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(Article begins on next page)



# UNIVERSITÀ DEGLI STUDI DI TORINO

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**An interdomain network: The endobacterium of a mycorrhizal fungus promotes  
antioxidative responses in both fungal and plant hosts**

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36 **Summary**

37

38 • Arbuscular mycorrhizal fungi (AMF) are obligate plant biotrophs that contain  
39 endobacteria in their cytoplasm. Genome sequencing of *Candidatus Glomeribacter*  
40 *gigasporarum* revealed a reduced genome and dependence on the fungal host.

41

42 • RNA-seq analysis of the AMF *Gigaspora margarita* in the presence and  
43 absence of the endobacterium indicated that endobacteria have an important role in  
44 the fungal pre-symbiotic phase by enhancing fungal bioenergetic capacity. To  
45 improve the understanding of fungal-endobacterial interactions, iTRAQ quantitative  
46 proteomics was used to identify differentially expressed proteins in *G. margarita*  
47 germinating spores with endobacteria (B+), without endobacteria in the cured line (B-  
48 ), and after application of the synthetic strigolactone GR24.

49

50 • Proteomic, transcriptomic, and biochemical data identified several fungal and  
51 bacterial proteins involved in interspecies interactions. Endobacteria influenced fungal  
52 growth, calcium signalling, and metabolism. The greatest effects were on fungal  
53 primary metabolism and respiration, which was 50% higher in B+ than in B-. A shift  
54 towards pentose phosphate metabolism was detected in B-. Quantification of  
55 carbonylated proteins indicated that the B- line had higher oxidative stress levels,  
56 which were also observed in two host plants.

57

58 • This study shows that endobacteria generate a complex interdomain network  
59 that affects AMF and fungal-plant interactions.

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62 Keywords: antioxidant status, Arbuscular Mycorrhizal Fungi, carbonylated proteins,  
63 endosymbiotic bacteria, plant host, proteome profiling.

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70 **Introduction**

71

72 Many bacteria can replicate inside eukaryotic cells. This intracellular life style results  
73 in a wide range of interactions with their hosts (Toft & Andersson, 2010).  
74 Endobacteria have an impressive diversity in their genomic traits and can establish  
75 parasitic or mutualistic relationships with their host, which can deeply affect host cell  
76 function. Insect endosymbiosis is one of the best investigated examples of mutualism  
77 between endobacteria and animal cells: genomic sequencing of both partners revealed  
78 functional compatibility that controls nutrient strategies and insect development  
79 (Moran & Bennett, 2014; Wernegreen, 2012).

80 Historically, observations of endobacteria inhabiting fungi were considered rare and  
81 primarily limited to some mycorrhizal and pathogenic fungi (Bonfante & Anca, 2009).  
82 However, extensive sequencing of environmental samples and detailed analysis of  
83 fungal genomes have indicated that these are not rare events. For example, a nitrogen-  
84 fixing bacterium was detected inside the pathogenic fungus *Ustilago* (Ruiz-Herrera *et*  
85 *al.*, 2015), the genomes of *Mollicutes*-related endobacteria (MRE) living inside many  
86 arbuscular mycorrhizal fungi (AMF) have been sequenced (Torres-Cortés *et al.*, 2015;  
87 Naito *et al.*, 2015), as well as the genome of a beta proteobacterium living inside  
88 *Mortierella* (Fujimura *et al.*, 2014). The adaptation mechanisms involved in bacterial-  
89 fungal symbiosis have not been elucidated. To obtain insights into these  
90 interrelationships, we investigated the symbiotic relationship between *Candidatus*  
91 *Glomeribacter gigasporarum* (*Ca. G. gigasporarum*) and the AMF *Gigaspora*  
92 *margarita*. *Ca. G. gigasporarum* is an obligate, stable, and structurally integrated  
93 endosymbiont of *G. margarita*, which -on its part- forms symbiotic associations with  
94 roots of most land plants. This three-way interrelationship provides a very interesting  
95 example of a meta-organism (Bosch & McFall-Ngai, 2011).

96 The *Ca. G. gigasporarum* genome lacks some crucial metabolic pathways, indicating  
97 that the endobacteria are metabolically dependent on the fungal host for nutrients and  
98 energy (Ghignone *et al.*, 2012). This result explains why *Ca. G. gigasporarum* cannot  
99 be cultured outside of the host. This situation severely limits experimental efforts to  
100 define the molecular mechanisms underlying host-symbiont interactions. However, a  
101 *G. margarita* line was developed that lacks its endobacteria (designated as B- or cured

102 line); this line is a stable wild-type (designated as B<sup>+</sup> or wt line) variant that is still  
103 able to establish mycorrhizal symbiosis (Lumini *et al.*, 2007).

104 To understand the bacterial effect on fungal fitness, we used next-generation  
105 sequencing to analyse the transcriptional profile of *G. margarita* in the presence and  
106 in the absence of its endobacterium (Salvioli *et al.*, 2015). Transcriptional analysis  
107 was performed using germinated spores with and without GR24 treatment, which is a  
108 synthetic analogue of strigolactone (SL). SLs are plant hormones that play a key role  
109 in plant-fungal signalling (Al-Babili & Bouwmeester, 2015; Bonfante & Genre, 2015).

110 In addition to the fungal sporification success, transcriptomic results indicate that the  
111 endobacterium affects a large number of fungal cell functions. In particular, it targets  
112 mitochondrial activity, upregulating genes involved in respiration, ATP production,  
113 and reactive oxygen species (ROS) detoxification.

114 Many studies have shown that mRNA levels could only partially correlate with  
115 protein abundance (Maier *et al.*, 2009; Haider & Pal, 2013) due to translational and  
116 post-translational regulation. In mammals, this is true for important regulators of  
117 cell development and differentiation (e.g., transcription factors and signalling  
118 proteins), whereas housekeeping proteins (e.g., ribosomal proteins, glycolytic proteins,  
119 and tricarboxylic acid cycle proteins) have a better correlation with mRNA levels  
120 (Schwanhäusser *et al.*, 2011). Focussing on plant microbe interactions, Feussner and  
121 Polle (2015) underlined how proteomics may increase the spatial resolution of RNA-  
122 based analyses, revealing for example basal immunity components. Due to the  
123 technical challenges presented by our experimental system (neither AMF nor  
124 endobacteria can be cultivated or genetically transformed), we reasoned that  
125 identification and quantitation of proteins expressed during the fungal-endobacterial  
126 interaction might provide a further level of understanding of our previous  
127 transcriptomic analysis (Salvioli *et al.*, 2015), providing a more realistic picture of  
128 gene function.

129 The aim of the present work was to analyse the proteome profile of AMF *G.*  
130 *margarita* and its endobacterium, with and without GR24 treatment, in order to  
131 validate the hypothesis that proteomics may be closer to phenotype (Feussner & Polle,  
132 2015) and could better explain some morphological traits of the cured line (Lumini *et*  
133 *al.*, 2007). A preliminary analysis of proteomic profiles used classical two-  
134 dimensional gel electrophoresis (Salvioli *et al.*, 2010). We wanted to complement the  
135 previous study and improve the coverage of protein changes associated with

136 endosymbiosis. Therefore, we employed the alternative proteomic approach iTRAQ  
137 (isobaric tags for relative and absolute quantification). This non-gel-based technique  
138 enabled unbiased evaluation of protein expression in complex biological samples and  
139 has wide application in the biological and biomedical sciences (Cox & Mann, 2011).  
140 Data obtained via iTRAQ analysis were supported by transcriptomic and  
141 physiological analyses. The results provide new insights into the molecular  
142 mechanisms mediating endosymbiosis and on how bacteria provide direct and/or  
143 indirect ecological benefits not only for their fungal host, but also for the plant. The  
144 study shows in fact that the endosymbiont can enhance the fungal response to  
145 endogenous ROS, increasing the total antioxidant activity of the fungus as well its  
146 glutathione content. This event also can influence the antioxidant status of  
147 mycorrhizal roots. This suggests the presence of a specific interdomain network  
148 involving the bacterial-mediated increase in fungal antioxidant capacity, which is  
149 subsequently transmitted to the mycorrhizal host plant.

150

151

## 152 **Materials and Methods**

153

### 154 *Biological materials*

155 Spores of *Gigaspora margarita* Becker and Hall (BEG 34, deposited at the European  
156 Bank of Glomeromycota) containing (B+) or not (B-) the *Ca. G. gigasporarum*  
157 endobacteria were used in this study. All the details concerning the propagation of B+  
158 and B- spores, the protocol for spore germination, the treatment with the solution  
159 10<sup>-7</sup> M of the synthetic strigolactone (SL) analogue GR24, and the mycorrhization  
160 procedure are detailed in Salvioli *et al.* (2015). *Lotus japonicus* (Regel) K. Larsen  
161 seedlings were inoculated with the fungal spores by using the 'Millipore sandwich'  
162 method (Novero *et al.*, 2002). Mycorrhizal status was checked after 4 weeks.  
163 Mycorrhizal clover plants (*Trifolium pratense* L.) were maintained in pots containing  
164 sterilized quartz sand; roots were sampled after three months.

