

Pairwise Transcriptomic Analysis of the Interactions Between the Ectomycorrhizal Fungus *Laccaria bicolor* S238N and Three Beneficial, Neutral and Antagonistic Soil Bacteria

Aurélie Deveau · Matthieu Barret · Abdala G. Diedhiou ·
Johan Leveau · Wietse de Boer · Francis Martin ·
Alain Sarniguet · Pascale Frey-Klett

Received: 19 February 2014 / Accepted: 9 June 2014 / Published online: 2 August 2014
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Abstract Ectomycorrhizal fungi are surrounded by bacterial communities with which they interact physically and metabolically during their life cycle. These bacteria can have positive or negative effects on the formation and the functioning of ectomycorrhizae. However, relatively little is known about the mechanisms by which ectomycorrhizal fungi and associated bacteria interact. To understand how ectomycorrhizal fungi perceive their biotic environment and the mechanisms

supporting interactions between ectomycorrhizal fungi and soil bacteria, we analysed the pairwise transcriptomic responses of the ectomycorrhizal fungus *Laccaria bicolor* (Basidiomycota: Agaricales) when confronted with beneficial, neutral or detrimental soil bacteria. Comparative analyses of the three transcriptomes indicated that the fungus reacted differently to each bacterial strain. Similarly, each bacterial strain produced a specific and distinct response to the presence of the fungus. Despite these differences in responses observed at the gene level, we found common classes of genes linked to cell–cell interaction, stress response and metabolic processes to be involved in the interaction of the four microorganisms.

A. Deveau · A. G. Diedhiou · F. Martin · P. Frey-Klett
Interactions Arbres – Microorganismes, INRA UMR1136,
54280 Champenoux, France

A. Deveau · A. G. Diedhiou · F. Martin · P. Frey-Klett
Interactions Arbres – Microorganismes, Université de Lorraine
UMR1136, 54500 Vandoeuvre-lès-Nancy, France

M. Barret · A. Sarniguet
INRA UMR1349 IGEPP, 35653 Le Rheu, France

J. Leveau
Department of Plant Pathology, University of California, Davis,
CA 95616, USA

W. de Boer
Department of Microbial Ecology, Netherlands Institute of Ecology
(NIOO-KNAW), Wageningen, The Netherlands

A. Deveau (✉)
Interactions Arbres Micro-organismes, INRA Lorraine Université,
UMR 1136, 54280 Champenoux, France
e-mail: Aurelie.Deveau@nancy.inra.fr

Present Address:
M. Barret
INRA – IRHS, 42 rue Georges Morel, 49071 Beaucouze, France

Present Address:
A. G. Diedhiou
Laboratoire Commun de Microbiologie, IRD/ISRA/UCAD Centre
de Recherche de Bel-Air, BP1386, 18524 Dakar, Senegal

Introduction

Rhizospheres form a complex ecosystem in which roots interact with communities of highly diverse microorganisms, which is known as the microbiome [1, 2]. Similar to the human gut microbiome which can impact fundamental host processes ranging from physiology to immunology [3], plants cope with microorganisms living inside and outside their tissues and rely on some for nutrient acquisition and defence against pests [4]. Ectomycorrhizal fungi play a particularly important role by providing mineral nutrients in exchange for carbohydrates and by stimulating the plant's defence systems [5]. As a part of the rhizosphere microbiome, ectomycorrhizal fungi themselves are permanently surrounded by numerous microorganisms including bacteria with which they interact both physically and metabolically [6]. Our knowledge is relatively limited about how ectomycorrhizal and bacterial consortia establish and maintain themselves as well as the identity and characteristics of molecular interactions between these microorganisms. Ectomycorrhizal fungi perceive the presence of host roots and are chemoattracted towards these

roots [7]. Ectomycorrhizal fungi also modulate their physiology and respond at the transcription level to the presence of mycorrhiza helper bacteria which stimulate mycorrhiza formation [8–11]. However, whether the fungal response is specific to beneficial bacteria or extends towards neutral or detrimental bacteria is not known. Surprisingly, relatively little is known about the molecular mechanisms involved in the interaction between ectomycorrhizal fungi and bacteria. To understand the specificities of these molecular pathways, we conducted a simultaneous analysis of transcriptomic responses of the ectomycorrhizal fungus *Laccaria bicolor* S238 with three soil bacteria during their interactions. The three different soil bacterial strains were chosen for their contrasting effects on the growth and morphology of the fungus [8]: *Pseudomonas fluorescens* BBc6R8, a mycorrhiza helper strain of *L. bicolor* S238N which had been isolated from a sporocarp of the fungus [8], *P. fluorescens* Pf29Arp, a strain commensal to *L. bicolor* S238N [12, 13] and *Collimonas fungivorans* Ter331, a strain antagonistic to *L. bicolor* S238N [8, 14]. While strain BBc6R8 stimulates radial growth, the apex density and the branching angle and density of the fungal mycelium, strain Pf29Arp only enhances the branching angle of the hyphae. In contrast, the antagonist strain Ter331 inhibits radial growth of the fungus but stimulates hyphal branching and density. In parallel, we also analysed the differential responses of the three bacterial strains to the presence of the ectomycorrhizal fungus at the transcriptomic level to test whether and how the bacteria reacted to the interaction with the fungus.

Materials and Methods

Fungal–Bacterial Confrontation Bioassay

The ectomycorrhizal basidiomycete *L. bicolor* S238N was maintained on Pachlewski agar medium P5 [15] at 25 °C for 3 weeks. *P. fluorescens* BBc6R8, *P. fluorescens* Pf29Arp and *C. fungivorans* were stored at –80 °C in LB medium [16] with 20 % glycerol added. To prepare the bacterial inocula for the in vitro bioassays with *L. bicolor*, the bacterial strains were first grown on 10 % TSA plates (tryptic soy broth from Difco and 15 g l⁻¹ of agar) at 25 °C for 65 h. The bioassay was prepared as described by Deveau et al. [8]. A plug of *L. bicolor* S238N was cut out from the edge of a colony grown on P5 medium (0.5 g Di-NH₄⁺ tartrate, 1 g KH₂PO₄, 0.5 g MgSO₄, 20 g glucose, 5 g maltose, 1 ml 1/10 diluted Kanieltra microelement solution, 1 ml thiamine 100 mg/l⁻¹ solution, and 20 g agar l⁻¹ at pH 5.5 for 1 l) and transferred into the centre of a P20Th– plate (0.5 g Di-NH₄⁺ tartrate, 1 g KH₂PO₄, 0.5 g MgSO₄, 1 g glucose, 1 ml 1/10 diluted Kanieltra microelement solution, and 20 g agar l⁻¹ at pH 5.5 for 1 l). Four 10- μ l droplets bacterial suspension (OD_{600 nm}

0.7 in sterile water) were distributed at 1.2 cm from the centre of the fungal plug of mycelium (Fig. 1). Control treatments were performed, either without fungus or where bacteria were replaced by sterile water droplets. Plates were sealed with plastic tape and incubated at 10 °C in the dark. For each fungal treatment and corresponding mock control, mycelia of *L. bicolor* S238N from 50 plates were collected, frozen in liquid nitrogen and pooled. For each bacterial treatment and corresponding mock control, half of the four colonies facing the mycelium were collected from ten plates, frozen in liquid nitrogen and pooled. Fungal and bacterial samples were collected from independent plates to avoid changes in transcriptomic responses caused by handling of the plates during the isolation of the interacting partner. The full experiment was performed three times independently.

