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# 1 At the nexus of three kingdoms: the genome of the mycorrhizal fungus Gigaspora margarita

# 2 provides insights into plant, endobacterial and fungal interactions

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- 39 **Running title:** Fungal Genome Adaptations: nexus of three kingdoms

# 40 Originality-Significance Statement

Many exciting studies have recently provided a wealth of data on the arbuscular mycorrhizal (AM) establishment and functioning. However, our knowledge is mostly plant- centric, and the biology of AM fungi is still enigmatic, notwithstanding some isolates have been recently sequenced. Here, we provide the genome sequence of the AM Gigaspora margarita, which resulted to be the largest fungal genome so far sequenced with the 64% of transposable elements. By crossing genomics with transcriptomics and experimental evidences, we offer new ideas on the biology of a fungus which is indeed a "holobiont", since it contains endobacterial populations and viral sequences, and constantly interacts with its host plant. We have identified novel elements (from immunity genes-never identified before in AM fungi- to horizontal transfer events) supporting the hypothesis that the presence of the endobacteria - together with the transposable elements- has shaped the fungal genome. Our genomic data open a new window on the evolutionary, environmental, and ecological meaning of an AM fungus which on one hand has a such huge genome and on the other an apparent limited distribution.

# 64 Summary

As members of the plant microbiota, Arbuscular Mycorrhizal Fungi (AMF, Glomeromycotina) symbiotically colonize plant roots. AMF also possess their own microbiota, hosting some uncultivable endobacteria. Ongoing research has revealed the genetics underlying plant responses to colonization by AMF, but the fungal side of the relationship remains in the dark. Here, we sequenced the genome of Gigaspora margarita, a member of the Gigasporaceae in an early diverging group of the Glomeromycotina. In contrast to other AMF, G. margarita may host distinct endobacterial populations and possesses the largest fungal genome so far annotated (773.104 Mbp), with more than 64% transposable elements. Other unique traits of the G. margarita genome include: the expansion of genes for inorganic phosphate metabolism, the presence of genes for production of secondary metabolites and a considerable number of potential horizontal gene transfer events. The sequencing of G. margarita genome reveals the importance of its immune system, shedding light on the evolutionary pathways that allowed early diverging fungi to interact with both plants and bacteria.

### 87 Introduction

Arbuscular mycorrhizal symbiosis involves 72% of vascular plants and an ancient group of fungi 88 89 (arbuscular mycorrhizal fungi, AMF) that enhance host fitness by improving mineral nutrition and 90 resistance to biotic and abiotic stresses (Bonfante, 2018). The large number of sequenced plant 91 genomes and availability of mutants allowed many of the mechanisms underlying plant responses to colonization by AMF to be deciphered (Pimprikar and Gutjahr, 2018; MacLean et al., 2017; 92 Lanfranco et al., 2018). By contrast, the biology of the fungal partners remains largely unexplained. 93 AMF are important members of the plant microbiota, present in the rhizosphere and inside roots 94 95 tissues as obligate symbionts (Davison et al., 2015). As many other fungi, AMF also possess their own microbiota (Bonfante et al, 2019), often hosting uncultivable endobacteria inside their 96 97 cytoplasm (Bonfante and Desirò, 2017, Pawlowska et al., 2018). AMF genomes therefore offer an unexplored source for investigating the evolution of inter-kingdom interactions among plants, fungi 98 and bacteria. 99

100 The genome of the model fungus Rhizophagus irregularis was the first AMF to be sequenced 101 (Tisserant et al., 2013; Lin et al., 2014; Chen et al., 2018), offering novel insights into the genetics 102 of a member of the Glomeromycotina, such as the surprising absence of plant cell wall-degrading enzymes and the unexpected presence of genes potentially involved in sexual reproduction. 103 104 Genome sequences of other AMF followed: Rhizophagus clarus (Kobayashi et al., 2018), 105 Rhizophagus cerebriforme and Rhizophagus diaphanus (Morin et al., 2019), as well as two 106 members of the Diversisporales: Diversispora epigea (Sun et al., 2019) and Gigaspora rosea 107 (Morin et al., 2019). Irrespective of their phylogenetic position in the AMF tree, these novel 108 genomes consistently confirmed the obligate biotrophic nature of AMF, revealing the loss of genes 109 involved in plant polysaccharide degradation and fatty acid biosynthesis. Most of the sequenced 110 Rhizophagus strains, as well as G. rosea, do not possess endobacteria (Bonfante and Desirò, 2017), 111 but D. epigea and R. clarus contain Mycoplasma-Related Endobacteria (MRE) (Naumann et al.,

2010; Naito et al., 2015). Here, we present the assembly and annotation of the genome of 112 113 Gigaspora margarita, which belongs to the Gigasporaceae (Krüger et al., 2012), as G. rosea, and is a member of an early diverging AMF group, well separated from Glomeraceae It presents a life 114 115 cycle with a conspicuous extra-radical phase consisting of huge spores, auxiliary cells and extraradical mycelium, as well as an intra-radical phase where intercellular hyphae support production of 116 117 arbuscules inside cortical cells (Supporting information Fig. S1). Intra-radical vesicles are not 118 formed and its presence in natural environments is mostly limited to sandy soils (Stürmer et al., 2018). G. margarita may host Burkolderia- and MRE communities (Bianciotto et al., 1996; Desirò 119 et al., 2014), as well as viral sequences (Turina et al., 2018). The isolate used here contains a single 120 121 population of non-cultivable endobacteria, identified as Candidatus Glomeribacter gigasporarum 122 (*Ca*Gg), whose genome has been sequenced (Ghignone et al., 2012).

