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Metabolome changes are induced in the arbuscular mycorrhizal fungus *Gigaspora margarita* by germination and by its bacterial endosymbiont

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Short title

Metabolite profiling of Gigaspora margarita

Abstract

Metabolomic profiling is becoming an increasingly important technique in the larger field of systems biology by allowing the simultaneous measurement of thousands of small molecules participating in and resulting from cellular reactions. In this way, metabolomics presents an opportunity to observe the physiological state of a system, which may provide the ability to monitor the whole of cellular metabolism as the technology progresses. The arbuscular mycorrhizal fungus, *Gigaspora margarita* has not previously been explored with regards to metabolite composition. To develop a better understanding of *G. margarita* and the influences of its endosymbiont *Candidatus* Glomeribacter gigasporarum, a metabolomic analysis was applied to quiescent and germinated spores with and without endobacteria. Over 100 metabolites were identified and greater than 2,600 unique unidentified spectral features were observed. Multivariate analysis of the metabolomes was performed, and a differentiation between all metabolic states of spores and spores hosting the endobacteria was observed. The known metabolites were recruited to many biochemical pathways, with many being involved in maintenance of the antioxidant potential, tyrosine metabolism, and melanin production. Each of the pathways had higher metabolite abundances in the presence of the

endosymbiont. These metabolomics data also agree with previously reported transcriptomics results demonstrating the capability of this technique to confirm hypotheses and showing the feasibility of multi-omic approaches for the study of arbuscular mycorrhizal fungi and their endobacterial communities. Although challenges still exist in metabolomic analysis, e.g. the identification of compounds is still challenging due to incomplete libraries. A metabolomics technique to probe the effects of bacterial endosymbionts on fungal physiology is presented herein, and this method is useful for hypothesis generation as well as testing as noted above.

Keywords:
Metabolomics; bacterial-fungal interaction; arbuscular mycorrhizal fungi; germination; endobacteria; endosymbiosis; high resolution mass spectrometry; untargeted; ultra performance liquid chromatography; polar, water-soluble metabolite, Oribtrap, metabolome

Introduction

Metabolomics has emerged as a powerful tool in the field of chemical ecology to investigate metabolic regulation in a large number of organisms by detecting and quantifying the products that mediate interactions within and between organisms (Kuhlisch and Pohnert 2015). As the intermediates and final products of biochemical reactions, information regarding metabolite profiles provide an accurate way to characterize the phenotype (Patti et al. 2012a). Studies on tritrophic interactions, like those represented by *Arabidopsis thaliana*, herbivores and aphids, or those involving *A. thaliana* and beneficial plant growth promoting microbes have pioneered the field of metabolic interplays between species (Tenenboim and Brotman 2016).

Arbuscular mycorrhizas (AMs) are among the most ecologically relevant symbiotic plant-fungal associations, since they are present in more than 80% of the land plants, and play a crucial role in nutrient cycling (Smith and Read 2010). However, information on the metabolite profiles of AMs is still limited and mostly restricted to the metabolic profile of the photosynthetic partners (Abdallah et al. 2014; Laparre et al. 2014). Arbuscular mycorrhizal fungi (AMF) belonging to the Glomeromycotina (Spatafora et al. 2016) have not yet been studied using metabolomic approaches. Due to their obligate biotrophy (Bonfante and Genre 2010), the possibilities for experimentation are limited. At the moment the genome of only one species, *Rhizophagus irregularis*, has been sequenced (Chen et al. 2018; Liu et al. 2014; Tisserant et al. 2013), while the transcriptomes of three species, *R. irregularis, Gigaspora margarita,* and *G. rosea*, have been determined (Chen et al. 2018; Salvioli et al. 2016; Tang et al. 2016; Tisserant et al. 2013). Proteomic analyses were carried out on *R. irregularis* and *G. margarita* (Recorbet et al. 2013; Salvioli et al. 2010; Vannini et al. 2016). Focusing on the transcriptome and proteome features, these data sets point to some common characteristics of AMF: the absence of enzymes coding for cell wall degrading enzymes, the absence of genes coding for fatty acid synthesis (Tisserant et al. 2013; Wewer et al. 2014), and secretion of proteins that can act as effectors (Kamel et al. 2017; Sędzielewska Toro and Brachmann 2016). Recently, AMF have been demonstrated to not only be auxotrophic for sugars, but also for lipids. Even if AMF store lipids mainly as tri-palmityl-triacylglyerol (16:0 - TAG) and desaturate the 16:0 370 fatty acyl chain at a specific ω5 position, they do not possess genes for fatty acid synthesis in their genome (Wewer et al.

2014). By contrast, colonization of roots by AMF has been shown to elicit a specific lipid metabolism in the arbusculated root cortical cells, where lipids are delivered to the fungus (Bravo et al. 2017; Jiang et al. 2017; Keymer et al. 2017; Luginbuehl et al. 2017).

The microbiota (i.e. the microbial communities which live associated to multicellular organisms, both plants and animals) have been shown to critically influence health, immunity, and regulation of many metabolic processes (Ash and Mueller 2016). It is intriguing to note that AMF are relevant members of plant microbiota (Bonfante and Genre 2010; Brundrett 2017) while possessing their own microbiota. The microbiome of AMF consists of microbes which live at the surface of extraradical hyphae (Bonfante and Anca 2009; Olsson et al. 2017) where they provide important services like phosphate solubilization (Wang et al. 2016), and the endobacteria which live inside the fungi (Desiro et al. 2014). The discovery of endobacteria has raised the question of whether and how endobacteria affect fungal metabolism.

