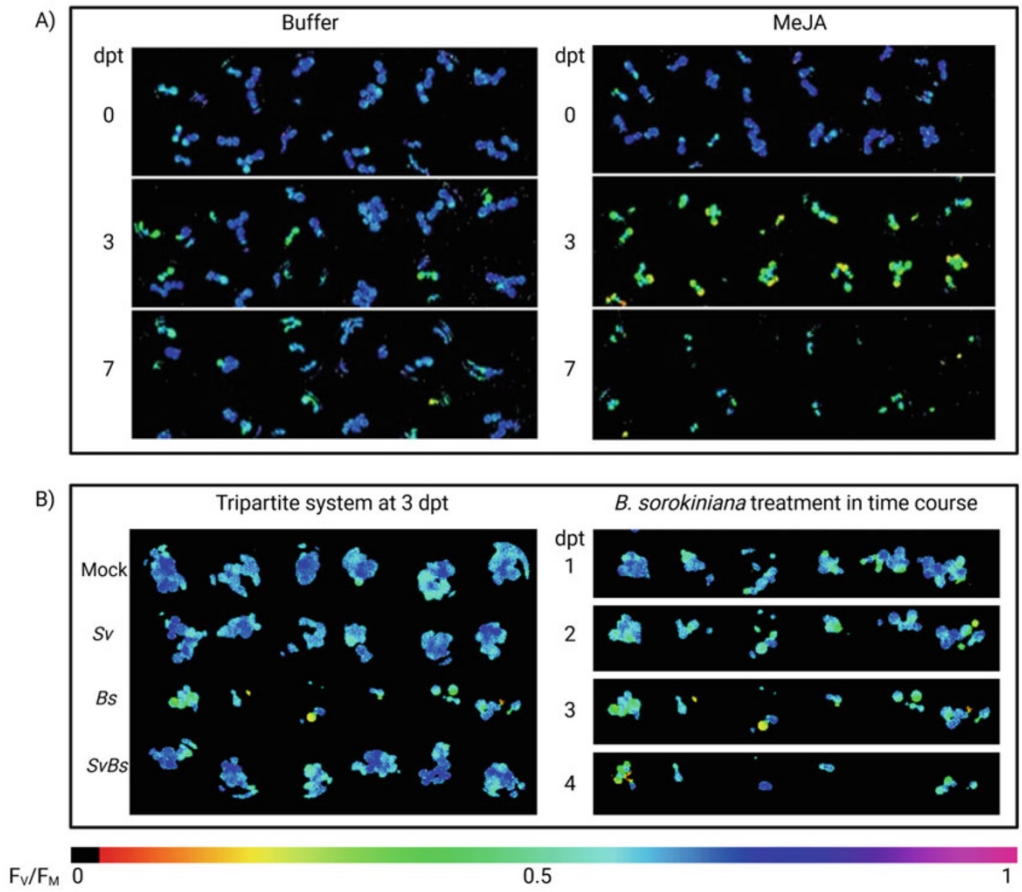

Fig. 2 PAM fluorometry pictures of (**a**) 7-day-old *Arabidopsis thaliana* seedlings treated with 50 mM MES buffer (pH 5.6) or 500 mM MeJA, respectively, at 0, 3, and 7 days post transfer (dpt). (**b**) 14-day-old *Arabidopsis thaliana* seedlings inoculated with sterile water (Mock), the beneficial fungal endophyte *S. vermifera* (Sv), the fungal pathogen *B. sorokiniana* (Bs) or a combination of both fungi (SvBs). At 7 days post inoculation (dpi) the plant roots were washed and the seedlings were transferred to deionized water. Pictures were taken every 24 h for 4 days. Blue color represents high photosynthetic activity while lighter colors represent a lower photosynthetic activity

6. Personal computer with a software suitable for PAM evaluation (e.g., ImagingWin, Walz, Germany) installed.
7. Growth cabinet or growth chamber for plant growth under controlled conditions.

2.2 Reagents and Consumables

1. *Arabidopsis thaliana* seeds.
2. Murashige-Skoog (MS) medium including vitamins.
3. Sucrose.
4. 70% Ethanol.

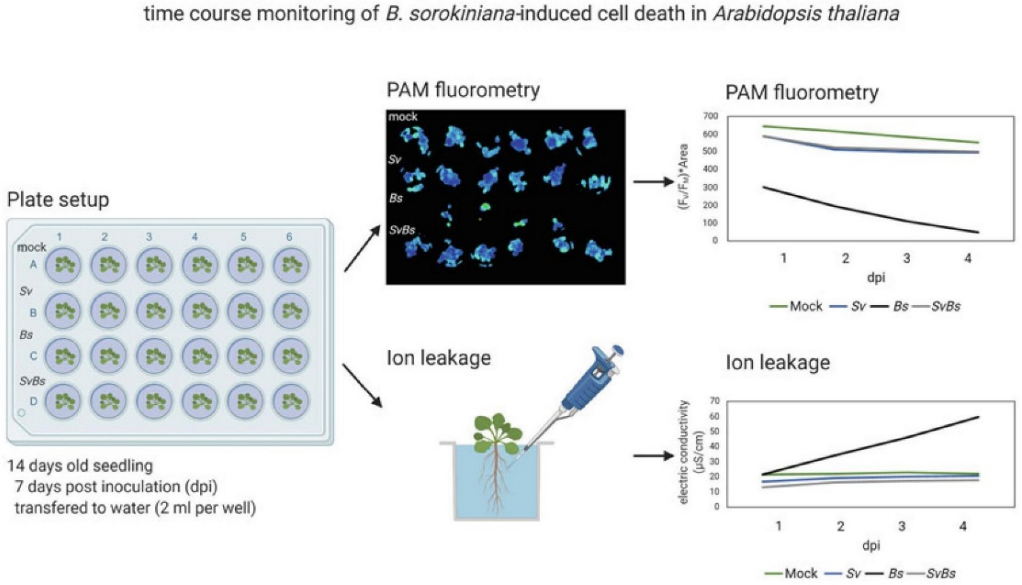

Fig. 3 Evaluation of *B. sorokiniana*-induced cell death via PAM fluorometry and ion leakage analysis. Both readouts are noninvasive and thus suitable to measure cell death progression over time on the same samples. While ion leakage represents a direct proxy, its measurement is more time-consuming. PAM fluorometry visualizes plant cell death indirectly via F_V/F_M-ratios but can represent a more efficient screening strategy and can be enhanced by additional analysis of the photosynthetic active area

