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Gigaspora margarita with and without its endobacterium shows adaptive responses to oxidative stress

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Abstract

Arbuscular mycorrhizal (AM) fungi experience oxidative stress during the plant-fungal interaction, due to endogenous reactive oxygen species (ROS) produced by fungal metabolism and exogenous ROS produced by plant cells. Here, we examine the responses to H_2O_2 in *Gigaspora margarita*, an AM fungus containing the endobacterial symbiont *Candidatus* Glomeribacter gigasporarum (*Ca*Gg). Previous studies revealed that *G. margarita* with its endobacterium produces more ATP and has higher respiratory activity compared to a cured line that lacks the endobacterium. This higher bioenergetic potential leads to higher production of ROS, and to a higher ROS-detoxifying capacity, suggesting a direct or indirect role of the endobacterium in modulating fungal anti-oxidant responses. To test the hypothesis that the fungal-endobacterial symbiosis may enhance the fitness of the AM fungus in the presence of oxidative stress, we treated the fungus with a sublethal concentration of H_2O_2 and performed RNA-seq analysis. Our results demonstrate that: i) irrespective of the endobacterium presence, *G. margarita* faces oxidative stress by activating multiple metabolic processes (methionine oxidation, sulfur uptake, the pentose phosphate pathway, activation of ROS-scavenger genes), ii) in the presence of its endobacterium, *G. margarita* upregulates some metabolic pathways, like chromatin status modifications and iron metabolism; iii) contrary to our hypothesis, the cured line responds to H_2O_2 by activating the transcription of specific ROS scavengers. We confirmed the RNA-seq findings by measuring the glutathione and ascorbate content, which was the same in both lines after H_2O_2 treatment. We conclude that both fungal lines may face oxidative stress, but they activate alternative strategies.

Key words

Arbuscular mycorrhizal fungi; Endobacteria; RNA-seq; Oxidative stress; Reactive oxygen species

69**Introduction**

70As obligate biotrophs that colonize the roots of most land plants and enhance the host's ability to 71acquire nutrients, arbuscular mycorrhizal (AM) fungi have important roles among the plant 72microbiota. Some AM fungi possess obligate endobacteria inside their cytoplasm, giving rise to an 73endosymbiosis which is not essential for the fungal host (Bonfante and Desirò 2017; Olsson et al. 742017). The genome sequences of rod- and coccoid-shaped endobacteria revealed that both have a 75reduced genome and a strong dependence on the fungal host (Ghignone et al. 2012; Torres-Cortés et 76al. 2015). The rod-shaped bacterium, *Candidatus* Glomeribacter gigasporarum (*Ca*Gg), has been 77more deeply investigated: as an obligate endosymbiont of the AM fungus *Gigaspora margarita*, 78*Ca*Gg relies on its fungal host for nutrition, thus representing a physiological cost to the fungus. 79However, the bacterial population has been maintained in the fungus over many generations in an 80association that is believed to be as ancient as the mycorrhizal symbiosis (Mondo et al. 2012; 81Bonfante and Desirò 2017).

82The benefits that the endobacteria provide to the fungal partner remain largely unknown, although 83recent studies on other fungal-bacterial interactions have given some hints (Uehling et al. 2017; Li 84et al. 2017). In a previous study, we combined next-generation sequencing, molecular biology, and 85cell physiology analyses to compare *G. margarita* containing *Ca*Gg (B+ line) with a "cured" line of 86the fungus that lacks *Ca*Gg (B- line) obtained under laboratory conditions (Lumini et al. 2007). We 87demonstrated that *Ca*Gg can enhance its host's bioenergetic potential in terms of ATP production, 88by increasing mitochondrial respiration rates in spores germinated in H_2O (Salvioli et al. 2016). In a 89comparison between B+ and B- germinating spores, RNA-seq data revealed that the B+ line showed 90overall higher expression of genes involved in the response to reactive oxygen species (ROS) 91(Salvioli et al. 2016), including several ROS-scavenging enzymes. The results pointed to a 92relationship between cellular respiration and superoxide radical production (as a direct byproduct of 93the electron transport chain). Taking in account the results discussed above, we argued that *Ca*Gg 94may have a protective effect on *G. margarita*, making the fungal host more efficient in detoxifying 95endogenous ROS. Consistent with this, proteomic data confirmed that the B- spores produced less 96thioredoxin and other ROS-detoxifying proteins, and showed signs of oxidative damage (Vannini et 97al. 2016).

98ROS cause detrimental chemical changes in proteins, lipids, polysaccharides, DNA, RNA, and even 99in small metabolites (Winterbourn 2008). Oxidative stresses can produce diverse responses in 100filamentous fungi, ranging from mitochondria-mediated ageing and programmed cell death 101(Osiewacz 2011) to signaling that may regulate mycelial development and sexual reproduction 102(Breitenbach et al. 2015). Oxidative stresses can originate from endogenous or exogenous sources