Fungal RNA Isolation and cDNA *L. bicolor* S238N Array Hybridization

After 14 days of incubation in the dark at 10 °C, the whole mycelium from 50 plates was collected, frozen in liquid nitrogen, pooled and stored at –80 °C. RNA from 50 mg of frozen mycelium was then extracted using the RNeasy Plant mini kit (Qiagen, Hilden, Germany) as recommended by the

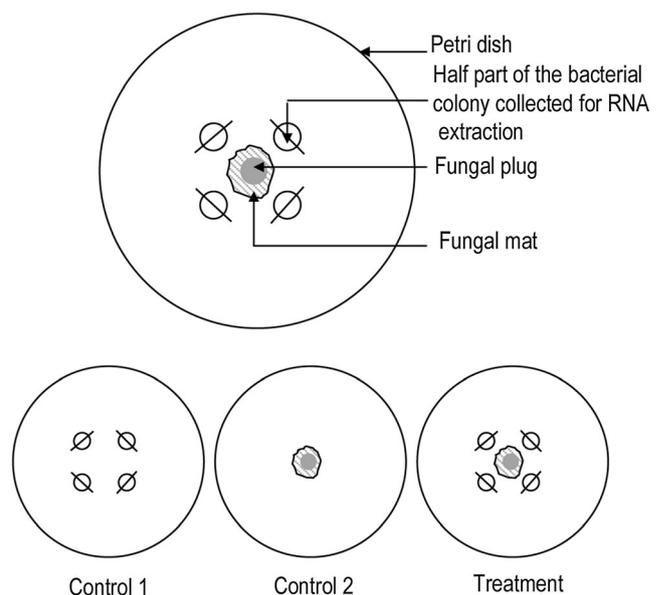


Fig. 1 Scheme of the in vitro confrontation assay and of the sampling strategy for transcriptomic analyses. Four droplets of bacterial suspension (white circles) were distributed at 1.2 cm from the center of the fungal plug of mycelium (grey circle). Control treatments without fungus (control 1) or in which bacteria were replaced by sterile water droplets (control 2) were performed. For each fungal treatment and corresponding mock control, mycelium of *L. bicolor* S238N from 50 plates was collected, frozen in liquid nitrogen and pooled. For each bacterial treatment and corresponding mock control, the half part of the four colonies facing the mycelium was collected from ten plates, frozen in liquid nitrogen and pooled

manufacturer for fungal material. Quality of RNA was checked by RNase-free 1 % agarose electrophoresis. Synthesis of complex cDNA probes from 400 ng of fungal RNA and cDNA hybridization on a Nylon membrane containing 4,992 cDNAs were performed as described in Deveau et al. [8]. Identification of cDNA clones corresponding to up- and down-regulated transcripts was carried out as described by Deveau et al. [8].

Validation of Expression Array Data

Validity and reproducibility of array data were checked by calculating the Pearson correlation coefficient which was over 0.95 for all set of arrays (data not shown). Data quality assessment was performed through analysis of variance (*t*-test), and a Bayesian statistical framework was implemented in the Cyber-T web interface [17] as described by Duplessis et al. [18]. Regulated transcripts were identified by the following criteria: (1) when significant modification (Bayesian *t*-test) of gene expression was detected in at least two of the three replicates and (2) when the average of fold changes (treatment vs. control) in the expression level was observed at plus or minus twofolds (Duplessis et al., 2005). A Posterior Probability of Differential Expression test (PPDE test) was used to determine the global false positive and negative levels. The complete expression data set is available as a series (accession numbers GSE53608 and GSE53609) at the Gene Expression Omnibus at NCBI (<http://www.ncbi.nlm.nih.gov/geo/>). Finally, quantitative RT-PCR measurements were performed on five fungal transcripts with altered levels of expression in response to at least one of the three bacterial strains (tectonin 2, hypothetical protein 456319, hypothetical protein 656382, glutathione-*S*-transferase 674829, hypothetical protein 304792). Eight non-regulated genes were tested as controls and the most stable of the two (hypothetical protein 701050-CloneLB17E10 and trehalose phosphorylase) were used for data normalization as previously described by

Deveau et al. [8]. Primer design and qRT-PCR were performed as described by Deveau et al. [8]. Primer sequences are provided in Table 1.

Bacterial RNA Isolation

All samples were collected after 14 days of incubation before contact occurred between bacterial cells and fungal hyphae. The semicircular section of the bacterial colonies from *P. fluorescens* BBc6R8, Pf29Arp or *C. fungivorans* Ter331 which faced the mycelium (Fig. 1) was collected from ten plates, frozen in liquid nitrogen and then pooled. Three independent biological replicates per treatment were performed except for *C. fungivorans* Ter331 for which two replicates were obtained. RNA was then extracted using Trizol (Invitrogen, Stockholm, Sweden) as recommended by the manufacturer. One hundred micrograms of total RNA were further purified using the RNeasy Plant mini kit (Qiagen, Hilden, Germany) as recommended by the manufacturer. Quality of RNAs was checked by electrophoresis using an Experion automated electrophoresis system (Biorad, USA). RNAs were then used for hybridization against a microarray specific for BBc6R8 (this paper), Ter331 [19] or Pf29Arp [20].

BBc6R8 Microarray Design, Hybridization and Analysis

BBc6R8 cDNA microarray synthesis, array hybridization and scanning were performed by NimbleGen (Roche NimbleGen Systems, Iceland). The cDNA were synthesized from 20 µg total RNA. The *P. fluorescens* BBc6R8 NimbleGen expression array was designed based on the initial genome annotation (available on Genbank—referenced under accession numbers AKXH01000001–AKXH01000163). The array was designed as follows: four oligomers of 60-nucleotides-long probes were designed for 6,469 of the predicted open reading frames. For each probe, three replicates were synthesized on the array. Probes for 16 genes from *L. bicolor* S238N were then added and used to quantify the background signal.