5. 100% Ethanol.
6. Plant Agar.
7. Deionized water or sterile 2.5 mM 2-(N-morpholino) ethanesulfonic acid (MES) adjusted to pH 5.6 (*see* **Note 1**).
8. Chemical or organism to be tested, here: 10 mM MeJA solved in 2.5 mM MES (pH 5.6) or fungal inoculum in water.

3 Method

3.1 Seedling Sterilization, Germination, and Treatment

1. Sterilize *Arabidopsis thaliana* seeds by washing them two times with 70% EtOH for 5 min followed by one washing step with 100% EtOH for 5 min.
2. Let the seeds dry under sterile conditions.
3. Sow sterilized seeds on ½ MS, 4% plant agar, and 1% sucrose square plates.
4. Stratify the seeds for 2 days at 4 °C in darkness.
5. Then transfer plates to a growth chamber at a day/night cycle of 8/16 h at 22/18 °C, 60% humidity and a light intensity of 125 µmol m^{-2} s^{-1}.

6. Let seedlings germinate for 7 days in these conditions prior to the treatment.
7. *For chemical treatment*: Fill each of the 24 wells of a sterile culture plate with 1.9 mL of 2.5 mM MES buffer (pH 5.6).
8. Transfer 3–5 7-day-old seedlings to each well and let them regenerate in the growth chamber at the abovementioned conditions (*see* **Note 2**).
9. After regeneration, take first measurements of ion leakage and PAM fluorometry as a time point 0.
10. Treat the 8-day-old seedlings with 100 μL of 20× chemical solution (e.g., 10 mM MeJA; end concentration: 500 μM) per well.
11. *For tripartite treatment*: transfer 20 seedlings per plate to ½ MS plates, 4% plant agar without added sucrose.
12. Inoculate the plants with the respective fungi or fungal combination, here with 1 mL of a 1 g/L *S. vermifera* mycelium solution per plate, 1 mL of a 5000 spores/L *B. sorokiniana* spore solution per plate, or a combination of both fungi.
13. Distribute the fungal solution on the roots and the plate area below the roots. For this, distribute the solution on the roots and let it run down the plate. Recollect it with a pipette and repeat until a homogenous inoculation is achieved.
14. Incubate the plates in the growth chamber (conditions see above) for 7 days.
15. At 7 days post inoculation, when the seedlings are 14 days old, fill each well with 2 mL deionized water, wash the colonized roots to remove the extraradical hyphae, and transfer 3–5 seedlings to each well.
16. For washing, hold the seedlings carefully with forceps, dip the roots into autoclaved water, and slip the wet root over tissue paper. Extraradical hyphae will stick to the tissue paper.

3.2 Time Course PAM and Ion Leakage Measurement

1. Measure ion leakage for each well according to the manufacturer instructions. Be careful not to touch the seedlings while measuring (*see* **Note 3**).
2. Before measuring PAM, transfer the plates to darkness for 15 min to enable measurement of the maximum fluorescence (F_M) as well as the ground fluorescence (F_0) in the dark-adapted leaves (*see* **Note 4**). For dark adaption, the plates can be kept in a closed drawer or covered with aluminum foil. Afterward, measure the fluorescence by taking pictures with the fluorometer plate by plate in darkness according to the manufacturer's instructions.
3. Continue the measurements every 24 h for 4–8 days.

3.3 Evaluation of PAM with and Without Area

1. The evaluation differs depending on the Imaging PAM fluorometer and the respective evaluation-software. Here, we explain the evaluation with ImagingWin (Walz) in more detail.
2. Open the first PAM file (.pim or .xpim) in ImagingWin. Photosynthetically active leaf tissues are visualized in blurry colors (Fig. 2).
3. Click on Options and uncheck "Mean over Area of interest (AOI)."
4. Reset the AOI and calculate the F_V/F_M value of each well by adding AOI circles around each well.
5. Extract the F_V/F_M values (denoted as Y(II) by the program) using the "Report" tab. The resulting F_V/F_M values represent the mean PSII activity of living leaves of the 3–5 seedlings in each well.
6. Export a JPEG file of the F_V/F_M channel and measure the photosynthetic active leaf area via a suitable software (e.g., ImageJ [12]) to include the dimension of the living leaf area into the evaluation (*see* **Notes 5** and **6**).
7. Multiply the F_V/F_M value for each well with the photosynthetic active leaf area.

4 Notes

1. For chemical treatments, the buffer is of high importance for the experiment. Many chemicals have a strong effect on the pH of the solution. The buffer ensures that the plant health is not affected by an extremely acidic or basic pH.
2. When transferring and washing the seedlings, be careful not to injure the plants, as this induces stress and tampers the results. An additional recovery day between picking and treatment is thus recommended (*see* Subheading 3.1).
3. 24 h of regeneration before starting measuring are recommended to avoid artifacts in ion leakage measurements due to wounding during the seedling transfer.
4. Be sure not to expose the dark adapted plants to light before measuring. Also, do not measure the plates twice without repeating the adaption process.
5. During evaluation, be aware that dead cells are no longer photosynthetic active and are thus excluded from the program calculation. Including the area in your calculation will allow you to take the dead leaf area into account. For a cell death response such as observed upon *B. sorokiniana* treatment (Figs. 1, 2b and 3) the area is essential for quantification. For a cell death that spreads and proceeds uniformly on the plant

such as in case of MeJA (Figs. 1 and 2a), the area calculation is not necessarily needed. However, it still increases the measurement sensitivity.