123 By means of an extended comparative genomic analysis, we reveal that the genome sequence of G. 124 margarita isolate BEG34 shares the peculiarities of the other AMF species (conserved core genes, 125 absence of plant cell wall-degrading enzymes, absence of type I fatty acid synthase), but is also 126 remarkably different from all other sequenced fungal genomes in its genome size (predicted to be 127 831 Mbp by flow cytometry) large number of transposable elements (TEs); in addition, several 128 horizontal gene transfer (HGT) events have been detected similar to other Diversisporaceae (Sun et 129 al., 2019). Thanks to the availability of OMICs data generated by using fungal lines with and 130 without bacteria (Salvioli et al., 2016; Vannini et al., 2016; Dearth et al., 2018), the genome 131 sequence of G. margarita sheds light on the evolutionary signatures of adaptation that have allowed 132 this early diverging fungus to interact with both plants and bacteria since Devonian times (Strullu-133 Derrien et al., 2018). Novel features, i.e. presence of an immune system; secondary metabolite production, and refined tuning of proteins and enzymes that target chitin, offer insights into the 134 biotrophic lifestyle of G. margarita. By providing a deeper understanding of inter-kingdom 135 136 interactions, the genomic information of G. margarita allows us to advance hypotheses on the 137 ecological and evolutionary meaning of AMF genomes.

#### 138 **Results and Discussion**

# Separation of the *G. margarita* nuclear genome sequence from mitochondria and endosymbiont genomes

We sequenced the genome of *G. margarita* BEG34 using one paired-end library (Illumina TruSeq
Nano) and two mate-pair (3 Kbp and 8 Kbp) libraries (Illumina Nextera). Sequencing of *G. margarita* BEG34 produced a first assembly of ~875 Mbp consisting of 6,453 scaffolds.

By using BlobTools (Laetsch and Blaxter, 2017) analysis for the visualization and partitioning of 144 metagenome assemblies, scaffolds were visualized as circles of variable size, separated on the basis 145 146 of their coverage and G/C content, and their taxonomic affiliation was inferred through sequence 147 homology. This analysis allowed us to separate the nuclear component, which is the focus of this 148 study (Fig. 1), from the other compartments containing DNA (mitochondria and CaGg 149 endobacteria, Supporting information Fig. S2). The mitochondrial reads were re-assembled into a complete circular chromosome (96,998 bp), agreeing with the results of Pelin et al. (2012), while 150 the CaGg reads led to an improved assembly (2.07 Mbp) compared with the previous version 151 (Ghignone et al., 2012). The nuclear genome, obtained by reassembling the nuclear reads, consists 152 153 of 6,490 scaffolds and is ~758 Mbp (Table 1), almost six times larger than that of the model species R. irregularis, and significantly larger than those of the saprotrophic and phylogenetically related 154 155 Mortierella elongata that has a genome size of around 49 Mbp (Uehling et al., 2017). According to NCBI and JGI Mycocosm (Supporting information Table S1), the size of the G. margarita genome 156 157 is second only to that of Austropuccinia psidii (Pucciniomycotina, Basidiomycota) at 1.2 Gbp, for 158 which no functional annotation is available (McTaggart et al., 2018).

This result was supported by flow cytometry, which revealed a DNA quantity for *G. margarita* corresponding to a genome of about 831 Mbp (Supporting information Fig. S3). Indeed, previous static cytometry suggested a comparable value (Bianciotto and Bonfante, 1992). Similar to other AMFs, the *G. margarita* genome has a G+C content below 30% (27.68%).

## 163 Transposable elements dominate the *G. margarita* genome

164 The nuclear assembly revealed that repeated sequences cover 64 to 86% of the assembled genome sequence, depending on the filtering of highly degenerated copies (Fig. 2, Supporting information 165 166 Fig. S4). These repeats can be ascribed to 4,264 consensus sequences, representing the putative ancestral TEs sequences from which they originated. The proportion of TEs is very high when 167 168 compared with the generally low value found in fungal genomes (Castanera et al., 2016), usually 169 ranging from 0 to 25% with the exception of some plant pathogens (Spanu et al., 2010) and 170 ectomycorrhizal species that have undergone massive TE amplifications (Martin et al., 2010). 171 Among the latter, Tuberaceae show a genome expansion which correlates with TEs increase, 172 accounting for 50% of the genomic content (Murat et al., 2018). Similar to the related G. rosea (Morin et al, 2019), G. margarita confirms this relationship well: the huge genome size is 173 174 accompanied by the highest proportion of TEs detected so far in AMF; transposable elements in the 175 model R. irregularis (Chen et al., 2018) represent 22 to 26% of the total assemblies (from 122 to 176 138 Mbp).

Overall, 63% of the *G. margarita* TE content (40.6% of genome) remains unclassified. Of the remainder, the largest group of TEs is classified as class I retrotransposons (12% of the genome) (Fig. 2, Supporting information Fig. S4), where the major classes are LINE (8%) and LTR Gypsy (2.5%) elements. At least one full length Gypsy element (g2860) was found to be active based on gene expression data (Supporting information Fig. S5). Since these groups are well represented in *G. rosea* (Morin et al., 2019) and *D. epigaea* (Sun et al., 2019) compared to *Rhizophagus* species, a Diversisporaceae-specific expansion might be hypothesized.

Among the DNA-repeats, at least 15 full-length TIRs were expressed, revealing a differential expression across the fungal life stages (Supporting information Fig. S5, Table S2). Another peculiar feature of *G. margarita* DNA-TEs consists in the presence of repeats with homology to the *sola* class, which is present in plants, bacteria and metazoa, but previously considered to be absent in fungi, except for *R. irregularis* (Gladyshev and Kleckner, 2017). Lastly, *G. margarita* genome harbours Helitrons, which are characterized by a rolling circle replication mechanism and which
may contain genes captured from other organisms. They have been demonstrated to be mediators of
horizontal gene transfer in other fungi (HGT, see specific paragraph) (Castanera et al., 2014).

192 Usually, TE presence is counterbalanced by host genome defences, including fungal repeat-induced point mutation (RIP), methylation induced pre-meiotically (MIP), meiotic silencing of unpaired 193 194 DNA (MSUD) and quelling (Muszewska et al., 2017). When compared to their ancestor sequences 195 (consensus sequences), the G. margarita TEs fragments had low frequency of nucleotide transitions (C to T, specifically; Supporting information Fig. S6a), which are operated by DNA-methylases and 196 are the outcome of RIP activity (Amselem et al., 2015). Furthermore, AT-rich regions could not be 197 198 statistically detected (Supporting information Fig. S6b), suggesting that G. margarita lacks RIP activity, while the impact of MIP remains to be evaluated. We inferred the absence of MSUD 199 200 activity as no homology could be found with the associated genes so far characterized in 201 Neurospora crassa; by contrast, small RNA-mediated TEs silencing (quelling) cannot be excluded, 202 as G. margarita genome encodes for the genes related to this pathway (see the Immune System 203 section), such as Dicer and Argonaute proteins.