165

### 166 *Protein extraction*

167 Proteins have been extracted from the four lines: B+, B+GR24, B-, B-GR24, starting  
168 from 500 spores for each one. Protein extractions from roots were performed starting  
169 from 1g of fresh material. Finely ground samples were suspended in 2,5 ml of

170 extraction buffer (Tris-HCl 0.5M pH8, sucrose 0.7M, NaEDTA 10mM, Ascorbic acid  
171 4mM,  $\beta$ -mercaptoethanol 0.4%, PMSF 1mM, leupeptin 1 $\mu$ M, pefabloc 0.1mg/ml). An  
172 equal volume of Tris-saturated Phenol was added. The samples were mixed and  
173 incubated for 30 min at 4°C. The phenol phase was collected after 15 min of  
174 centrifugation at 5000xg at 4°C. Proteins were precipitated overnight with 5 volumes  
175 of ice-cold 0.1M ammonium acetate in 100% methanol at -20°C. After 40 min of  
176 centrifugation at 9.500 rpm, the protein pellet was washed twice in 0.1M ammonium  
177 acetate and twice in ice-cold 80% acetone. The resulting pellets were dried and stored  
178 at -80°C until further processing. Three independent protein extractions were  
179 performed for each condition tested.

180

#### 181 *Protein digestion and iTRAQ labelling*

182 An equal amount of spore proteins was prepared for each biological replication.  
183 Protein samples were reduced with 10mM DTT, alkylated with 55mM iodoacetamide,  
184 digested using sequencing grade trypsin (Promega) at a ratio of 1:10 (w:w) for 12 h at  
185 37°C, and labeled using iTRAQ 4-plex kit (AB Sciex Inc., Framingham, MA, USA)  
186 according to the manufacturer's protocol. Samples were labeled with iTRAQ tags 114,  
187 115, 116 and 117, respectively.

188

#### 189 *LC-MS/MS analysis*

190 LC-MS/MS was performed using an EASY-nLC capillary system (ThermoFisher  
191 Scientific, San Jose, CA), coupled to an LTQ-Orbitrap XL hybrid mass spectrometer  
192 (ThermoFisher Scientific, San Jose, CA). Sample concentration and desalting were  
193 performed online using a column (180 $\mu$ m by 20mm; packed with 5- $\mu$ m, 100-Å-pore-  
194 size Symmetry C18 material; ThermoFisher Corp.) at a flow rate of 15 $\mu$ l/min for 1  
195 min. Separation was accomplished on a capillary column (100 $\mu$ m by 100 mm; packed  
196 with 1.7- $\mu$ m, 130-Å-pore-size bridged ethyl hybrid [BEH] C18 material;  
197 ThermoFisher Corp.). A linear gradient of A and B buffers (buffer A, 3% acetone  
198 [ACN]-0.1% formic acid [FA]; buffer B, 97% ACN-0.1% FA) from 7% to 45%  
199 buffer B over 124 min was used at a flow rate of 0.5 $\mu$ l/min to elute peptides into the  
200 mass spectrometer. Columns were washed and re-equilibrated between LC-MS/MS  
201 experiments. Electrospray ionization was carried out at 1.7kV, with the LTQ heated  
202 capillary set to 150°C.



203 Mass spectra were acquired in the Orbitrap in the positive-ion mode over the range of  
204 m/z 300 to 2,000 at a resolution of 60,000. Mass accuracy after internal calibration  
205 was within 4 ppm. Simultaneously, tandem MS spectra were acquired using the LTQ  
206 for the five most abundant, multiply charged species in the mass spectrum with signal  
207 intensities of >8,000 noise levels. MS/MS collision energies were set at 35%, using  
208 helium as the collision gas, and MS/MS spectra were acquired over a range of m/z  
209 values dependent on the precursor ion. Dynamic exclusion was set such that MS/MS  
210 for each species was acquired a maximum of twice. All spectra were recorded in  
211 profile mode for further processing and analysis. Xcalibur software was used for MS  
212 and MS/MS data analysis.

213

#### 214 *iTRAQ protein identification and quantification*

215 For protein identification, MS/MS data were searched using in house MASCOT  
216 version 2.3.02 (Matrix Science, London, United Kingdom) against the “Fungi” and  
217 “Bacteria” subsets of NCBI. The search parameters were as follows: threshold set-off  
218 at 0.05 in the ion-score cutoff (with 95% confidence); MS/MS fragment ion mass  
219 tolerance of  $\pm 0.6$ Da; enzyme specificity was set to trypsin with one missed cleavage;  
220 peptide tolerance was set at 10 ppm; fixed modifications of carbamidomethylation at  
221 Cys and iTRAQ 4plex at Lys and the N-terminal amino group of peptides; variable  
222 modifications of oxidation at methionine and glutamine as pyroglutamic acid; charge  
223 states of peptides were set to +2 and +3. Only peptides with significance scores  
224 greater than “identity\_score” were counted as identified. MASCOT analyzed three  
225 biological replicates of the iTRAQ data; only data with a false discovery rate (FDR)  
226 less than 5% were used for subsequent data analysis.

227 To demonstrate repeatability, the protein abundances between various biological  
228 replicates were compared and the ratios for the proteins in each comparison were  
229 compared with 1. The difference was plotted against the percentage of the proteins  
230 quantified. For quantitative changes, a 1.2-fold cutoff was set to determine up-  
231 accumulated and down-accumulated proteins, with a p-value < 0.05 present in at least  
232 two replicates. Quantitative analysis was performed by Scaffold software (version  
233 3.0).

234 A comparison between *Rhizophagus irregularis* and *G. margarita* transcriptomes has  
235 revealed that notwithstanding their deep differences in phylogeny, life cycle and  
236 ecological strategies, both the AM fungi have a strict genetic relatedness (Salvioli *et*

237 *al.*, 2015). According to this and in the absence of reference proteome, the identified  
238 *G. margarita* proteins were blasted against the NCBI database for *R. irregularis* (E-  
239 value <  $e^{-40}$ , identity  $\geq 40\%$ ). The proteins identified as bacterial proteins were blasted  
240 against the NCBI database of *Ca. G. gigasporarum* (E-value <  $e^{-40}$ , identity  $\geq 40\%$ ).  
241 For Venn diagrams we used the open source program Venny 2 (*Oliveros, J.C. (2007-*  
242 *2015) Venny. An interactive tool for comparing lists with Venn's diagrams.*  
243 <http://bioinfogp.cnb.csic.es/tools/venny/index.html>)

244

#### 245 *Respiratory activity*

246 O<sub>2</sub> consumption was measured using a Clark-type electrode (Hansatech Ltd,  
247 Hardwick Industrial, Norfolk, UK) calibrated between 0% and 100% with  
248 atmospheric oxygen. The respiration chamber was connected with a water circulator  
249 to maintain constant temperature of 30°C. The reaction was carried out at a constant  
250 stirrer speed in a 1 ml chamber volume. Recording of oxygen consumption was  
251 started by adding 1ml of dH<sub>2</sub>O into the chamber followed by 100 fungal spores.  
252 Spores were germinated for 3 days at 30°C in the dark. For GR24 treatment, the  
253 spores were incubated for 3 h with 10<sup>-7</sup>M of GR24 before polarographic  
254 measurement. O<sub>2</sub> consumptions were read for 15 min. Increase of O<sub>2</sub> consumption in  
255 spores was calculated by comparison of the slope with distilled water in equilibrium  
256 with the O<sub>2</sub> atmospheric (control).

257

#### 258 *H<sub>2</sub>O<sub>2</sub>, glutathione and total antioxidant activity*

259 After three days of germination 2700 spores of each line were collected by filtration  
260 on Whatman 3MM paper and separately weighed for the determination of H<sub>2</sub>O<sub>2</sub>, total  
261 antioxidant activity (TAA) and total glutathione (GSH). For TAA and GSH 0.1 g of  
262 spores were ground in a mortar in liquid nitrogen with 10 volumes of acidified  
263 methanol and 5% metaphosphoric acid, respectively. After centrifugation at 20000 g  
264 the levels of TAA and GSH were measured in the supernatants according to Locato *et*  
265 *al.* (2008).

266 For intracellular H<sub>2</sub>O<sub>2</sub> determination, 0.1g of spores were homogenized with 10  
267 volumes of ice-cold 5% trichloroacetic acid. The extracts were centrifuged for 20min  
268 at 1400g, and the supernatant was neutralized in the presence of an 0.1M phosphate  
269 buffer (pH 7.0) with 2M KOH to approximately pH 7.0. H<sub>2</sub>O<sub>2</sub> was measured in the

270 extract through the oxidation of 3,3',5,5'-tetramethylbenzidine in the presence of  
271 peroxidase according to Sgobba *et al.* (2015).