6. As it is hard to perform absolute size measurements of the photosynthetic active leaf area due to the lack of a size standard, relative sizes can be compared. For this, the plates have to be continuously measured in the same setup (same height, zoom, etc.)

Acknowledgments

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Discussion

Anthropocene driven pathogen intensification and plant microbiota dysbiosis

The United Nations (UN) sustainable development agenda for the 21st century aims to “end hunger, achieve food security, improve nutrition and promote sustainable agriculture”. A major threat to food security are infections with plant pathogens, which account for around 15 % of global yield losses, and this figure is rising (Delgado-Baquerizo et al., 2020). Yield losses caused by pathogens are often associated with the disruption of the plant microbiota, leading to dysbiosis within the plant holobiont (Arnault et al., 2022). This dysbiosis is thought to be exacerbated by the Anthropocene, the present human-dominated epoch (Berg and Cernava, 2022; Perreault and Laforest-Lapointe, 2022). The rise in temperature caused by climate change increases the occurrence of pests (Delgado-Baquerizo et al., 2020). At the same time, environmental pollution and the use of pesticides lead to changes in the plant microbiota (Sangiorgio et al., 2022; Vitali et al., 2019). Large-scale monocultures are associated with an impoverishment of host-associated microbes and lower soil biodiversity (Berg and Cernava, 2022). Breeding aimed at altering secondary metabolites, such as the removal of compounds toxic to humans, leads to changes in the complexity of the microbiota network and the abundance of keystone taxa (Pérez-Jaramillo et al., 2018). Similarly, breeding resistant plant varieties can alter the overall potential of plant hosts for microbial colonization due to overlapping genes that are relevant for the colonization of both, pathogenic and beneficial microbes (Jacott et al., 2020; Zuccaro and Langen, 2020b).

New agricultural strategies and the use of biocontrol agents

Preventing dysbiosis, plant diseases, and associated yield losses requires a deep understanding of the factors and dynamics shaping the plant holobiont. In this context, plant-microbe research has emerged as a promising area to explore these dynamics and develop strategies for biocontrol and increasing crop production (Compant et al., 2019; de Souza et al., 2020a; Finkel et al., 2017; Köhl et al., 2019; Vannier et al., 2019; Vishwakarma et al., 2020). Newer strategies in agriculture include the use of biocontrol agents or synthetically produced microbial communities with beneficial traits (de Souza et al., 2020b; Song et al., 2020). To date, about 300 fungi from 113 genera have been

identified as biological control agents that protect their hosts through very different mechanisms, ranging from competition for space and nutrients to direct antagonism or mycroparasitism (Guetsky et al., 2002; Thambugala et al., 2020). Among them, the impotance of Sebacinales in natural systems has been frequently highlighted (Dietrich et al., 2022; Lee and Hawkes, 2021; Oberwinkler et al., 2013; Shekhawat et al., 2021; Verbruggen et al., 2014; Weiß et al., 2011).

Sebacinales-mediated host protection

The Sebacinales representative *S. vermifera* acts against several pathogens, including *B. sorokiniana*, reducing pathogen colonization and disease symptoms on host plants (Mahdi et al., 2022; Sarkar et al., 2019). This biocontrol activity is strong enough to functionally replace key members of the bacterial microbiota and has several features. First, the antagonism is largely host-independent. *Sv*-mediated bioprotection functions in distantly related plant hosts and is not associated with extensive host transcriptional reprogramming (Mahdi et al., 2022; Sarkar et al., 2019). Second, antagonism is based on direct contact in the plant rhizosphere. *Sv* hyphae wrap around and occasionally invade *Bs* hyphae. At the same time, the spatial separation of the two fungi within the host limits this required direct contact, making the host rhizosphere the central *SvBs* interaction zone (Mahdi et al., 2022; Sarkar et al., 2019). Third, antagonism shows signs of mycoparasitism. Confrontation with fungi does not induce stress-related genes in *Sv* but does in *Bs*. *Sv* cell wall degrading enzymes and CAZymes such as the *Sv*CHIT-CBM5 studied here are induced, while secretion of *Sv* secondary metabolites was not significantly involved (Mahdi et al., n.d.; Sarkar et al., 2019). Similarly, *Si* exerts biocontrol effects against *Bs* in distantly related plant hosts. In both fungi, antagonism and plant biocontrol are likely associated with the expression of the homologous chitinases *Sv*CHIT-CBM5 and *Si*CHIT-CBM5, which impair spore germination of *Bs* (Mahdi et al., n.d.)

The bioprotective capabilities of Sebacinales are not limited to direct antagonism. Colonization by *Sv* and *Si* leads to systemic resistance of plants to various fungal infections (Deshmukh et al., 2006; Pedrotti et al., 2013; Sarkar et al., 2019; Waller et al., 2008). In Arabidopsis, *Sv* counteracts bacterial-mediated ion leakage and *Si* reduces flg22 triggered callose deposition, growth retardation, MAMP responsive gene expression, and oxidative burst, suggesting that Sebacinales fungi have the potential to counteract bacterial triggered host immune responses (Jacobs et al., 2011; Mahdi

et al., 2022). These regulatory capabilities enable Sebacinales to dynamically adapt to changes in the biotic environment and to persist within diverse microbiota across different geographical locations, environmental conditions, and plant hosts (Lee and Hawkes, 2021; Mahdi et al., 2022; Weiß et al., 2016, 2011). Considering that the poor suitability of current biocontrol inoculants under field conditions is often attributed to their inability to cope with changes in the biotic or abiotic environment (Pirttilä et al., 2021), these adaptive capabilities indicate a great potential of Sebacinales for future agricultural use.