204 It has been suggested that gene-sparse regions in fungal genomes contain small secreted proteins (SSPs) which are often associated with TEs, providing a favourable environment for the 205 206 diversification of fungal effector repertoire (Sánchez-Vallet et al., 2018). The G. margarita genome 207 is also gene-sparse (Supporting information Fig. S7a); a further screening revealed a significant 208 spatial association between G. margarita candidate SSPs (Supporting information Table S3) and 209 CRYPTON, TIR and MITE (Supporting information Table S4, Fig. S7b). In conclusion, a glimpse 210 at the TEs present in G. margarita genome reveals many peculiar features: the heterogenous TEs groups suggest that Diversisporaceae experienced some specific TE bursts; Helitrons could be 211 involved in HGT events; lastly, the TE abundance mirrors the absence of fungal genome defense 212 213 barriers, confirmed by the expression of some TE typologies.

### 215 Genomic features of *Gigaspora margarita*

216 The gene prediction process combined *ab initio* prediction and hints from physical evidence (see the Methods section). Exon coordinates were determined by genome mapping of the entire set of 217 218 UniProt Mucoromycota proteins; intron/exon boundaries were defined by spliced alignment of ten G. margarita RNA-seq libraries from different biological conditions (Supporting information Table 219 220 S5). Finally, non-exon regions were identified using coordinates of repeated regions. We identified 221 26,603 coding genes (Table 1). The PASA pipeline refined 11,487 gene models, detecting 4,965 transcript isoforms, leading to 31,568 non-redundant transcripts. BUSCO assessment (Waterhouse 222 223 et al., 2018) detected 98.5% of the conserved fungal gene set as complete sequences, indicating that 224 this gene catalogue is highly comprehensive. The total number of genes found for G. margarita was 225 close to that of the other Glomeromycotina so far sequenced, R. irregularis, R. clarus, R. cerebriforme, R. diaphanus D. epigea and G. rosea (Chen et al., 2018; Kobayashi et al., 2018; 226 227 Morin et al., 2019; Sun et al., 2019). Fig. 3a highlights the exceptionally high genome size of the 228 two Gigaspora species, when compared with other AMF, ectomycorrhizal fungi and free-living 229 Mucoromycota. Alongside G. margarita and G. rosea, the ten largest fungal genomes (Supporting 230 information Table S1) include other obligate biotrophs (rusts from Pucciniomycotina), an 231 endophytic fungus and a gut fungus (Zoophthora radicans and Neocallimastix californiae, 232 respectively). Several of these genomes lack annotation, leaving open the question of whether their 233 expansions also implied an increase in gene number. AMF possess more genes than their free-living 234 Mucoromycota relatives; however, plant symbiotic lifestyle and gene number increase do not seem 235 to be strictly related, since ectomycorrhizal truffles (Murat et al., 2018) and obligate plant 236 pathogens (Spanu et al., 2010) possess a reduced gene number. Coding space, defined as the space occupied by the protein-coding genes, is another informative parameter: as expected, among the 237 238 analyzed AMF, D. epigaea and G. rosea possess the largest coding space, in line with their higher 239 gene number (Fig. 3a). A similarity network built up on groups of orthologs (Fig. 3b), and based on 240 gene composition rather than gene number, revealed a considerable distance between AMF genera

(*Gigaspora*, *Diversispora*, *Rhizophagus*), while the intra-genus diversity was low. These
 quantitative parameters suggest that, irrespectively of genome size and comparable gene number,
 Gigasporaceae and Glomeraceae are characterized by different genes.

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# <sup>245</sup> Crossing genomics with transcriptomics to describe gene expression depending on fungal life <sup>246</sup> cycle and endobacterial presence

247 Having predicted the gene repertoire of G. margarita, we combined gene information with a large set of transcriptomic data (Supporting information Table S5). In G. margarita native line containing 248 249 the endobacterium (B+), ~82% of the predicted genes were expressed at the transcript level at least 250 in one of the examined biological conditions: both asymbiotic (spores germinated in H<sub>2</sub>O, or in the 251 presence of a synthetic analogue of plant strigolactones, GR24) and symbiotic (intraradical and 252 extraradical mycelium) stages (Supporting information Fig. S8; Table S6). Around 57% (15,205) of genes were expressed under all biological conditions. Pre-symbiotic and symbiotic stages were 253 characterized by 2,431 and 885 unshared genes, respectively. Genes encoding enzymes (such as 254 255 oxidases, carbohydrate-active enzymes and peptidases), transporters and even SSPs were specifically expressed in these two stages, suggesting a transcriptional shift involved in 256 257 development, nutrient acquisition and communication with the host. Most of the expressed 258 symbiotic genes have no known function, as observed in other AMF (Morin et al., 2019). A 259 significant number of genes (444) which were expressed at least in one of the examined conditions 260 in the B+ line, were never expressed in the cured fungal line (B-, without the endobacteria) (Supporting information Table S6). Similarly, 38 genes were exclusively expressed in the B- line. 261 While most of these B+ or B- specific genes (~63%) are functionally uncharacterized, others 262 263 encode for enzymes putatively involved in DNA binding, replication and transcription, such as zinc 264 finger domain-containing proteins, far1-related proteins and HMG-box transcription factors, which 265 are indicated as regulators of cryptic sexuality events in AMF (Ropars et al., 2016). A number of 266 genes encoding for peptidases are exclusively expressed in the B+ line, as well as two genes

267 encoding for hydrolases with peptidoglycan as predicted substrate (glycoside hydrolase family 25
268 proteins).

The following sections will highlight the evolution of gene families and diversification of relevant and /or novel gene categories in the context of *G. margarita* lifestyle, including biotrophy and endobacterium presence.