272

### 273 *Detection of carbonylated proteins in fungal and plant hosts*

274 The proteins were extracted as described above, 20µg of proteins were derivatized  
275 with DNPH (2,4-Dinitrophenylhydrazine) as previously described with some  
276 modifications (Levine *et al.*, 1994). Briefly, the proteins were denatured adding SDS  
277 at the final concentration of 6%. The derivatization was performed by adding 1 volume  
278 of 10mM DNPH in 2N HCl. Only 2N HCl was added to the negative control. After  
279 30' of incubation at room temperature, the mixture was neutralized by adding 1  
280 volume of Neutralization Solution (2M Tris, 30% Glycerol). Proteins were separated  
281 by 12% SDS-PAGE and transferred to PVP membrane (SERVA Electrophoresis  
282 GmbH, USA). The oxidatively modified proteins were detected using anti-DNPH  
283 antibodies (anti-dinitrophenyl-group antibodies, Sigma, USA) and visualized by a  
284 chemiluminescence detection kit (SuperSignal, Pierce Biotechnology, Rockford, IL,  
285 USA). Colloidal Coomassie Brilliant Blue (CCBB) was used to stain a duplicate gel.  
286 Alternatively, after electrophoresis, the proteins were stained with Bio-Safe  
287 Coomassie (Bio-Rad) and then processed for immunoblotting. Gel and immunoblot  
288 images were acquired by using GS-800 (Bio-Rad) and analyzed using ImageJ  
289 software (<http://imagej.nih.gov/ij/>).

290

### 291 *Real-time PCR assays*

292 For RT-qPCR validation, total RNA was extracted from batches of 100 *G. margarita*  
293 spores with the Rneasy Plant Mini Kit (Qiagen, Hilden, Germany) and treated with  
294 the TURBO DNA-free kit (Life Technologies, Carlsbad, CA, USA). The samples  
295 were then reverse-transcribed using Superscript II Reverse Transcriptase (Life  
296 Technologies). Quantitative real-time PCR experiments and data analysis were carried  
297 out as described in Salvioli *et al.* (2012), using as a reference gene for transcript  
298 normalisation the *G. margarita* translation elongation factor (EF1- $\alpha$ ). The primer  
299 names and corresponding sequences are listed in Table S1.

300

301

## 302 **Results and Discussion**

303 A total soluble proteome data set from the arbuscular mycorrhizal fungus *G.*  
304 *margarita* was generated using iTRAQ analysis under the following four conditions:  
305 *G. margarita* B+ (wild type) or B- (cured) germinating spores, treated with or without  
306  $10^{-7}$ M of GR24. Each of the four samples (B+, B-, B+GR24, and B-GR24) was  
307 labelled with one of four reagents of the iTRAQ four-plex, and then samples were  
308 combined into one aliquot. Triplicate labelling was performed, which showed a high  
309 level of reproducibility (data not shown). Data from the three replicates were merged  
310 and used for protein identification. MASCOT analysis detected a false discovery rate  
311 (FDR)<5%. For the second step, protein profile changes in the four samples were  
312 analysed by Scaffold software and used to generate a proteomic data set consisting of  
313 156 fungal proteins (Table S2, Fig. S1a). This approach complements the genome  
314 data and protein prediction analysis of *Rhizophagus irregularis*, and provides a novel  
315 platform for investigating AMF function (Recorbet *et al.*, 2013; Tisserant *et al.*, 2013;  
316 Lin *et al.*, 2014).

317

#### 318 *Ca. G.igasporarum* affects a core set of fungal proteins

319 The proteomes of B+ and B- lines were analysed after 7 days of germination. We  
320 quantified and identified 127 unique fungal proteins. Statistical analysis indicated that  
321 61 proteins differed in the two lines: the levels of 26 proteins were higher and 35  
322 proteins were lower in B- than in B+. These differentially expressed proteins are  
323 showed in Table S3 and Fig. S1b. The proteins were annotated into ten functional  
324 categories based on gene ontology, BLAST alignment, and information in the  
325 literature. The most abundant classes were “metabolic processes” (33%), “protein  
326 synthesis and degradation” (16%), and “unknown function” (24%).

327 When we compared the two germinating spore proteomes of B-GR24 and B+GR24, a  
328 total of 89 proteins were quantified and identified. Statistical analysis indicated that  
329 49 proteins differed in the two lines: the levels of 26 proteins were higher and 23  
330 proteins were lower in B-GR24 than in B+GR24. These differentially expressed  
331 proteins are showed in Table S4 and Fig S1b. The proteins were classified into seven  
332 functional categories based on gene ontology, BLAST alignment, and information in  
333 the literature. The most abundant classes were “metabolic processes” (27%), “protein  
334 folding” (20%), “protein synthesis and degradation” (18%), and “unknown function”  
335 (18%). Among the 49 differentially expressed proteins in these two samples, 19  
336 overlapped with the 61 proteins previously identified as differentially expressed in B+

337 and B- proteomes. We conclude that these 19 proteins (listed in bold in Tables S3 and  
338 S4) are good candidates for involvement in specific fungal responses to the  
339 endobacterium.

340 The combined results indicate that endobacteria modulate fungal protein expression  
341 and metabolism in the presence/absence of SL, and identify some of the primary  
342 molecular determinants involved in host adaptation responses (discussed in the  
343 subsequent section). Our analysis also detected and identified 24 bacterial proteins in  
344 the B+ and B+GR24 fungal proteomes. Of these, the levels of eight proteins were  
345 higher and nine were lower after GR24 treatment, indicating that SLs affect  
346 endobacteria (Table S5). However, only 5 of these 17 proteins found a direct match  
347 by using Blast analysis in the *Ca. G. gigasporarum* genome (Ghignone *et al.*, 2012);  
348 four of these five proteins were directly attributable to *Ca. G. gigasporarum*, and  
349 expression levels were modulated by SL. These proteins might function in  
350 extracellular bacterial communication. One of them was an outer membrane protein  
351 containing a YadA domain; the trimeric autotransporter adhesin YadA is considered  
352 one of the most important virulence factors in *Yersinia enterocolitica* (Pepe *et al.*,  
353 1995). Current models suggest that YadA mediates *Yersinia* adhesion to host cells,  
354 thus facilitating the injection of effectors via the type III secretion system (Keller *et*  
355 *al.*, 2015, Mühlenkamp *et al.*, 2015). It is tempting to fit this model to the *Ca. G.*  
356 *gigasporarum*-fungal interaction because endobacterial genes encoding T3SS  
357 components display specific expression patterns throughout the different stages of the  
358 fungal life cycle (Ghignone *et al.*, 2012). The outer membrane protein belonging to  
359 the OmpA/MotB family is required for pathogenesis and host interactions in  
360 *Escherichia coli* (Selvaraj *et al.*, 2007), and accumulates in response to GR24  
361 treatment. Two other endobacterial proteins possessing interesting features are  
362 influenced by SL treatment: the osmotically-inducible protein OsmY, which is a  
363 periplasmic sensory protein that confers stress resistance (e.g. low phosphate  
364 conditions) to *Salmonella* when living in macrophage vesicles (Zheng *et al.*, 2015),  
365 and a protein with unknown function belonging to the ElaB family of membrane-  
366 anchored ribosome-binding protein.

367 In summary, these proteomic data provide experimental evidence for the hypothesis  
368 that endobacteria communicate with fungal hosts via membrane proteins such as the  
369 T3S system (Ghignone *et al.*, 2012) and those involved in sensing nutrient

370 concentration. The data also suggest that plant signals are directly or indirectly  
371 perceived by endobacteria (Anca *et al.*, 2009).

372

373 *Ca. G. gigasporarum* affects proteins involved in fungal growth, morphology and  
374 calcium signalling

375 Germinating spores of B+ and B+GR24 accumulated proteins involved in DNA  
376 replication, transcription, and protein synthesis (Tables S3 and S4), suggesting that  
377 endobacteria enhance fungal growth. This result is consistent with the higher growth  
378 rate of the B+ line than the B- line (Lumini *et al.*, 2007). One of the most strongly  
379 differentially expressed proteins was a Rho-GDP-dissociation inhibitor (Rho-GDI),  
380 which was downregulated in the B- and B-GR24 proteomes. The *Rho-GDI* transcript  
381 (comp37206\_c0\_seq1) level was also lower in the B- line (Salvioli *et al.*, 2015). Rho-  
382 GDI represses monomeric Rho-GTPases, which control many fundamental cellular  
383 processes such as cytoskeletal organisation, vesicle trafficking, and bud site selection  
384 (DerMardirossian & Bokoch, 2005). Curing the colonial marine bryozoan *Bugula*  
385 *neritina* from its endosymbiont *Candidatus* Endobugula sertula also resulted in Rho-  
386 GDI downregulation and disrupted cytoskeletal organisation (Mathew & Lopanik,  
387 2014). Curing *G. margarita* from its endobacterium caused phenotypic changes in the  
388 cell wall, lipid drops, and cytoplasmic viscoelasticity (Lumini *et al.*, 2007). The B-line  
389 has a denser and more extensively aggregated cytoplasm than the B+ line; this could  
390 be due to Rho-GDI downregulation and accumulation of actin and tubulin proteins  
391 (Table S3 and S4).