An integrated proteomics and metabolomics approach was recently used to characterize metabolic changes in *Mortierella elongata*, a fungus phylogenetically related to AMF (Li et al. 2017). *M. elongata* was investigated when cured of its endobacterium, *Mycoavidus cysteinexigens*, which revealed significant correlations between these two -omics approaches. Genome sequencing revealed that the genome of *M. cysteinexigens* (AG77) is reduced in size and function, yet still affects the growth of its fungal host (Uehling et al. 2017).

Aiming for a comprehensive systems biology framework for AMF and to better decipher the relationships between AMF and their endobacteria, we used *G. margarita* as a model fungal system since transcriptomics and proteomics data are already available (Salvioli et al. 2016; Vannini et al. 2016). Here, we evaluated whether metabolomics would allow the identification of metabolites involved in crucial stages of the fungal life cycle, namely dormancy and germination (Bonfante and Requena 2011). *G. margarita* harbors an intracellular microbiota consisting of hundreds of endobacteria, identified as *Candidatus* Glomeribacter gigasporarum (*Ca*Gg) and phylogenetically related to the genus *Burkolderia* taxon (Bonfante and Desiro 2017) of which the genome has been sequenced (Ghignone et al. 2012). The establishment of a cured line without the endobacterium has allowed the direct comparison of this cured line (B-) with the wild-type strain hosting the endobacterium (B+). Interestingly, in spite of its success in colonizing the plant host, the B- line is impaired in the mycelial growth, has a different spore wall structure, and may produce fewer spores than the B+ line (Lumini et al. 2007). Given the profound effects on the fungal transcriptome and proteome (Salvioli et al. 2016; Vannini et al. 2016), we hypothesized that the bacterial endosymbiosis

with *Ca*Gg along with spore germination will change the metabolome; and the premise was tested using an Ultra Performance Liquid Chromatography-High Resolution Mass Spectrometry (UPLC-HRMS) metabolomics technique.

Material and methods

Fungal propagation and spore collection

Spores of *G. margarita* Becker and Hall (BEG 34, deposited at the European Bank of Glomeromycota) were collected from trap cultures of white clover (*Trifolium repens*), divided in separate Eppendorf tubes containing 200 spores. Spores were surface sterilized with chloramine T (3% W/V) and streptomycin sulphate (0.03% W/V) using the following procedure: one wash (10 mins) in the sterilizing solution, one wash (10 mins) in sterile distilled water, one wash (10 mins) in the sterilizing solution, and three washes (10 mins each) in sterile distilled water. Immediately after sterilization a portion of the spores were frozen in liquid nitrogen and stored at -80°C denoted as quiescent spores with or without the endosymbiont (Q-B+ or Q-B-). Others were placed in multiwell plates (200 spores per well) and allowed to germinate in the dark at 30°C for 7 days. Spores were then checked under a stereomicroscope to evaluate the germination rate and confirm absence of contamination. Spores with the endosymbiont, *Ca*Gg, were denotated as G-B+ and without as G-B-. Batches of well germinated spores (number of germinated spores per well >50%) and void of contaminants were frozen in liquid nitrogen and stored at -80°C until metabolomics could be performed. In total, five batches of 200 quiescent (Q) spores with (Q-B+) and without (Q-B-) the bacterial endosymbiont as well as germinated (G) spores with (G-B+) and without (G-B-) the bacterial endosymbiont were analyzed (Fig. 1). This corresponds to five replicates for each of the four treatments (Q-B+, Q-B-, G-B+, G-B-).

Metabolite extraction

The extraction of the polar, water soluble metabolites was carried out at 4 ˚C unless otherwise stated. Six hundred fifty microliters of extraction solvent (40:40:20 HPLC grade methanol: acetonitrile: water, with formic acid added to a final concentration of 0.1 M). was added to the tubes with spores (Rabinowitz and Kimball 2007). The spores were crushed in the extraction solvent using a micro pestle. Excess material was washed from the pestle into the microcentrifuge tubes using additional 650 µL of extraction solvent, bringing the total volume of extraction solvent to 1.3 mL. Samples were vortexed briefly to suspend the spore particles then the solution was incubated at 4°C on an orbital shaker for 20 minutes. Samples were centrifuged at 16,100 rpm for 5 min before the supernatant was

transferred to a new vial. An additional 200 µL of extraction buffer was added to the spore debris for a further extraction at 4 °C for 20 minutes. Following centrifugation, this supernatant was combined with the previous supernatant, and the solvent was evaporated to dryness under N_2. The solid residues were resuspended in 300 µL of sterile water before being transferred to autosampler vials, and immediately placed in the Ultimate 3000 RS autosampler (Dionex, Sunnyvale, CA) cooled to 4 ˚C until aliquots could be analyzed.