Fungal biocontrol and growth promoting properties within multi-kingdom environments

Microbe-mediated biocontrol is influenced by a plethora of internal and external variables. Biotic and abiotic stresses affect biocontrol agents either directly or indirectly by triggering plant host responses that in turn, affect the rhizosphere (Jacoby et al., 2021; Liu et al., 2020; Schmidt and Saha, 2021). The combination of biocontrol agents can result in additive or synergistic beneficial effects on the host but also in microbial competition between biocontrol agents which has negative effects on the plant host (Guetsky et al., 2002, 2001; Xu et al., 2011; Xu and Jeger, 2013). Without a deep understanding of the underlying dynamics, it is difficult to predict the impact on plant health by applying or combining known biocontrol agents in natural systems (Guetsky et al., 2002, 2001; Xu et al., 2011; Xu and Jeger, 2013). Studying the stability of microbe-transmitted traits within the plant holobiont requires new scientific strategies that go beyond classical molecular approaches. In recent years, the identification, collection, and reconstruction of microbiota members from different host plants has enabled the study of microbial biocontrol agents in more complex yet controlled environments (Bai et al., 2015; Bulgarelli et al., 2015; Edwards et al., 2015; Niu et al., 2017; Ofek-Lalzar et al., 2014; Peiffer et al., 2013; Robertson-Albertyn et al., 2022; Thiergart et al., 2020; Vorholt, 2012).

By expanding the tripartite system to include individual bacterial strains as well as host-adapted and non-adapted SynComs, we found a high susceptibility of *Bs* to other microbes and a resilient colonization of *Sv* across all treatments. Most importantly, we observed synergistic host protection and growth promotion in the combined presence of *Sv* and bacteria (Mahdi et al., 2022). These beneficial effects on the host are

stabilized or enhanced in more complex microbial communities (Mahdi et al., 2022). Consistent with our findings, previous studies describe host beneficial properties conferred by inter-kingdom microbial consortia to be more resilient than those of single strains, while pathogenic traits are often suppressed in the presence of other microbes (Durán et al., 2018; Finkel et al., 2017; Mesny et al., 2021; Pascale et al., 2020; Vannier et al., 2019). These observations have contributed to the idea that the plant microbiota plays an important role in plant health and productivity (Song et al., 2020; Vannier et al., 2019). However, the mechanisms underlying inter-kingdom mediated beneficial host traits are still largely unknown. Synergistic host effects mediated by *Sv* and bacteria cannot be explained by changes in *Sv* growth or colonization alone (Mahdi et al., 2022). Instead, the synergistic effects likely result from a combination of direct microbial effects, inter-microbial dynamics, and host factors (Mahdi et al., 2022). Despite a low host transcriptional response, even largely host-independent, microbe-mediated traits are not fully reproducible in the absence of the host (Mahdi et al., 2022), underscoring that microbe-mediated host benefits cannot be studied exclusively from a microbial perspective.

Modulation of plant immunity in multispecies interactions

Plant host genetics is an important determinant of microbiota composition (Brachi et al., 2022; Deng et al., 2021; Escudero-Martinez et al., 2022; Peiffer et al., 2013; Wagner et al., 2016; Walters et al., 2018). Plants actively shape their microbiota by regulating plant immunity and the secretion of secondary metabolites that alter the rhizosphere environment and serve as infochemicals for intra-kingdom communication (Jacoby et al., 2021; Schmidt and Saha, 2021; Schütz et al., 2021; Zipfel and Oldroyd, 2017). Plant immunity is an integral part of the plant-microbe interface and influences the interaction between the host and individual microbes as well as the microbiota (Bodenhausen et al., 2014; Pfeilmeier et al., 2021; Wolinska et al., 2021). Ultimately, plant immunity must be overcome to establish long-lasting associations (Dodds and Rathjen, 2010; el Yahyaoui et al., 2004; Jamet et al., 2007; Pel and Pieterse, 2013; Puppo et al., 2013; Yu et al., 2019; Zipfel and Oldroyd, 2017).

How plants engage with beneficial microorganisms while restricting the growth of pathogens remains an important question in the field of plant microbiota research (Thoms et al., 2021). Associations with beneficial microorganisms are thought to be

mediated by mutual suppression or evasion of plant immunity, while recognition of pathogens leads to the induction of plant immunity. (Boyle and Martin, 2015; Gourion et al., 2015; Wang et al., 2021; Zipfel and Oldroyd, 2017; Zuluaga et al., 2017). Similarly, *Bs* colonization triggers a strong barley immune response, upregulation of PR genes and genes related to diterpenoid production (Mahdi et al., 2022; Sarkar et al., 2019). Diterpenoids have previously been identified as defense compounds in other monocotyledonous plants such as switchgrass, maize, and wheat, while no diterpenoids have beed described in barley (Schmelz et al., 2011; Toyomasu et al., 2020). The characterization of the first diterpenoid biosynthesis cluster in barley and the detection of diterpenoid secretion in barley during pathogen colonization is a first step to investigate the function of these compounds in plant-pathogen and plant-multipartite interactions (Liu et al., 2021). Preliminary data suggests that exposure to the main secreted barley diterpene does not inhibit but rather promotes germination and growth of *Bs* (Chapter 4; AdditionalFig. 1). Higher tolerance to plant-derived defense compounds or the ability to metabolize and detoxify these compounds is often observed in adapted pathogens, suggesting a similar mechanism in the interaction between *Bs* and barley (Buxdorf et al., 2013; Ökmen et al., 2013; Westrick et al., 2021). In contrast, the same barley diterpenoid inhibited the germination and growth of other root-associated fungi, making it a phytoalexin (Jeandet, 2015; Liu et al., 2021) (Chapter 4; AdditionalFig.1). The opposing effects of this diterpene on different fungi suggests that barley diterpenoid secretion has an impact on the plant microbiota. Indeed, changes in the composition of the microbiota have already been reported upon secretion of maize diterpenoids and other pathogen-induced plant metabolites (Jacoby et al., 2021; Liu et al., 2020; Murphy et al., 2021; Schmidt and Saha, 2021; Schütz et al., 2021).