Table 1 List of the primers used for quantitative RT-PCR analyses

Gene name	Laccaria v2 protein ID	Primer sequences	Reference
Hypothetical protein with atypical PIKK/TRRAP protein kinase domain	456319	5' AGGGCAATACACTGAGGACAA 3' 5' TAGAGGGTGATCCGTTTCCA 3'	Deveau et al. 2007
Glutathione S transferase	674829	5' CCTTTTCGAGTTCCACAGCAT 3' 5' GATTAGGCCCGGTGTACCTT 3'	Deveau et al. 2007
Hypothetical protein 304792	304792	5' CCCGAGCCTTCTCTCTCTT 3' 5' TGTGCTTGTGGAGTCTGAGG 3'	This study
Tectonin 2	399271	5' GGCGGTAGCCTGGTTGAT 3' 5' CGTCGGGAACGGACAC 3'	Deveau et al. 2007
Trehalose phosphorylase	293441	5' ACCCGACTCTGGCTGGAC 3' 5' AGTACGGGGTATGGGGAGAC 3'	Deveau et al. 2007
Hypothetical protein clone Lb17E10	701050	5' TTCCACCGTCTCCGTTCC 3' 5' GGTTACCAAGTCGGGAGAGC 3'	Deveau et al. 2007

BBc6R8 cDNA microarray synthesis, array hybridization and scanning were performed by NimbleGen (Roche NimbleGen Systems, Iceland). The cDNA were synthesized from 20 µg total RNA. Average expression levels were calculated for each gene from the independent probes on the array and then used for further analysis. Raw array data were normalized by the robust multiarray average (RMA) routine using the ARRA YSTAR software (Dnastar Inc., Madison, WI, USA). A Student's *t*-test with FDR correction (Benjamini–Hochberg) was applied to the data using ARRAYSTAR. Transcripts with a significant *P*-value (<0.05) and \geq twofold change in transcript level were considered to be differentially expressed. The complete expression data set is available as a series (accession number GSE38243) on the Gene Expression Omnibus at NCBI (<http://www.ncbi.nlm.nih.gov/geo/>).

Pf29A DNA Microarray Hybridization and Data Analysis

Shotgun DNA microarrays used are fully described in Barret et al. [20]. Hybridization, scan and analyses were performed as previously described [20]. The contribution of each experimental factor (gene, fungus, biological replicate, dye) and the interactions between them were evaluated using a global ANOVA. Differentially expressed genes were selected by imposing stringent cutoffs (see “Results” section) on the local ANOVA graphic of the interaction ‘gene–fungus’.

The complete expression data set is available as a series (accession number GSE55147) on the Gene Expression Omnibus at NCBI (<http://www.ncbi.nlm.nih.gov/geo/>).

Ter331 Microarray Hybridization and Data Analysis

Ter331 cDNA microarray synthesis, array hybridization and scanning were performed by NimbleGen (Roche NimbleGen Systems, Iceland) as previously described in Mela et al. [19]. The cDNA were synthesized from 20 µg total RNA. Chip information is available via the MIAMExpress database (accession number A-MEXP-1876). Average expression levels and raw data analysis were the same as for BBc6R8 analysis. The complete expression data set is available as a series (accession number GSE54533) on the Gene Expression Omnibus at NCBI (<http://www.ncbi.nlm.nih.gov/geo/>).

Results

Bacterial Strains Differentially Impact Gene Expression in *L. bicolor* S238N

In S238N, 22, 31 and 83 cDNAs representing 16, 14 and 70 transcripts were found to be differentially expressed (\leq or $>$ twofold) in the presence of *P. fluorescens* BBc6R8, Pf29Arp

and *C. fungivorans* Ter331, respectively, compared to unexposed cultures of the fungus (Table 2). Levels of regulation varied between two- and 11 folds, the highest levels of regulation being observed for strain *C. fungivorans* Ter331. All S238N transcripts were up-regulated by *P. fluorescens* BBc6R8 and Pf29Arp, while 12 and 58 transcripts were up- and down-regulated in response to *C. fungivorans* Ter331, respectively. Functional categories of S238N transcripts regulated by the three bacteria are presented in Fig. 2. A Venn diagram was used to visualize how similar the responses of *L. bicolor* S238N were between the three types of interactions (Fig. 3). Most of the transcripts fell into the outer “unique” zones of the Venn diagram, implying that their regulations were strain specific. Only three transcripts were co-regulated by the different bacterial strains. Those three responsive transcripts encoded for a metallothionein (transcript ID 388066), a small hypothetical protein of 51 amino acids specific to *L. bicolor* S238N (308883) and a hypothetical protein Tral (456319) potentially involved in transcription regulation (Table 2). All were down-regulated in response to Ter331 and up-regulated in response to BBc6R8 and Pf29Arp. A transcript encoding for tectonin 2 was up-regulated in the presence of BBc6R8 and Pf29Arp. The transcript was not found to be significantly up-regulated in the presence of Ter331 on arrays because of high variability in the signal intensity between arrays for this transcript. However, quantitative RT-PCR indicated that it was also overexpressed in response to Ter331 (*t*-test, $P < 0.01$; Table 2). In addition, two transcripts encoding two hypothetical proteins only found so far in the genome of *L. bicolor* S238N (608752 and 656382) were co-regulated in the presence of BBc6R8 and Pf29Arp (Fig. 3). Transcripts induced only by BBc6R8 were involved in transcription (splicing factor 3b), chromatin structure (histone H4), stress response (one other metallothionein) and transport (major facilitator super family 1 transport protein), while transcripts responsive to Pf29Arp were predicted to encode proteins whose function was unknown (Table 2). Finally, Ter331 induced the down-regulation of several transcripts involved in protein synthesis (e.g. ribosomal proteins, translation elongation factor), mitochondrial activity (e.g. cytochrome c oxidases, ADP/ATP carrier, coenzyme Q biosynthesis) and stress response (glutathione-*S*-transferases). Two transcripts encoding proteins potentially involved in the regulation of cell wall integrity (*pil1*) and response to pH (predicted protein with PalI/Rim9 domain) were down-regulated. Three other transcripts encoding proteins potentially involved in signal transduction were either up- (phosphoesterase) or down-regulated (protein kinases). Validation of regulation levels of five S238N-responsive transcripts was performed by quantitative RT-PCR (Fig. 4).

Table 2 Transcripts of *L. bicolor* S238N with altered expression in the presence of strains *P. fluorescens* BBc6R8 (adapted from Deveau et al. [8]), Pf29Arp, *C. fungivorans* Ter331 (fold changes >2.0<-2.0)

