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This is the author's manuscript

Original Citation:

Availability:

This version is available http://hdl.handle.net/2318/1645707 since 2017-11-14T15:25:22Z

Published version:

DOI:10.1007/s00572-017-0790-z

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30 August 2024

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This is the author's final version of the contribution published as:

Venice, Francesco; de Pinto, Maria Concetta; Novero, Mara; Ghignone, Stefano; Salvioli, Alessandra; Bonfante, Paola. Gigaspora margarita with and without its endobacterium shows adaptive responses to oxidative stress. MYCORRHIZA. None pp: 1-13.
DOI: 10.1007/s00572-017-0790-z

The publisher's version is available at:
http://link.springer.com/10.1007/s00572-017-0790-z

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

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Acknowledgments

This work was supported by funding from the University of Torino to PB (60%). The authors thank Luisa Lanfranco and Simone Belmondo for their support in the library construction, Marcella Bracale and Candida Vannini for advice on metabolite analyses as well as Jennifer M Mach for the critical reading of the manuscript.

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

Introduction

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

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

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

and may play different roles. On the one hand, H_2O_2 may act as a signal in response to injury in fungi such as *Trichoderma* and *Rhizophagus irregularis*, suggesting that fungi share a defense mechanism based on ROS-related molecules, as is common in plants and animals (Hernández-Oñate et al. 2012; Fester and Hause 2005). On the other hand, environmental stimuli can lead to oxidative stress; for example, a recent study reported that the glutathione peroxidase redox system is directly involved in the resistance of *Alternaria alternata* to the fungicides fludioxonil and vinclozolin (Yang et al. 2016). Current information on how AM fungi respond to exogenous oxidative stress is restricted to the intraradical stages of fungal development (Belmondo et al. 2016; Lanfranco et al. 2005), and define a scenario in which host plant-produced ROS are part of a fine-tuned signaling pathway necessary for fungal development. Similar experiments have described how *Beauveria bassiana* (an entomopathogenic fungus) responds to insect-produced ROS (Zhang et al. 2015).

Few studies have examined the response of AM fungi to induced oxidative stress when the AM fungi are living in the soil as extraradical hyphae. A recent investigation (Tamayo et al. 2016) revealed that five *R. irregularis* glutaredoxin genes respond to H_2O_2 and may have a role in iron homeostasis. By using an omics approach, we wanted to test the hypothesis that *G.. margarita* can respond to exogenous oxidative stress by activating specific anti-oxidants and that the presence of its endobacterium enhances the fungal response to oxidative stress. Preliminary results (Salvioli et al. 2016) suggested that both the bacterial and fungal symbionts perceived the oxidative stress. Here, we set up an RNA-seq approach to describe the response of an AM fungus to H_2O_2 treatment, validating the transcriptomic data with antioxidant measurements. We demonstrate that *G. margarita* with its endobacterium is equipped to respond to environmental oxidative stresses, but also the cured line successfully responds to H_2O_2 with the expression of genes involved in specific ROS- related pathways.

Materials and Methods

Spore production

Gigaspora margarita Becker and Hall (isolate BEG 34, deposited at the European Bank of Glomeromycota) was used in this study. This strain naturally contains endobacteria (Bianciotto et al. 1996) and is identified here as the B+ line. The cured line, i.e. without the endobacterium (B-line), was obtained from the B+ line as described in Lumini et al. 2007. To maintain and propagate both 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).

Differential expression analysis

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

Extraction and analysis of ascorbate and glutathione

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

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

The glutathione pool was assayed utilizing 0.4 ml aliquots of supernatant neutralized with 0.6 ml of 0.5 M phosphate buffer pH 7.5. For glutathione disulphide (GSSG) assay, the reduced form of glutathione (GSH) was masked adding to the neutralized supernatant 20 µl of 2-vinylpyridine, whereas 20 µl H_2O were added in the aliquots utilized for total glutathione pool assay. Tubes were mixed until an emulsion was formed. Glutathione content was measured in a 1 ml reaction mixture containing 0.2 mM NADPH, 100 mM phosphate buffer pH 7.5, 5 mM EDTA, 0.6 mM 5,5'-dithiobis(2-nitrobenzoic acid) and 0.1 ml of sample obtained as described above. The reaction was started by adding 3 units of glutathione reductase and was monitored by measuring the change in absorbance at 412 nm for 1 min. GSH was estimated as the difference between the amount of total glutathione and that of GSSG. A standard curve in the range 0-30 µM ml^{-1} GSSG was prepared. Ascorbate and glutathione data were expressed as the average of three independent experiments. After checking for normality and homoscedasticity with Shapiro-Wilk and Levene's mean tests, respectively, the differences between groups were assessed by using one-way analysis of variance (ANOVA) with a post-hoc Tukey's test, assuming $P<0.05$ as statistically significant. The statistical analyses were performed using Sigma Plot software 12.0 (Systat Software, Inc., CA, USA).

