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This is a pre print version of the following article:

Original Citation:

Availability:

This version is available http://hdl.handle.net/2318/1557227 since 2017-05-15T11:00:48Z

Published version:

DOI:10.1111/nph.13895

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13 November 2024

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This is an author version of the contribution published on:
Questa è la versione dell'autore dell'opera:
[New Phytologist, 2016, DOI: 10.1111/nph.13895]

The definitive version is available at:
La versione definitiva è disponibile alla URL:
[http://onlinelibrary.wiley.com/doi/10.1111/nph.13895/full]

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

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Summary

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

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

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

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

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

Introduction

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

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

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

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

To understand the bacterial effect on fungal fitness, we used next-generation sequencing to analyse the transcriptional profile of *G. margarita* in the presence and in the absence of its endobacterium (Salvioli *et al.*, 2015). Transcriptional analysis was performed using germinated spores with and without GR24 treatment, which is a synthetic analogue of strigolactone (SL). SLs are plant hormones that play a key role in plant-fungal signalling (Al-Babili & Bouwmeester, 2015; Bonfante & Genre, 2015). In addition to the fungal sporification success, transcriptomic results indicate that the endobacterium affects a large number of fungal cell functions. In particular, it targets mitochondrial activity, upregulating genes involved in respiration, ATP production, and reactive oxygen species (ROS) detoxification.

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

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

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

Materials and Methods

Biological materials

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

Protein extraction

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

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

Protein digestion and iTRAQ labelling

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

LC-MS/MS analysis

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

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

iTRAQ protein identification and quantification

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

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

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

al., 2015). According to this and in the absence of reference proteome, the identified *G. margarita* proteins were blasted against the NCBI database for *R. irregularis* (E-value<e^{-40}, identity≥40%). The proteins identified as bacterial proteins were blasted against the NCBI database of *Ca.* G. gigasporarum (E-value<e^{-40}, identity≥40%).

For Venn diagrams we used the open source program Venny 2 (*Oliveros, J.C. (2007-2015) Venny. An interactive tool for comparing lists with Venn's diagrams.* http://bioinfogp.cnb.csic.es/tools/venny/index.html)

Respiratory activity

O_2 consumption was measured using a Clark-type electrode (Hansatech Ltd, Hardwick Industrial, Norfolk, UK) calibrated between 0% and 100% with atmospheric oxygen. The respiration chamber was connected with a water circulator to maintain constant temperature of 30°C. The reaction was carried out at a constant stirrer speed in a 1 ml chamber volume. Recording of oxygen consumption was started by adding 1ml of dH_2O into the chamber followed by 100 fungal spores. Spores were germinated for 3 days at 30°C in the dark. For GR24 treatment, the spores were incubated for 3 h with 10-7M of GR24 before polarographic measurement. O_2 consumptions were read for 15 min. Increase of O_2 consumption in spores was calculated by comparison of the slope with distilled water in equilibrium with the O_2 atmospheric (control).

H_2O_2, glutathione and total antioxidant activity

After three days of germination 2700 spores of each line were collected by filtration on Whatman 3MM paper and separately weighed for the determination of H_2O_2, total antioxidant activity (TAA) and total glutathione (GSH). For TAA and GSH 0.1 g of spores were ground in a mortar in liquid nitrogen with 10 volumes of acidified methanol and 5% metaphosphoric acid, respectively. After centrifugation at 20000 g the levels of TAA and GSH were measured in the supernatants according to Locato *et al.* (2008).

For intracellular H_2O_2 determination, 0.1g of spores were homogenized with 10 volumes of ice-cold 5% trichloroacetic acid. The extracts were centrifuged for 20min at 1400*g*, and the supernatant was neutralized in the presence of an 0.1M phosphate buffer (pH 7.0) with 2M KOH to approximately pH 7.0. H_2O_2 was measured in the