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# 273 Evolution of gene families in Gigasporaceae

We used the Orthofinder (Emms and Kelly, 2015) and CAFE (De Bie et al., 2006) algorithms and a 274 275 set of fungi to reconstruct the AMF phylogeny and compare close relatives possessing different 276 lifestyles (Supporting information Table S7). Alongside AMF, we included free-living 277 Mucoromycota, plus two ectomycorrhizal fungi (Tuber melanosporum and Laccaria bicolor) from 278 distant clades and a saprotroph/opportunistic pathogen (Aspergillus fumigatus), considered as 279 outgroup. The pipeline first performed a homology search between the entire proteomes of the 280 selected species; MCL clustering (Enright et al., 2002) was then used to process the homology 281 results, generating 17,341 orthogroups, i.e. gene families gathering orthologs, incorporating ~81% 282 of the 287,540 analyzed proteins. Roughly 84% of G. margarita proteins were clustered within 283 these orthogroups. Inter-species distances were calculated through multiple sequence alignment for 284 each orthogroup containing one protein per species (533 single-copy orthogroups). These distances 285 were summarized in a phylogenetic tree, converted into evolutionary time, and finally used by 286 CAFE to detect, for each node and leaf of the tree, orthogroups that underwent accelerated gene gains or losses, interpreted in this context as the effects of evolutionary pressure. The tree was 287 288 calibrated using the divergence date of 434 MYA between *M. elongata* and *R. irregularis* (Uehling et al., 2017). The reconstructed phylogenetic tree (Fig. 4) indicates that G. margarita and G. rosea 289 290 diverged very recently, at around 13 MYA Different from Glomerales, for which all the sequenced 291 fungi belong to the genus Rhizophagus within the Glomeraceae, a divergence node (~174 MYA) 292 between Diversisporaceae (D. epigaea) and Gigasporaceae (G. margarita and G. rosea) is present

293 in Diversisporales. Most of the estimated phylogenetic distances resemble those obtained by Chang 294 and colleagues (Chang et al., 2019) in the context of a deeper phylogenetic analysis, which was designed for the Endogonales family, also belonging to Mucoromycota. Looking for 295 296 Gigasporaceae-specific traits, we focused on expansion/contraction events that took place after divergence from the Diversisporaceae. Of the 271 expansion events observed in the Gigaspora, 120 297 originated in Gigaspora vs D. epigaea differentiation (Supporting information Table S8a, Table S9 298 299 and Fig. S9 a-m). Among them, the Gigaspora species possess two expanded gene families 300 containing alcohol oxidases which, in brown rot fungi, participate in Fenton chemistry (Hernández-301 Ortega et al., 2012; Guillén et al., 2000), and contribute to the non-enzymatic degradation of plant 302 cell walls. The analysis also highlighted the presence of a Gigaspora-specific feature, i.e. the 303 potential to synthesize secondary metabolites due to the presence of Polyketide synthases, which are 304 normally absent in basal fungi (see also the paragraph on HGT). A rapid gene gain for Gigaspora 305 species was particularly dramatic in the families of oligopeptide (OPT) transporters and of patatins, 306 which can be related to fungal immunity (see the specific paragraph).

307 After the divergence between G. rosea and G. margarita (Fig. 4), our pipeline identified 117 308 families that underwent rapid expansion and 133 that underwent rapid contraction in G. margarita (Supporting information Table S8b, Table S9 and Fig. S9 a-m). Gene family evolution in G. 309 310 margarita vs G. rosea may have been shaped by the interaction of G. margarita with CaG, which -311 so far- has never been detected in G. rosea. Gene loss events in G. margarita include proteins 312 related to DNA replication and repair, while among the 117 rapidly expanded families, we found a family of fungal-like Nod-like receptors identified by a central NACHT domain and putatively 313 314 involved in non-self recognition (discussed later), and MATA-HMG proteins (OG0000316). This latter family of highly diverse transcription factors is widespread among AMF genomes (Morin et 315 316 al., 2019) and is suggested to regulate cryptic sexuality events in AMF (Ropars et al., 2016). 317 According to our pipeline, MATA-HMG of AMF are clustered in more than one orthogroup, but in 318 OG0000316 G. margarita possesses the highest number of proteins; furthermore, two of these 319 genes (g24252 and g7196) are up-regulated at the transcript level in germinating spores containing 320 CaGg, when compared to cured spores (Supporting information Table S9). While it is already demonstrated that the expression of MATA-HMG can be influenced by Burkholderia-related 321 322 endobacteria in their Mucoromycota hosts (Mondo et al., 2017), the putative effects of such regulation remains elusive for G. margarita. The expanded family of multicopper oxidases of the 323 AA1 class (OG0000106) gathers two genes (g3919 and g11237) that are strongly up-regulated in 324 325 the B+ line during the mycorrhizal symbiosis, and are also putatively secreted (Supplemental information Table S3). These extracellular oxidases are particularly abundant in plant-interacting 326 327 Basidiomycota (Kües and Rühl, 2011), where they mediate plant cell wall depolymerazation. We 328 speculate that multicopper oxidases might have a role during the interaction of G. margarita with the cell wall of its plant host, and hypothesize that the endobacterial presence may have shaped this 329 330 symbiotic interplay. Finally g11471, present in the expanded gene family of immunoreactive 331 mannoproteins (OG0000217) has the highest up-regulation in response to the endobacterial presence 332 during the germination phase (Supporting information Table S9).

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In conclusion, the genomes of Gigasporaceae have diversified not only from those of the Glomerales (Mondo et al., 2017), but also from that of *D. epigaea* (Sun et al., 2019). In addition, *G. margarita* shows specific evolution of its gene repertoire when compared with the related *G. rosea*: since the sequenced *G. rosea* isolate does not host bacteria, such events could be related to the presence of the endobacterium CaGg.

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As the other AMF, whose obligate biotrophy has been shaped by lack of specific pathways (Tisserant et al., 2013), *G. margarita* genome does not possess invertase as well as genes involved in fatty acid and thiamine biosynthesis (Supplementary text). The genetic basis for the unculturability in fungi may also involve the lack of other genes, for example those for spermidine and biotin biosynthesis (Ahrendt et al., 2018). This does not seem to be the case of *G. margarita*, since its genome encodes for spermidine and biotin synthases (g1668, g3373 and g11043), all apparently functional on the basis of their consistent expression throughout the life cycle (Supporting information Table S6). However, a closer inspection revealed that these sequences are all intersected by TEs: the upstream genomic region of both spermidine synthases contain fragments of TIR transposons, while biotin synthase carries the exonic insertion of an unclassified repeat. We conclude that these insertion events did not disrupt gene functionality in *G. margarita*.