392 It is interestingly to note that also the Pmt6 protein mannosyltransferase accumulated  
393 in B- cured line. Pmt proteins initiate *O*-glycosylation of secreted fungal proteins and  
394 are involved in fungal cell wall rigidity. *Candida albicans* mutants lacking one or two  
395 *Pmt6* alleles grow normally, but exhibit morphogenetic defects, indicating that Pmt6  
396 regulates secreted proteins that are involved in morphogenesis (Timpel *et al.*, 2000).  
397 Being involved in cell wall metabolism and cytoplasm viscoelasticity, the commented  
398 proteins offer a mechanistic explanation for the thick, rigid cell wall and dense  
399 cytoplasm observed in the cured fungi (Lumini *et al.*, 2007).

400 The vacuolar calcium-transporting ATPase PMC1 strongly accumulated in the B- line  
401 treated with GR24. In plant and yeast cells, the vacuole serves as the principal site of  
402 Ca<sup>2+</sup> sequestration and contains 95% of total cellular Ca<sup>2+</sup> stores (Cunningham, 2011).  
403 Deletion of *PMC1* in yeast effectively reduces cell growth in high-Ca<sup>2+</sup> environments,

404 suggesting that PMC1 has a significant role in vacuolar Ca<sup>2+</sup> sequestration. Elevations  
405 in cytosolic Ca<sup>2+</sup> increase *PMC1* expression (Cunningham & Fink, 1996). Many  
406 fungal genes related to Ca<sup>2+</sup> homeostasis and signalling have been identified in the  
407 *Glomus intraradices* genome (Liu *et al.*, 2013), and their transcripts are differentially  
408 regulated. This is consistent with our proteomic data. Transcripts of these same genes  
409 and the putative Ca<sup>2+</sup>-transporting ATPase were also detected in the *G. margarita*  
410 transcriptome (Salvioli *et al.*, 2015). These transcripts were slightly upregulated in the  
411 GR24-treated B- line. Higher cytosolic Ca<sup>2+</sup> levels have been detected in germinating  
412 spores of the cured line (Salvioli *et al.*, 2015) using a cell-permeant aequorin peptide  
413 (Moscatiello *et al.*, 2014), and SL treatment further enhances cytosolic Ca<sup>2+</sup>  
414 concentrations in the cured line.

415 In summary, PMC1 upregulation in GR24-treated B- line suggests that Ca<sup>2+</sup>  
416 homeostasis changes in fungi cured of the endobacterium. *Ca. G. gigasporarum* might  
417 act as a specific calcium store; in its absence, calcium accumulates in the cytoplasm  
418 and in the vacuole. Therefore, the observed reduction in ATP content in the cured line  
419 could be explained by ATP consumption required by PMC1 to store calcium inside  
420 the vacuole and by the negative interference of cytoplasmic calcium on ATP  
421 production (Case *et al.*, 2007).

422

423 *Curing G. margarita of its endobacterium induces a metabolic shift towards*  
424 *alternative reducing pathways*

425 Approximately 33% of the differentially expressed proteins in B+ and B- lines were  
426 involved in metabolic processes (Table S3). Proteins that are differentially expressed  
427 in the B+ line include a subunit of NADH-ubiquinone reductase, which is involved in  
428 mitochondrial oxidative phosphorylation; the mitochondrial malate dehydrogenase  
429 (MDH1), which converts malate to oxaloacetate in the tricarboxylic acid cycle; and  
430 triose phosphate isomerase (TPI). These results are supported by transcriptomic data  
431 indicating that genes involved in oxidative phosphorylation are upregulated and ATP  
432 production increases in the B+ line, which suggests that endobacteria increase the  
433 bioenergetic potential of host fungi (Salvioli *et al.*, 2015). To obtain evidence for this  
434 hypothesis, we measured fungal respiration in the B+ and B- lines. Polarography was  
435 sensitive enough to detect the O<sub>2</sub> consumption rate in 100 *G. margarita* spores after 3  
436 days of germination. The results showed that O<sub>2</sub> consumption was approximately 50%  
437 higher in the B+ line than in the B- line. As expected, GR24 treatment for 3 hours

438 increased O<sub>2</sub> consumption for both lines, but the respiratory capacity in the B+ line  
439 was approximately 50% higher than in the B- line (Table 1).

440 These results raised the question of what metabolic pathways were used for energy  
441 production in the cured *G. margarita* line, as energy demands are crucial during plant  
442 colonisation. Proteomic analysis provided some insight into an alternative reducing  
443 pathway in the cured line. The following two proteins upregulated in the B- line were  
444 involved in the pentose phosphate pathway (PPP): the phosphogluconate  
445 dehydrogenase (decarboxylating enzyme) GND2 and the D-glyceraldehyde-3-  
446 phosphate transaldolase TAL1. These proteins operate during oxidative and non-  
447 oxidative phases of the PPP. This central pathway produces reduced equivalents in the  
448 form of NADPH during the oxidative PPP phase, and produces precursors for nucleic  
449 acid and aromatic amino acid biosynthesis during the non-oxidative PPP phase. Ralser  
450 *et al.* (2007) showed that dynamic re-routing of metabolic flux to the PPP, with  
451 concomitant NADPH generation, was a conserved response to oxidative stress.  
452 NADPH provides the reducing potential for most antioxidant and regulatory enzymes  
453 controlling cellular redox homeostasis.

454 Another NADPH source in the cured line is NADP<sup>+</sup>-dependent isocitrate  
455 dehydrogenase (IDP1). The enzyme has been studied in *Saccharomyces cerevisiae*,  
456 where it catalyses the conversion of *D-threo*-isocitrate to 2-oxoglutarate in  
457 mitochondria. IDP has also been localised to the cytosol (IDP2) and peroxisome  
458 (IDP3). These three IDPs are involved in defence against oxidative stress in yeast  
459 (Contreras-Shannon & McAllister-Henn, 2004; Minard & McAllister-Henn, 2001). In  
460 the current study, IDP differentially accumulated in the cured line as confirmed by  
461 transcriptional results (Fig. 1), suggesting that the B- line had defective regulation of  
462 oxidative status.

463 GR24 treatment induced the expression of some proteins involved in maintaining the  
464 cellular redox balance in the B- line, including a cytosolic aldehyde dehydrogenase  
465 (ALDH), an alcohol dehydrogenase (ADH), and a cytosolic glycerol-3-phosphate  
466 dehydrogenase (GPD). RT-qPCR analyses confirmed the differential expression of  
467 *ALDH* and *GPD* transcripts in the B+ and B- lines, while changes in ADH transcript  
468 levels were not detected (Fig. 1). Cytosolic ALDH oxidises acetaldehyde to acetic  
469 acid and produces NADPH. The ability to act as an aldehyde scavenger during lipid  
470 peroxidation is another universal ALDH function found across species. Upregulation  
471 of ALDH is a stress response in bacteria, plants, yeast, and mammals (Singh *et al.*,



472 2013). ADH and GPD are reported to maintain redox balance in *S. cerevisiae* under  
473 limited respiratory capacity. Under aerobic conditions, oxidation of NADH produced  
474 during glycolysis occurs via the respiratory chain, which transfers the reducing  
475 equivalents to oxygen. Under limited respiratory capacity, *S. cerevisiae* strongly  
476 increases alcohol fermentation and glycerol production via GPD to accommodate  
477 non-respiratory oxidation of NADH to NAD<sup>+</sup> (Valadi *et al.*, 2004; Snoek & de  
478 Steensma, 2007). In light of the current study, it appears that the cured line may have  
479 a deficit in reducing power and greater oxidative stress; consequently, this line  
480 specifically upregulates proteins in alternative pathways that can remediate the redox  
481 balance (Fig. 2). Interestingly, substantial modifications in the energy metabolic  
482 pathways were already reported to occur as a consequence of symbiosis establishment,  
483 as in the nitrogen fixing Rhizobium-legume association (Karunakaran *et al.*, 2009).

