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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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35

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.

60

61

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⁺ 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 10⁻⁷ 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, β -mercaptoethanol 0.4%, PMSF 1mM, leupeptin 1 μ 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 μ m by 20mm; packed with 5- μ m, 100-Å-pore-
194 size Symmetry C18 material; ThermoFisher Corp.) at a flow rate of 15 μ l/min for 1
195 min. Separation was accomplished on a capillary column (100 μ m by 100 mm; packed
196 with 1.7- μ 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 μ 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 ± 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 O₂ 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 dH₂O 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 10⁻⁷M of GR24 before polarographic
254 measurement. O₂ consumptions were read for 15 min. Increase of O₂ consumption in
255 spores was calculated by comparison of the slope with distilled water in equilibrium
256 with the O₂ atmospheric (control).

257

258 *H₂O₂, 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 H₂O₂, 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 H₂O₂ 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. H₂O₂ 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- α). 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 Ca²⁺ sequestration and contains 95% of total cellular Ca²⁺ stores (Cunningham, 2011).
403 Deletion of *PMC1* in yeast effectively reduces cell growth in high-Ca²⁺ environments,

404 suggesting that PMC1 has a significant role in vacuolar Ca²⁺ sequestration. Elevations
405 in cytosolic Ca²⁺ increase *PMC1* expression (Cunningham & Fink, 1996). Many
406 fungal genes related to Ca²⁺ 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 Ca²⁺-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 Ca²⁺ 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 Ca²⁺
414 concentrations in the cured line.

415 In summary, PMC1 upregulation in GR24-treated B- line suggests that Ca²⁺
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 O₂ consumption rate in 100 *G. margarita* spores after 3
436 days of germination. The results showed that O₂ consumption was approximately 50%
437 higher in the B+ line than in the B- line. As expected, GR24 treatment for 3 hours

438 increased O₂ 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⁺-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⁺ (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⁺ and B⁻ 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 β -oxidation pathway, the enoyl-CoA hydratase
495 FOX2, was upregulated in the B⁻ line compared with that in the B⁺ line, also after
496 GR24 treatment. This result was confirmed by qRT-PCR analysis (Fig. 1). In the
497 GR24-treated B⁻ line, we also detected higher levels of one thiolase and one acyl-CoA
498 dehydrogenase, which are involved in the β -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⁺ 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 β -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 H₂O₂ concentration in both
541 systems. In B- the H₂O₂ 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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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.

941

942

943 **Table 1. O₂ consumption in *G. margarita* lines**

944

945 Relative differences in O₂ 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 ^{a,b}	0.95	0.48 ^{a,b}
Standard deviation	0.2	0.01	0.17	0.05

950

951

952

953 **Table 2 Intracellular levels of H₂O₂, 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)

958

959

	H ₂ O ₂ nmoles g ⁻¹ FW	TAA nmoles Trolox eq g ⁻¹ FW	GSH nmoles g ⁻¹ FW
B+	6.1 ± 0.6	884 ± 35	189 ± 14

B- 8.5 ± 0.4 543 ± 30^a 107 ± 11^a

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963 **Supporting Information**

964

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.