Ultra Performance Liquid Chromatography- High Resolution Mass Spectrometry (UPLC–HRMS) analysis

An injection volume of 10 µL was separated through a Synergi 2.5 µm Hydro-RP, 100 Å, 100 x 2.00 mm liquid chromatography column (Phenomenex, Torrance, CA) kept at 25 ˚C. The mass spectrometer was run in full scan mode following a protocol adapted from Rabinowitz (Lu et al. 2010). The chromatographic eluent was ionized via an electrospray ionization (ESI) source in negative mode, and coupled to a Thermo Scientific Exactive Plus Orbitrap mass spectrometer through a 0.1 mm internal diameter fused silica capillary tube. The samples were run with a spray voltage of 3 kV, nitrogen sheath gas of 10 (arbitrary units), capillary temperature of 320 ˚C, and automatic gain control (AGC) target set to $3x10^6$ ions The samples were analyzed at a resolution of 140,000 and a scan window of 85-800 *m/z* from 0-9 min and 110-1000 *m/z* from 9 to 25 min. Solvent A consisted of 97:3 water:methanol, 10 mM tributylamine, and 15 mM acetic acid. Solvent B was 100% methanol. The solvent gradient from 0-5 min was 100%A 0% B, from 5-13 min 80%A 20% B, from 13-15.5 min 45%A 55% B, from 15.5-19 min 5%A 95% B, and from 19-25 min 100%A 0% B with a flow rate of 200 µL/min.

The raw data files generated by the mass spectrometer's software, Xcalibur (Thermo Fisher Scientific, Waltham, MA), were converted to the open-source mzML format (Martens et al. 2011) via the msconvert software that is part of the ProteoWizard package (Chambers et al. 2012). MAVEN v.682 software (Clasquin et al. 2002) was used to automatically align the total ion chromatograms based on the retention times for each sample. This method is untargeted and therefore detects both molecules of known chemical structure as well as spectral features from molecules of unknown structure (Patti et al. 2012b). Known metabolites were manually selected and integrated by exact mass (± 5 ppm) and known retention time. The 275 compound, metabolite library used in this study was constructed to cover a broad range of the water-soluble metabolome, with a particular focus on pathways involved in the biosynthesis of metabolites and macromolecular precursors that are

conserved among the domains of life. Initially, *Escherichia coli (E.coli)* was used for identification and validation of the metabolites, and the library has been expanded to include additional compounds as standards have become available (Lu et al. 2010). Unidentified peaks were automatically selected via MAVEN's automated peak detection algorithms using the following settings: mass domain resolution: 10 ppm, time domain resolution: 10 scans, EIC smoothing: 5 scans with 0.5 min peak binning, and baseline smoothing: 5 scans. Peak scoring was done using a trained classifier algorithm that looked for a minimum of four peaks per group, 5:1 signal to noise minimum, 5:1 signal to blank minimum, 10,000 signal intensity minima, and 5 scan peak width minimum. All the known and unknown chromatographic peaks were integrated for relative quantification.

Heatmaps and cloudplots were generated in R, version 3.4.2 (R Core Team 2016) using the ggplot2 (Wickham 2009), reshape2 (Wickham 2007), and colorspace (Ihaka et al. 2016; Zeileis et al. 2009) packages. Partial least squares discriminant analysis (PLS-DA) and Variable Importance in Projection (VIP) scores were calculated using MetaboAnalyst (Xia et al. 2015) with inner quartile range data filtering and cube root transformation preprocessing. PLS–DA was used to assess whether the differences in sample compositions were reflected in metabolite abundance using biological condition as the *Y* categorical matrix. This analysis was performed on the knowns (Fig. 2A) and the unidentified features (Fig. 2B). Pathway maps were modeled based on the information in the Kyoto Encyclopedia of Genes and Genomics (KEGG), (Kanehisa et al. 2017; Kanehisa and Goto 2000; Kanehisa et al. 2016). Slices of heat maps were overlaid on the pathways using an open source vector graphics software, Inkscape V0.92.2.

Results and Discussion

Herein, the first metabolomics investigation of the endosymbiotic relationship between *Gigaspora margarita* and *Candidatus* Glomeribacter gigasporarum is presented, and these data are compared to known transcripts and proteins corresponding to this interaction. This effort provides a first step towards understanding the metabolic impact of this endosymbiont on the fungus and provides a rich set of data that can be used to corroborate existing hypotheses and/or generate new ones.

The metabolomics profile of Gigaspora margarita: an overview

The mass spectrometric measurements identified a total of 108 metabolites that were included in the library of knowns, defined by *m/z* and retention times (Supplemental Table 1). Nine additional

metabolites were putatively annotated (based on their exact masses) to molecules with antioxidant activity (Supplemental Table 2). Finally, 2,610 *m/z*–retention time pairs for metabolites of unknown identity that displayed chromatographic peak-like qualities (referred to as unidentified spectral features) were recorded (Supplemental Table 3). The multivariate analysis of the profiles of the known metabolites differentiated the germinated and quiescent spores in addition to those with and without the endobacteria (Fig. 2A).