Results

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

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

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

To examine the effect of the endobacterium (B+ and B-) and the treatment (with and without H_2O_2) in shaping the fungal transcriptome, we performed Principal Component Analysis (PCA) and Variance Partitioning Analysis (VPA) on the whole dataset of normalized read counts (Fig. 1a and b, respectively). PCA analysis highlighted a strong differentiation between the control and treated samples (PC1, H_2O_2 treatment), but the separation between the data for the B+ and B- lines (PC2, endobacterial presence) was lower in both control and treated conditions. The lowest degree of separation was observed between the B+ and B- treated lines. Indeed, the direct comparison between the treated B+ fungus versus the treated B- fungus showed the fewest DEGs among all the comparisons (2423). The distance between the control and treated B- fungal samples was lower than the distance between the control and treated B+ fungal samples. This observation was consistent with the number of DEGs from the two comparisons (5543 *vs* 8291, respectively). VPA revealed the treatment with H_2O_2 as the factor mostly influencing the overall variability (~65%), while the presence/absence of the bacterium explained ~6% of the overall variability (Fig.1 b). These results confirm the PCA results, where the PC1 axis represents 72% of the variance *vs* the 18% represented by the PC2 axis.

Since the H_2O_2 treatment seemed to be the driving parameter, all the control fungal samples (B+ control and B- control) were compared with the treated samples (B+ treated and B- treated), irrespective of the presence of *Ca*Gg. This comparison identified 5458 DEGs, corresponding to about 15% of the *G. margarita* transcriptome (2398 up-regulated *vs* 3060 down-regulated genes).

Hydrogen peroxide treatment: identification of the main Gene Ontology categories

The results of GO enrichment analysis on this data set are shown in Fig 2. Among the enriched categories, the pentose-phosphate shunt, nicotinamide metabolic process, zinc ion transport, methionine metabolic process, and sulfur metabolic process categories were up-regulated overall in the treated samples.

The up-regulation of metabolic processes relative to sulfur and methionine is not surprising, since methionine containing proteins directly participate in defense against oxidative stress. Several types of ROS react with methionine, forming methionine sulfoxide, which is reduced by methionine sulfoxide reductase (Luo and Levine 2009). We previously demonstrated that under normal growth conditions, the B- line contains a higher quantity of carbonylated proteins (Vannini et al. 2016), which are considered a major hallmark of oxidative damage (Fedorova et al. 2014) and specifically involve methionine oxidation. The gene coding for methionine sulfoxide reductase showed a significant up-regulation in our data set upon H_2O_2 treatment (comp18281_c1, $\log_2$ fold change: 12.69). Consistent with this, sulfur metabolism was among the enriched and up-regulated categories.

The re-routing of carbon flux to the pentose-phosphate pathway has been described as a conserved response to oxidative stress (Ralser et al. 2007). In yeast, the pentose phosphate pathway is essential for sulfur assimilation, being the main reservoir of NADPH used by the cell for sulfite to sulfide conversion (Slekar et al. 1996). In *G. margarita* both the absence of *Ca*Gg (Salvioli et al. 2016; Vannini et al. 2016) and the H_2O_2 treatment seemed to induce the shift towards the pentose phosphate pathway. Taken in the whole, the GO enrichment analysis reveals that the H_2O_2 treatment pushes *G. margarita* to deeply change its transcriptome profile, shifting towards alternative metabolic pathways that are directly linked to mitigation of oxidative stress.