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

Detection of carbonylated proteins in fungal and plant hosts

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

Real-time PCR assays

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

Results and Discussion

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

Ca. G. gigasporarum affects a core set of fungal proteins

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

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

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

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

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

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

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

Germinating spores of B+ and B+GR24 accumulated proteins involved in DNA replication, transcription, and protein synthesis (Tables S3 and S4), suggesting that endobacteria enhance fungal growth. This result is consistent with the higher growth rate of the B+ line than the B- line (Lumini *et al.*, 2007). One of the most strongly differentially expressed proteins was a Rho-GDP-dissociation inhibitor (Rho-GDI), which was downregulated in the B- and B-GR24 proteomes. The *Rho-GDI* transcript (comp37206_c0_seq1) level was also lower in the B- line (Salvioli *et al.*, 2015). Rho-GDI represses monomeric Rho-GTPases, which control many fundamental cellular processes such as cytoskeletal organisation, vesicle trafficking, and bud site selection (DerMardirossian & Bokoch, 2005). Curing the colonial marine bryozoan *Bugula neritina* from its endosymbiont *Candidatus* Endobugula sertula also resulted in Rho-GDI downregulation and disrupted cytoskeletal organisation (Mathew & Lopanik, 2014). Curing *G. margarita* from its endobacterium caused phenotypic changes in the cell wall, lipid drops, and cytoplasmic viscoelasticity (Lumini *et al.*, 2007). The B- line has a denser and more extensively aggregated cytoplasm than the B+ line; this could be due to Rho-GDI downregulation and accumulation of actin and tubulin proteins (Table S3 and S4).

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

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

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

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

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

Approximately 33% of the differentially expressed proteins in B+ and B- lines were involved in metabolic processes (Table S3). Proteins that are differentially expressed in the B+ line include a subunit of NADH-ubiquinone reductase, which is involved in mitochondrial oxidative phosphorylation; the mitochondrial malate dehydrogenase (MDH1), which converts malate to oxaloacetate in the tricarboxylic acid cycle; and triose phosphate isomerase (TPI). These results are supported by transcriptomic data indicating that genes involved in oxidative phosphorylation are upregulated and ATP production increases in the B+ line, which suggests that endobacteria increase the bioenergetic potential of host fungi (Salvioli *et al.*, 2015). To obtain evidence for this hypothesis, we measured fungal respiration in the B+ and B- lines. Polarography was sensitive enough to detect the O_2 consumption rate in 100 *G. margarita* spores after 3 days of germination. The results showed that O_2 consumption was approximately 50% higher in the B+ line than in the B- line. As expected, GR24 treatment for 3 hours

438 increased O_2 consumption for both lines, but the respiratory capacity in the B+ line
439 was approximately 50% higher than in the B- line (Table 1).
440 These results raised the question of what metabolic pathways were used for energy
441 production in the cured *G. margarita* line, as energy demands are crucial during plant
442 colonisation. Proteomic analysis provided some insight into an alternative reducing
443 pathway in the cured line. The following two proteins upregulated in the B- line were
444 involved in the pentose phosphate pathway (PPP): the phosphogluconate
445 dehydrogenase (decarboxylating enzyme) GND2 and the D-glyceraldehyde-3-
446 phosphate transaldolase TAL1. These proteins operate during oxidative and non-
447 oxidative phases of the PPP. This central pathway produces reduced equivalents in the
448 form of NADPH during the oxidative PPP phase, and produces precursors for nucleic
449 acid and aromatic amino acid biosynthesis during the non-oxidative PPP phase. Ralser
450 *et al.* (2007) showed that dynamic re-routing of metabolic flux to the PPP, with
451 concomitant NADPH generation, was a conserved response to oxidative stress.
452 NADPH provides the reducing potential for most antioxidant and regulatory enzymes
453 controlling cellular redox homeostasis.
454 Another NADPH source in the cured line is NADP+-dependent isocitrate
455 dehydrogenase (IDP1). The enzyme has been studied in *Saccharomyces cerevisiae*,
456 where it catalyses the conversion of D-*threo*-isocitrate to 2-oxoglutarate in
457 mitochondria. IDP has also been localised to the cytosol (IDP2) and peroxisome
458 (IDP3). These three IDPs are involved in defence against oxidative stress in yeast
459 (Contreras-Shannon & McAllister-Henn, 2004; Minard & McAllister-Henn, 2001). In
460 the current study, IDP differentially accumulated in the cured line as confirmed by
461 transcriptional results (Fig. 1), suggesting that the B- line had defective regulation of
462 oxidative status.
463 GR24 treatment induced the expression of some proteins involved in maintaining the
464 cellular redox balance in the B- line, including a cytosolic aldehyde dehydrogenase
465 (ALDH), an alcohol dehydrogenase (ADH), and a cytosolic glycerol-3-phosphate
466 dehydrogenase (GPD). RT-qPCR analyses confirmed the differential expression of
467 *ALDH* and *GPD* transcripts in the B+ and B- lines, while changes in ADH transcript
468 levels were not detected (Fig. 1). Cytosolic ALDH oxidises acetaldehyde to acetic
469 acid and produces NADPH. The ability to act as an aldehyde scavenger during lipid
470 peroxidation is another universal ALDH function found across species. Upregulation
471 of ALDH is a stress response in bacteria, plants, yeast, and mammals (Singh *et al.*,