Unidentified components of the metabolome

The advantage of using an Orbitrap mass analyzer in full scan mode is that one obtains near full coverage of all the ions being generated, while the identifiable features are limited to known standards in the database analyzed with the same chromatographic parameters. The utilized extraction and analysis procedure is capable of profiling a large number of small molecules present in both the fungal and endobacterial metabolome. Although, it is possible that a number of fungus-specific metabolites were not identified from the current data set due to lack of database annotations. Aside from these identified metabolites, valuable information regarding the metabolome changes can be seen when looking at the unidentified features. Unidentified *m/z* features exhibit metabolite like behavior in terms of intensity, S/N, and chromatographic elution. These features (Supplementary Table 3) can provide a better understanding of the overall trends in the data and help to further classify experimental treatments. In this case, 2610 were annotated. For example, the PLS-DA of the unidentified features (Fig. 2B) clearly separated the four biological conditions (Q-B+, Q-B–, G-B+, and G-B-) from each other with a high clustering within each group. Even though we currently are not currently able to identify these features, it is possible to go back to the data at a later date and begin assigning compound names and biological function as future experiments reveal more about the complicated endosymbiosis. However, it is not possible to determine the organism origin of any molecule by metabolomics alone.

The analysis of the unidentified spectral features allowed a profiling of the overall characteristics among the different sample conditions, emphasizing uniqueness between spore status (quiescent and germinated) and the presence or absence of the endosymbiont. Since the identified metabolites (polar and water soluble) in the experiment only represent a small portion of all small molecules present, this could potentially bias the separation of the metabolomes among the four biological conditions. Analysis of the totality of unidentified features, provides a more holistic view of the complete metabolomes. However, the extraction (acidic acetonitrile and resuspension in water) and ionization

(ESI) conditions bias the analysis towards polar and water-soluble metabolites. Whereas this paper is focused on the detection of polar metabolites, future profiling of the lipidome is also likely to yield valuable information, and previous reports have shown that lipids play a role in the endosymbiosis between the fungi and bacteria (Desirò et al. 2018; Lastovetsky et al. 2016; Pétriacq et al. 2017).The detected features could be assigned to a number of compound classes: amino acids, nucleotides, organic acids, and sugars. Unidentified features that showed a high level of differentiation between groups could be further characterized to determine biological significance.

The identified metabolites which contributed most to the differences seen in the PLS-DA were identified by assaying the variable importance in projection (VIP) scores (Fig. 3A). This revealed strong differences between the spores with and without the *Ca*Gg during the quiescent status. For example, the abundance of 2-Hydroxy-2-methylsuccinate, arginine, and asparagine were higher in presence of the endosymbiont while spores were in the quiescent phase. Orotate, 2-Hydroxy-2-methylsuccinate, arginine, citraconate, and asparagine were other compounds that strongly accumulated in the presence of the bacterium during quiescent and germination (Q-B+ and G-B+). Interestingly, germination led to a clear decrease in all 30 top VIP scored metabolites in the endosymbiont free AMF. A similar but less drastic decrease is seen in spores with the endosymbiont, except for pantothenate which showed an increase. All in all, the alterations in amino acid metabolism were strongly associated with the changes seen in the metabolomes by the multivariate analysis.

Furthermore, the unidentified spectral features contributing most to the PLS-DA, can be seen by *m/z* with high VIP scores (Fig. 3B) which reveals at least 6 features important to the distinction between Q-B+ *versus* Q-B- (445.0887, 791.1539, 396.0765, etc.) and all 24 differentiating quiescent *versus* germinated spores (170.028, 146.0457, 162.9804, etc.). Only one of these features (213.0155) showed a relative increase in both, the endosymbiont hosting and free spores, compared to the respective quiescent spores While additional work is necessary to identify the compounds represented by the *m/z*, VIP score analysis provides a viable path to find potential biomarkers for the different metabolic and symbiotic states of the spores (Bogdanov et al. 2008). A comparison of the values of the VIP scores (approximate values of 4-8 for the unidentified features and 1-4 for the identified metabolites) show the unidentified spectral features have a higher level of impact in describing the metabolic changes between the spores.

An analysis of the shared and unique unidentified metabolic features (Fig. S2 and Table S3), revealed that the majority (2,449, 93.8%) of the 2,610 spectral features were observed in all four conditions. Sixty-three of the features (2.4%) were only present in both quiescent groups of spores (Q-B+ and Q-B-) and may be unique to the spore developmental status. Further, 0.3% of

metabolites (8 features) were unique to germinating spores. Each individual biological condition has very few spectral features (<3) that were detected exclusively in that set of spores. None of the features represented in figure S2 were represented in the top 30 VIP scores generated from the PLS-DA analysis of the unidentified features (Fig.3B), and further work will be necessary to determine their biological relevance. This shows that the presence or absence of a unique feature doesn't drive the uniqueness of the metabolome, but it's more centered around changes in abundances of features that are present across all biological conditions.