To describe the oxidative stress response of *G. margarita* more in depth, DEGs from the treated *vs* control comparison (pooling results for B+ and B-) were further divided on the basis of their functional annotation, and we manually selected some genes known to be involved in ROS detoxification in fungi (Table 1). Among them, genes encoding glutathione peroxidase, glutathione reductase, and glutathione s-transferase, as well as a peroxiredoxin and a thioredoxin reductase were strongly up-regulated after the hydrogen peroxide treatment. Notwithstanding the central role that the gluthatione cycle plays as a cellular redox buffer in yeasts and filamentous fungi, knowledge of its role and regulation in AM fungi is still limited (Benabdellah et al. 2009; Tamayo et al. 2016). Four glutaredoxin genes of *R. irregularis* have been characterized and demonstrated to be upregulated in the extraradical and intraradical mycelium following exposure to H_2O_2. The homologues of glutaredoxin *RiGRX1* (Benabdellah et al. 2009), *RiGRX4* and *RiGRX6* (Tamayo et al. 2016), were found in the *G. margarita* transcriptome (E-values: 3e-25, 9e-105 and 3e-63, respectively) and seemed to respond to H_2O_2. The homologue for *RiGRX5* was also found (E-value: 2e-48), but its expression did not change significantly after H_2O_2 treatment. *G. margarita* catalase was also up-regulated upon treatment, but its expression level was not above the applied $\log_2$ fold change threshold. Other players of the ROS-scavenging system, such as thioredoxin reductase, have already been identified as markers of *G. margarita* endogenous oxidative stress response (Salvioli et al. 2016; Vannini et al. 2016). Taken as a whole, these observations indicate that *G. margarita* modulates the expression levels of many redox enzymes in a way comparable to other filamentous fungi, as well as the model AM fungus *R. irregularis*. Moreover, given the number and the expression of the genes reported in Table 1, the glutathione/thioredoxin systems could be identified as key players in the oxidative stress response of *G. margarita*.

To gather information on how G. margarita senses oxidative stress, we specifically searched for DEGs annotated as mitogen-activated protein kinases (MAPK) and transcription factors. This identified 14 DEGs encoding transcription factors and 160 encoding MAPKs (73 up-regulated vs 87 down-regulated). Thirteen of these kinases possessed a PAS domain, which is common to bacteria,

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

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

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

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

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

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

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

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

Glutathione and ascorbate content analysis

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

both lines. The transcript encoding for glutamate-cysteine ligase (comp33867_c0), an enzyme participating in the first steps of glutathione biosynthesis, showed a tendency to up-regulation in the all treated *vs* all control comparison. The same trend was observed while considering the B- and the B+ treated lines separately (log_2 fold change: 1.20 for the B- treated line and 0.82 for the B+ treated line). Despite the increase in total glutathione, the GSH/GSSG ratio was only slightly changed in the treated B+ *vs* the control B+ samples. By contrast, the increase in glutathione content in the treated B- fungus deeply affected the GSH/GSSG ratio, which reached values comparable to those of the B+ line. In treated conditions, the maintenance or the increase of GSH/GSSG ratios in B+ and B- lines, respectively, seem to be due to the increase in the transcript encoding glutathione reductase (Table 1).

In plants, ascorbate plays a critical role in protecting cells against damaging ROS produced by the chloroplast during photosynthesis (Wheeler et al. 2015); however, our knowledge of the biosynthesis and role of ascorbate in fungi is much more limited. Fungi synthesize a range of ascorbate analogues, including 6-deoxy-L-ascorbate, ascorbate glycosides, and the five-carbon analogue, D-erythroascorbate (Loewus 1999). However, molecular information on the involved genes is limited to yeast, and the description of the pathways leading to the different fungal ASC analogues is incomplete (Wheeler et al. 2015). Since at least two enzymes, D-arabinono-1,4-lactone oxidase (ALO1) and L-gulonolactone oxidase (GULO), are known to be involved in ascorbate biosynthesis in fungi, as a first step we used TBLASTN to look for homologues in the transcriptome of *R. irregularis* (Tisserant et al. 2013; Lin et al. 2014) and of *G. margarita* (Salvioli et al.2016). We found that *G. margarita* has distinct transcripts as homologues for the two genes, but *R. irregularis* has one homologue that matched both GULO and ALO1 (Table S5). In our datasets, the expression of the two *G. margarita* putative homologues did not show an expression pattern that can clearly suggest their involvement in the response to hydrogen peroxide.