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

Lipid catabolism provides an important energy resource for arbuscular mycorrhizal fungi

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

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

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

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

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

measurement of the cellular ROS, we analysed the H_2O_2 concentration in both systems. In B- the H_2O_2 content was 30% higher than the one of B+. However the very low concentrations measured do not allow to obtain results with statistical significance.

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

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

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

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

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

Conclusions

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

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

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

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

Acknowledgements

The Authors express their thanks to: Stefano Ghignone and Francesco Venice for bioinformatics support; Antonella Faccio for electron microscope observations; Maria Teresa Della Beffa and Sara Torrielli (University of Torino) for the taking care of the spore production; Alberto Vianelli and Simone Barera (Insubria University) for their very helpful support on polarographic measurements; the Functional Genomics Center Zürich (FGCZ) for highly valuable technical support.

Research was funded by the Ateneo Project (ex 60%) to PB. AS fellowship was granted by Piedmont Region and University of Torino. FO is a student of the *PhD*

School in “Biotechnology Life Sciences and Surgical Technologies” at the Insubria University.

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Legends

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

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

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

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

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

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

939 way analysis of variance (ANOVA). Bars not accompanied by the same letter are
940 significantly different at the 5% level using Tukey's test.
941
942
943 **Table 1. O_2 consumption in *G. margarita* lines**
944
945 Relative differences in O_2 consumption were measured by polarography in spores
946 germinated for three days and treated for 3 h with or without GR24.
947 a indicates values significantly different from B+ (t test, $p<0.01$)
948 b indicates values significantly different from B+GR24 (t test, $p<0.01$
949

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

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953 **Table 2 Intracellular levels of H_2O_2, antioxidant activity (TAA), and total**
954 **glutathione (GSH) in *G. margarita* lines.**
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956 The values are the mean ± ES of three independent experiments. a indicates values
957 significantly different from B+ (t test, $p<0.05$)
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959

	H_2O_2 nmoles g^{-1}FW	TAA nmoles Trolox eq g^{-1}FW	GSH nmoles g^{-1}FW
B+	6.1 ± 0.6	884 ± 35	189 ± 14

B-	8.5 ± 0.4	543 ± 30 [a]	107 ± 11 [a]

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963 **Supporting Information**
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965 Additional supporting information may be found in the online version of this article.
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967 **Table S1** Primers used for qRT-PCR analyses
968 **Table S2** List of the fungal proteins identified by iTRAQ analysis
969 **Table S3** List of the differentially expressed proteins in B- line in comparison with
970 B+ line
971 **Table S4** List of the differentially GR24-responsive proteins in B- line in comparison
972 with B+ line
973 **Table S5** Bacterial proteins differentially expressed in B+GR24 sample in
974 comparison with B+ sample
975 **Table S6** List of differentially carbonylated proteins in B-Myc in comparison with
976 B+Myc
977 **Fig. S1** Venn diagrams showing: a) the identified proteins in each analyzed condition;
978 b) the degree of overlap between differentially regulated proteins obtained by
979 comparing B+ versus B- and B+GR24 versus B-GR24.
980 **Fig. S2** Protein carbonylation profiles of Lotus japonicus roots after mycorrhizal
981 colonisation with the B+ or B- lines: a) protein stain, b) anti-DNP immunoassay c)
982 relative protein carbonylation values (referred to B+ sample) expressed as
983 carbonylation index, after normalization for protein amounts. Data (means ± SD, n=
984 3) were subjected to one-way analysis of variance (ANOVA). Bars not accompanied
985 by the same letter are significantly different at the 5% level using Tukey's test.
986 **Fig. S3** Representative two dimensional profiles of protein abundance and oxidation
987 in B+Myc and B- Myc clover roots. Protein stain (A,C) and anti-DNP immunoassay
988 (B, D) are shown. Selected protein undergoing differential carbonylation are labeled
989 with arrows. They are listed in Table S6.