Information provided by unidentified features

Cloud plots are a means to visualize large numbers of unidentified features similarly as heatmaps, while retaining the information of *m/z*, retention time, and significance (*p*-value) (Patti et al. 2013). In this way, chromatographic retention, molecular sizes, and global changes can be assessed at a glance. A cloud plot comparing the average intensities of all unidentified features for germinating spores to quiescent spores revealed a number of large and significant ($p \leq 0.1$) changes. Interestingly, combined quiescent spores (with and without endobacteria, Q-B±) showed a larger number of unidentified metabolites that were more abundant than the combined germinated spores (with and without endobacteria, G-B±) (Fig. 4A), reflecting a similar trend as seen in the heat map of the identified metabolites (Fig. 5 column 3). The distribution of these significant features is spread out across retention times suggesting that there is not a single group of chemically similar compounds that are changing based on spore status (quiescence or germination). This shows that regardless of the presence of the endosymbiont, quiescent spores have a global metabolome that is much different from that of germinated spores. When comparing quiescent spores with and without the endobacterium (Q-B+/Q-B–), the number of compounds that are more abundant in the spores with the endobacteria (red circles) is roughly the same as the number of compounds that are more abundant in the spores without the endobacteria (blue circles) (Fig. 4B). However, the G-B+/G-B-, comparison (Fig. 4C) revealed larger and more significant changes in abundance in the germinated spores with the endobacterium. This points to the endosymbiont having a greater influence on the abundance of metabolites in the germinating stage. While presently the identity of these metabolite like features remains unknown, they present opportunity for molecular discovery via in-detail analyses such as gene expression, on the bacterial or fungal side. These data can be revisited, and unidentified features can then be identified.

Spore germination alters the metabolome

The big spores of *G. margarita* (400 micrometer in diameter) are rich in storage compounds like protein bodies, glycogen granules, and lipid droplets, which surround hundreds of nuclei (Bonfante et al. 1994). During germination these macromolecules are catabolized to provide energy for emission of the germ tubes and the growth of the extraradical mycelium. Because all the processes took place in water, major changes are expected in the metabolic processes under nutrient limiting conditions. In the experiment, quiescent and germinated spores with and without the bacterial endosymbiont (Q-B±, G-B±) were investigated using heat maps (Fig. 5, Supplemental Table 4). A comparison of the germinating spores to the quiescent spores (G-B±/Q-B±) revealed a similar but more intense pattern to what was seen in the individual comparisons (G-B+/Q-B+ and G-B-/Q-B-). Except for phenylpyruvate and guanine, G-B+ contained significantly higher amounts of metabolites when compared to G-B-. The B+ spores, whether quiescent or germinated, contained higher relative amounts of metabolites, suggesting that presence of the bacterium is either the direct or indirect cause of increased metabolic activity.

The purine pathway is of interest because it contains many metabolites whose abundances were significantly different (Fig. 5). Except for guanine, metabolites belonging to this pathway showed a significant decrease during B± germination, as seen by column three of figure 5. Only a few papers report the role of purine metabolism in fungal germination; a purine-auxotrophic strain of *Magnaporthe oryzae* is able to germinate and form appressorium, but not to complete the infection process in rice (Fernandez et al. 2013), while *Aspergillus nidulans* possesses a purine transporter that is strongly expressed during germination (Vlanti and Diallinas 2008). Supplying purines to the culture media also induced spore germination of a plasmid-less strain of *Bacillus anthracis* (Ireland and Hanna 2002). The role of the purine metabolism in germination of obligate biotrophic fungi remains unresolved, but our data suggest that guanine could play a role during germination as a result of interactions between *Ca*Gg and *G. margarita*. A screening in the genome of *Ca*Gg (Ghignone et al. 2012) revealed that the endobacterium does not possess the genes involved in the last biosynthetic steps of guanine. By investigating the expression of ftsZ, a marker gene for bacterial division, and quantifying the dividing endobacteria, we demonstrated that *Ca*Gg preferentially divides in the germinating spores after strigolactone treatment (Anca et al. 2009). We can therefore suggest that that, in the B+ line of germinating *G. margarita*, the precursors of guanine could be consumed not only by the fungus, but also by *Ca*Gg to sustain its own cell division. This could explain why, in absence of the bacterium, guanine accumulates in germinating spores (G-B-/Q-B-).

The urea cycle (Supplemental Fig. 1) has already been demonstrated to play an important role during the asymbiotic growth of *Glomus intraradices* (now *R. irregularis*), since carbon backbones are mobilized through this pathway for amino acid biosynthesis (Bago et al. 1999). Our dataset showed a consistent reduction of glutamine, citrulline, and glutamate suggesting that these newly synthesized molecules could be quickly incorporated or transformed into their derivatives for mycelial growth.

Spore metabolites responsive to the presence of the bacterial endosymbiont

The coupling of transcriptomics and proteomics approaches demonstrated that *Ca*Gg raises the bioenergetic capacity of the fungus, increasing ATP production, respiration, and elicits mechanisms to detoxify endogenous Reactive Oxygen Species (ROS) (Salvioli et al. 2016; Vannini et al. 2016). The synthesis of proteins which are specifically involved in ROS detoxification were found to be upregulated in the B+ spores, which indeed produced more H_2O_2, but also had higher antioxidant capacities. We investigated whether our metabolomics data set could confirm this trend for the B+ spores. The data set was examined for specific metabolites of heavily represented metabolic pathways. Starting from those involved in the Tricarboxylic Acid (TCA) cycle (Fig 5 and Fig. 6), the metabolites have comparable values during the quiescence, but they were more abundant in the germinating B+ spores when compared to the B- spores (citrate, malate, aconitate, fumarate, etc). However, when looking at the metabolite profiles for each biological condition (G-B+/Q-B+ and G-B-/Q-B-), the Q-B- spores revealed the strongest decrease in such metabolites, suggesting that the metabolites undergo a stronger consumption without replacement in the absence of the endobacterium.