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# Not only phosphate transporter genes: *G. margarita* has a large number of phosphate-related genes

354 The iconic function of AMF is characterized by their capacity to take up phosphate from the soil 355 and transfer it to plants through their phosphate transporters (PTs) (Smith and Read, 2008; Ezawa 356 and Saito, 2018). Surprisingly, however, the molecular characterization of fungal PTs is very 357 limited (Harrison and van Buuren, 1995; Xie et al., 2016). Mining the G. margarita genome revealed a very rich genetic machinery related to phosphate metabolism, sensing, transport and 358 359 signalling (Supporting information Table S10). We first focused on phosphate signal transduction 360 (PHO) genes since, different from metazoa where cells are regularly supplied with phosphate and, 361 as a consequence, lack a PHO network (Lev and Djordjevic, 2018), AMF preferentially thrive in 362 phosphate-deprived conditions and their capacity to successfully interact with their host plants depends strictly on the P content. Under certain conditions, high Pi concentrations block symbiosis 363 364 establishment (Balzergue et al., 2013; Fiorilli et al., 2013). Comparison of PHO proteins belonging to many fungal groups revealed that some members of the PHO cascade are absent or poorly 365 366 represented in the G. margarita genome (for instance, SPL2, PHO4 and PHO89 homologs), while 367 other components are enriched (Supporting information Table S10). G. margarita encodes for at 368 least 11 PT isoforms (Fig. 5, red dots), which are grouped into two distinct lineages. Accordingly, 369 nine Pi transporters, GigmPT (g21463) to GigmPT5 (g26234) and their three paralogs GigmPT8 370 (g19323) to GigmPT10 (g6532), are closely related to the fungal PHO84 PTs and clustered with 371 Glomeromycotina PTs belonging to the Mucoromycota PHO84-like subfamily. Two other PTs, 372 GigmPT6 (g10792) and GigmPT7 (g26017), which contain SPX domains, are very closely related to the PHO87/90/91 PTs derived from yeast. On the basis of the classification (Fig. 5) and 373 374 expression (Supporting information Fig. S10, Table S11) of these G. margarita PTs, we speculate that GigmPT1 and PT2, as well as PT4 and PT9, may contribute to Pi uptake from the environment, 375 while PT6-PT7 might be responsible for Pi homeostasis during symbiosis, similar to the SPX-Pi 376 377 transporters in yeast (Secco et al., 2012). The heatmap drawn on G. margarita PTs expression 378 (Supporting information Fig. S10) revealed that most of the transporters belonging to the 379 Glomeromycotina lineage were expressed in the extra-radical phase, confirming the largely 380 acknowledged concept that AMF uptake Pi from the soil. GigmPT (g21463) is also consistently expressed during the symbiotic phase. Pho1 (g17792), encoding PHO1-type Pi transporter, is 381 382 heavily expressed during the intraradical stage, where it has a predicted role in Pi unloading (or 383 export) from fungus to the plant (Xie et al., 2016). These data might confirm results from laser-384 dissected cells revealing the unexpected expression of fungal PTs in arbusculated cells (Balestrini et 385 al., 2009). Pi uptake is driven by H<sup>+</sup>-ATPases (Ezawa and Saito, 2018), which are expressed in G. 386 margarita, not only at the fungal/substrate interface, but also during the symbiotic phase (g4412, 387 g3891; Supporting information Fig. S10). Once inside the fungus, Pi is polymerized into vacuolar 388 polyphosphate granules maybe thanks to vacuolar transporter chaperons (VTC), which are evenly 389 expressed throughout the G. margarita life cycle (Supporting information Table S11, Fig. S10). 390 On the basis of the genomic and transcriptomic data, we conclude that G. margarita possesses a consistent number of functional PTs, confirming its capacity to uptake Pi from the soil; cellular Pi 391

homeostasis is probably maintained through a rather homogenous expression of two SPX-PT transporters (g10792-PT6, g26017-PT7), which could re-uptake Pi from the peri-arbuscular and intercellular spaces (Fig. 6, Supporting information Fig. S10). Interestingly, some ATPases and PT transporters are more expressed when the fungus contains its endobacterium (Supporting information Table S11); in particular the HA2 ATPase (g4412) is up-regulated by the presence of the endobacterium in both germinating spores and intraradical mycelium. Furthermore, the vacuolar VTC1-2 (g24765) is up-regulated by the presence of *Ca*Gg in germinating spores and GR24-treated spores, suggesting a role in moving the Pi from the fungus to bacterium, which is compartmented inside fungal vacuoles (Supporting information Fig. S2), and which possesses its own Pi transporter (Ghignone et al., 2012; Ruiz-Lozano and Bonfante, 1999). These results are in agreement with previous data showing that *G. margarita* with its endobacterium has a general more active metabolism, eventually leading to a higher Pi concentration in the plant host (Salvioli et al., 2016).

Chitin-related genes: molecular tools forming the basis of fungal growth and communication
 with the plant

407 Chitin, beta-1,3-glucans, beta-1,6-glucans and mannoproteins are usually listed as the major components of fungal cell walls (Gow et al., 2017). Biochemical analyses are not available for 408 409 AMF. In situ labeling showed chitin in the cell wall in all steps of the AMF life cycle (Bonfante, 410 2018), while monoclonal antibodies failed to detect beta-1,3-glucans (Lemoine et al., 1995; Ligrone 411 et al. 2007). Indeed, homologs of FSK, the beta-1,3-glucan synthase, were not detected among the 412 protein-coding genes of the G. margarita genome, while mannoproteins could be biosynthesized by the numerous enzymes present in the GT15 family (Supporting information Table S12) and 413 involved in mannosyltransferase activity. The GT2 family of G. margarita has 38 members, which 414 415 is high compared with the other AMF sequenced so far. Among these, 15 show characteristics of 416 chitin synthases: presence of the CON1 region (Liu et al., 2017), and domain organization leading to further divisions and types (Supporting information Fig. S11). The number of secreted 417 418 Carbohydrate Active Enzymes (CAZymes) involved in fungal wall deconstruction is very high in G. margarita (Supporting information Table S3) and probably guarantees the cell wall dynamics 419 420 described morphologically (Bonfante, 2018); in addition to many 1,2-alpha-mannosidases (GH92), 421 which seem to be specific for Gigasporaceae (Supporting information Table S12), many CAZymes

are devoted to chitin breakdown. Chitinases are encoded by genes present in the family GH18,which are abundant in Gigasporales genomes.