484

485 *Lipid catabolism provides an important energy resource for arbuscular mycorrhizal*  
486 *fungi*

487 Another important change in fungal basal metabolism in the B<sup>+</sup> and B<sup>-</sup> lines involves  
488 lipid catabolism. Lipid metabolism in AMF has been extensively studied. Although  
489 lipids are the most important energy storage form, the *R. irregularis* genome does not  
490 contain any gene involved in de novo fatty acid synthesis (Tisserant *et al.*, 2013;  
491 Wewer *et al.*, 2014). Gluconeogenesis, which catabolises lipids into hexoses, has been  
492 reported in the AMF extraradical mycelium (Pfeffer *et al.*, 1999), and has been  
493 confirmed for *R. irregularis* (Wewer *et al.*, 2014) and our *G. margarita* isolates. One  
494 of the key enzymes of the fatty acid  $\beta$ -oxidation pathway, the enoyl-CoA hydratase  
495 FOX2, was upregulated in the B<sup>-</sup> line compared with that in the B<sup>+</sup> line, also after  
496 GR24 treatment. This result was confirmed by qRT-PCR analysis (Fig. 1). In the  
497 GR24-treated B<sup>-</sup> line, we also detected higher levels of one thiolase and one acyl-CoA  
498 dehydrogenase, which are involved in the  $\beta$ -oxidation pathway. Therefore, catabolism  
499 of fatty acids into hexoses appears to increase in the cured line. This was further  
500 supported by the accumulation in the B<sup>+</sup> line of a WD repeat-containing protein  
501 homologue to the glucose-induced degradation complex subunit GID7 of *R.*  
502 *irregularis*. GID7 is involved in proteasomal degradation of fructose-1,6-  
503 biphosphatase (FBPase), which is a key regulatory enzyme of gluconeogenesis.  
504 FBPase is degraded via the ubiquitin proteasome system when cells are replenished  
505 with glucose (Regelmann *et al.*, 2003). In fungi, FOX2 is repressed by glucose

506 (Ebbole, 1998). The observed change in lipid catabolism could also be associated with  
507 higher bioenergetic potential in the B+ line due to the upregulation of oxidative  
508 phosphorylation. Our proteomic and genetic evidence for increased  $\beta$ -oxidation in the  
509 B- line is consistent with other morphological and biochemical studies showing that  
510 cured spores have reduced lipid storage (Lumini *et al.*, 2007; Salvioli *et al.*, 2010).

511

512 *Endobacteria elicit fungal antioxidative activity, which is subsequently transmitted to*  
513 *mycorrhizal host plants*

514 Transcriptomic results suggested that higher respiration was associated with greater  
515 ROS detoxification in the B+ line (Salvioli *et al.*, 2015). Our proteomic data identified  
516 proteins that could be involved in this process. The peroxiredoxin (Prx) Tsa1, which is  
517 the most abundant Prx in yeast, accumulated in the B+ line and in the GR24-treated  
518 B+ line. This result was confirmed by transcriptomic analysis of the GR24-treated B+  
519 line (data not shown). Tsa1 is crucial for resistance to ROS, and it is required during  
520 normal aerobic growth conditions (Iraqi *et al.*, 2009). Tsa1 protects cells against  
521 oxidative stress caused by misfolding and aggregation of nascent proteins. Protein  
522 aggregation is accompanied by mitochondrial fragmentation, and Tsa1 localises to  
523 sites of protein aggregation. Disruption of mitochondrial function rescues the ROS  
524 sensitivity of *tsa1* mutants (Weids & Grant, 2014). Tsa1 accumulation in the B+ line  
525 reflects the need to remove excess ROS generated during respiration. ROS  
526 detoxification processes were also activated in the B- line due to the induction of  
527 cysteine Prx and glutathione-S-transferase (GST). GR24 treatment further stimulates  
528 ROS detoxification in the B+ line and induces the accumulation of Tsa1, GST, and a  
529 copper- and zinc-containing superoxide dismutase (Cu/Zn-SOD). Treatment of *G.*  
530 *margarita* with root exudate was reported to induce Cu/Zn-SOD (Lanfranco *et al.*,  
531 2005).

532 Transcriptomics and proteomics data indicated that *G. margarita* exhibited different  
533 responses to ROS depending on whether *Ca. G. gigasporarum* was present or absent.  
534 We tested the hypothesis that endobacteria promote fungal responses to oxidative  
535 stress. The total antioxidant activities (TAA) of the soluble extracts were separately  
536 analysed in the B+ and B- spores. The TAA was lower in B- (Table 2). Among the  
537 hydrophilic antioxidants, attention was focused on the changes in the level of GSH.  
538 The fungus without endobacteria had 43% of total GSH content (reduced plus  
539 oxidized forms) lower than the B+ line (Table 2). Moreover, to obtain a direct

540 measurement of the cellular ROS, we analysed the H<sub>2</sub>O<sub>2</sub> concentration in both  
541 systems. In B- the H<sub>2</sub>O<sub>2</sub> content was 30% higher than the one of B+. However the  
542 very low concentrations measured do not allow to obtain results with statistical  
543 significance.

544 Oxidative damage can occur when there is an imbalance between ROS production and  
545 antioxidant defence. Therefore, we examined the accumulation of oxidatively  
546 modified polypeptides by performing immunoblot analysis of carbonylated proteins.  
547 Protein carbonylation is one of the most harmful and irreversible oxidative protein  
548 modifications, and is considered as a major hallmark of oxidative damage (Fedorova  
549 *et al.*, 2014). Protein carbonylation level was higher in the B- line than in the B+ line,  
550 indicating that the absence of endobacteria leads to a higher level of oxidative damage  
551 (Fig. 3). Surprisingly, we also detected a significant increase in protein carbonylation  
552 levels in the GR24-treated B+ line (Fig. 3). This suggests that there is an increased  
553 imbalance between ROS levels and the capacity of antioxidant scavengers due to  
554 GR24-mediated stimulation of respiration, or that SLs are perceived by AMF as  
555 xenobiotics that may cause transient oxidative damage (Salvioli *et al.*, 2015).  
556 However, with this exception, the results support the hypothesis that redox  
557 homeostasis is disrupted in the cured fungal line under constitutive conditions.

558 The key question arising from this study is whether the higher antioxidant capacity of  
559 the B+ line helps the host plant cells to maintain its cellular redox homeostasis during  
560 the symbiosis. To answer this question, we compared the protein carbonylation  
561 profiles of clover roots after mycorrhizal colonisation with the B+ (B+Myc) or B- (B-  
562 Myc) lines. The levels of oxidatively modified proteins were higher in roots colonised  
563 by the B- line (Fig. 4). This result was confirmed in a parallel experiment testing  
564 *Lotus japonicus* roots colonized by the B+ or B- lines (Fig. S2).

565 To understand whether the carbonylated proteins detected in the mycorrhizal roots  
566 were of plant or fungal origin, proteins were submitted to carbonylation analysis after  
567 two dimensional separation (2DE). Among the differentially carbonylated proteins we  
568 randomly selected and identified seven of them by MS/MS (Suppl Mat, Fig. S3). All  
569 the selected proteins correspond to *Medicago truncatula* proteins (Table S6); they  
570 exhibited at least one peptide with oxidized methionine residues. Putting together  
571 these results and data from transcriptomic analysis of mycorrhizal roots revealing that  
572 only a low number of fungal transcripts (2.5%) are detectable (Ruzicka *et al.*, 2013),

573 we may conclude that the the carbonylated proteins detected in the mycorrhizal roots  
574 are mostly of plant origin.

575 Overall results indicate that endobacteria may affect the host plant through the  
576 intermediary of an AM fungus. The enhanced detoxification of ROS and resistance to  
577 oxidative stress may help plant roots to adapt to complex soil environments  
578 characterised by strong fluctuations in abiotic and biotic parameters.

579

580

## 581 **Conclusions**

582 Endobacterial symbionts of insects, invertebrates, and vertebrates are excellent  
583 models for investigations of the molecular links between bacteria, bacterial  
584 metabolites, and host physiologies (Lee & Hase, 2014). Bacteria and endobacteria  
585 also associate with fungi, either as extracellular microbes (Frey-Klett *et al.*, 2011) or  
586 as endobacteria in symbiotic and pathogenic fungi (Bonfante, 2014; Ruiz-Herrera *et*  
587 *al.*, 2015). Previous research efforts focused on identifying endobacteria rather than  
588 defining the mechanisms that regulate the symbiotic interactions. The molecular  
589 relationships among fungal endobacteria, bacterial metabolites, fungal signalling  
590 pathways, and fungal physiology are largely unknown. An exception is the *Rhizopus*  
591 system; *Rhizopus* hosts the endobacterium *Burkholderia rhizoxinica*, which produces  
592 a deleterious phytotoxin affecting the infected plant (Lackner & Hertweck, 2011).

593 In this study, we examined the relationship between the mycorrhizal fungus *G.*  
594 *margarita* and its obligate endobacterium *Ca. G. gigasporarum*. This symbiotic  
595 relationship appears to be stable and evolutionarily maintained for 400 million years  
596 (Mondo *et al.*, 2012). We used a combination of proteomic, physiological, molecular,  
597 and cellular approaches to conclusively demonstrate that the endobacterium affects  
598 fungal growth and development via its effects on lipid catabolism, cell wall  
599 organisation, and cytoplasmic characteristics. Proteomic analysis indicated that the  
600 endobacterium promoted fungal oxidative phosphorylation and increased respiratory  
601 activity. By contrast, fungi cured of the endobacterium exhibited metabolic shifts  
602 favouring the PPP as an alternative method to acquire reducing power. These results  
603 are consistent with those for another group of mycorrhizal fungi, the ericoid fungi,  
604 which were subjected to heavy-metal stress (Chiapello *et al.*, 2015). Our results using  
605 cured fungi clearly demonstrate that the endobacterium is crucial for optimum fungal  
606 cell homeostasis.