Protein ID	Gene product	Function	BBc6R8	Pf29Arp	Ter331
Transcripts regulated in response to the three strains					
456319	Hypothetical protein with atypical PIKK/TRRAP protein kinase domain	3	4.2	3.8	-2.7
388066	Metallothionein	6	3.3	3.2	-3.2
308883	Hypothetical protein	9	2.3	2.2	-3.1
Transcripts regulated in response to two strains					
608752	Hypothetical protein	9	6.4	9.2	na
399271	Tectonin 2	1	3.1	4.1	na
656382	Hypothetical protein	9	2.4	3.1	bs
Transcripts regulated in response to BBc6R8 only					
708567	Splicing factor 3b. subunit 4	3	8.7	na	na
380651	Hypothetical protein	9	8.1	na	na
298211	Major facilitator super family MFS-1 protein	7	6.4	na	na
399683	Putative metallothionein	6	5.2	na	na
399510	Cipc1 protein, concanamycin Induced protein C	8	3.2	na	na
699477	Hypothetical protein	9	2.7	na	na
293272	Hypothetical protein, one of the most abundant transcript in <i>Laccaria</i>	9	2.5	na	na
649344	Hypothetical protein	9	2.4	na	na
191150	Histone H4	4	2.1	na	na
609611	Hypothetical protein	9	2	na	na
Transcripts regulated in response to Pf29Arp only					
190777	mismatched base pair and cruciform DNA recognition protein	4	na	431.8	na
475510	Hypothetical protein	9	na	10.9	na
656016	Hypothetical protein	9	na	2.9	na
705946	Hypothetical protein with Ras small GTPase domain	5	na	2.9	na
455347	60S ribosomal protein L24e	3	na	2.8	na
666006	Hypothetical protein	9	na	2.6	na
678566	Hypothetical protein	9	na	2.5	na
622774	Conserved alpha-helical protein	9	na	2.2	na
Transcripts regulated in response to Ter331 only					
318749	Cysteine proteinase inhibitor; mycocypin family; clitocypin analog	8	na	na	11
693382	Predicted protein	9	na	na	9.1
307960	Hypothetical protein	9	na	na	8.2
318727	clitocypin cysteine proteinase inhibitor	8	na	na	5.8
490044	Hypothetical protein	9	na	na	5.8
617245	Hypothetical protein	9	na	na	5.4
436967	DNA polymerase	4	na	na	5.1
489617	Predicted protein with F-BOX domain	8	na	na	5
671387	Hypothetical protein	9	na	na	4.7
297361	Phosphoesterase	5	na	na	4.5
399685	metallothionein	6	na	na	3.9
313608	Hypothetical protein	9	na	na	3.6
491035	Ribosomal large subunit biogenesis-related protein	3	na	na	-2.6
190640	Histone H2A	4	na	na	-2.6
189031	Glycosyltransferase family 2 protein	8	na	na	-2.6

Table 2 (continued)

Protein ID	Gene product	Function	BBc6R8	Pf29Arp	Ter331
292014	Hypothetical protein	9	na	na	-2.6
644165	Hypothetical protein	9	na	na	-2.7
229432	Carbohydrate esterase family 4 protein	8	na	na	-2.8
291441	Hypothetical protein	9	na	na	-2.8
587159	Ribosomal protein S3Ae	3	na	na	-2.9
673249	Hypothetical protein	9	na	na	-2.9
657662	Ribosomal protein S10	3	na	na	-3
185105	Histone H2A	4	na	na	-3
391051	Hypothetical protein, one of the most abundant transcript in <i>Laccaria</i>	9	na	na	-3
649549	Predicted protein with Pall/Rim9 pH responsive domain	5	na	na	-3.1
655530	Predicted protein with Mov34 domain	8	na	na	-3.1
308883	Hypothetical protein	9	na	na	-3.1
578839	Hypothetical protein	9	na	na	-3.1
605247	Hypothetical protein	9	na	na	-3.1
388066	Metallothionein	6	na	na	-3.2
686081	Cytochrome C oxidase subunit Vb	2	na	na	-3.2
326534	Hypothetical protein	9	na	na	-3.2
438154	Glutathione S-transferase	6	na	na	-3.3
674829	Glutathione S-transferase	6	na	na	-3.3
487613	Hypothetical protein	9	na	na	-3.3
694105	Hypothetical protein	9	na	na	-3.3
694245	Hypothetical monooxygenase involved in coenzyme Q biosynthesis	2	na	na	-3.4
292159	Secreted proline-rich protein; similarity to predicted protein of Coprinopsisiscinerea. The most highly expressed transcript in free-living mycelium of <i>L. bicolor</i> S238N. Single copy gene	8	na	na	-3.4
628541	Hypothetical protein	9	na	na	-3.4
527109	Protein kinase	5	na	na	-3.5
441220	Mitochondrion membrane protein	8	na	na	-3.5
446662	Ribosomal protein L7Ae	3	na	na	-3.7
306303	Tyrosine protein kinase	5	na	na	-3.7
678241	Hypothetical protein	9	na	na	-3.7
296671	Glutathione S-transferase	6	na	na	-3.8
568550	Translation elongation factor 1a	3	na	na	-3.8
682114	Predicted protein with mitochondrial chaperon in GroEL-like domain	8	na	na	-3.8
293525	Hypothetical protein	9	na	na	-3.8
304792	Conserved hypothetical protein, one of the most abundant transcript in <i>L. bicolor</i>	8	na	na	-3.9
576039	Hypothetical protein	9	na	na	-3.9
656016	Hypothetical protein	9	na	na	-3.9
656016	Hypothetical protein	9	na	na	-3.9
293300	Hypothetical protein	9	na	na	-4
487774	Hypothetical protein	9	na	na	-4
577351	Hypothetical protein	9	na	na	-4
660224	Putative oxidoreductase	8	na	na	-4.1
685903	Hypothetical protein	9	na	na	-4.1
625241	Mitochondrial ADP/ATP carrier protein	2	na	na	-4.3

Table 2 (continued)

Protein ID	Gene product	Function	BBc6R8	Pf29Arp	Ter331
691268	Predicted protein with F-BOX domain	8	na	na	-4.3
473056	Putative metalloprotease	8	na	na	-4.4
622412	Hypothetical protein with small nuclear ribonucleoprotein motif	3	na	na	-4.5
709535	Hypothetical protein with small nuclear ribonucleoprotein motif	3	na	na	-4.6
708959	Mitochondrial solute carrier protein	2	na	na	-4.6
710288	Cytochrome C oxidase subunit I	2	na	na	-4.8
291511	Sphingolipid long chain base-responsive protein PIL1	5	na	na	-4.8
638697	Cytochrome C oxidase subunit I	2	na	na	-5
636228	Hypothetical protein with DNA binding domain	8	na	na	-5.1
638697	Cytochrome C oxidase subunit I	2	na	na	-5.3
628012	Predicted protein	9	na	na	-5.7
638697	Cytochrome C oxidase subunit I	2	na	na	-6.1

Protein ID in the v2 annotation of the genome sequence, gene product description, functional class and fold change are given. The functional classes correspond to : 1, cell-cell interaction; 2, energy metabolism; 3, protein synthesis and transcription regulation; 4, replication and chromatin structure; 5, signal transduction; 6, stress response; 7, transport; 8, others; 9, hypothetical proteins

na not significantly regulated, bs background signal

Bacterial Strains React Differently in the Presence of *L. bicolor* S238N at the Transcriptomic Level

Twenty-nine and 14 transcripts from strain BBc6R8 and Ter331, and seven genomic regions corresponding to 12

transcripts of Pf29Arp were found to be significantly regulated after 14 days of incubation with *L. bicolor* S238N, respectively. Regulation levels were observed as no higher than 12 folds. All genes differentially regulated from strain Pf29Arp in the presence of *L. bicolor* S238N were down-regulated, while