424 Chitin can be deacetylated by chitin deacetylases encoded by CE4 family (Fig. 7). Glucosaminidase
425 encoded by GH20 genes might lead to production of glucosamine residues.

GT2, GH18, and CE4 family members, which are particularly expanded in the *G. margarita* genome (Supporting information Table S12) seem to be expressed homogeneously during the *G. margarita* life cycle (Supporting information Fig. S12, Table S13). However, some revealed specific behaviour: g25383 (GH18) was particularly activated in the pre-symbiotic phase and sensitive to strigolactones; expression of others was enhanced during the symbiotic phase, preferentially in the presence of the endobacterium (g19206, g23868); some CE4 members, g7314, g19924 and g19811, had their highest expression in the intra-radical phase.

433 Chitin has multiple roles in fungi as a structural and signalling molecule (Pusztahelyi, 2018; 434 Schmitz and Harrison, 2014). This is particularly relevant in AMF, where chitin organization is 435 modulated from the extra-radical to the intra-radical phase (Bonfante, 2018) and 436 chitooligosaccharides (COs) are the main signaling molecules (Zipfel and Oldroyd, 2017). During the pre-symbiotic phase and in the extra-radical symbiotic mycelium, G. margarita produces COs, 437 438 which may or not be decorated by lipid chains. This is enabled by the GH18 family, supported by 439 the action of CBM14, which represent the chitin-binding domains of chitinases. N-Acetylated COs 440 are recognized by plant chitin receptors, (Miyata et al., 2014; Zhang et al., 2015; Carotenuto et al., 441 2017), activating a conserved downstream symbiotic signal transduction pathway. Here, one of the 442 first signatures is a calcium spiking response, which is activated by N-acetylated COs and enhanced 443 by treatment with the synthetic strigolactone GR24 (Genre et al., 2013). However, chitin not only 444 acts as a signal for symbiosis, but also for pathogenicity (Sánchez-Vallet et al., 2015). N-Acetyl 445 COs released by AMF activate host plant defenses, inducing immunity signaling (Pozo and Azcón-Aguilar, 2007; Martinez-Medina et al., 2016; Chialva et al., 2018). Many studies have demonstrated 446 447 that such plant defenses are limited to the first interaction phase (Giovannetti et al., 2015),

questioning whether N-acetyl COs are no longer active during intra-radical colonization. The 448 449 presence of expressed CE4 members suggests a new hypothesis: the deacetylation process could be more important during the intra-radical phase when changes in molecular organization (loss of 450 451 acetyl groups) would correspond to loss of chitin fibrillar structure, as seen under the electron microscope. Gow and colleagues (Gow et al., 2017) wrote that deacetylated chitin is not recognized 452 453 by plant chitin receptors, allowing a deep colonization of plant tissues without any evident rejection. 454 Another alternative hypothesis to explain the stealth colonization of plant tissues by AMF, is based on LysM domain containing proteins. AMF could sequestrate chitin oligosaccharides to elude 455 456 host's immunity, as already reported for pathogenic Ascomycetes (de Jonge and Thomma, 2009); 457 all the sequenced AMF (except for G. rosea) possess one or more of such enzymes, which are gathered in the CBM50 CAZy class (Supporting information Table S12). Lastly, the progressive 458 459 thinning of the arbuscular cell wall may guarantee passage of SSPs, which act as fungal effectors 460 (Kloppholz et al., 2011). In G. margarita, 41 SSPs had absolute expression levels around 10 times 461 greater in the intra-radical mycelium than at all other life stages (Supporting information Fig. S13, 462 Table S14). While no experimental evidence is available of the secretion of these proteins, our 463 results point to the arbuscule as a preferential site for effector production.

Combining genomics, transcriptomics and morphological data reveals the finely tuned activity of
 genes related to the fungal cell wall, and in particular of chitin metabolism, with potential feedback
 on signaling and defense mechanisms in plant hosts.

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### 468 Between plant and bacterial cells: HGT events

During its life cycle, *G. margarita* lives in intimate contact with both its plant host and its endobacteria (Supporting information Fig. S2). Since this inter-kingdom interaction is expected to have been stable for more than 400 million years (Mondo et al., 2012), we hypothesized that HGT events might have occurred. The predicted gene models were analysed using a pipeline for HGT discovery (Li et al., 2018). Following this method, two candidate lists were generated, representing
sequences putatively transferred to *G. margarita* from plants and bacteria, respectively. Among
genes possibly derived from plants (Supporting information Table S15), we found two sequences
related to fucosyltransferases. The BLASTX top hits for these sequences included *Chara braunii, a*green alga that is considered the ancestor of AMF-hosting green plants (Delaux et al., 2015), as well
as *Spizellomyces punctatus*, a basally branching chytrid fungus in phylum Chytridiomycota.