607 The second novel result of our investigation is that curing the fungi of its endobacteria  
608 induced increased oxidative stress, which was also subsequently transmitted to the  
609 third partner of the system: the host plant. Carbonylated proteins are considered as  
610 specific markers of oxidative stress, and have been identified in many plant species at  
611 different stages of growth and development (Debska *et al.*, 2012). This suggests that  
612 protein carbonylation may be involved in cellular signalling. Recent work reported a  
613 link between ROS-based protein carbonylation and reactive nitrogen species (RNS)-  
614 based protein nitrosylation (Lounifi *et al.*, 2013). Our data open the way to investigate  
615 redox proteomics in mycorrhizal plants. Recent studies reported that ROS-related  
616 pathways are important for both pathogenic and symbiotic plant-fungal interactions  
617 (Samalova *et al.*, 2014), but the molecular mechanisms regulating these interactions  
618 are largely unknown.

619 In conclusion, this study showed that the presence or absence of an endobacterium in  
620 a colonising arbuscular mycorrhizal fungus can modulate the redox status of a host  
621 plant root system. This could be the indirect result of the AM symbiosis established  
622 by the cured fungal line: even if the latter does not cause a clear mycorrhizal  
623 phenotype, it has some growth defect (Lumini *et al.*, 2007), and the symbiotic  
624 functionality in term of phosphate content is negatively impacted (Salvioli *et al.*,  
625 2015). These results open new questions about interspecies molecular interactions that  
626 occur under field conditions when the whole plant interacts with highly diverse  
627 microbiota (Bulgarelli *et al.*, 2013). The biodiversity of plant microbiota has been the  
628 subject of many studies, but limited attention has been given to plant responses.

629

630

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642

## 643 **References**

644

645 **Al-Babili S and Bouwmeester HJ. 2015.** Strigolactones, a Novel Carotenoid-  
646 Derived Plant Hormone *Annual Review of Plant Biology* **66**:161–86

647

648 **Anca IA, Lumini E, Ghignone S, Salvioli A, Bianciotto V, Bonfante P. 2009.** The  
649 *ftsZ* gene of the endocellular bacterium ‘*Candidatus Glomeribacter gigasporarum*’ is  
650 preferentially expressed during the symbiotic phases of its host mycorrhizal fungus.

651 *Molecular Plant Microbe Interactions* **22**: 302–310.

652

653 **Bonfante P. 2014.** The endless tale of endobacteria: a conversation with Paola  
654 Bonfante. *Trends in Plant Science* **19(12)**: 744-746

655

656 **Bonfante P, Anca IA. 2009.** Plants, Mycorrhizal Fungi, and Bacteria: A Network of  
657 Interactions. *Annual Review in Microbiology* **63**: 363–83.

658

659 **Bonfante P, Genre A. 2015.** Arbuscular mycorrhizal dialogues: do you speak  
660 'plantish' or 'fungish'? *Trends Plant Science* **20(3)**:150-4.

661

662 **Bosch TC, McFall-Ngai MJ. 2011.** Metaorganisms as the new frontier. *Zoology* **114**:  
663 185–190.

664

665 **Bulgarelli D, Schlaeppi K, Spaepen S, Ver Loren van Themaat E, Schulze-Lefert  
666 P. 2013.** Structure and functions of the bacterial microbiota of plants. *Annual Reviews  
667 in Plant Biology* **64**: 807-838.

668

669 **Case RM, Eisner D, Gurney A, Jones O, Muallem S, Verkhatsky A. 2007.**  
670 Evolution of calcium homeostasis: From birth of the first cell to an omnipresent  
671 signalling system. *Cell Calcium* **42**: 345–350.

672

673 **Chiapello M, Martino E, Perotto S. 2015.** Common and metal-specific proteomic  
674 responses to cadmium and zinc in the metal tolerant ericoid mycorrhizal fungus  
675 *Oidiodendron maius*. *Metallomics* DOI: 10.1039/C5MT00024F

676 **Contreras-Shannon V, McAlister-Henn L. 2004.** Influence of compartmental  
677 localization on the function of yeast NADP<sup>+</sup>-specific isocitrate dehydrogenases.  
678 *Archives of Biochemistry and Biophysics* **423**: 235–246.

679

680 **Cox J, Mann M. 2011.** Quantitative, high-resolution proteomics for data-driven  
681 systems biology *Annual Review Biochemistry* **80**: 273–299.

682

683 **Cunningham K, Fink G. 1996.** Calcineurin inhibits VCX1-dependent H<sup>+</sup>/Ca<sup>2+</sup>  
684 exchange and induces Ca<sup>2+</sup> ATPases in *Saccharomyces cerevisiae*. *Molecular &*  
685 *Cellular Biology* **16**: 2226–2237.

686

687 **Cunningham KW. 2011.** Acidic calcium stores of *Saccharomyces cerevisiae*. *Cell*  
688 *Calcium* **50**: 129–138.

689

690 **Debska K, Bogatek R, Gniazdowska A. 2012.** Protein carbonylation and its role in  
691 physiological processes in plants. *Biochemistry* **58**: 34-43.

692

693 **DerMardirossian C, & Bokoch GM. 2005.** GDIs: central regulatory molecules in  
694 Rho-GTPase activation. *Trends in Cellular Biology* **15**: 356-363.

695

696 **Ebbole DJ. 1998.** Carbon catabolite repression of gene expression and conidiation in  
697 *Neurospora crassa*. *Fungal Genetics & Biology* **25**: 15-21.

698

699 **Fedorova, M., Bollineni, R.C. & Hoffmann, R. 2014.** Protein carbonylation as a  
700 major hallmark of oxidative damage: update of analytical strategies. *Mass*  
701 *Spectrometry Reviews* **33**: 79-97.

702

703 **Feussner I, Polle A. 2015.**What the transcriptome does not tell - proteomics and  
704 metabolomics are closer to the plants' patho-phenotype. *Current Opinion in Plant*  
705 *Biology* **26**:26-31

706

707 **Frey-Klett P, Burlinson P, Deveau A, Barret M, Tarkka M, Sarniguet A. 2011.**  
708 Bacterial-fungal interactions: hyphens between agricultural, clinical, environmental,  
709 and food microbiologists. *Microbiology and Molecular Biology Reviews* **75**: 583–609.

710 **Fujimura R, Nishimura A, Ohshima S, Sato Y, Nishizawa T, Oshima K,**  
711 **Masahira Hattori M, Narisawa K and Ohtaa H. 2014.** Draft Genome Sequence of  
712 the Betaproteobacterial Endosymbiont associated with the Fungus *Mortierella elongata*  
713 FMR23-6 *Genome Announcements* **2(6)** e01272-14.

714

715 **Ghignone S, Salvioli A, Anca I, Lumini E, Ortu G, Petiti L, Cruveiller S,**  
716 **Bianciotto V, Piffanelli P, Lanfranco L et al. 2012.** The genome of the obligate  
717 endobacterium of an AM fungus reveals an interphylum network of nutritional  
718 interactions. *The ISME Journal* **6**:136–145.

719

720 **Haider S, Pal R. 2013.** Integrated Analysis of Transcriptomic and Proteomic Data.  
721 *Current Genomics* **14**: 91–110. doi: 10.2174/1389202911314020003.

722

723 **Iraqi I, Kienda G, Soeur J, Faye G, Baldacci G, Kolodner RD, Huang ME. 2009.**  
724 Peroxiredoxin Tsa1 is the key peroxidase suppressing genome instability and  
725 protecting against cell death in *Saccharomyces cerevisiae*. *PLoS Genetics* **5**,  
726 e1000524 10.1371/journal.pgen.1000524.

727

728 **Karunakaran R, Ramachandran VK, Seaman JC, East AK, Mouhsine B,**  
729 **Mauchline TH, Prell J, Skeffington A, Poole PS. 2009.** Transcriptomic analysis of  
730 *Rhizobium leguminosarum* Biovar *viciae* in symbiosis with host plants *Pisum sativum*  
731 and *Vicia cracca* *Journal of Bacteriology* **191**: 4002-4014.

732

733 **Keller B, Mühlenkamp M, Deuschle E, Siegfried A, Mössner S, Schade J,**  
734 **Griesinger T, Katava N, Braunsdorf C, Fehrenbacher B, Jiménez-Soto LF,**  
735 **Schaller M, Haas R, Genth H, Retta SF, Meyer H6, Böttcher RT, Zent R, Schütz**  
736 **M, Autenrieth IB, Bohn E. 2015.** *Yersinia enterocolitica* exploits different pathways  
737 to accomplish adhesion and toxin injection into host cells. *Cellular Microbiology*  
738 **17(8)**: 1179-1204. doi: 10.1111/cmi.12429.

739



740 **Lackner G, Hertweck C. 2011.** Impact of Endofungal Bacteria on Infection Biology,  
741 Food Safety, and Drug Development. *PLoS Pathogens* **7(6)**: e1002096

742 **Lanfranco L, Novero M, Bonfante P. 2005.** The mycorrhizal fungus *Gigaspora*  
743 *margarita* possesses a CuZn superoxide dismutase that is up-regulated during  
744 symbiosis with legume hosts. *Plant Physiology* **137**: 1319-1330.