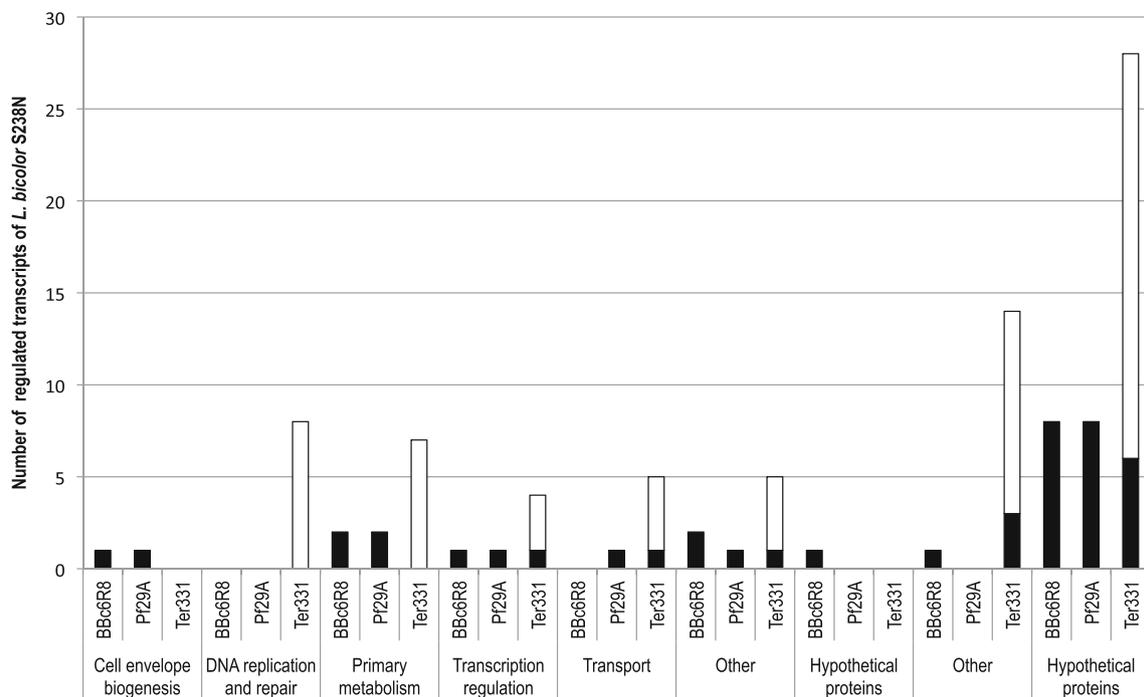


Fig. 2 Gene ontology of *L. bicolor* S238N transcripts differentially expressed in the presence of the three bacterial strains. Data are expressed as the number of transcripts down-regulated (*open*) or up-regulated (*closed*) in the presence of strains *P. fluorescens* BBc6R8, Pf29Arp, *C. fungivorans* Ter331

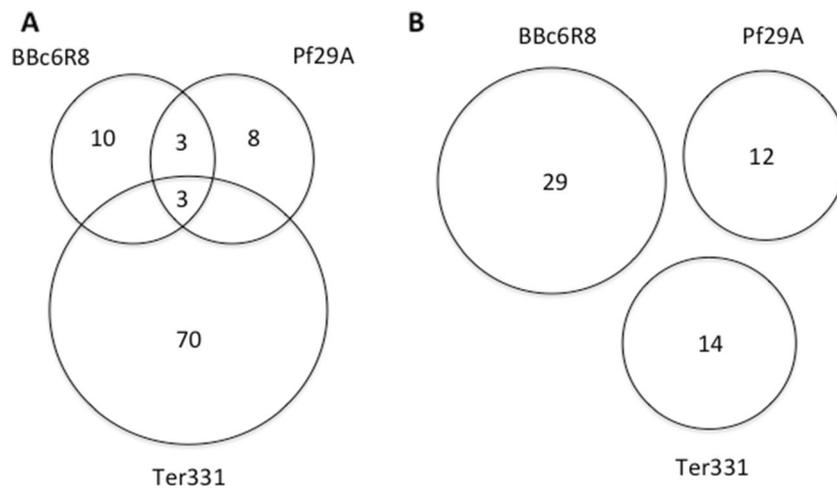


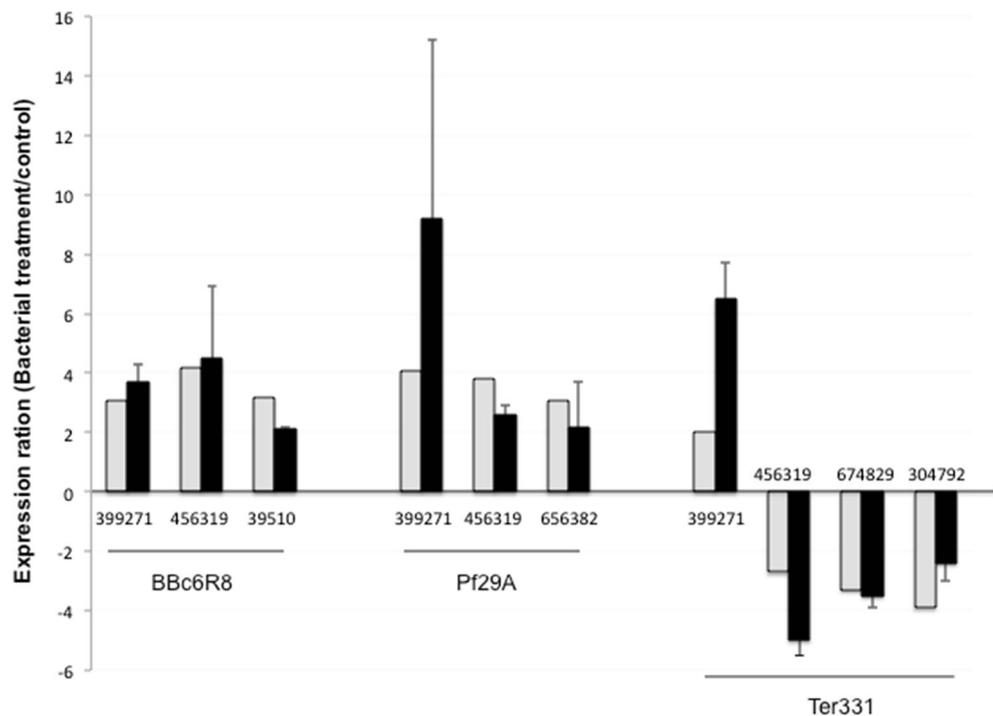
Fig. 3 Venn diagram showing the number of transcripts differentially expressed in *L. bicolor* S238N (a) in the presence of *P. fluorescens*-BBc6R8, Pf29Arp, *C. fungivorans* Ter331 and in the three bacterial strains (b) in the presence of *L. bicolor* S238N. Values represent the

number of transcripts that were >2-fold induced or repressed during the interaction compared to control treatment without interacting microorganisms. Intersects show the number of transcripts which were regulated in response to several bacterial strains

expression of 16 and 13 genes was down-regulated in strains BBc6R8 and Ter331, respectively. Moreover, the expression of four and ten genes of BBc6R8 and Ter331, respectively, were up-regulated in the presence of S238N. Regulated transcripts in the three strains were distributed in seven functional categories (Fig. 5, Table 3): primary metabolism (nine transcripts), cell envelop biogenesis (eight), transport (seven), transcription regulation (seven), DNA replication and repair (two), other functions (seven) and hypothetical proteins (13). No orthologous gene was commonly regulated between strains. However, some functional categories were shared between bacteria:

transcription factors were regulated in all strains but none was encoding for the same type of transcription regulator (Table 3). Regulation of the transcription of genes involved in primary metabolism was observed in all strains. Transcripts involved in lipid metabolism (cyclopropane-fatty-acyl-phospholipid synthase), arginine catabolism (carbamate kinase) and NAD production (nicotinatephosphoribosyltransferase, NAD synthetase) were up-regulated in BBc6R8, while transcript involved in amino acid metabolism were down-regulated in Pf29Arp (B12-dependent methionine synthase) and Ter331 (saccharopine dehydrogenase). In addition, transcripts encoding