Previous transcriptome analyses observed an increase in pentose phosphate pathway activity, suggesting an alternative method for producing reducing equivalents in the spores without the endobacteria (Salvioli et al. 2016; Vannini et al. 2016). In the case of the germinated spores, our metabolomics analysis revealed a trend towards higher concentrations of metabolites of the pentose phosphate pathway in G-B+/G-B–, as seen by ribose-5-phosphate (2.70, p = 0.0023), 3-phosphoglycerate (2.85, p = 0.0182), glucono-1,5-lactone (2.57, p = 0.0005), gluconate, 6-phosphogluconate (1.91, p = 0.0262), and 2-dehydrogluconate (2.55, p = 0.0006). Conversely, in the case of the quiescent spores (Q-B+/Q-B–), the majority of pentose phosphate pathway metabolites showed little change between Q-B+ and Q-B-spores, with the exception of a few negative fold-changes; pyruvate (0.75, p = 0.0268) and 6-phosphogluconate (0.55, p = 0.0016). Pentose phosphate metabolites were more abundant in the quiescent spores than the germinated spores (Q-B+ > G-B+

and Q-B– > G-B–), indicating that the pentose phosphate metabolite usage in germinating spores may be higher than in quiescent spores. This is consistent with germinating spores having an increased consumption of these metabolites. Increased transcript and protein concentrations in the pentose phosphate pathway of B- mycelium could result in lower metabolite concentrations as consequence of metabolite consumption (Salvioli et al. 2016; Vannini et al. 2016).

Fifteen metabolites that are potentially involved in the ROS metabolism were identified, and some of them were found to be more abundant in the presence of the fungus hosting the bacterium, irrespectively of the whether the spores were quiescent or germinated (Fig. 5 and Supplemental Table 2). Among them, the glutathione tripeptide (GSH) acts as the main redox buffer in yeast and filamentous fungi for both endogenous and exogenous oxidative stress adaptation (Gostimskaya and Grant 2016; Sato et al. 2009; Segal and Wilson 2018). Glutathione is oxidized to glutathione disulfide (GSSG) when its cysteine groups are exposed to oxidants, and is restored to the reduced form by glutathione reductase. An increase in the transcript levels of glutathione peroxidase, an enzyme which uses glutathione as a substrate to reduce H_2O_2, was found in *G. margarita* upon hydrogen peroxide and strigolactone treatment (Salvioli et al. 2016; Venice et al. 2017). The metabolomics data presented here seems to confirm RNA-seq data. Both quiescent and germinated spores, showed higher levels of GSH and GSSG than the cured fungal spores. The role of glutathione during spore germination is less clear, since only the B+ spores showed a relevant increase in GSSG upon germination. Interestingly, a preliminary evaluation of glutathione quantity by using a colorimetric assay had already led to similar results (Vannini et al. 2016). Ophtalmate is a tripeptide analogue to glutathione; studies in mammal models have reported that ophtalmate is mainly synthetized in cells where GSH depletion takes place (Soga et al. 2006). In the *G. margarita* metabolome, ophtalmate is highly abundant when GSH seems to undergo depletion when B+ and B- spores are germinating.
Lipoic acid and ascorbic acid are molecules whose role in oxidative stress defense emerged for fungi (Branduardi et al. 2007; Georgiou and Petropoulou 2001; Spalding and Prigge 2010). The lipoate – dehydrolipoate redox couple quenches several cellular radicals, including singlet oxygen species and superoxide radical. Dehydrolipoate also has a synergistic effect with other antioxidants such as glutathione and ascorbic acid, acting as substrate for the reduction of their oxidized forms (Spalding and Prigge 2010). Ascorbic acid acts as a cofactor for ascorbate oxidase in the detoxification of ROS, and is restored to the active form by ascorbate reductase. Both lipoic and ascorbic acids are more abundant in the B+ quiescent and germinating spores, in comparison with the B- spores. However, the role and biosynthesis of ascorbic acid in fungi is more important. Information on the genes

involved is limited to yeast, and the description of the pathways leading to the different fungal ASC analogues is incomplete (Wheeler et al. 2015). Two *G. margarita* genes involved in the biosynthesis of ascorbic acid (or its analogues) were identified, whose expression did not change after hydrogen peroxide treatment (Salvioli et al. 2016). Ascorbic acid seems to undergo depletion in B- spores during germination. The dipyridyl assay, which measures all the ascorbate-like reductants revealed other dynamics in the ascorbate content of the B+ and B- sporeas following a different experimental timing (Venice et al. 2017), but eventually the treatment with H_2O_2 led to a decrease of ascorbic acid in the cured spores only.

The metabolism of sulfur amino acids is potentially related with the oxidation-reduction potential (Missall et al. 2004) since sulfur amino acids are directly and reversibly oxidized by ROS, with consequent formation of disulfide bridges. Previous studies (Salvioli et al. 2016; Venice et al. 2017) demonstrated the potential role of methionine-rich proteins in oxidative stress defense in *G. margarita*. In particular, methionine is restored to its reduced form by methionine sulfoxide reductase. The importance of the methionine redox system in the protection from oxidative stress has been demonstrated in yeast (Campbell et al. 2016; Sideri et al. 2009) as well as in filamentous fungi (Soriani et al. 2009; Yin et al. 2015). B- germinated spores have a higher content in the amino acid methionine, when compared with the quiescent B- spores. Such an increase upon germination is not observed for the B+ spores, which by contrast, are already richer in methionine than the B- spores during the quiescent stage. Cysteine showed a similar pattern and increased in abundance during germination in the B+ spores.