103and may play different roles. On the one hand, H_2O_2 may act as a signal in response to injury in
104fungi such as *Trichoderma* and *Rhizophagus irregularis*, suggesting that fungi share a defense
105mechanism based on ROS-related molecules, as is common in plants and animals (Hernández-
106Oñate et al. 2012; Fester and Hause 2005). On the other hand, environmental stimuli can lead to
107oxidative stress; for example, a recent study reported that the glutathione peroxidase redox system
108is directly involved in the resistance of *Alternaria alternata* to the fungicides fludioxonil and
109vinclozolin (Yang et al. 2016). Current information on how AM fungi respond to exogenous
110oxidative stress is restricted to the intraradical stages of fungal development (Belmondo et al. 2016;
111Lanfranco et al. 2005), and define a scenario in which host plant-produced ROS are part of a fine-
112tuned signaling pathway necessary for fungal development. Similar experiments have described
113how *Beauveria bassiana* (an entomopathogenic fungus) responds to insect-produced ROS (Zhang et
114al. 2015).
115Few studies have examined the response of AM fungi to induced oxidative stress when the AM
116fungi are living in the soil as extraradical hyphae. A recent investigation (Tamayo et al. 2016)
117revealed that five *R. irregularis* glutaredoxin genes respond to H_2O_2 and may have a role in iron
118homeostasis. By using an omics approach, we wanted to test the hypothesis that *G.. margarita* can
119respond to exogenous oxidative stress by activating specific anti-oxidants and that the presence of
120its endobacterium enhances the fungal response to oxidative stress. Preliminary results (Salvioli et
121al. 2016) suggested that both the bacterial and fungal symbionts perceived the oxidative stress.
122Here, we set up an RNA-seq approach to describe the response of an AM fungus to H_2O_2 treatment,
123validating the transcriptomic data with antioxidant measurements. We demonstrate that *G.*
124*margarita* with its endobacterium is equipped to respond to environmental oxidative stresses, but
125also the cured line successfully responds to H_2O_2 with the expression of genes involved in specific
126ROS- related pathways.
127
128**Materials and Methods**
129
130**Spore production**
131*Gigaspora margarita* Becker and Hall (isolate BEG 34, deposited at the European Bank of
132Glomeromycota) was used in this study. This strain naturally contains endobacteria (Bianciotto et
133al. 1996) and is identified here as the B+ line. The cured line, i.e. without the endobacterium (B-
134line), was obtained from the B+ line as described in Lumini et al. 2007. To maintain and propagate
135both lines, we used pot cultures with white clover (*Trifolium repens*) as a trap plant, inoculating

100–150 spores for each pot. Second-generation spores were produced within 3 months, and were then collected by the wet sieving technique (Gerdemann and Nicolson 1963).

Treatment with hydrogen peroxide

6000 B+ and 6000 B- spores were surface sterilized with a solution containing 3% P/V Chloramine T and 0.03% P/V streptomycin sulphate, and subsequently washed with sterile water three times for 10 minutes each. The sterilized spores were divided into batches of 100 (each batch corresponding to one biological replicate), placed in 10 wells of 12 multi-well plates, and incubated for 3 days at 30°C in the dark, for germination pre-conditioning. After this step, the B+ and B- spores of half of the wells were treated with 0.3 mM H_2O_2, previously identified as the highest concentration at which the fungus could successfully germinate (Salvioli et al. 2016). Sterile water was replaced in the other half of the wells. After 3 hours of incubation at 30°C in the dark, 3 biological replicates (300 spores) from each condition were collected and frozen in liquid nitrogen for RNA extraction.

RNA seq experiments

RNA extraction, library preparation, and sequencing were performed according to previously described protocols (Salvioli et al. 2016). Total RNA was extracted using the RNeasy Microarray Tissue Mini Kit (Qiagen, Germany). To measure the concentration and quality of the extracted samples, we used the Nanodrop1000 (Thermo Scientific, Wilmington, NC, USA) and RNA integrity was assessed with the Bioanalyzer instrument (Agilent Technologies, Santa Clara, CA, USA). Three Illumina single-end libraries, corresponding to three biological replicates, were generated for each condition (B+ control spores, B- control spores, B+ treated spores, and B- treated spores, for a total of 12 libraries). Illumina HiScanSQ sequencing (50-bp single-end reads) was performed at the Genomic facility of the HuGeF (http://www.hugef-torino.org/site/index.php?id=102&t=articolo) and of DBios, University of Torino http://www.dbios.unito.it/do/home.pl/View?doc=servizi_esterno/analisi_genomica.html) following ENCODE project standard protocols (http://encodeproject.org/ENCODE/protocols/dataStandards/ENCODE_RNAseq_Standards_V1.0.pdf). Libraries were processed with Trimmomatic V.0.36 (settings: LEADING:10, TRAILING:10, SLIDINGWINDOW:4:25, MINLEN:25, Q32, ILLUMINACLIP:TruSeq3-PE.fa:2:30:10) to remove low-quality reads and Illumina adapters (Bolger et al. 2014). Raw reads were then mapped to *G. margarita* assembled transcripts (Salvioli et al. 2016) using Bowtie2 (Langmead and Salzberg 2012).

170**Differential expression analysis**

171DESeq2 1.12.4 Bioconductor package was used for the identification of differentially expressed 172genes (DEGs), setting local fit and betaPrior parameters to TRUE. Independent filtering was 173enabled (Anders and Huber 2010; Love et al. 2014). A threshold of 0.05 was applied to false 174discovery rates (FDR) to filter for significant DEGs. An additional filter was applied on log_2 fold 175changes, and only genes with a |log2 FC| higher than 1.5 where selected for each comparison. The 176following 4 comparisons were investigated: treated spores *vs* control spores (regardless of the 177endobacterial presence); B+ treated spores *vs* B+ control spores; B- treated spores *vs* B- control 178spores; and B+ treated spores *vs* B- treated spores. Gene Onthology (GO) enrichment for each 179comparison was performed with the AgriGO web platform (http://bioinfo.cau.edu.cn/agriGO/) and 180visualized with Goplot v.1.0.2 (Walter et al. 2015). BLAST searches were performed with the 181BLAST+ suite (Camacho et al. 2009).

