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

Debika Sarkar1*, Hanna Rovenich1*, Ganga Jeena1*, Shadab Nizam1, Alain Tissier2, Gerd U. Balcke2, Lisa K. Mahdi1, Michael Bonkowski3, Gregor Langen1, and Alga Zuccaro1

1Botanical Institute, Cluster of Excellence on Plant Sciences (CEPLAS), University of Cologne, 50674, Cologne, Germany; 2Department of Cell and Metabolic Biology, Institute of Plant Biochemistry, 06120, Halle (Saale), Germany; 3Institute of Zoology, Terrestrial Ecology, Cluster of Excellence on Plant Sciences (CEPLAS), University of Cologne, 50674, Cologne, Germany

Author for correspondence:
Alga Zuccaro
Tel: +49 221 470 7170
Email: azuccaro@uni-koeln.de

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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 *tvhyd1* 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* (Laut et al., 2018). However, the...
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; Fakhiro 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 sequenc-

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.
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 (BpPNM) generated more biomass than S. vermifera (SpPNM) 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 + BpPNM, 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, 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 log2-fold expression changes (logFC) ≥ |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,
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.
with *B. sorokiniana* (Fig. S4a; Table S4). Correspondingly, an over-representation of *S. vermifera* genes encoding carbohydrate-active enzymes (CAZymes, 8.2%, $\chi^2$ P-value < 0.0001) and secreted proteins (24.8%, $\chi^2$ 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 carbohydrate-active enzymes (CAZymes, 8.2%, $\chi^2$ P-value < 0.0001) and secreted proteins (24.8%, $\chi^2$ P-value < 0.0001) were 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 with *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.
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   |
| Colisation in planta                | SvLOC     | 781    | 5.1   | 868    | 5.67   |
| Colisation in planta                | SvLOC + BSSYS | 629 | 4.11 | 667    | 4.36   |
| Colisation in planta                | SvLOC + BSLOC | 802 | 5.24 | 747    | 4.88   |
| Genes in *B. sorokiniana*          |           |        |       |        |        |
| Confrontation in soil              | Sv + Bs   | 1275   | 10.41 | 974    | 7.95   |
| Colisation in planta                | BsLOC     | 1481   | 12.09 | 1079   | 8.81   |
| Colisation in planta                | SvsYS + BsLOC | 1301 | 10.62 | 1081   | 8.82   |
| Colisation in planta                | SvLOC + BsLOC | 1341 | 10.95 | 1064   | 8.69   |

Numbers of differentially expressed genes with a log2-fold change ≥|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; $\chi^2$ P-value < 0.0001). CAZymes, particularly chitinases and β-1,6-gluconases, are fungal cell wall-degrading enzymes (CWDEs) that are important facilitators of mycoparasitism (Druzhinina *et al*., 2011). The expression of the *S. vermifera* gene Sevch1_06375 (hereafter referred to as *SvCHIT1*), which encodes a chitinase belonging to the glycosyl hydrolase (GH) family 18 with a ChBD3 chitin-binding domain found specifically in Agaricomycota and chitinolytic bacteria, increased 13-fold only during direct confrontation with the pathogen in soil (Table S5). The hydrolytic domain of *SvCHIT1* 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*, Bips1_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; $\chi^2$ 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
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.