479 Of the genes putatively transferred from bacteria (Supporting information Table S15), the ones possessing the highest alien index were non-ribosomal peptide synthetases-polyketide synthases 480 481 (NRPS-PKS). Since these sequences also possess a high alien index when considering a potential 482 plant origin, they might confirm an extensive HGT from bacteria to a wide variety of eukaryotes (Lawrence et al., 2011). NRPS-PKSs are involved in the biosynthesis of secondary metabolites 483 484 including antibiotics, toxins and siderophores. Similar sequences belong to a Gigaspora specific 485 expanded gene family (Supporting information Table S8a), but are absent in R. irregularis genome 486 (Tisserant et al., 2013). These findings, together with the limited potential of early diverging fungi 487 to biosynthesize secondary metabolites (Voigt et al., 2016), suggest that the Gigasporaceae lineage might have acquired such a peculiar ability via HGT from bacteria. 488

Two gene models (g16267 and g8176) share similarity with bacterial Toll/interleukin-1 489 490 receptor/resistance domain-containing proteins (TIR). This domain is a protein-protein interaction 491 domain widely distributed in animals, plants and bacteria but considered absent in fungi (Ve et al., 492 2015). In plants and animals, TIRs play roles in innate immunity, while in bacteria some of them 493 interfere with the innate immune pathways of the host (Ve et al., 2015). The TIR-like G.margarita 494 sequences found homologs in MREs, such as the endobacteria of Dentiscutata heterogama (Torres-Cortés et al., 2015; Naito et al., 2015) and D. epigaea (Sun et al., 2019) but not in CaGg (Ghignone 495 496 et al., 2012). G. margarita BEG34 does not host MREs; furthermore TIR-like G.margarita 497 sequences were also expressed in the cured fungal line, ruling them out as bacterial contaminants. 498 In G. margarita, transcription of g8176 is strongly increased in germinating spores in the presence

499 of CaGg, and is inactivated upon GR24 treatment (Supporting information Table S16). By contrast, 500 g16267 shows uniform expression across biological conditions. According to Sun and colleagues 501 (Sun et al., 2019), we suggest that such sequences originated from MREs, which were probably 502 present in the common ancestors of Glomeromycotina and Mucoromycotina (Bonfante and Desirò, 2017). Finally, as Helitrons-mediated HGT has been reported in fungi (Castanera et al., 2014), we 503 504 searched for HGT candidates whose genomic locations overlap those of Helitrons: we found two 505 similar sequences encoding S1 peptidases (g18889 and g8238) and an amidohydrolase, both having putative bacterial origin, along with an a-mannosidase (g17903) with high homology with both 506 507 bacteria and plants.

508 In conclusion, HGT events from plants to the G. margarita genome appear to be limited and represent ancient events related to the algal origin of land plants (Delaux et al., 2015) and to the 509 510 interactions with basal Chytridiomycota fungi, which may feed on plants (Berbee et al., 2017). HGT 511 events from bacteria to AMF are multilayered; on the one hand, they reflect the interactions 512 occurring on the surface of AMF, which - in the soil - is regularly colonized by multiple bacterial 513 communities (Agnolucci et al., 2015) with different metabolic capacities. On the other, these HGT 514 events reflect the ancient presence of endobacteria, which are hosted in the G. margarita cytoplasm, 515 making genetic exchange between bacteria and their fungal hosts easier.

516

# 517 *G. margarita* genome and the fungal immune system

Since *G. margarita* interacts with viruses, bacteria and plants, we mined its genome to identify genetic determinants potentially involved in the fungal immune system. Nod-like-receptors (NLRs) are the main actors of the immune system in plants and animals, but are also present in fungi, in a number ranging from 0 to 200 (Dyrka et al., 2014). They are involved in allorecognition processes, mediating multiple processes from heterokaryon incompatibility to restriction of mycovirus transfer along the hyphae, as well as xenorecognition, working during pathogen attack or symbiosis establishment (Heller et al., 2018). These proteins harbour a nucleotide-binding domain of the

525 NACHT or AAA family, flanked by an N-terminal recognition domain and C-terminal repeats for 526 protein-protein interactions. In addition to the 2 TIR-like sequences (HGT events), we found 18 proteins in G. margarita where both the central domain and the terminal repeats were present, most 527 528 of them belonging to an expanded gene family; the central portion consisted of a NACHT domain in 17 cases, while an NB-ARC domain was found in one case (g12905) (Supporting information 529 Table S16, Fig. S14). NB-ARCs are normally found in plant and animals NLR, where they are 530 531 associated with resistance and cell death, respectively (Van der Biezen and Jones, 1998). The Cterminal of the identified proteins was always composed of pentapeptide repeats. By contrast, their 532 N-terminal recognition domain could not be functionally annotated, probably due to the lack of 533 534 information in public databases, or to the modular structure of these proteins, where such modules could be present in separate proteins (Uehling et al., 2017). The N-terminal domains associated with 535 536 fungal NLRs can possess enzymatic activity such as lipase (patatins) and peptidase (subtilisin-537 related) activity: in G. margarita, the expression of four patatins seemed to be sensitive to the 538 presence of the endobacterium, while three subtilisins were up-regulated upon treatment with the 539 plant signal analogue GR24 (Supporting information Table S16), suggesting their involvement in 540 plant or bacterial interaction. These patatins belong to expanded protein families in the Gigaspora genus (OG0000175), while subtilisins (OG0000164) are expanded exclusively in G. margarita, 541 542 when compared to G. rosea (Supporting information Table S8b).

Finally, *G. margarita* possesses several genes that may be involved in defence against viruses,
including PIWI domain-containing proteins, a DICER-like protein and three argonaute-binding
proteins, all related to RNA-mediated gene silencing (Supporting information Fig. S14).

In conclusion, the genome of *G. margarita* reveals novel genetic determinants that might form the basis of the fungal immune system, which must activate non-self recognition events throughout the life cycle.

549

### 551 Conclusions

552 The genome sequencing of G. margarita BEG34 revealed key genomic traits shedding new light on the biology of the Glomeromycotina (Spatafora et al., 2016). G. margarita possesses the largest 553 554 fungal genome annotated so far. Its genome expansion is mostly driven by a TE explosion, also characterizing many other mycorrhizal fungi, from truffles with their low gene number (Murat et 555 556 al., 2018) to the related G. rosea (Morin et al., 2019). Diversification time of Gigasporaceae has 557 been placed at around 170 MYA (Davison et al., 2015), being congruent with phylogenetic trees reconstructed using genome data, which suggest that divergence of Gigasporaceae and Glomeraceae 558 559 from the last common ancestor was around 300 MYA (Sun et al., 2019). These different speciation times suggest diverse evolution pathways among AMF and provide strong support for the many 560 561 unique features reported in the G. margarita genome.