182

183Extraction and analysis of ascorbate and glutathione

184A new set of B+ and B- control and treated spores was obtained as described in the "Treatment with 185hydrogen peroxide" section. For each condition ascorbate and glutathione were measured in 186independent experiments by using three biological replicates of 1000 spores. The material was 187ground in liquid nitrogen and homogenized with 1 mL of cold 5% meta-phosphoric acid at 4°C in a 188porcelain mortar. The homogenate was centrifuged at 20,000 g for 15 min at 4°C, and the 189supernatant was collected for analysis of glutathione and ascorbate (or its analogues) according to 190de Pinto et al. (1999).

191The dipyridyl assay was used for the measurement of all the ascorbate-like reductants (Spickett et 192al. 2000). Briefly, total content of ascorbate or its analogues was determined after reduction of the 193oxidized forms (DHA) with DTT, and the concentration of DHA was estimated from the difference 194between total pool of ascorbate and its analogues (ASC+DHA) and the reduced forms (ASC). The 195reaction mixture for ASC+DHA determination contained a 0.1 ml aliquot of the supernatant, 0.25 196ml of 150 mM phosphate buffer (pH 7.4) containing 5 mM EDTA, and 0.05 ml of 10 mM DTT. 197After incubation for 10 min at room temperature, 0.05 ml of 0.5% N-ethylmaleimide was added to 198remove excess DTT. ASC was determined in a similar reaction mixture except that 0.1 ml H2O was 199added rather than DTT and N-ethylmaleimide. Colour was developed in both reaction mixtures after 200addition of the following reagents: 0.2 ml of 10% trichloroacetic acid, 0.2 ml of 44% ortho-201phosphoric acid, 0.2 ml of 4% α,α'-dipyridyl in 70% ethanol and 0.3% (w/v) FeCl3. After 202vortexing, the mixture was incubated at 40°C for 40 min and the A525 was read. A standard curve 203was developed based on ascorbate in the range 0-50 μg ml-1.

204The glutathione pool was assayed utilizing 0.4 ml aliquots of supernatant neutralized with 0.6 ml of 2050.5 M phosphate buffer pH 7.5. For glutathione disulphide (GSSG) assay, the reduced form of glu-206tathione (GSH) was masked adding to the neutralized supernatant 20 µl of 2-vinylpyridine, whereas 20720 µl H_2O were added in the aliquots utilized for total glutathione pool assay. Tubes were mixed un-208til an emulsion was formed. Glutathione content was measured in a 1 ml reaction mixture contain-209ing 0.2 mM NADPH, 100 mM phosphate buffer pH 7.5, 5 mM EDTA, 0.6 mM 5,5'-dithiobis(2-ni-210trobenzoic acid) and 0.1 ml of sample obtained as described above. The reaction was started by 211adding 3 units of glutathione reductase and was monitored by measuring the change in absorbance 212at 412 nm for 1 min. GSH was estimated as the difference between the amount of total glutathione 213and that of GSSG. A standard curve in the range 0-30 µM ml^{-1} GSSG was prepared. Ascorbate and 214glutathione data were expressed as the average of three independent experiments. After checking for 215normality and homoscedasticity with Shapiro-Wilk and Levene's mean tests, respectively, the differ-216ences between groups were assessed by using one-way analysis of variance (ANOVA) with a post-217hoc Tukey's test, assuming $P<0.05$ as statistically significant. The statistical analyses were per-218formed using Sigma Plot software 12.0 (Systat Software, Inc., CA, USA).

219

220

221**Results**

222A previous study assessed the tolerance of the B+ and B- lines of *G. margarita* to a range of 223concentrations of hydrogen peroxide (Salvioli et al. 2016) from 0.2 to 2 mM. The study identified 2240.3 mM H_2O_2 as the highest concentration at which the fungus could successfully germinate. To 225describe the mechanisms that allow *G. margarita* to face exogenous oxidative stress, and to 226determine whether the presence of *Ca*Gg affects these dynamics, we selected the 0.3 mM H_2O_2 227concentration to treat B+ and B- germinating spores for the RNA-seq experiment.

228

229**RNA-seq quantitative data: the overall response of *G. margarita* to hydrogen peroxide**

230To examine the response to H_2O_2 on the transcriptional level, we first conducted transcriptome 231analysis by RNA-seq. To this end, we generated twelve Illumina single-end libraries, which yielded 232~10 to 17 million filtered reads (Table S1), giving a ~93% overall mapping rate on the *G. margarita* 233reference transcriptome (Salvioli et al. 2016). For each set of differentially expressed genes (DEGs), 234we applied a ±1.5 $\log_2$ fold change filter, and a total of 12527 genes among all the considered 235comparisons (of the 35029 currently predicted *G. margarita* genes) passed the filter. DEGs for each 236comparison are listed in Table S2, while Table S3 shows only the most up- or down- regulated 237genes.