The dipyridyl assay, which measures all the ascorbate-like reductants (Spickett et al. 2000), showed that the B- control spores had an higher content of ascorbate or its analogues (hereafter, collectively indicated as ASC in the reduced form, and DHA in the oxidized form) than the B+ control spores; this indicates that the presence of *Ca*Gg is involved in the decrease of these compounds (Fig. 6b). Treatment with H_2O_2 decreased ASC and DHA levels in the cured lines and had no effect on the content of these metabolites in the B+ lines. As for glutathione, H_2O_2 treatment reduced the differences between the two lines, which after the treatment had the same ASC and DHA levels.

Discussion

To verify whether the fungal-endobacterial symbiosis established between *G. margarita* and *Ca*Gg may enhance the fitness of the fungus in the presence of environmental oxidative stress, we treated the germinating fungus with a sublethal hydrogen peroxide concentration and performed a detailed RNA-seq analysis. Our transcriptomic results indicate that *G. margarita* may efficiently face exogenous oxidative stress, and in the presence of its endobacterium upregulates many metabolic pathways. However, and contrary to our hypothesis, the cured line responds to H_2O_2 by activating the transcription of genes encoding specific ROS scavengers. We confirmed the efficacy of this strategy by measuring the glutathione content and redox state of cured and uncured lines, finding that, at the end of the treatment, these were the same for both lines.

We further suggest that glutathione could constitute the main antioxidant molecule for *G. margarita*, making the role of ascorbate secondary. Indeed, although the ASC and DHA contents in the B+ and B- lines subjected to H_2O_2 treatment are lower than in the control B- line, no changes in the redox state of these metabolites has been observed, suggesting that in *G. margarita* ascorbate and its analogues could play different metabolic roles from the antioxidative one, as already suggested for yeast (Spickett et al. 2000).

Gigaspora margarita has adaptive mechanisms to face oxidative stress

Hydrogen peroxide has often been used to mimic environmental oxidative stress in many model organisms. Our experiments reveal that *G. margarita* is highly sensitive to H_2O_2 treatment, leading to a consistent number of DEGs (5458 out of 35029 potential *G. margarita* genes) that respond to the treatment, irrespective of the presence of the endobacterium. *G. margarita* therefore is similar to yeast and many other filamentous fungi, where H_2O_2 treatment induces a strong detoxifying response with the activation of glutathione and thioredoxin metabolism, but can also trigger cellular differentiation and development (Breitenbach et al. 2015). Some activated genes in the treated AM fungus were expected, such as the genes encoding ROS-detoxifying enzymes listed in Table 1, some of which have already been characterized in *R.. irregularis* (Tamayo et al. 2016). Other genes coding for reducing enzymes linked to glutathione and thioredoxin, and known as the most important redox buffers for eukaryotic cells, were up-regulated upon H_2O_2 treatment. The changes observed in the expression of several genes coding for iron/heme-binding enzymes and for other mitochondrial proteins confirm the role of this organelle in the bacterial-fungal interaction (Salvioli et al. 2016). By contrast, the HOG pathway, which is considered a marker for H_2O_2 response in yeast (Alonso-Monge et al. 2003), was weakly activated, suggesting that symbiotic filamentous fungi have probably elaborated different mechanisms. The upregulation of the gene coding for methionine sulfoxide reductase has significance, since methionine-containing proteins directly

participate in oxidative stress responses and methionine itself is often described as a defense against oxidative stress in eukaryotic cells (Luo and Levine 2009). Together with the upregulation of sulfur metabolism and the pentose phosphate pathway, which is essential for sulfur assimilation, being the main source of reducing power (Slekar et al. 1996), we can conclude that the main actors involved in detoxification are at work when *G. margarita* is exposed to H_2O_2 (Fig.7). Given the relatively high number of MAPKs regulated by H_2O_2 in our data set, we also suggest that *G. margarita* strongly relies on this type of signal transduction to face the oxidative stress, as seen for *Candida albicans* (de Dios et al. 2010) and other filamentous fungi (Ding et al. 2015; Jacob et al. 2014).

Curiously, a comparable succession of events (activation of pentose phosphate pathway, production of reducing power, recycling of GSSG to GSH, and relief of oxidative damage) has been described when pollinators ingest nectar (Martinez del Rio and Dillon 2017; Levin et al. 2017). This could suggest that the use of the ancient pentose phosphate pathway to avoid oxidative damage may be a conserved strategy for living organisms.