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). Log₂ 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.
GO enrichment analysis of upregulated \textit{S. vermifera} genes during bipartite (SvLOC, not shown) or tripartite (SvLOC + BsLOC) 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, \textit{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; \( \chi^2 \) P-values < 0.0001). Notably, the groups of \textit{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 \textit{S. indica} showed that genes encoding hexose transporters are significantly induced during later colonisation stages (Zuccaro \textit{et al.}, 2011), including the high-affinity glucose transporter HXT5 (Rani \textit{et al.}, 2016). Our analysis showed that the closest homologue of \textit{SiHXT5}, SevH1_09710 (here referred to as \textit{SeHXT}), is among the most highly expressed barley-responsive genes at 6 dpi (27-fold; Table S8). Quantitative RT-PCR confirmed that \textit{SeHXT} is induced during barley root colonisation on PNM and in soil and its expression is independent of the presence of \textit{B. sorokiniana} (Fig. S5c, right panel), representing a valuable \textit{S. vermifera} symbiosis marker gene. GO enrichment analysis of downregulated \textit{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 \textit{S. indica}, \textit{S. vermifera} is experiencing nitrogen starvation at the onset of saprophytism, at c. 6 dpi in barley (Lahrman \textit{et al.}, 2013).

A similar situation was found for \textit{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, \( \chi^2 \) P-value < 0.0001), secreted protein (17.8% vs 4.9% in genome, \( \chi^2 \) P-value < 0.0001) and transporter (7.4% vs 4.7% in genome, \( \chi^2 \) 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 \textit{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 \textit{Bipolaris} genus (Finking & Marahiel, 2004; Condon \textit{et al.}, 2013). Five of the induced \textit{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 \textit{B. sorokiniana} infecting leaves of barley cv Bowman (Condon \textit{et al.}, 2013). We confirmed by RT-PCR that the expression of Bipso1_12059, referred to as \textit{BsNPS1}, was significantly induced during barley root infection on PNM and in soil, and this induction was independent of the presence of \textit{S. vermifera} (Fig. S5d, right panel), confirming \textit{BsNPS1} as a robust virulence marker gene (Condon \textit{et al.}, 2013). A closer inspection of barley-responsive genes of \textit{B. sorokiniana} (upregulated in BsLOC and SvLOC + BsLOC) 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 glitoxin biosynthetic enzymes of \textit{Aspergillus fumigatus} (Schreitl \textit{et al.}, 2010; Dolan \textit{et al.}, 2015), was 16- to 2000-fold higher during infection compared with \textit{B. sorokiniana} growing in soil (Table S8). GO term enrichment analysis identified distinct processes among downregulated \textit{B. sorokiniana} genes during infection that are different from those found for \textit{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 \textit{et al.}, 2018), \textit{B. sorokiniana} utilises secondary metabolites and potentially detoxifies phytoalexins to successfully infect host roots. Interestingly, by contrast with the gene expression changes in \textit{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 \textit{B. sorokiniana} from non-\textit{B. sorokiniana}-infected roots. Strikingly, samples isolated from mock-treated and \textit{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 \( \geq 1 \)) compared with mock-treated roots (Table 2). By contrast, infection with \textit{B. sorokiniana} resulted in the differential expression of 2741 barley genes (1643 with logFC \( \geq 1 \)) 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 \textit{S. vermifera} locally or systemically (Figs 4b, S8; Table S9). Accordingly, GO term enrichment analysis on barley genes upregulated during \textit{B. sorokiniana} infection in absence and presence of \textit{S. vermifera} (BsLOC and SvLOC + BsLOC, 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 \textit{B. sorokiniana}-induced genes were several \textit{PATHOGENESIS-RELATED} (\textit{PR}) genes including \textit{HvPR17a} (HORVU1Hr1G043910), \textit{HvPR17b} (HORVU7Hr1G013170),...
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 genes.
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 expression during host colonisation is affected by the presence of *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).

### 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 | Down (logFC ≤ −1) | %Down (logFC ≤ −1) |
|-----------|------|----|-----|------|-------|-----------------|----------------|-----|-------------------|-------------------|
| SvSYS     | 8    | 7  | 0.009 | 1 | 0.001 | 6              | 5              | 0.006 | 1                  | 0.001             |
| SvLOC     | 41   | 31 | 0.038 | 10 | 0.012 | 29             | 24             | 0.029 | 5                  | 0.006             |
| BsSYS     | 149  | 112| 0.137 | 37 | 0.045 | 91             | 68             | 0.083 | 23                 | 0.028             |
| BsLOC     | 2741 | 1872| 2.292 | 869 | 1.064 | 1643           | 1238           | 1.516 | 405                | 0.496             |
| SvLOC + BsSYS | 166 | 108| 0.132 | 58 | 0.071 | 143            | 95             | 0.116 | 48                 | 0.059             |
| SvSYS + BsLOC | 1540 | 1210| 1.481 | 330 | 0.404 | 1110          | 927            | 1.135 | 183                | 0.224             |
| SvLOC + BsLOC | 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 log2 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.