238To examine the effect of the endobacterium (B+ and B-) and the treatment (with and without H_2O_2)
239in shaping the fungal transcriptome, we performed Principal Component Analysis (PCA) and
240Variance Partitioning Analysis (VPA) on the whole dataset of normalized read counts (Fig. 1a and b,
241respectively). PCA analysis highlighted a strong differentiation between the control and treated
242samples (PC1, H_2O_2 treatment), but the separation between the data for the B+ and B- lines (PC2,
243endobacterial presence) was lower in both control and treated conditions. The lowest degree of
244separation was observed between the B+ and B- treated lines. Indeed, the direct comparison
245between the treated B+ fungus versus the treated B- fungus showed the fewest DEGs among all the
246comparisons (2423). The distance between the control and treated B- fungal samples was lower
247than the distance between the control and treated B+ fungal samples. This observation was
248consistent with the number of DEGs from the two comparisons (5543 *vs* 8291, respectively). VPA
249revealed the treatment with H_2O_2 as the factor mostly influencing the overall variability (~65%),
250while the presence/absence of the bacterium explained ~6% of the overall variability (Fig.1 b).
251These results confirm the PCA results, where the PC1 axis represents 72% of the variance *vs* the
25218% represented by the PC2 axis.
253Since the H_2O_2 treatment seemed to be the driving parameter, all the control fungal samples (B+
254control and B- control) were compared with the treated samples (B+ treated and B- treated),
255irrespective of the presence of *Ca*Gg. This comparison identified 5458 DEGs, corresponding to
256about 15% of the *G. margarita* transcriptome (2398 up-regulated *vs* 3060 down-regulated genes).
257
258**Hydrogen peroxide treatment: identification of the main Gene Ontology categories**
259The results of GO enrichment analysis on this data set are shown in Fig 2. Among the enriched
260categories, the pentose-phosphate shunt, nicotinamide metabolic process, zinc ion transport,
261methionine metabolic process, and sulfur metabolic process categories were up-regulated overall in
262the treated samples.
263The up-regulation of metabolic processes relative to sulfur and methionine is not surprising, since
264methionine containing proteins directly participate in defense against oxidative stress. Several types
265of ROS react with methionine, forming methionine sulfoxide, which is reduced by methionine
266sulfoxide reductase (Luo and Levine 2009). We previously demonstrated that under normal growth
267conditions, the B- line contains a higher quantity of carbonylated proteins (Vannini et al. 2016),
268which are considered a major hallmark of oxidative damage (Fedorova et al. 2014) and specifically
269involve methionine oxidation. The gene coding for methionine sulfoxide reductase showed a
270significant up-regulation in our data set upon H_2O_2 treatment (comp18281_c1, $\log_2$ fold change:
2712.69). Consistent with this, sulfur metabolism was among the enriched and up-regulated categories.

272The re-routing of carbon flux to the pentose-phosphate pathway has been described as a conserved
273response to oxidative stress (Ralser et al. 2007). In yeast, the pentose phosphate pathway is essential
274for sulfur assimilation, being the main reservoir of NADPH used by the cell for sulfite to sulfide
275conversion (Slekar et al. 1996). In *G. margarita* both the absence of *Ca*Gg (Salvioli et al. 2016;
276Vannini et al. 2016) and the H_2O_2 treatment seemed to induce the shift towards the pentose
277phosphate pathway. Taken in the whole, the GO enrichment analysis reveals that the H_2O_2 treatment
278pushes *G. margarita* to deeply change its transcriptome profile, shifting towards alternative
279metabolic pathways that are directly linked to mitigation of oxidative stress.
280To describe the oxidative stress response of *G. margarita* more in depth, DEGs from the treated *vs*
281control comparison (pooling results for B+ and B-) were further divided on the basis of their
282functional annotation, and we manually selected some genes known to be involved in ROS
283detoxification in fungi (Table 1). Among them, genes encoding glutathione peroxidase, glutathione
284reductase, and glutathione s-transferase, as well as a peroxiredoxin and a thioredoxin reductase
285were strongly up-regulated after the hydrogen peroxide treatment. Notwithstanding the central role
286that the gluthatione cycle plays as a cellular redox buffer in yeasts and filamentous fungi,
287knowledge of its role and regulation in AM fungi is still limited (Benabdellah et al. 2009; Tamayo et
288al. 2016). Four glutaredoxin genes of *R. irregularis* have been characterized and demonstrated to be
289upregulated in the extraradical and intraradical mycelium following exposure to H_2O_2. The
290homologues of glutaredoxin *RiGRX1* (Benabdellah et al. 2009), *RiGRX4* and *RiGRX6* (Tamayo et
291al. 2016), were found in the *G. margarita* transcriptome (E-values: 3e-25, 9e-105 and 3e-63,
292respectively) and seemed to respond to H_2O_2. The homologue for *RiGRX5* was also found (E-value:
2932e-48), but its expression did not change significantly after H_2O_2 treatment. *G. margarita* catalase
294was also up-regulated upon treatment, but its expression level was not above the applied $\log_2$ fold
295change threshold. Other players of the ROS-scavenging system, such as thioredoxin reductase, have
296already been identified as markers of *G. margarita* endogenous oxidative stress response (Salvioli
297et al. 2016; Vannini et al. 2016). Taken as a whole, these observations indicate that *G. margarita*
298modulates the expression levels of many redox enzymes in a way comparable to other filamentous
299fungi, as well as the model AM fungus *R. irregularis*. Moreover, given the number and the
300expression of the genes reported in Table 1, the glutathione/thioredoxin systems could be identified
301as key players in the oxidative stress response of *G. margarita*.
302To gather information on how G. margarita senses oxidative stress, we specifically searched for
303DEGs annotated as mitogen-activated protein kinases (MAPK) and transcription factors. This
304identified 14 DEGs encoding transcription factors and 160 encoding MAPKs (73 up-regulated vs 87
305down-regulated). Thirteen of these kinases possessed a PAS domain, which is common to bacteria,