Future research directions

The continous development of new scientific methods and concepts has led to enormous advances in the study of the composition, interconnections, and functions of plant-microbe associations (del Barrio-Duque et al., 2019; Durán et al., 2018; Mahdi et al., 2022; Meena et al., 2010). The results presented in this work contribute to answering some of the most current questions in the field of plant-microbe interactions by providing insights into the processes and dynamics underlying interactions between a beneficial root endophyte, a pathogen and different plant hosts in the absence and

presence of bacterial SynComs (Harris et al., 2020). We also present several large transcriptome data sets, new methods, and experimental designs that can be used to answer future research questions (Fig 2).

First, genetic variation between and within plant species has high ecological importance and comparable influence on community dynamics (Albert et al., 2010; des Roches et al., 2018). Nevertheless, the host genetic factors that shape the holobiont and its accompanying traits have only just been expored (Escudero-Martinez et al., 2022; Pérez-Izquierdo et al., 2019). Future projects may use natural species variation or mutant libraries to associate microbe-conferred traits with specific host loci or genes. Correlations between host genotypes, their ability to accommodate microbes and their affinity for microbiota-driven traits will broaden the basis for future breeding strategies (Fig.2; Q1). In this regard, the inter-kingdom mediated host beneficial effects identified here, and the developed screening method could serve as excellent model systems (Dunken et al., 2021; Mahdi et al., 2022).

Second, little is known about how interspecies dynamics within the holobiont change upon pathogen invasion. The study presented here on the function of barley diterpenoids in multi-kingdom interactions could not only be used to understand adaptation between pathogen and host, but also how the induction of plant immunity upon pathogen colonization affects the surrounding microbiota that must cope with the resulting environmental changes (Fig.2; Q2). The factors that determine niche adaptation, niche competition, and niche invasion in natural systems are still largely unknown (Fig. 2; Q3-5). It is important to understand how resilient endophytes interact with the host (Fig. 2; Q3) and surrounding microbes (Fig. 2; Q4) and dynamically adapt to the environment to sustain colonization (Mahdi et al., n.d., 2022; Sarkar et al., 2019). The prevalence of the main members of the microbiota within the holobiont might be related to their bioprotective properties. However, the underlying mechanisms are still largely unknown (Zheng et al., 2021)(Fig. 2; Q3-5). Further, it remains to be elucidated how successful pathogens overcome this protective microbiota barrier (Fig. 2; Q5).

Uncovering interkingdom interactions requires not only the identification of microbial or host-derived effectors, but also their underlying cues and regulatory mechanisms (Fig. 2; Q6). This requires the identification of species-specific transcription factors as well as their activity and regulation across biotic and abiotic stimulants. Such studies are

made possible by recent technological advances, including deep learning approaches and DAP-seq analyses, which allow the prediction of transcription factors within whole communities based on (meta)genomes and the identification of transcription factor binding sites (Bartlett et al., 2017; Oliveira Monteiro et al., 2022).

Answering these questions will bring us closer to the holistic understanding of plant-microbe interactions required to meet the agricultural needs of the future.