Fig. 4 Quantitative RT-PCR validation of macroarray data. Comparison of the expression ratio [bacterial treatment/water control] measured by quantitative PCR (black) or macroarray analyses (grey). Each value represents the average value of three biological replicates ± standard error



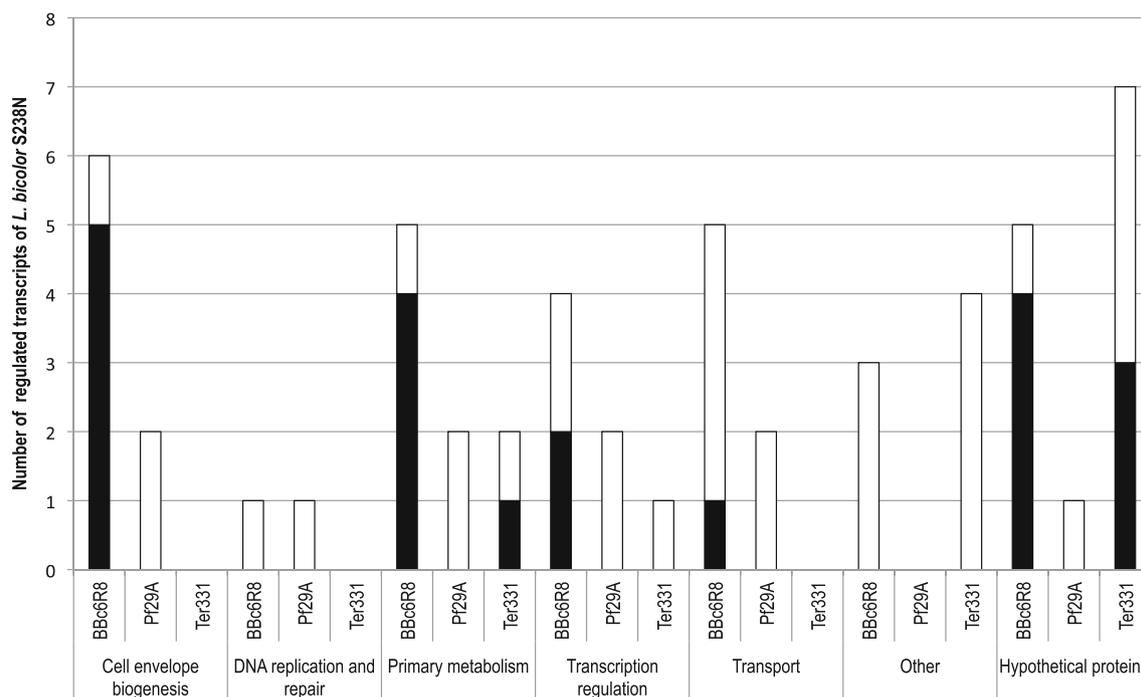


Fig. 5 Gene ontology of *P. fluorescens* BBc6R8, Pf29A, *C. fungivorans* Ter331 transcripts differentially expressed in the presence of *L. bicolor* S238N. Data are expressed as the number of transcripts down-regulated (*open*) or up-regulated (*closed*) in the presence of *L. bicolor* S238N

proteins related to transport of nutrients were regulated in BBc6R8 and Pf29A: transcripts encoding for one MFS transporter, an oligopeptide transporter (Pf29A) and a glucose ABC transporter (BBc6R8) were down-regulated, while one transcript encoding a glycine/betain ABC transporter was up-regulated in BBc6R8. Finally, several transcripts encoding proteins which could be associated to cell membrane biogenesis were regulated in the three strains: observed among the most highly up-regulated transcripts (seven fold) of BBc6R8 was a cluster of four genes (Pfr8_560-565) related to capsular polysaccharide biosynthesis, while one transcript associated with membrane-derived oligosaccharide synthesis was down-regulated in Pf29A as well as an endochitinase potentially involved in cell wall remodelling in Ter331.

Discussion

Ectomycorrhizal fungi interact with bacterial strains which can have beneficial, neutral or harmful effects on fungal physiology. Mechanisms by which ectomycorrhizal perceive and react to surrounding microorganisms are to date underexplored. However, previous related studies have brought out attention to a molecular dialog involving recognition processes within the ectomycorrhizal biotic environment by the fungus and subsequent production of an adapted response [8, 9, 11]. To determine how this molecular dialog

occurs between the microorganisms and its degree of specificity, we performed a dual-transcriptomic analysis of the interaction between the ectomycorrhizal fungus *L. bicolor* S238N and three soil bacterial strains related to the *Pseudomonas fluorescens* and *Collimonas* genus. Bacteria belonging to *P. fluorescens* group were enriched in the mycorrhizosphere of *L. bicolor* [21], while *Collimonas* strains were detected in forest soils typically colonized by *L. bicolor* [14, 22]. These bacterial strains were chosen for their contrasting effects on the growth and morphology of *L. bicolor* S238N [8]. This experiment allowed us to further characterize transcriptomes from the four microorganisms while comparing their behavioural patterns. To the best of our knowledge, this is the first study in which the transcriptomes from both an ectomycorrhizal fungus and interacting bacteria have been analysed at the same time.

Although changes in *L. bicolor* transcriptome were observed in response to the presence of the three bacterial strains, the strain Ter331 modified the expression of numerous fungal genes when compared to BBc6R8 and Pf29A strains. Contrasting responses were observed, suggesting that the ectomycorrhizal fungus responded specifically to each bacterium. Such differential response had previously been described in the case of the interaction between filamentous bacterial *Streptomyces* strains [23]. Highly specific interactions between *Aspergillus nidulans* and *Streptomyces* bacteria were also observed [24], reinforcing the hypothesis that microbes have the potential to precisely recognize their biotic

Table 3 Transcripts of *P. fluorescens* BBc6R8, Pf29Atp, *C. fungivorans* Ter331 with a statistically significant modulation of expression in the presence of *L. bicolor* S238N (fold changes >2.0 <-2.0)