Cytologically the fungus with the endobacterium contained more electron dense melanin granules (Bonfante et al. 1994; Lumini et al. 2007). Melanins have been implicated in fungal pathogenesis, due to their capacity to reduce oxidants, and may protect fungi from diverse oxidative stresses (Heinekamp et al. 2013; Mandal et al. 2005). The heatmap (Fig. 5) revealed that a number of metabolites involved in melanin synthesis (tyrosine, L-DOPA, indole-5-6 quinone) were present in higher concentrations when the fungus was in symbiosis with the endobacterium. Finally, orotate which is the metabolite with the largest change in abundance in the G-B+/G-B- and Q-B+/Q-B- comparisons, was always more abundant in B+ spores, irrespective of germination status. *Ca*Gg has all the enzymes required to form orotate from glutamine that was potentially obtained from the import of fungal peptides. We therefore, suggest orotate to be strictly related to the bacterial metabolism, although we do not know its role.

Summary and Conclusion

Numerous recent studies have identified endobacteria in several lineages of Mucoromycota, opening many questions about their impacts on fungal physiology (Bonfante and Desiro 2017). In this context, metabolomic profiles of *G. margarita* with and without its natural bacterial endosymbionts were analyzed using ultra performance liquid chromatography–high resolution mass spectrometry (UPLC-HRMS). The spores of the two *G. margarita* lines showed significant differences in their pools of metabolites depending on germination status. Identified metabolites originated from a number of major metabolic pathways including the TCA and urea cycles, purine, pyrimidine, and pentose phosphate metabolisms. Additionally, certain metabolites with inferred antioxidant capabilities were identified in the spores that hosted the endosymbiont.

While metabolomic approaches have already been used to characterize pathways of primary and secondary metabolites in many saprotrophic and pathogenic fungi (Kluger et al. 2015), AMF have not yet been analyzed for their metabolite composition. Metabolomics has also been used as a tool to support classical taxonomy (Ząbek et al. 2017), but its use in the analysis of metabolites to describe interactions between fungi and bacteria is still limited. Good examples are provided by *Rhizopus oryzae*, where the pathways for the main fermentation products are linked to each other by the availability of pyruvate (Meussen et al. 2012), and where the coculture with *Burkolderia gladioli* leads to the biosynthesis of novel antifungal and antibacterial polyketides (Ross et al. 2014). Data focused on fungal-endobacterial interactions are even more limited. A pioneering example is a study combining proteomics and metabolomics to describe *Mortierella elongata* and its endobacterium (Li et al. 2017). These authors suggested that the proportion of fungal to bacterial metabolites should be similar to that of the proteins. Making the same assumption, the large majority of metabolites should have been of *G. margarita* origin. A previous proteomic analysis (Vannini et al. 2016) on the *G. margarita*/*Ca*Gg system detected only 24 bacterial proteins compared to over 127 fungal proteins.

For these reasons, this study focused on comparing the fungal metabolic pathways in the presence and absence of the bacterial endosymbiont. We found that the products of the TCA cycle are depleted during the spore germination, irrespectively of the presence of *Ca*Gg bacteria. Since the mitochondrial metabolism is known to increase in AMF during germination (Besserer et al. 2008; Besserer et al. 2006), we consider our results in line with previous observations. The impact of *Ca*Gg on this fungal metabolism must be considered as well: the endobacterium does not possess pyruvate kinase, suggesting that, similarly to its close relative *Mycoavidus*, it cannot rely on glucose as primary carbon source (Ghignone et al. 2012; Uehling et al. 2017). *Mycoavidus* has been shown to possess 12

genes encoding malate/citrate transporters. It was suggested that the bacterium imports malate from its fungal host and potentially converts it to pyruvate (by malic enzyme activity) and CoA (by pyruvate dehydrogenase activity), both precursors for the TCA cycle (Li et al. 2017). *Ca*Gg, on the contrary, possesses only one malate/citrate transporter (Ghignone et al. 2012). Our metabolomics dataset shows that, although malate is consumed during germination in both B+ and B- spores, it remains more abundant in the G-B+ condition, compared to the G-B-. In conclusion, we suggest that, contrary to *Mycoavidus*, *Ca*Gg may not consume host-produced malate for its TCA cycle. This interpretation fits well to the previous transcriptomics study (Salvioli et al. 2016), which revealed that *G. margarita* malate synthase was not differentially expressed, in G-B+ and G-B- spores.