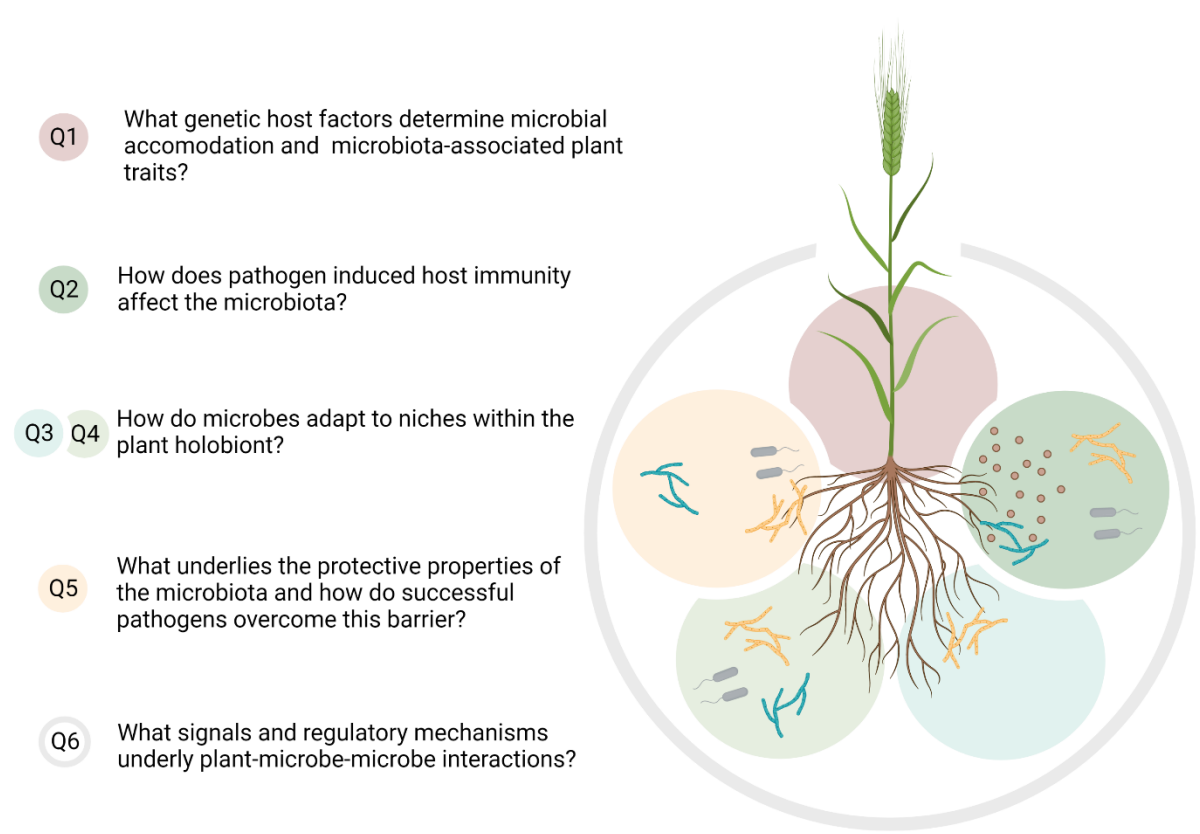

Figure 2: Future questions in the field of plant-microbiota research. The studies in this thesis provide insights into the dynamics underlying plant-microbe interactions. The established systems, methods, and data sets will help to answer several future research questions. What genetic host factors determine microbial accomodation and microbiota-associated plant traits? (Q1) How does pathogen induced host immunity affect the microbiota? (Q2) What microbe-host (Q3) and microbe-microbe (Q4) interactions underlie microbial niche adaptation within the holobiont? What underlies the protective properties of the microbiota and how do successful pathogens overcome this barrier? (Q5) What signals and regulatory mechanisms underlie these interactions between plants and microbes? (Q6).

Summary

Plant-microbe interactions are an emerging area of research with a great potential for developing future sustainable agricultural solutions. However, the outcome of such complex interactions can rarely be predicted due to the large number of underlying variables and influencing factors. The studies conducted in this thesis analyzed the multipartite interactions between plants and microbes from different perspectives to gain a comprehensive understanding of their outcomes and dynamics.

We describe the Sebacinales fungus *Serendipita vermifera* as a potential biocontrol organism against fungi and bacteria in largely distinct host species. *Sv*-mediated beneficial effects on host protection and growth are stable or potentiated in more complex environments. These beneficial effects are mediated mainly by the modulation of effector gene expression in *Sv* that executes interaction partner-specific transcriptional responses. On the plant side, we characterized the first diterpenoid synthesis pathway in barley and determined barley diterpenoid production and secretion as part of an immune response to the adapted pathogen *Bipolaris sorokiniana*. In contrast, colonization of *Sv* elicited only a moderate host immune response. *Sv* growth and colonization is highly resilient, regardless of the presence of other microbes or their impact on the host immune response, increasing its potential for biocontrol. In addition, we developed a screening method to quantify plant health and cell death and have provided several large transcriptome data sets that will help to unravel the molecular mechanisms underlying these multipartite interactions.

Zusammenfassung

Pflanzen-Mikrobiota Interaktionen bilden ein aufstrebendes Forschungsfeld mit einem hohen Potential für die Entwicklung nachhaltiger Lösungen für die Landwirtschaft. Aber die Auswirkungen solch komplexer Interaktionen können aufgrund einer Vielzahl von Variablen und Einflussfaktoren kaum vorhergesagt werden. Im Rahmen der hier vorgestellten Studien wurden Pflanze-Mikroben Interaktionen von verschiedenen Perspektiven untersucht, um ein möglichst umfassendes Verständnis von den Auswirkungen und Dynamiken dieser Interaktionen zu gewinnen. Wir beschreiben den Sebacinales Pilz *Serendipita vermifera* als potenziellen Biokontroll-Organismus gegen Pilze und Bakterien in unterschiedlichen Wirtspflanzen. Die vorteilhaften Effekte von *Sv* in den Bereichen Wirtsschutz und Wirtswachstum sind in komplexen Systemen stabilisiert oder intensiviert. Diese vorteilhaften Effekte hängen zu einem Großteil mit der regulierten Genexpression von *Sv* Effektoren zusammen, die je nach Interaktionspartner und Zeitpunkt sehr spezifische transkriptionelle Antworten generieren. In Bezug auf die Wirtspflanze, haben wir den ersten Diterpen-Syntheseweg in Gerste charakterisiert und belegt, dass diese Diterpene als Reaktion auf den Befall mit dem adaptierten Schädling *Bipolaris sorokiniana* produziert und sekretiert werden. Wir zeigen, dass im Vergleich dazu, eine Kolonisierung mit *Serendipita vermifera* nur eine geringe Immunantwort der Pflanze auslöst. Außerdem sind Wachstum und Kolonisierung von *Serendipita vermifera* sehr widerstandsfähig, sowohl gegenüber der Präsenz anderer Mikroben als auch gegenüber deren Einfluss auf die Immunantwort der Wirtspflanze. Diese Eigenschaften erhöhen die Chance, *Serendipita vermifera* zukünftig als Biokontrollorganismus nutzen zu können. Daneben haben wir eine optimierte experimentelle Methode zur Quantifizierung von Pflanzengesundheit und -zelltod entwickelt und eine Reihe umfangreicher Transkriptomik-Datensätze erstellt, die zukünftig dafür genutzt werden können die molekularen Mechanismen zu entschlüsseln, die diesen multilateralen Interaktionen zu Grunde liegen.

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- 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 extendet plant protection barrier in the rhizosphere. New Phytologist doi: 10.1111/nph.15904

AZ, GL, MB, and DS conceived the project and planned the experiments. DS, **LM**, and HR carried out inoculations, RNA extractions, RT-PCRs and confocal microscopy. DS, GJ, HR, SN, AT, GUB and GL analyzed the data. HR, DS and AZ wrote the manuscript. DS, HR and GJ contributed equally to this work.

- **Mahdi LK**, Miyauchi S, Uhlmann C, Garrido-Oter R, Langen G, Wawra S, Niu Y, Robertson-Albertyn S, Bulgarelli D, Parker JE, Zuccaro A (2021) The fungal root endophyte Serendipita vermifera displays inter-kingdom synergistic beneficial effects with the microbiota in Arabidopsis thaliana and barley. ISME Journal doi: 10.1038/s41396-021-01138-y

LM and AZ designed the experiments. **LM**, CU, DB, and SRA performed the experiments, isolated the barley bacterial strains, and designed the *Hv*SynCom. **LM**, SM, GL, YN, RGO, RG, and AZ analyzed the data. **LM**, AZ, JP, CU, and SM wrote the paper with editorial input from all other authors.