745

746 **Lee W-J, Hase K. 2014.** Gut microbiota-generated metabolites in animal health and  
747 disease *Nature Chemical Biology* **10**: 416–424.

748

749 **Levine RL, Williams JA, Stadtman ER, Shacter E. 1994.** Carbonyl assays for  
750 determination of oxidatively modified proteins. *Methods in Enzymology* **233**: 346-357.

751

752 **Lin K, Limpens E, Zhang ZH, Ivanov S, Saunders DGO, Mu DS et al. 2014.**  
753 Single nucleus genome sequencing reveals high similarity among nuclei of an  
754 endomycorrhizal fungus. *PLoS Genetics* **10**: e1004078.

755

756 **Liu Y, Gianinazzi-Pearson V, Arnould C, Wipf D, Zhao B, Van Tuinen D. 2013.**  
757 Fungal genes related to calcium homeostasis and signalling are upregulated in  
758 symbiotic arbuscular mycorrhiza interactions. *Fungal Biology* **117**: 22–31.

759

760 **Locato V, Gadaleta C, De Gara L, De Pinto MC 2008.** Production of reactive  
761 species and modulation of antioxidant network in response to heat shock: critical  
762 balance for cell fate. *Plant Cell and Environment* **31**: 1606–1619.

763

764 **Lounifi I, Arc E, Molassiotis A, Job D, Rajjou L, Tanou G. 2013.** Interplay  
765 between protein carbonylation and nitrosylation in plants. *Proteomics* **13**: 568–578.

766

767 **Lumini E, Bianciotto V, Jargeat P, Novero M, Salvioli A, Faccio A, Bécard G,**  
768 **Bonfante P. 2007.** Presymbiotic growth and sporal morphology are affected in the  
769 arbuscular mycorrhizal fungus *Gigaspora margarita* cured of its endobacteria. *Cell*  
770 *Microbiology* **9**: 1716-1729.

771

772 **Maier T, Guell M, Serrano L. 2009.** Correlation of mRNA and protein in complex  
773 biological samples. *FEBS Letters* **583**: 3966–3973.

774  
775 **Mathew M, Lopanik N. 2014.** Host differentially expressed genes during association  
776 with its defensive endosymbiont. *Biology bulletin* **226**: 152-163.  
777  
778 **Minard KI, McAlister-Henn L. 2001.** Antioxidant function of cytosolic sources of  
779 NADPH in yeast. *Free Radical Biology and Medicine* **31**: 832-843.  
780  
781 **Mondo SJ, Toomer KH, Morton JB, Lekberg Y, Pawlowska TE. 2012.**  
782 Evolutionary stability in a 400-million-year-old heritable facultative mutualism.  
783 *Evolution* **66**: 2564–2574.  
784  
785 **Moran NA, Bennett GM. 2014.** The tiniest tiny genomes. *Annual Review in*  
786 *Microbiology*. **68**: 195-215. doi: 10.1146/annurev-micro-091213-112901.  
787  
788 **Moscatiello R, Sello S, Novero M, Negro A, Bonfante P, Navazio L. 2014.** The  
789 intracellular delivery of TAT-aequorin reveals calcium-mediated sensing of  
790 environmental and symbiotic signals by the arbuscular mycorrhizal fungus *Gigaspora*  
791 *margarita*. *New Phytologist* **203**: 1012–1020. doi:10.1111/nph.12849  
792  
793 **Mühlenkamp M, Oberhettinger P, Leo JC, Linke D, Schütz MS. 2015.** *Yersinia*  
794 adhesin A (YadA)--beauty & beast. *International Journal of Medical Microbiology*  
795 **305(2)**: 252-258.  
796  
797 **Naito M, Morton JB, Pawlowska TE. 2015.** Minimal genomes of mycoplasma-  
798 related endobacteria are plastic and contain host-derived genes for sustained life  
799 within Glomeromycota. *Proceedings of the National Academy of Sciences of the*  
800 *United States of America* **112(25)**: 7791–7796.  
801  
802 **Novero M, Faccio A, Genre A, Stougaard J, Webb KJ, Mulder L, Parniske M,**  
803 **Bonfante P. 2002.** Dual requirement of the *LjSym4* gene for mycorrhizal development  
804 in epidermal and cortical cells of *Lotus japonicus* roots *New Phytologist* **154**: 741–749.  
805

806 **Pepe JC, Wachtel MR, Wagar E, Miller VL. 1995.** Pathogenesis of defined  
807 invasion mutants of *Yersinia enterocolitica* in a BALB/c mouse model of infection.  
808 *Infection and Immunity* **63**: 4837–4848.

809 **Pfeffer PE, Douds DD, Bécard G, Shachar-Hill Y. 1999.** Carbon uptake and the  
810 metabolism and transport of lipids in and arbuscular mycorrhiza. *Plant Physiology*  
811 **120**: 587–598.

812

813 **Ralser M, Walmelink MM, Kowald A, Gerisch B, Heeren G, Struys EA, Klipp E,**  
814 **Jakobs C, Breitenbach M, Lehrach H, Krobitsch S. 2007.** Dynamic rerouting of  
815 the carbohydrate flux is key to counteracting oxidative stress. *Journal of Biology*. **6**:10.  
816 doi: 10.1186/jbiol61.

817

818 **Recorbet G, Abdallah C, Renaut J, Wipf D, Dumas-Gaudot E. 2013.** Protein  
819 actors sustaining arbuscular mycorrhizal symbiosis: underground artists break the  
820 silence. *New Phytologist* **199(1)**: 26-40.

821

822 **Regelmann J, Schüle T, Josupeit FS, Horak J, Rose M, Entian KD, Thumm M,**  
823 **Wolf DH. 2003.** Catabolite degradation of fructose-1,6-bisphosphatase in the yeast  
824 *Saccharomyces cerevisiae*: a genome-wide screen identifies eight novel GID genes  
825 and indicates the existence of two degradation pathways. *Molecular Biology of the*  
826 *Cell* **14**:1652–63. doi: 10.1091/mbc.E02-08-0456.

827

828 **Ruiz-Herrera J, León-Ramírez C, Vera-Nuñez A, Sánchez-Arreguín A, Ruiz-**  
829 **Medrano R, Salgado-Lugo H, Sánchez-Segura L, Peña-Cabriaes JJ. 2015.** A  
830 novel intracellular nitrogen-fixing symbiosis made by *Ustilago maydis* and *Bacillus*  
831 spp. *New Phytologist* **207**:769-77.

832

833 **Ruzicka D, Chamala S, Barrios-Masias FH, Martin F, Smith S, Jackson LE,**  
834 **Brad Barbazuk W, Schachtman DP. 2013** Inside Arbuscular Mycorrhizal Roots –  
835 Molecular Probes to Understand the Symbiosis. *The Plant Genome* **6(2)**: 1:13

836

837 **Salvioli A, Chiapello M, FontaineJ, Lounes Hadj-Sahraoui A, Grandmougin-**  
838 **Ferjani A, Lanfranco L, Bonfante P. 2010.** Endobacteria affect the metabolic profile

839 of their host *Gigaspora margarita*, an arbuscular mycorrhizal fungus. *Environmental*  
840 *Microbiology* **12(8)**: 2083–2096.

841 **Salvioli A, Zouari I, Chalot M, Bonfante P. 2012.** The arbuscular mycorrhizal status  
842 has an impact on the transcriptome profile and amino acid composition of tomato fruit.  
843 *BMC Plant Biology* **27**: 12–44.

844

845 **Salvioli A, Ghignone S, Novero M, Navazio L, Venice F, Bagnaresi P, Bonfante P.**  
846 **2015.** Symbiosis with an endobacterium increases the fitness of a mycorrhizal fungus,  
847 raising its bioenergetic potential. *The ISME Journal* doi: 10.1038/ismej.2015.91.

848

849 **Samalova M, Meyer AJ, Gurr SJ, Fricker MD. 2014.** Robust anti-oxidant  
850 defences in the rice blast fungus *Magnaporthe oryzae* confer tolerance to the host  
851 oxidative burst. *New Phytologist* **201**: 556–573.

852

853 **Schwahnhauser B, Busse D, Li N, Dittmar G, Schuchhard T, Wolf J, Chen W,**  
854 **Selbach M. 2011.** Global quantification of mammalian gene expression control.  
855 *Nature* **473**: 337–342.

856

857 **Selvaraj SK, Periandythevar P, Prasadarao NV. 2007.** Outer membrane protein A  
858 of *Escherichia coli* K1 selectively enhances the expression of intercellular adhesion  
859 molecule-1 in brain microvascular endothelial cells. *Microbes and Infection* **9**: 547–  
860 557.

861

862 **Sgobba A, Paradiso A, Dipierro S, De Gara L, de Pinto MC 2015.** Changes in  
863 antioxidants are critical in determining cell responses to short- and long-term heat  
864 stress. *Physiologia Plantarum* **153**: 68–78.