One of the main questions in relation to endosymbionts are possible benefits that these bacteria could provide their fungal hosts (Bonfante and Desiro 2017). It was suggested that the endobacterium raises the bioenergetic status of the fungus and thus creates benefits, like faster growth during the presymbiotic phase (Salvioli et al. 2016; Vannini et al. 2016) . Moreover, the treatment with H_2O_2 identifies the activation of specific ROS scavenging genes, with a differential regulation between the B+ and the B- spores (Venice et al. 2017). The metabolomics study identified many metabolites which are closely related to antioxidants such as cysteine, GSH, GSSG, and lipoic acid and are present in higher amount in the B+ spores. In addition, on the basis of genomic data, neither *Mycoavidus*, nor *Ca*Gg seem to possess gamma-glutamylcysteine synthetase (GCS) and glutathione synthetase (GSS). However, due to the presence of a glutathione S-transferase (GST), they could use the fungal glutathione as a substrate (Ghignone et al. 2012; Uehling et al. 2017; Venice et al. 2017). It seems that these fungal endobacteria play a limited, if any, role in the antioxidant capacities of *M. elongata* and *G. margarita*.

In short, for the first time, metabolomic analysis was successfully applied to the characterization of *G. margarita* and its endobacterium, *Ca*Gg. Using profiles from identified metabolites and unidentified spectral features, characteristic differences in *G. margarita* were clearly observed between quiescent and germinated fungal spores with and without endosymbiotic bacteria. It was evident that the fungal life cycle stage (quiescent versus germinated spores) and symbiotic relationship with its endosymbiont, had significant effects on metabolic and antioxidant pathway utilization and overall metabolite concentrations. In particular, as evidenced from cloud plots, certain shifts in metabolisms had profound impacts on the overall accumulation of metabolites (*e.g.* concentrations in G-B+>>G-B-). Although this specific analysis is focused on the water-soluble fraction of the metabolome, future directions that employ a total lipid analysis would allow for the

further exploration of the spore status and the relationship with its endosymbiont. These findings, together with previously reported transcriptomics and proteomics datasets, revealed the applicability of utilizing metabolomics to better understand the physiological plasticity of AMF and its endobacteria.

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List of figures

Fig. 1 Schematic representation of the four biological conditions investigated for *Gigaspora margarita* with and without their endosymbiont *Candidatus* Glomeribacter gigasporarum: quiescent spores with and without endosymbiont respectively (Q-B+, Q-B-) and germinated spores with and without endosymbiont, respectively (G-B+, G-B-).

Fig. 2 Partial least squares-discriminant analysis of identified metabolites (A) and unidentified spectral features (B) showing differentiation among the biological conditions: the quiescent spores of *G. margarita* with and without the bacterium (Q-B+, Q-B–) and after germination with and without the bacterium (G-B+, and G-B-).

Fig. 3 Top 30 Variable importance in projections (VIP) scores generated from partial least squares-discriminant analysis of identified metabolites (A) and unidentified spectral features (B) differentiating the spores based on biological conditions (Q-B+, Q-B–, G- B+, and G-B-).

Fig. 4 Cloud plots of unidentified spectral features with and without the bacterial endosymbiont during germination and quiescent status (G-B±/Q-B±) (A), quiescent spores with and without the endosymbiont (Q-B+/Q-B-) (B), and germinated spores with and without the endosymbiont (G-B+/G-B-) (C). The color and intensity of the circle represents the direction and degree of change for a specific unidentified feature. The size the circle represents the significance (Student's T-test) for that unidentified feature.

Fig. 5 Heatmap depicting log_2-transformed fold-changes for relative abundances of known metabolites. Red indicates a positive and blue a negative fold change. Different significant levels (Student's T test) are indicated with dots.

Fig. 6 Tricarboxylic acid cycle (TCA) with fold changes of abundances for metabolites across comparisons of the four biological conditions: the quiescent spores of *G. margarita* with and without the endosymbiont (Q-B+, Q+B–) and after germination with and without the endosymbiont (G+B+, and G-B–). Rectangles indicate a log_2-transformed fold change in intensity and directionality, with red and blue indicating a positive and negative fold change in metabolite abundance, respectively. Significance (Student's T test) is indicated with dots.

Supplemental materials

Supplemental Table 1 Identified metabolites: average ion intensities for five replicates, standard deviation, and relative standard deviation at all four biological conditions (Q-B+, Q-B-, G-B-, G-B+).

Supplemental Table 2 Mass/charges, average ion intensities for five replicates, standard deviation, and relative standard deviation corresponding to metabolites that are related to antioxidant activity. The .1, or .2 following the mass denotes that two peaks were found at the same exact *m/z*.

Supplemental Table 3 All unidentified *m/z* spectral features as recorded with MAVEN software (version 682), average ion intensities for 5 replicates, standard deviation, and relative standard deviation. Rows are colored to match the data presented in Fig. S3.

Supplemental Table 4 Fold-changes of metabolite abundances and corresponding p-values (Student's T-test), and for identified metabolites. Green: $p<0.01$, blue: $0.05<p<0.01$, yellow: $0.1<p<0.05$.

Supplemental Fig. 1 Metabolomics data for the urea cycle at all four biological conditions: the quiescent spores of *Gigaspora margarita* with and without the bacterium (Q-B+, Q-B–) and after germination with and without the bacterium (G-B+, and G-B-). Rectangles represent log_2-transformed fold-change intensity and directionality Red: positive, blue: negative. Significant differences are indicated by dots.

Supplemental Fig. 2 Venn diagram showing the number of unique and shared features among the four biological conditions: the quiescent spores of *Gigaspora margarita* with and without the bacterium (Q-B+, Q-B–) and after germination with and without the bacterium (G-B+, and G-B-).

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