- **Mahdi LK***, Eichfeld R*, Miyauchi S*, Concetta De Quattro, Vivian Ng, Daniel Peterson, Igor Grigoriev, Langen G, Zuccaro, A (*in prep.*) Plant host and microbe specific gene expression in the mutualistic root endophytic fungus *Serendipita vermifera* In prep

LM, GL and AZ planed the experiments. **LM** performed the inoculations and sample preparations for the RNA seq analysis. YZ, ST, DP, VN and IG organized and performed the RNA sequencing. SM and CDQ performed the bioinformatic analysis and prepared the bioinformatic figures. **LM** interpreted the data. RE performed *Si* tripartite assays, the cloning, and the chitinase-activity assay. RE and **LM** performed the fungal toxicity assay. **LM** wrote the manuscript.

- Liu Y, Balcke GU, Porzel A, **Mahdi LK**, Scherr-Henning A, Bathe U, Zuccaro A, Tissier A (2021) A barley gene cluster for the biosynthesis of diterpenoid phytoalexins. BioRXiv doi: 10.1101/2021.05.21.445084

YL performed the LC-MS, GC-MS, yeast and *N. benthamiana* expression and gene expression analysis. GB provided supervision for LC-MS and data analysis. AS-H provided assistance for LC-MS sample preparation. AP performed the NMR measurements and analyses. UB supervised the yeast expression experiments. **LM** and AZ provided infected barley samples, media samples and transcriptomic data. **LM** established protocols and performed experiments to harvest fungal free media and test antifungal activity of the purified diterpene via multiple readouts. AT and GB designed the project. **LM** performed colonization experiments with barley KSL4 and CPS2 mutants. AT designed and supervised the project. AT wrote the abstract, introduction, results and discussion. YL wrote the materials and methods, except the NMR part (AP) and LC-MS (GB). AT, YL and GB prepared the figures. All authors read and approved the manuscript. The final version of the manuscript will contain the full set of the above-mentioned data.

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ND, **LM** and AZ developed the protocol and wrote the manuscript, RH corrected the manuscript.

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Erklärung

Hiermit versichere ich an Eides statt, dass ich die vorliegende Dissertation selbstständig und ohne die Benutzung anderer als der angegebenen Hilfsmittel und Literatur angefertigt habe. Alle Stellen, die wörtlich oder sinngemäß aus veröffentlichten und nicht veröffentlichten Werken dem Wortlaut oder dem Sinn nach entnommen wurden, sind als solche kenntlich gemacht. Ich versichere an Eides statt, dass diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; dass sie -abgesehen von den oben angegebenen Teilpublikationen- noch nicht veröffentlicht worden ist sowie, dass ich eine Veröffentlichung der Dissertation vor Abschluss der Promotion nicht ohne Genehmigung des Promotionsausschusses vornehmen werde. Die Bestimmungen dieser Ordnung sind mir bekannt. Darüber hinaus erkläre ich hiermit, dass ich die Ordnung zur Sicherung guter wissenschaftlicher Praxis und zum Umgang mit wissenschaftlichem Fehlverhalten der Universität zu Köln gelesen und sie bei der Durchführung der Dissertation zugrundeliegenden Arbeiten und der schriftlich verfassten Dissertation beachtet habe und verpflichte mich hiermit, die dort genannten Vorgaben bei allen wissenschaftlichen Tätigkeiten zu beachten und umzusetzen. Ich versichere, dass die eingereichte elektronische Fassung der eingereichten Druckfassung vollständig entspricht.

Die von mir vorgelegte Dissertation ist von Prof. Alga Zuccaro betreut worden.

Köln, den 18.12.2022

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• Personal Information

Date of Birth:	30.08.1991
Place of Birth:	Saarlouis, Germany
Nationality:	German

• University Educational History

2018-2022	University of Cologne, and Max Planck Institute for Plant Breeding Research Cologne, Germany **PhD thesis:** Characterization of the mechanisms underlying plant-microbe multi-kingdom interactions AG Zuccaro, University of Cologne, Germany
2015-2018	University of Cologne and Max Planck Institute for Plant Breeding Research Cologne, Germany Degree: Master of Science **Master thesis:** The HeLo-domain: evolutionary link between animal and plant cell death AG Schulze-Lefert, Max Planck Institute for Plant breeding research, Germany
2012-2015	University of Cologne, Germany Degree: Bachelor of Science **Bachelor thesis:** Functional Analysis of GRAS Proteins RAD1 and RAM1 AG Bucher, University of Cologne, Germany

• Work and Research History

05.2018 – 09.2018	**Student helper contract** AG Paul Schulze-Lefert, MPIPZ Cologne, Germany

	Continuation of my Master Project: The HeLo-domain: evolutionary link between animal and plant cell death **Techniques:** Protoplast assay (in *Arabidopsis Thaliana*), Microscopy (mainly inverted LSM880 Zeiss), pathogen assays (*Pseudomonas syringae, Botrytis cinerea, Golovinomyces orontii*)
03.2017-05.2017	**Internship** AG Höcker, University of Cologne, Germany **Title:** Fine mapping CRY1 C-terminal domain motifs for interaction with COP1 in *Arabidopsis thaliana* **Techniques:** Yeast-two-hybrid
02.2017-05.2017	**Internship** AG Schmitz, University Duisburg - Essen, Germany **Title:** "Metabolom analysis of *Hedyotis diffusa* and *Sutellaria barbata* extracts used in traditional Chinese medicine" **Techniques:** LC-IM-MS
10.2016-12.2016	**Project** AG Plomann, Colonge, Germany **Title:** "Role of PACSIN3 in differentiation and autophagy of keratinocytes" **Techniques:** model cells: primary mouse keratinocytes and HaCaT cell line, Westernblot, siRNA knockdown generation, cell transfection and different staining
05.2016-07.2016	**Project** AG Bucher, Cologne, Germany **Title:** "Quantification of fungal isolate 229 colonization in *Arabis Alpina* under low and high phosphate conditions using quantitative PCR and microscopy" **Techniques:** Go-Taq PCR, qRT-PCR (absolute quantification), various fungal staining methods
03.2015-04.2015	**Internship** Julius Kühn Institute, Quedlinburg, Germany **Title:** "Untersuchungen zur Fungizidtoleranz von *Fusarium Culmorum*" **Techniques:** RT-PCR, GoTaq PCR, growth experiments, Microscope
10.2014-11.2014	**Internship** AG Kopriva, Cologne, Germany