306fungi, and animals and is associated with osmotic and redox sensing (Taylor and Zhulin 1999; 307Moye-Rowley 2003). We then performed a BLAST search to find putative components of the High 308Osmolarity Glycerol Pathway (HOG), which is important for the response to oxidative stress in 309yeasts and filamentous fungi (Duran et al. 2010). Four top scoring G. margarita transcripts were 310differentially expressed upon H2O2 treatment, but without a clear expression pattern (Table S4). Given the 311high number of putative redox sensing elements whose expression is modulated by the treatment in 312a relatively short time (3h), we suggest that G. margarita evolved some specific mechanisms to 313perceive ROS also when growing outside the plant. Indeed, previous works demonstrated that AM 314fungi are exposed to H2O2 produced by the plant during the symbiotic phase, and that H2O2 315concentrations could change the outcome of the symbiosis by being perceived as a signaling 316molecule (Kiirika et al. 2012; Arthikala et al. 2014; Belmondo et al. 2016).

317

318**The effect of *Ca*Gg on the response of *G. margarita* to hydrogen peroxide**

319

320PCA (Fig. 1a) revealed that the treatment with H_2O_2 reduced the differences between the B+ and the 321B- fungal line. Since the B- transcriptional profile seemed to be "triggered" by H_2O_2 becoming 322closer to the treated B+ profile, we analyzed the fungal B+ and B- responses in more detail. The 323Venn diagram (Fig. 3) highlighted a subset of 1716 genes that were identified as DEGs in all the 324three comparisons (all treated *vs* all control, B+ treated *vs* B+ control, B- treated *vs* B- control). This 325subset can be referred to as the 'core' response of *G. margarita* to H_2O_2. Among this common gene 326core, the transcripts directly involved in ROS- related metabolism are listed in Table 1. Additionally, 327the B+ and B- lines had a non-shared set of genes responding to H_2O_2. In more detail, 3386 DEGs 328responded to the H_2O_2 uniquely in the B+ line, but fewer DEGs (2054) responded uniquely in the B- 329line.

330Assuming that the areas of the Venn diagram (3386 B+ and 2054 B- non shared DEGs) may 331efficiently summarize a part of the variability explained by the fungal-bacterium interaction, GO 332enrichment was performed on both the subsets (Figs. 4 and 5). The analyses revealed that among 333the enriched categories of the B+ treated *vs* B+ control comparison there was a tendency to 334upregulation, while the B- treated *vs* B- control comparison had prevalently down regulated 335categories. Phosphate and phosphorus metabolic processes were highly represented in both the 336enriched GO sets, but were up-regulated in the B+ and down-regulated in the B- treated spores. 337Interestingly, response to other organism (GO:0051707) was among the enriched terms only for the 338B+ line upon treatment. Similarly, in the B+ treated line two processes associated with DNA and 339chromatin conformation changes were well represented and up-regulated, while proton transport

340and electron transport chain were down-regulated. It has already been demonstrated that, at least in 341*Candida*, the exposure to H_2O_2 triggers a conformational chromatin change that finely modulates 342the antioxidant responses (da Silva Dantas et al. 2015).

343Two functional categories associated with sporulation (GO:0043937 and GO:0034306) were down-344regulated in the B- line after treatment. Nitrate assimilation and metabolism were enriched uniquely 345for the B- line, as well as the response to iron ion. An iron transporter was reported to be one of the 346most upregulated transcripts in the B+ control line *vs* the B- control line (Salvioli et al. 2016.). We 347could thus hypothesize an active role of *Ca*Gg in regulating the fungal iron homeostasis: in the 348absence of the endobacterium, and in particular under oxidative stress, this process could be 349strongly impaired.

350Processes involved in modification of proteins and macromolecules were also enriched in the B+ 351line: since chaperones, heat shock proteins, and ubiquitination/proteasome related genes belong to 352this category, the activation of such pathways suggests that fungal proteins were indeed damaged by 353oxidative stress, and that these processes were essential to maintain the functionality of the cell.

354Finally, some of the transcripts encoding for ROS scavengers illustrated in Table 1, and mainly 355upregulated in the all treated *vs* all control comparison were down-regulated in the treated B+, when 356compared to the treated B- line. These data were consistent with glutathione S-transferase, 357peroxiredoxin, and glutathione peroxidase (Table 2). This result may be explained by considering 358that the expression of these ROS scavengers is already higher in the B+ control fungus, when 359compared to the B- control (B+ control *vs* B- control comparison). A similar expression pattern has 360already been described for glutathione S-transferase and peroxiredoxin, which were shown to be 361specifically up-regulated at transcript and protein levels in the B+ line under normal growth 362conditions (Salvioli et al. 2016; Vannini et al. 2016). Thus, given that *G. margarita* B+ efficiently 363elicits the ROS scavengers, it potentially needs a lower activation of these genes when compared to 364the less-efficient B- line upon H_2O_2 treatment.