865

866 **Singh S, Brocker C, Koppaka V, Chen Y, Jackson BC, Matsumoto A, Thompson**  
867 **DC, Vasiliou V. 2013.** Aldehyde dehydrogenases in cellular responses to  
868 oxidative/electrophilic stress. *Free Radical Biology & Medicine* **56**: 89–101.

869

870 **Snoek ISI, H. Steensma. 2007.** Factors involved in anaerobic growth of  
871 *Saccharomyces cerevisiae*. *Yeast* **24**: 1-10.

872

873 **Timpel C, Zink S, Strahl-Bolsinger S, Schroppel K, Ernst J. 2000.** Morphogenesis,  
874 adhesive properties, and antifungal resistance depend on the Pmt6 protein  
875 mannosyltransferase in the fungal pathogen *Candida albicans*. *Journal of*  
876 *Bacteriology* **182(11)**:3063-71.

877

878 **Tisserant E, Malbreil M, Kuo A, Kohler A, Symeonidi A, Balestrini R et al. 2013.**  
879 Genome of an arbuscular mycorrhizal fungus provides insight into the oldest plant  
880 symbiosis. *Proceeding of National Academy of Science USA* **110**: 20117–20122

881

882 **Toft C & Andersson SG. 2010.** Evolutionary microbial genomics: insights into  
883 bacterial host adaptation. *Nature Reviews Genetics* **11**: 465–475.

884

885 **Torres-Cortés G, Ghignone S, Bonfante P, Schüßler A. 2015.** Mosaic genome of  
886 endobacteria in arbuscular mycorrhizal fungi: Trans-kingdom gene transfer in an  
887 ancient mycoplasma-fungus association. *Proceedings of National Academy of Science*  
888 **112**: 7785–7790

889

890 **Valadi A, Granath K, Gustafsson L. 2004.** Distinct intracellular localization of  
891 Gpd1p and Gpd2p, the two yeast isoforms of NAD<sup>+</sup>-dependent glycerol-3-phosphate  
892 dehydrogenase, explains their different contributions to redox-driven glycerol  
893 production. *Journal of Biological Chemistry* **279(38)**: 39677-85.

894

895 **Wernegreen J. 2012** Strategies of genomic integration within insect-bacterial  
896 mutualisms. *Biological Bulletin* **223(1)**: 112–122.

897

898 **Wewer V, Brands M, Dörmann P. 2014.** Fatty acid synthesis and lipid metabolism  
899 in the obligate biotrophic fungus *Rhizophagus irregularis* during mycorrhization of  
900 *Lotus japonicus*. *The Plant Journal* **79(3)**: 398-412

901

902 **Weids AJ, Grant CM. 2014.** The yeast peroxiredoxin Tsa1 protects against protein-  
903 aggregate induced oxidative stress. *Journal of Cell Science* **127**: 1327–1335  
904 doi:10.1242/jcs.144022.

905

906 **Zheng X, Ji Y, Weng X, Huang X. 2015.** RpoS-dependent expression of OsmY in  
907 *Salmonella enterica* serovar typhi: activation under stationary phase and SPI-2-  
908 inducing conditions. *Current Microbiology* **70(6)**: 877-882.

## 909 **Legends**

910

911 **Fig. 1** Relative quantification of gene expression as obtained for a subset of  
912 metabolism-related sequences. Expression data were obtained for B+ germinating  
913 spores (B+G), B- germinating spores (B-G), B+ strigolactone-treated spores  
914 (B+GR24) and B- strigolactone-treated spores (B-GR24).

915 For each transcript, fold changes were calculated considering the B+G as reference  
916 basal condition (for this latter the Fold change is=1). Statistically supported  
917 differences are indicated with different letters according to a Kruskal-Wallis non  
918 parametric test at  $p < 0.05$ .

919 **Fig. 2** Schematic overview of metabolic pathways differentially regulated in B- lines  
920 in comparison with the B+ lines on the basis of the proteins identified in the current  
921 study. Proteins that were up-regulated are indicated in red, those that were down-  
922 regulated are indicated in green.

923 G6P, glucose6-phosphate; F6P, fructose6-phosphate; F1,6P, fructose 1,6-diphosphate;  
924 DHAP, dihydroxyacetone phosphate; GAP, glyceraldehyde-3- phosphate; ALDH,  
925 aldehyde dehydrogenase; ADH, alcohol dehydrogenase; GPD, glycerol-3-phosphate  
926 dehydrogenase; IDP1, NADP+-dependent isocitrate dehydrogenase; MDH1, malate  
927 dehydrogenase; TPI, triose phosphate isomerase; GND2, phosphogluconate  
928 dehydrogenase; TAL, transaldolase.

929 **Fig. 3** Protein carbonylation profiles of B+ and B- lines without and with GR24  
930 treatment: a) protein stain, b) anti-DNP immunoassay c) relative protein carbonylation  
931 values (referred to B+ sample) expressed as carbonylation index, after normalization  
932 for protein amounts. Data (means  $\pm$  SD,  $n = 3$ ) were subjected to one-way analysis of  
933 variance (ANOVA). Bars not accompanied by the same letter are significantly  
934 different at the 5% level using Tukey's test.

935 **Fig. 4** Protein carbonylation profiles of clover roots after mycorrhizal colonisation  
936 with the B+ or B- lines: a) protein stain, b) anti-DNP immunoassay c) relative protein  
937 carbonylation values (referred to B+ sample) expressed as carbonylation index, after  
938 normalization for protein amounts. Data (means  $\pm$  SD,  $n = 3$ ) were subjected to one-

939 way analysis of variance (ANOVA). Bars not accompanied by the same letter are  
 940 significantly different at the 5% level using Tukey's test.

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943 **Table 1. O<sub>2</sub> consumption in *G. margarita* lines**

944

945 Relative differences in O<sub>2</sub> consumption were measured by polarography in spores  
 946 germinated for three days and treated for 3 h with or without GR24.

947 a indicates values significantly different from B+ (t test, p<0.01)

948 b indicates values significantly different from B+GR24 (t test, p<0.01)

949

Experiments	Slope values			
	B+	B-	B+ GR24	B- GR24
1	0.9	0.4	0.83	0.5
2	1.1	0.4	0.85	0.5
3	0.65	0.42	0.9	0.5
4	0.7	0.4	1.2	0.4
Mean	0.84	0.40 <sup>a,b</sup>	0.95	0.48 <sup>a,b</sup>
Standard deviation	0.2	0.01	0.17	0.05

950

951

952

953 **Table 2 Intracellular levels of H<sub>2</sub>O<sub>2</sub>, antioxidant activity (TAA), and total  
 954 glutathione (GSH) in *G. margarita* lines.**

955

956 The values are the mean ± ES of three independent experiments. a indicates values  
 957 significantly different from B+ (t test, p<0.05)

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959

	H <sub>2</sub> O <sub>2</sub> nmoles g <sup>-1</sup> FW	TAA nmoles Trolox eq g <sup>-1</sup> FW	GSH nmoles g <sup>-1</sup> FW
B+	6.1 ± 0.6	884 ± 35	189 ± 14

B- 8.5 ± 0.4 543 ± 30<sup>a</sup> 107 ± 11<sup>a</sup>

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963 **Supporting Information**

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965 Additional supporting information may be found in the online version of this article.

966

967 **Table S1** Primers used for qRT-PCR analyses

968 **Table S2** List of the fungal proteins identified by iTRAQ analysis

969 **Table S3** List of the differentially expressed proteins in B- line in comparison with  
970 B+ line

971 **Table S4** List of the differentially GR24-responsive proteins in B- line in comparison  
972 with B+ line

973 **Table S5** Bacterial proteins differentially expressed in B+GR24 sample in  
974 comparison with B+ sample

975 **Table S6** List of differentially carbonylated proteins in B-Myc in comparison with  
976 B+Myc

977 **Fig. S1** Venn diagrams showing: a) the identified proteins in each analyzed condition;  
978 b) the degree of overlap between differentially regulated proteins obtained by  
979 comparing B+ versus B- and B+GR24 versus B-GR24.

980 **Fig. S2** Protein carbonylation profiles of *Lotus japonicus* roots after mycorrhizal  
981 colonisation with the B+ or B- lines: a) protein stain, b) anti-DNP immunoassay c)  
982 relative protein carbonylation values (referred to B+ sample) expressed as  
983 carbonylation index, after normalization for protein amounts. Data (means ± SD, n=  
984 3) were subjected to one-way analysis of variance (ANOVA). Bars not accompanied  
985 by the same letter are significantly different at the 5% level using Tukey's test.

986 **Fig. S3** Representative two dimensional profiles of protein abundance and oxidation  
987 in B+Myc and B- Myc clover roots. Protein stain (A,C) and anti-DNP immunoassay  
988 (B, D) are shown. Selected protein undergoing differential carbonylation are labeled  
989 with arrows. They are listed in Table S6.