BBc6R8 Gene ID	Predicted gene product	Predicted function	Fold change
Operon 1			
PfR8_2782	Binding-protein-dependent transport systems inner membrane component	Carbohydrate transport	-3.7
PfR8_2783	Binding-protein-dependent transport systems inner membrane component	Carbohydrate transport	-2.8
PfR8_2784	Glucose ABC transporter, ATP-binding protein	Carbohydrate transport	-2.3
Operon 2			
PfR8_560	Hypothetical protein	Cell envelope biogenesis, outer membrane	2.3
PfR8_561	Cyclopropane-fatty-acyl-phospholipid synthase (EC 2.1.1.79)	Cell envelope biogenesis, outer membrane	12.1
PfR8_563	Amine oxidase, flavin-containing	Cell envelope biogenesis, outer membrane	6.3
PfR8_564	Oxidoreductase, short-chain dehydrogenase/reductase family (EC 1.1.1.-)	Cell envelope biogenesis, outer membrane	7.7
PfR8_565	Putative transcriptional regulator	Cell envelope biogenesis, outer membrane	7.5
Operon 3			
PfR8_416	Nicotinamidephosphoribosyltransferase (EC 2.4.2.11)	Coenzyme metabolism	3
PfR8_417	NAD synthetase (EC 6.3.1.5)	Coenzyme metabolism	2.2
Operon 4			
PfR8_567	Transcriptional regulator, MerR family	Transcription regulation	6.7
PfR8_568	COG1683: uncharacterized conserved protein/hypothetical protein ybgA	Unknown function	7.4
PfR8_60	Hypothetical protein	Hypothetical protein	4.9
PfR8_6128	Hypothetical protein	Hypothetical protein	3.4
PfR8_6070	Carbamate kinase (EC 2.7.2.2)	Coenzyme metabolism	2.3
PfR8_747	Cyclopropane-fatty-acyl-phospholipid synthase (EC 2.1.1.79)	Coenzyme metabolism	2.3
PfR8_4137	Transcriptional regulator, GntR family	Transcription regulation	2.2
PfR8_4682	Substrate-binding region of ABC-type glycine betaine transport system	Carbohydrate transport	2
PfR8_633	Hypothetical protein	Hypothetical protein	2
PfR8_5721	2-Keto-3-deoxy-d-manno-octulosonate-8-phosphate synthase (EC 2.5.1.55)	Cell envelope biogenesis, outer membrane	-2
PfR8_278	Peptidyl-prolylis-trans isomeraseppID (EC 5.2.1.8)	Coenzyme metabolism	-2
PfR8_1253	MotA/ToIQ/ExbB proton channel family protein	Carbohydrate transport	-2
PfR8_3366	Flagellar basal-body rod protein flgB	Mobility	-2
PfR8_4508	Transcriptional regulatory protein	Transcription regulation	-2.5
PfR8_1599	Dienelactone hydrolase family protein	Other	-2.5
PfR8_2500	DNA recombination-dependent growth factor C	DNA Repair	-2.5
PfR8_1066	Hypothetical protein	Hypothetical protein	-2.5
PfR8_1516	Response regulator	Transcription regulation	-3.3
PfR8_584	NADH-dependent flavinoxidoreductase, Oye family	Other	-3.3
Pf29Atp CloneID			
11909	Predicted gene product	Predicted function	Fold change
1371	5-Methyltetrahydrofolate-homocysteine methyltransferase, Meth	Methionine biosynthesis	-4.3
1371	Transcriptional regulator, AsnC family	Transcription	-2.5
1371	Hypothetical protein	Unknown	-2.5
		Accession number (contigs)	
		ANOR01000052.1	
		ANOR01000097.1	
		ANOR01000097.1	

Table 3 (continued)

8541	Glucan biosynthesis protein G, MdoG	ANOR01000101.1	Cell envelopbiogenesis	AC_Number of best blast and E-value	Fold change
8541	Glucosyltransferase, MdoH	ANOR01000101.1	Cell envelopbiogenesis	ZP_00421146 (4.00E-05)	2.2
5459	Major facilitator superfamily transporter	ANOR01000115.1	Transport	No hit	2.1
5459	Transcriptional regulator, CdaR	ANOR01000115.1	Transcription	ABA50150 (2.00E-100)	2
6808	Putative camphor resistance protein CrcB	ANOR01000124.1	Unknown	CAI06681 (2.00E-28)	2
6808	Seryl-tRNA synthetase, SerS	ANOR01000124.1	Protein synthesis	P24091 (1.00E-80)	-1.8
268	Transcriptional regulator, AraC family	ANOR01000136.1	Transcription	YP_432774 (2.00E-118)	-2
3575	Uracil-DNA glycosylase superfamily	ANOR01000137.1	DNA replication and repair	YP_363381 (3.00E-20)	-2.2
3575	Oligopeptide transporter membrane protein	ANOR01000137.1	Transport	NP_396378 (2.00E-75)	-2.3
				No hit	-2.4
				No hit	-2.4
				YP_771542 (4.00E-117)	-2.6
				YP_546970 (2.00E-92)	-2.6
				P42097 (2.00E-63)	-2.1
				NP_891025 (6.00E-15)	-4
Ter331	Gene ID (locus tag)				
Cf_1761	Best blast identity		Predicted function		
Cf_3082 (CFU_3014)	Hypothetical protein		Hypothetical protein		
Cf_1669 (CFU_1610)	Hypothetical protein		Hypothetical protein		
Cf_4141 (CFU_4060)	3-Oxoadipate CoA-succinyltransferaseScoA		Benzoate degradation		
Cf_1676 (CFU_1617)	Hypothetical protein		Hypothetical protein		
Cf_3266 (CFU_3201)	Endochitinase B		Other		
Cf_1679 (CFU_1620)	Saccharopine dehydrogenase		Amino acid metabolism		
Cf_2155 (CFU_2092)	Hypothetical protein		Hypothetical protein		
Cf_1589	Short chain dehydrogenase		Other		
Cf_1681 (CFU_1622)	Hypothetical protein		Hypothetical protein		
Cf_3770 (CFU_3689)	Zinc-binding oxidoreductase		Hypothetical protein		
Cf_3771 (CFU_3690)	Short-chain dehydrogenase/reductase SDR		Other		
Cf_3772 (CFU_3691)	HTH-type transcriptional regulator		Other		
Cf_153 (CFU_0094)	Hypothetical protein		Transcription regulation		
			Hypothetical protein		

environment and are able to react to it accordingly. Our data suggests that the ectomycorrhizal fungus *L. bicolor* S238N might also have this ability. Interestingly, even two closely related strains belonging to the same taxonomic group induced different morphological and transcriptomic responses, suggesting that this process is strain specific. Although both BBc6R8 and Ter331 stimulated branching density and angle, no gene was found to be commonly regulated within these two strains. This incongruence between macroscopic observation and gene regulation could be explained either by the existence of distinct molecular pathways involved in hyphal branching or by earlier transcriptomic responses not included in this study. In contrast, the respective stimulation and inhibition of the radial growth of the fungus by strain BBc6R8 and Ter331 correlated with the respective increase and decrease in gene transcription linked to primary metabolism such as protein synthesis, transcription regulation and DNA replication (Fig. 2). These opposing transcriptional responses could therefore signify that regulation of the fungal metabolic activity is linked to its growth rate. Indeed an increase of cell growth requires elevated metabolic activity, while growth arrest in the presence of Ter331 would lead to a distinct decrease of metabolic activity. Such repression of the primary metabolism and growth in response to stressful conditions and starvation has been also described in *Saccharomyces* and *Neurospora* [25, 26].