365

366**Glutathione and ascorbate content analysis**

367To test whether the response observed at the transcriptional level allows the B+ and B- lines to 368maintain their redox homeostasis, we measured total glutathione and ascorbate, and ascorbate 369analogues, in control and treated fungal lines (Fig. 6). The B- control spores contained significantly 370less GSH than the B+ control spores but had more comparable GSSG content. As a consequence, 371the GSH/GSSG ratio was increased by the presence of the endobacterium (Fig. 6a), confirming 372previous observations that *Ca*Gg can influence the redox balance of its fungal host (Salvioli et 373al.2016; Vannini et al.2016). The H_2O_2 treatment led to an increase in total glutathione contents for

374both lines. The transcript encoding for glutamate-cysteine ligase (comp33867_c0), an enzyme
375participating in the first steps of glutathione biosynthesis, showed a tendency to up-regulation in the
376all treated *vs* all control comparison. The same trend was observed while considering the B- and the
377B+ treated lines separately ($\log_2$ fold change: 1.20 for the B- treated line and 0.82 for the B+ treated
378line). Despite the increase in total glutathione, the GSH/GSSG ratio was only slightly changed in
379the treated B+ *vs* the control B+ samples. By contrast, the increase in glutathione content in the
380treated B- fungus deeply affected the GSH/GSSG ratio, which reached values comparable to those
381of the B+ line. In treated conditions, the maintenance or the increase of GSH/GSSG ratios in B+
382and B- lines, respectively, seem to be due to the increase in the transcript encoding glutathione
383reductase (Table 1).
384In plants, ascorbate plays a critical role in protecting cells against damaging ROS produced by the
385chloroplast during photosynthesis (Wheeler et al. 2015); however, our knowledge of the
386biosynthesis and role of ascorbate in fungi is much more limited. Fungi synthesize a range of
387ascorbate analogues, including 6-deoxy-L-ascorbate, ascorbate glycosides, and the five-carbon
388analogue, D-erythroascorbate (Loewus 1999). However, molecular information on the involved
389genes is limited to yeast, and the description of the pathways leading to the different fungal ASC
390analogues is incomplete (Wheeler et al. 2015). Since at least two enzymes, D-arabinono-1,4-lactone
391oxidase (ALO1) and L-gulonolactone oxidase (GULO), are known to be involved in ascorbate
392biosynthesis in fungi, as a first step we used TBLASTN to look for homologues in the
393transcriptome of *R. irregularis* (Tisserant et al. 2013; Lin et al. 2014) and of *G. margarita* (Salvioli
394et al.2016). We found that *G. margarita* has distinct transcripts as homologues for the two genes,
395but *R. irregularis* has one homologue that matched both GULO and ALO1 (Table S5). In our
396datasets, the expression of the two *G. margarita* putative homologues did not show an expression
397pattern that can clearly suggest their involvement in the response to hydrogen peroxide.
398The dipyridyl assay, which measures all the ascorbate-like reductants (Spickett et al. 2000), showed
399that the B- control spores had an higher content of ascorbate or its analogues (hereafter, collectively
400indicated as ASC in the reduced form, and DHA in the oxidized form) than the B+ control spores;
401this indicates that the presence of *Ca*Gg is involved in the decrease of these compounds (Fig. 6b).
402Treatment with H_2O_2 decreased ASC and DHA levels in the cured lines and had no effect on the
403content of these metabolites in the B+ lines. As for glutathione, H_2O_2 treatment reduced the
404differences between the two lines, which after the treatment had the same ASC and DHA levels.
405
406**Discussion**

407To verify whether the fungal-endobacterial symbiosis established between *G. margarita* and *Ca*Gg
408may enhance the fitness of the fungus in the presence of environmental oxidative stress, we treated
409the germinating fungus with a sublethal hydrogen peroxide concentration and performed a detailed
410RNA-seq analysis. Our transcriptomic results indicate that *G. margarita* may efficiently face
411exogenous oxidative stress, and in the presence of its endobacterium upregulates many metabolic
412pathways. However, and contrary to our hypothesis, the cured line responds to H_2O_2 by activating
413the transcription of genes encoding specific ROS scavengers. We confirmed the efficacy of this
414strategy by measuring the glutathione content and redox state of cured and uncured lines, finding
415that, at the end of the treatment, these were the same for both lines.
416We further suggest that glutathione could constitute the main antioxidant molecule for *G.*
417*margarita*, making the role of ascorbate secondary. Indeed, although the ASC and DHA contents in
418the B+ and B- lines subjected to H_2O_2 treatment are lower than in the control B- line, no changes in
419the redox state of these metabolites has been observed, suggesting that in *G. margarita* ascorbate
420and its analogues could play different metabolic roles from the antioxidative one, as already
421suggested for yeast (Spickett et al. 2000).
422
423***Gigaspora margarita* has adaptive mechanisms to face oxidative stress**
424
425Hydrogen peroxide has often been used to mimic environmental oxidative stress in many model
426organisms. Our experiments reveal that *G. margarita* is highly sensitive to H_2O_2 treatment, leading
427to a consistent number of DEGs (5458 out of 35029 potential *G. margarita* genes) that respond to
428the treatment, irrespective of the presence of the endobacterium. *G. margarita* therefore is similar to
429yeast and many other filamentous fungi, where H_2O_2 treatment induces a strong detoxifying
430response with the activation of glutathione and thioredoxin metabolism, but can also trigger cellular
431differentiation and development (Breitenbach et al. 2015). Some activated genes in the treated AM
432fungus were expected, such as the genes encoding ROS-detoxifying enzymes listed in Table 1,
433some of which have already been characterized in *R.. irregularis* (Tamayo et al. 2016). Other genes
434coding for reducing enzymes linked to glutathione and thioredoxin, and known as the most
435important redox buffers for eukaryotic cells, were up-regulated upon H_2O_2 treatment. The changes
436observed in the expression of several genes coding for iron/heme-binding enzymes and for other
437mitochondrial proteins confirm the role of this organelle in the bacterial-fungal interaction (Salvioli
438et al. 2016). By contrast, the HOG pathway, which is considered a marker for H_2O_2 response in
439yeast (Alonso-Monge et al. 2003), was weakly activated, suggesting that symbiotic filamentous
440fungi have probably elaborated different mechanisms. The upregulation of the gene coding for
441methionine sulfoxide reductase has significance, since methionine-containing proteins directly