*Serendipita 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-Díez 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 Fo007 (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—fungus 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 AtTIP4;1 (Fig. S13), which transports small molecules and localises to the tonoplast in root epidermal cells (Gattolin et al., 2009). Generally, AtTIPs are implicated in transport of water, urea and H2O2 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 AtTIPs 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; Figueredo 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
Research 897

expressed fungal genes (DEGs) of S. vermifera highlighting the strong biotic stress the endophyte exerts onto the pathogen during saprotrophic growth. Organisms and categories of differentially expressed S. vermifera genes, especially those involved in DHN-melanin biosynthesis, are significantly upregulated in the pathogen during confrontation with B. sorokiniana. 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 over-representation 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,55a; Tables S4,55). 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 metabolism 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 664 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 gatekeeper. 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.

ORCID

Michael Bonkowski https://orcid.org/0000-0003-2656-1183
Gregor Langen https://orcid.org/0000-0002-8321-1756
Alain Tissier https://orcid.org/0000-0002-9406-4245
Alga Zuccaro https://orcid.org/0000-0002-8026-0114

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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 **Sersendipita vermifera** shows signs of mycoparasitism in direct confrontation with *B. sorokiniana*.

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

Fig. S3 *Sersendipita 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.

Fig. S8 UpSet graph of differentially expressed genes in barley.

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

Fig. S10 Pathogen infection induces putative terpenoid phytoalexin biosynthesis genes in barley.

Fig. S11 Phylogeny of barley enzymes with a putative role in gibberellin biosynthesis.

Fig. S12 Venn diagrams of differentially expressed fungal genes during colonisation and confrontation in soil or in planta.

Fig. S13 Phylogenetic analysis indicates that HORVU4Hr1G085250.5 belongs to the family of tonoplast intrinsic proteins.

Methods S1 Growth media and cultivation conditions.

Methods S2 Multipartite interaction experiments.

Methods S3 Bioinformatics analyses.

Methods S4 Phenotyping.

Table S1 List of primers used for qRT-PCR.

Table S2 Proportions of reads mapped onto annotated barley and fungal genes.

Table S3 Overview of differentially expressed genes in *S. vermifera*, *B. sorokiniana* and barley during bipartite and tripartite interactions as well as direct fungal confrontation.

Table S4 Gene ontology (GO) term enrichment for differentially expressed *S. vermifera* and *B. sorokiniana* genes during direct confrontation in soil.

Table S5 Annotation categories among differentially expressed fungal genes.

Table S6 List of *B. sorokiniana* genes encoding secreted proteins that are specifically repressed during direct confrontation with *S. vermifera* (C2; Fig. 3).

Table S7 Gene ontology (GO) term enrichment for differentially expressed *S. vermifera* and *B. sorokiniana* genes during barley root colonisation.

Table S8 Barley-responsive fungal gene expression.

Table S9 Fungal-responsive barley gene expression in infected root tissue.

Table S10 Gene ontology (GO) term enrichment for barley genes induced during *B. sorokiniana* infection in the absence or presence of *S. vermifera*.

Table S11 MEP pathway and CYP gene expression in infected barley roots.

Table S12 Fungal-responsive fungal gene expression during confrontation in planta.

Table S13 Gene ontology (GO) term enrichment analysis of *B. sorokiniana* genes downregulated during confrontation in planta.

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