Despite the high specificity of the fungal transcriptomic response to the three bacterial strains, four genes were found to be similarly regulated in response to the three bacterial strains. Therefore, we might conclude that some *L. bicolor* S238N pathways involved in the perception of bacteria could be commonly induced by all strains. Several earlier studies have suggested that some fungi could possibly have specific receptor systems for detecting other microorganisms [27, 28], although these receptors have yet to be discovered. The lipopolysaccharide binding-like protein encoding gene *tectonin II* is of particular interest. In other organisms, tectonins are able to bind bacteria through specific attachment to bacterial lipopolysaccharides [29, 30]. Preliminary data indicate that the protein is located on the cell wall of the fungus (data not shown) and that it is able also to agglutinate Gram-negative bacteria [31]. Physical adherence mediated by LPS and/or lectins has been observed in fungal–bacterial interaction [6, 32]. It would be tempting to speculate that this tectonin II is involved in physical interaction with bacteria and that it could be involved in the detection of bacteria. Further characterizations are currently being performed to determine its precise role in the physiology of the fungus and in the interaction with bacteria.

The three bacterial strains reacted differently to the presence of the ectomycorrhizal fungus with regulation of different sets of genes. Despite these differences, it should be noted that transcript regulations involved in nutrient acquisition and primary metabolism occurred in the three bacteria, suggesting

the existence of trophic interactions between the bacteria and the fungus. In the presence of strain BBc6R8, this regulation mirrored the sixfold over-expression of a *L. bicolor* gene encoding a MFS transporter at the same stage of the interaction. Considered together, these data suggest that the two microorganisms modulate their nutrient absorption and metabolism in the presence of each other. Whether the fungus and the helper bacterium compete for or exchange nutrients remains to be determined. In addition, transcripts potentially involved in cell wall remodelling were regulated in the three bacteria, although different transcripts were regulated for each strain. It is therefore possible that bacteria modify their cell wall structure in response to the presence of the ectomycorrhizal fungus. Further studies will be necessary to determine whether bacteria remodel their cell walls in response to the presence of the fungus and what the role is of this remodelling in the output of the interaction. Some interactions between fungi and bacteria only occur when the two partners have close physical interaction [6]. For example, the actinomycete *Streptomyces hygroscopicus* only produces orsellinic acid when it is adhering to hyphae of *A. nidulans* [24].

Strain Ter331 and Pf29Arp have been shown to react at the transcriptomic level during their interaction with the ascomycete *A. niger* [19] and the wheat pathogen *Gaeumannomyces graminis* [12, 20, 33, 34], respectively. Interestingly, both strains reacted quite differently when challenged with *L. bicolor* S238N. In contrast to the interaction with *A. niger* during which Ter331 strongly responded at the transcriptomic level to the presence of *A. niger* (8 % of the transcriptome), almost no modification of the transcriptome of the bacterium was observed in the presence of *L. bicolor* S238N (0.2 % of the transcriptome). As such, Ter331 may not perceive the presence of *L. bicolor* S238N and would constitutively produce antagonistic molecule(s). This “invisibility” of *L. bicolor* could be due to its reduced production of oxalic acid [35] which was involved in the interaction between Ter331 towards *A. niger* [19]. Indeed while *A. niger* can produce up to 70 mM of oxalic acid from 10 g of sugar [36], *L. bicolor* S238N only produces around 5 μ M of oxalic acid from the same amount of sugar [35]. While the mycophagous strain Ter331 induced the same macroscopic phenotype in *L. bicolor* S238N and *A. niger* by inhibiting their radial growth and by stimulating their hyphal branching, the molecular mechanisms of the interaction are probably different. The *A. niger*/Ter331 interaction seems to be based on a ping-pong interplay in which both organisms fight back through the production of secondary metabolites and competition for nitrogen [19]. *L. bicolor* S238N may also defend itself against Ter331 through the production of antibacterial compounds as suggested by the overexpression of clitocypin-encoding genes. Clitocypins are cysteine protease inhibitors with a broad inhibitory profile and which have been proposed to be involved in the defence against pathogens [37, 38]. This potential role in

defence against pathogens is supported by the nematicide activity of *L. bicolor* clitocybins (Plett and Martin, personal communication). However, while the ping-pong between *A. niger* and Ter331 resulted in reduced growth of both microorganisms, only the growth of *L. bicolor* S238N was inhibited during the interaction with Ter331 (data not shown). This growth inhibition correlated with a decrease in the expression of many genes linked to the primary metabolism of the ectomycorrhizal fungus. Whether this shutdown reflects a stress response due to the presence of the antagonist bacterium or a proactive response of the fungus to the activities of the bacterium remains to be determined.

No transcripts were found to be commonly regulated in Pf29Arp during the interaction with the pathogenic fungus *G. graminis* [12] compared to *L. bicolor* S238N, suggesting a specific response. However, in both cases, transcripts involved in metabolic processes and cell envelop biogenesis were regulated. Interestingly, a common pattern of response involving cell envelop biogenesis, transcription regulation and metabolic processes was observed, although different sets of genes were involved in each bacterium during the interaction with the fungus. More particularly, regulation of metabolic processes appeared to be a common point of the four microorganisms, suggesting the importance of trophic interactions in driving the interactions in our setup. However, the type of trophic interaction most likely changes, depending on the pair of microorganisms considered, since various types of metabolic processes were regulated. We can therefore expect the occurrence of a complex network of trophic interactions in the microbial communities selected in the ectomycorrhizosphere. Global food webs between mycorrhizal fungi bacteria and microfauna have yet to be described in greater detail [39]; to date, our knowledge about the metabolites exchanged and consumed in situ by interacting microorganisms is limited.

Conclusion

This study provides a glimpse of molecular mechanisms involved in ectomycorrhizal interaction with different soil bacteria. Based on analysis of transcriptomic datasets, we may conclude that a high degree of specificity exists within these molecular pathways. Hence, the ectomycorrhizal fungus *L. bicolor* S238N appears to differentially adapt its primary metabolism in response to distinct bacterial strains, which in turn modify the primary metabolism of these bacterial strains. Whether these changes are due to an exchange of metabolism or simply a competition for nutrients remains to be determined. In contrast to these specific modifications observed, the physical interactions between the fungus and the bacterial strains rely on conserved molecular mechanisms linked to cell wall modifications. Finally, a high proportion of differentially regulated genes was found to encode proteins with unknown

functions. As these genes could potentially play an important role in how microorganisms adapt to their environment, understanding how they function merits further investigation.

Acknowledgments A. Deveau was supported by a scholarship from INRA, Lorraine Region and the Foreign Office exchange program Van Gogh. This work was supported by the French National Research Agency through the Laboratory of Excellence ARBRE (ANR-11-LABX-0002-01). We would like to thank A. Kohler for her help in submitting data to the GEO database and Aimee Orsini for proofreading the manuscript.

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