The fungal responses to H_2O_2 change depending on the presence of the endobacterium

The variance partitioning analysis revealed a small effect of the endobacterium in shaping the transcriptome profile of its fungal host in the presence of an oxidative stress; however, we observed that some specific metabolic pathways changed depending on the presence/absence of the endobacterium.

When considering the general pattern of the transcripts, some processes were up-regulated in the B+ line and down-regulated in the B- line. We suggest that, for the B- line, the impairment of several aspects of the primary metabolism (Bonfante and Desirò 2017; Lumini et al. 2007) could impact the way the fungus reacts to stress induced by hydrogen peroxide. A crucial gene category showed an opposite pattern: all the ROS-scavenger genes shown in Table 2, were surprisingly upregulated in the B- line *vs* the B+ line after H_2O_2 treatment. Since we have previously demonstrated (Salvioli et al. 2016) that the B+ line had constitutively higher expression levels of such ROS scavenging enzymes than the B- line, we reasoned that the line with its endobacteria probably needs less of an increase in the expression of ROS-responsive elements compared with the B- line. Thus, the H_2O_2 treated B- line needs to invest more in eliciting the expression of ROS scavengers, in order to reach an antioxidant status that is comparable to that of the native fungal line.

In contrast to the cured line, the fungus with the endobacteria seems to activate a different strategy. It does not further elicit the transcription of the ROS-scavenger genes following the H_2O_2 exposure, and indeed (under similar conditions) the corresponding bacterial genes were downregulated (Salvioli et al. 2016), suggesting that the *Ca*Gg does not provide specific help in ROS

detoxification. However, regulatory systems of *Ca*Gg like the toxin-antitoxin systems are activated by H_2O_2 exposure (Salvioli et al. 2017). Therefore, we suggest that the bacterium provides a protection to its fungal host by using more complex mechanisms than the upregulation of ROS-scavanger genes. Differently from these genes, many other pathways were upregulated in the fungus with the endobacterium. In the treated B+ line processes related to MAPK signal transduction (such as phosphorus and phosphate metabolism categories, protein kinase cascade and phosphorylation processes) were overall up-regulated, while they were down-regulated in the treated B- line. Since the whole MAPK cascade is activated by diverse environmental stresses as a powerful actor to control gene expression and conferring cellular stress resistance (Morigasaki et al. 2013; Hagiwara et al. 2009), we hypothesize that the fungus with its endobacterium may more efficiently face oxidative stress, as well as high osmolarity and heat shock. Also DNA conformation/packaging GO categories were upregulated in the B+ treated line only. Given that H_2O_2 can act as a stimulus to activate some genomic areas (Breitenbach et al. 2015), the fungal–bacterial association could be more efficient in perceiving it.

Our work has demonstrated that an AM fungus actively responds to H_2O_2 used to mimic an exogenous source of oxidative stress. Some responses mirror mechanisms already described in yeast and filamentous fungi. The presence of the endobacterium leads to more tuned differences: the B- line mostly activates the expected ROS-scavenger genes, which are constitutively highly expressed in the B+ line, while the B+ line activates many other pathways. These pathways include MAPK cascades, which may lead to a more active and tightly regulated response to exogenous oxidative stress.

In conclusion, it seems that the mechanisms for adaptation to oxidative stress in *G. margarita* are diverse and differ depending on the presence/absence of the bacterium. The AM fungus with the bacteria strongly up-regulate many general processes, which probably also involve the endobacterium; the AM fungus without the bacteria show general down-regulation of such processes, but specifically activate a set of ROS-related genes. Eventually, the antioxidant status of the two fungal lines turns out to be very similar, demonstrating the unexpected capacity of the cured line to compensate for its original lower performance. We can speculate that for both the lines H_2O_2 also acts as a signal, as it does in dried seeds (Tommasi et al. 2001, El-Maarouf-Bouteau and Bailly 2008) and in fungal conidia also from pathogenic fungi, where changes in the cellular oxidative status trigger germination (Breitenbach et al. 2015). Since H_2O_2 is a strong antibacterial molecule, the question remains whether *Ca*Gg is affected by the treatment, also considering that some of the key 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

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

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

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

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

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

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

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

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

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

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

Supplemental materials

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

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

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

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

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