Title: “Interaktion von Pflanzen und Bakterien im Boden”
Techniques: model plant: *Arabidopsis Thaliana*, RT-PCR, DNA extraction

- Peer Reviewed Publication

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 doi: 10.1111/nph.15904

Mahdi LK, Schmitz OJ (2020) Herbal Medicine Using High Performance Liquid Chromatography Ion-Mobility Mass Spectrometry. The Column 16/5 (2020) 2-7

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Mahdi LK*, Miyauchi S*, Uhlmann C, Garrido-Oter R, Langen G, Wawra S, Niu Y, Robertson-Albertyn S, Bulgarelli D, Parker JE, Zuccaro A (2021) The fungal root endophyte *Serendipita vermifera* displays inter-kingdom synergistic beneficial effects with the microbiota in Arabidopsis and Barley. ISME Journal doi: 10.1038/s41396-021-01138-y

Dunken N*, **Mahdi LK***, Häusler ER, Zuccaro A (2022) Monitoring Cell Death via Ion leakage and PAM Fluorometry. Chapter in MiMB (Methods in Molecular Biology), Humana Press, Springer Nature in press

Liu Y, Balcke GU, Porzel A, **Mahdi LK**, Scherr-Henning A, Bathe U, Zuccaro A, Tissier A (2021) A barley gene cluster for the biosynthesis of diterpenoid phytoalexins. BioRXiv doi: 10.1101/2021.05.21.445084

Vanacore A, Vitiello G, Wanke A, Cavasso D, Clifton L, **Mahdi LK**, Campanero-Rhodes MA, Solís D, Wuhrer M, Nicolardi S, Molinaro A, Marchetti R, Zuccaro A, Paduano L, Silipo A (2022) Lipopolysaccharide O-antigen molecular and supramolecular modifications of plant root microbiota are pivotal for host recognition. Carbodhydrate Polymers doi: 10.1016/j.carbpol.2021.118839

Chandrasekar B, Wanke A, Wawra S, Saake P, **Mahdi LK**, Charura N, Neidert M, Malisic M, Thiele M, Pauly M, Dama M, Zuccaro A (2022) Fungi hijack a ubiquitous plant apoplastic endoglucanase to release a ROS scavenging β-glucan

decasaccharide to subvert immune responses. The Plant Cell doi:10.1093/plcell/koac114

• Scientific Meetings and Conferences

02.2019	annual Meeting SPP Meeting 1. Funding period, Cologne, Germany Poster Presentation
04.2019	Annual IMPRS PhD retreat, Hennef, Germany Oral Presentation
07.2019	18th biennial Congress on Molecular Plant- Microbe Interactions (MPMI XVIII Congress) in Glasgow, UK 2 Poster Presentations
09.2020	annual Meeting SPP DECRyPT online Oral Presentation
10.2020	Annual IMPRS PhD retreat, online Poster Presentation
03.2021	23. Jahrestagung des AK Biologischer Pflanzenschutz online Oral Presentation
04.2021	annual Meeting SPP DECRyPT online Oral Presentation
08.2021	Annual IMPRS PhD retreat, hybrid event in Colonge, Germany Oral Presentation
12.2021	biennial Congress on Molecular Plant- Microbe Interactions (MPMI Congress) online Poster Presentation
02.2022	Annual conference of the association for general and applied microbiology (VAAM) 2022 online
03.2022	annual Meeting SPP Meeting 2. Funding period, Cologne, Germany Oral Presentation
04.2022	Workshop on Biotrophy and Plant Immunity in Bonn, Germany Oral Presentation
08.2022	Botanik Tagung 2022 in Bonn Germany Poster Presentation

• Teaching History

Since 2018	Student supervision for internships, bachelor- and master thesis

Since 2019	Supervision of the master course “Molecular Plant-Microbe Interactions
Winter semester 2020	Supervision of the bachelor course Molekulare Pflanzenphysiologie (mPlant3)

- Work related Responsibilities

2019-2020	IMPRS Graduate School representative
Since 2018	Co-organization of all SPP DECRyPT meetings
07.2019 – 09.2020	Member of the Organizing committee of the University of Cologne Mini Symposium
08.2022	Speaker at the Soapbox Science Rhineland event in Bonn, Germany

- Courses and Career Development Workshops

12.2018	Scientific Writing (Avril Arthur-Goettig IMPRS)
11.2019	Self and Time Management (Sabine Lerch, IMPRS)
11.2019	Statistical Literacy (Rick Scavetta IMPRS)
04.2019	Data analysis with R (Rick Scavetta IMPRS)
07.2021-08.2021	Resilience for Scientists (Ben Hartwig, IMPRS)
09.2021	Basics of Science Communication (Richard Fuchs, NaWik, CEPLAS)
09.2021	Successfully communicating your research – The basis of science communication and journalistic writing for scientists (Ann-Lynn Wegener and Susanne Günther, CEPLAS)
12.2021	Biases in Science (Prof. Niels Gehring, GSfBS)

Bergisch Gladbach, 18.12.2022 

Lisa Katharina Mahdi