442participate in oxidative stress responses and methionine itself is often described as a defense against
443oxidative stress in eukaryotic cells (Luo and Levine 2009). Together with the upregulation of sulfur
444metabolism and the pentose phosphate pathway, which is essential for sulfur assimilation, being the
445main source of reducing power (Slekar et al. 1996), we can conclude that the main actors involved
446in detoxification are at work when *G. margarita* is exposed to H_2O_2 (Fig.7). Given the relatively
447high number of MAPKs regulated by H_2O_2 in our data set, we also suggest that *G. margarita*
448strongly relies on this type of signal transduction to face the oxidative stress, as seen for *Candida*
449*albicans* (de Dios et al. 2010) and other filamentous fungi (Ding et al. 2015; Jacob et al. 2014).
450Curiously, a comparable succession of events (activation of pentose phosphate pathway, production
451of reducing power, recycling of GSSG to GSH, and relief of oxidative damage) has been described
452when pollinators ingest nectar (Martinez del Rio and Dillon 2017; Levin et al. 2017). This could
453suggest that the use of the ancient pentose phosphate pathway to avoid oxidative damage may be a
454conserved strategy for living organisms.
455
456**The fungal responses to H_2O_2 change depending on the presence of the endobacterium**
457The variance partitioning analysis revealed a small effect of the endobacterium in shaping the
458transcriptome profile of its fungal host in the presence of an oxidative stress; however, we observed
459that some specific metabolic pathways changed depending on the presence/absence of the
460endobacterium.
461When considering the general pattern of the transcripts, some processes were up-regulated in the B+
462line and down-regulated in the B- line. We suggest that, for the B- line, the impairment of several
463aspects of the primary metabolism (Bonfante and Desirò 2017; Lumini et al. 2007) could impact the
464way the fungus reacts to stress induced by hydrogen peroxide. A crucial gene category showed an
465opposite pattern: all the ROS-scavenger genes shown in Table 2, were surprisingly upregulated in
466the B- line *vs* the B+ line after H_2O_2 treatment. Since we have previously demonstrated (Salvioli et
467al. 2016) that the B+ line had constitutively higher expression levels of such ROS scavenging
468enzymes than the B- line, we reasoned that the line with its endobacteria probably needs less of an
469increase in the expression of ROS-responsive elements compared with the B- line. Thus, the H_2O_2
470treated B- line needs to invest more in eliciting the expression of ROS scavengers, in order to reach
471an antioxidant status that is comparable to that of the native fungal line.
472In contrast to the cured line, the fungus with the endobacteria seems to activate a different strategy.
473It does not further elicit the transcription of the ROS-scavenger genes following the H_2O_2 exposure,
474and indeed (under similar conditions) the corresponding bacterial genes were downregulated
475(Salvioli et al. 2016), suggesting that the *Ca*Gg does not provide specific help in ROS

476detoxification. However, regulatory systems of *Ca*Gg like the toxin-antitoxin systems are activated
477by H_2O_2 exposure (Salvioli et al. 2017). Therefore, we suggest that the bacterium provides a
478protection to its fungal host by using more complex mechanisms than the upregulation of ROS-
479scavanger genes. Differently from these genes, many other pathways were upregulated in the
480fungus with the endobacterium. In the treated B+ line processes related to MAPK signal
481transduction (such as phosphorus and phosphate metabolism categories, protein kinase cascade and
482phosphorylation processes) were overall up-regulated, while they were down-regulated in the
483treated B- line. Since the whole MAPK cascade is activated by diverse environmental stresses as a
484powerful actor to control gene expression and conferring cellular stress resistance (Morigasaki et al.
4852013; Hagiwara et al. 2009), we hypothesize that the fungus with its endobacterium may more
486efficiently face oxidative stress, as well as high osmolarity and heat shock. Also DNA
487conformation/packaging GO categories were upregulated in the B+ treated line only. Given that
488H_2O_2 can act as a stimulus to activate some genomic areas (Breitenbach et al. 2015), the fungal–
489bacterial association could be more efficient in perceiving it.
490
491Our work has demonstrated that an AM fungus actively responds to H_2O_2 used to mimic an
492exogenous source of oxidative stress. Some responses mirror mechanisms already described in
493yeast and filamentous fungi. The presence of the endobacterium leads to more tuned differences: the
494B- line mostly activates the expected ROS-scavenger genes, which are constitutively highly
495expressed in the B+ line, while the B+ line activates many other pathways. These pathways include
496MAPK cascades, which may lead to a more active and tightly regulated response to exogenous
497oxidative stress.
498In conclusion, it seems that the mechanisms for adaptation to oxidative stress in *G. margarita* are
499diverse and differ depending on the presence/absence of the bacterium. The AM fungus with the
500bacteria strongly up-regulate many general processes, which probably also involve the
501endobacterium; the AM fungus without the bacteria show general down-regulation of such
502processes, but specifically activate a set of ROS-related genes. Eventually, the antioxidant status of
503the two fungal lines turns out to be very similar, demonstrating the unexpected capacity of the cured
504line to compensate for its original lower performance. We can speculate that for both the lines H_2O_2
505also acts as a signal, as it does in dried seeds (Tommasi et al. 2001, El-Maarouf-Bouteau and Bailly
5062008) and in fungal conidia also from pathogenic fungi, where changes in the cellular oxidative
507status trigger germination (Breitenbach et al. 2015). Since H_2O_2 is a strong antibacterial molecule,
508the question remains whether *Ca*Gg is affected by the treatment, also considering that some of the
509key enzymes for glutathione biosynthesis were not found in its genome (Ghignone et al, 2012).

Given the relevance of AM fungi in natural and agricultural ecosystems, we propose that the data set developed for *G. margarita* may be a starting point for studying environmental adaptation of AM fungi to the oxidative stress that originates from the application of fungicide or herbicide (Yang et al. 2016; Angelova et al. 2005).

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Figure Legends

757**Fig. 1:** (a) Principal Component Analysis (PCA) showing the distance between samples of each 758condition. Ellipses represent 95% confidence interval around each group; (b) Variance Partitioning 759Analysis (VPA) executed over the whole dataset, showing hydrogen peroxide treatment as the 760driving parameter contributing to overall variability.

761**Fig. 2:** GO enrichment analysis representation of the DEGs generated for the all treated *vs* all 762control comparison. Each slice of the circle is associated with an enriched GO category. The size of 763the inner circle slices is proportional to the significance of the related term (P<0.05), and their color 764indicates if the related category is globally up- or down-regulated. A dot is plotted in the outer circle 765slices for each gene belonging to a specific category. The color of the dots indicates if the genes are 766up- (red) or down- (blue) regulated, and their position on the gray spaces is a representation of their 767log2 fold change (i.e. genes with the strongest up-regulation are placed on the outer border of the 768spaces).

769**Fig. 3:** Venn diagram showing the number of DEGs that are in common between the all treated *vs* 770all control, B+ treated *vs* B+ control and B- treated *vs* B- control comparisons, and genes whose 771expression changes uniquely for each comparison.

772**Fig. 4:** GO enrichment analysis representation of the DEGs generated for the genes that are DE 773only for the B- treated *vs* B- control comparison. Each slice of the circle is associated with an 774enriched GO category. The size of the inner circle slices is proportional to the significance of the 775related term (P < 0.05), and their color indicates if the related category is globally up- or down-776regulated. A dot is plotted in the outer circle slices for each gene belonging to a specific category. 777The color of the dots indicates if the genes are up- (red) or down- (blue) regulated, and their 778position on the gray spaces is a representation of their log2 fold changes (genes with the strongest 779regulation are placed on the borders of the spaces).

780**Fig. 5:** GO enrichment analysis representation of the DEGs generated for the genes that are DE 781only for the B+ treated *vs* B+ control comparison. Each slice of the circle is associated with an 782enriched GO category. The size of the inner circle slices is proportional to the significance of the 783related term (P < 0.05), and their color indicates if the related category is globally up or down 784regulated. A dot is plotted in the outer circle slices for each gene belonging to a specific category. 785The color of the dots indicates if the genes are up- (red) or down- (blue) regulated, and their 786position on the gray spaces is a representation of their log2 fold changes (genes with the strongest 787regulation are placed on the borders of the spaces).

788**Fig. 6:** Glutathione (a) and ascorbate and/or its analogues (b) contents in control and treated spores 789of *G. margarita*. Bars indicate the total amount of the molecules (GSH and ASC) and the ratios 790between the reduced and the oxidised form. Values (expressed as picomoles per spore) are the mean

791of three independent biological replicates. Letters indicate whether the differences observed in the 792total amount of GSH and ASC are statistically significant ($p<0.05$).

793**Fig. 7:** Scheme showing the enzymes activated by *G. margarita* in response to hydrogen peroxide 794treatment and the connection between their activities and ROS detoxification. Methionine rich 795proteins actively participate in ROS scavenging, since methionine reacts with peroxide radicals and 796is oxidized to methionine sulfoxide, being successively reduced by methionine sulfoxide reductase. 797The oxidized enzyme is then restored to the reduced form in a reaction which involves the oxidation 798of thioredoxin, reduced by the enzyme thioredoxin reductase in a NADPH dependent reaction. 799Given its activation in response to hydrogen peroxide, pentose phosphate cycle is suggested as the 800main source of NADPH to counteract oxidative stress in *G. margarita*. NADPH derived from the 801pentose phosphate cycle could also be involved in the assimilation of sulfur, which is the element 802characterizing the functional groups of all the elements reported in the scheme.

803**Table 1:** *G. margarita* ROS detoxifying enzymes and their activation in the all treated *vs* all control 804comparison.

805**Table 2:** Expression of three *G. margarita* ROS scavengers in different conditions. The transcript 806levels for these genes are higher in the B+ control; therefore, after treatment, the B+ line requires 807less transcriptional activation to achieve the same detoxifying potential as the B-.

808

809**Supplemental materials**

810**Table S1:** Data for each Illumina single-end library, including the initial yield of raw reads and the 811percentage of reads that survived the trimming with Trimmomatic V.0.36.

812**Table S2:** summary of the differentially expressed genes (DEGs) identified for each comparison 813(FDR<0.05; |log2foldchange| >1.5).

814**Table S3:** list of the most up- and down- regulated genes in each comparison.

815**Table S4:** G. margarita homologues for the key genes involved in the yeast HOG1 signaling 816pathway and their expression.

817**Table S5:** *R. irregularis* and *G. margarita* candidates for GULO and ALO1, two enzymes known to 818catalyze the synthesis of ascorbate analogues in fungi. A single *R. irregularis* showed homology to 819both GULO and ALO1, but two distinct candidates were found for *G. margarita*