562 Similar to other AMF investigated, G. margarita is a strictly biotrophic fungus (Bonfante and 563 Genre, 2010), meaning that it cannot uptake complex sugars from the environment and cannot 564 biosynthesize fatty acids, being therefore dependent on its host plants. G. margarita and G. rosea 565 share an enriched set of CAZymes, which explains the capacity of Gigaspora to colonize different 566 plants, maybe using a combination of non-enzymatic and enzymatic mechanisms. The high number 567 of chitinase-encoding genes supports the dynamics of the fungal cell wall, which thins progressively 568 moving from the extra-radical phase to the thinner arbusculated branches. The discovery of highly 569 regulated chitin deacetylases during the symbiotic phase suggests that deacetylated chitin might 570 offer a good tool to escape from the plant immune system, avoiding the activation of plant defenses. 571 The absence of beta 1-3-glucans in the wall of the intra-radical hyphae might reflect the same 572 strategy, similar to that described for the pathogenic fungus Colletotrichum (Oliveira-Garcia and 573 Deising, 2013). By contrast, G. margarita has had stable interactions with its endobacterium CaGg 574 for a very long time (Mondo et al., 2012). The intimate contact that G. margarita establishes with 575 its host plants, its endobacterium and fungal-associated bacteria has provided the physical 576 possibility of HGT events. Indeed, mining the genome revealed a unique transfer event from an

algal ancestor towards *G. margarita*; many transfer events from a range of soil bacteria; and an interesting HGT event, involving TIRs, probably transferred from Mollicutes-related endobacteria detected in many AMF (Sun et al., 2019) and some African strains of *G. margarita* (Desirò et al., 2014). The presence of these TIRs together with a number of Nod-like-receptors allowed us to identify the first core genes of the immune system present in AMF, which probably allows these fungi to safely interact with plants, bacteria and viruses.

583 Lastly, the high number of phosphate transporters first provides a rationale for the experimental evidence of Pi transporters expressed during the symbiotic mycorrhizal phase, but also sheds a 584 585 different light on the biology of AMF, usually considered as biofertilizers. G. margarita has a 586 staggeringly high genome size: as for plant genomes (Pellicer et al., 2018), we suggest that this peculiar trait has shaped its evolution. Plants with small genomes seem to be more widespread than 587 588 those with large genomes, which persist only under conditions where selective pressures are more 589 relaxed (Pellicer et al., 2018). From a mechanistic point of view, nucleic acids are amongst the most 590 nitrogen- and phosphorous-rich molecules of the cell, so under limiting nutrient N and P conditions, 591 we predict that species with large genomes, which are more demanding and costly to build and 592 maintain than species with small genomes, would be less competitive. This reasoning fits well with G. margarita BEG34, which is commonly isolated from sand dunes (Stürmer et al., 2018), but 593 594 poorly represented in soils originating from many other diverse environments (Davison et al., 2015; 595 Davison et al. 2018). On the other hand, its spores are among the largest produced by AMF, a 596 feature which is commonly associated with long-term survival strategies (Aguilar-Trigueros et al., 597 2018). In conclusion, the genome description of G. margarita revealed novel and unique features, 598 showing its weaknesses (a huge genome may be a limiting factor in terms of ecological success), but also its biological strengths through the capacity to communicate with plants and endobacteria, 599 600 acting therefore as a hub of inter-kingdom interactions.

601

### 603 Materials and methods

# <sup>604</sup> AMF culture, Flow cytometry, and gDNA extraction

605 *G. margarita* strain BEG34 with (B+) and without (B-) the *Ca*Gg endobacterium was used for this 606 study. The spores were obtained as described in Salvioli et al., 2016 (19) (see Supplementary Text).

The genome size of *G. margarita* was first determined by flow cytometry using *Solanum lycopersicum* as an internal reference. For each set of isolated nuclei, 100 sterilized B+ spores and 0.5 cm<sup>2</sup> of young *S. lycopersicum* leaf tissues were processed as described in Sędzielewska et al., 2011 (Sędzielewska et al., 2011) and measured using a FACSAria (BD Biosciences, San Josè, CA, USA) with a 488 nm laser. The absolute DNA amounts of the samples were calculated based on the values of the G1 peak means. At least 10,000 nuclei per sample were analysed.

The gDNA was extracted from batches of 100 spores using the CTAB method. Sample purity was assessed at Nanodrop1000 (Thermo Scientific, Wilmington, NC, USA). Only samples with a 260/230 - 260/280 > 1.8 were kept, and quantified with Qubit (Broad Range Kit, Thermo-Fisher). In total, 15 µg of DNA, extracted from 16 batches of spores (~1600 spores), was sent to the sequencing facility.

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## 619 **DNA sequencing and genome assembly**

The genome of *G. margarita* was sequenced using Illumina platform. One fragment Paired-End library (PE; Illumina TruSeq Nano) and two long Mate-Pair libraries (MP; Illumina Nextera; inserts sizes 3 Kbp and 8 Kbp) were constructed in 2 X 150 bp format using one lane of Illumina HiSeq3000 by GeT-PlaGe GenoToul (Castanet]Tolosan, France). All libraries have been filtered for

adapter sequences and low-quality reads, using combinations of trim\_galore v.0.4.1
(http://www.bioinformatics.babraham.ac.uk/projects/trim\_galore/; last accessed 2019-02-28),
trimmomatic v.0.36 (Bolger et al., 2014) and 1, cutadapt v.1.2.1, (Martin, 2011). Genome assembly
was performed by Bison SeqTech ApS (Frederiksberg, Denmark) with ALLPATH-LG v.3 (Gnerre

et al., 2011). More details on assembly, quality check and refinements are provided in Supplementary Text.

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# 631 **RNA-seq experiments**

The single-end libraries used to reconstruct the *G. margarita* transcriptome (Salvioli et al., 2016) were used in this study along with new libraries obtained from *G. margarita* B+ symbiotic extraradical mycelium (JGI proposal ID 1450), and from *Lotus japonicus* B+ and the Bmycorrhizal roots (Supporting information Table S5). RNA for mycorrhizal roots was extracted with NucleoSpin RNA Plant and Fungi Kit (Macherey-Nagel), and checked at BioAnalyzer 2100 (Agilent). Samples with RIN<7.0 were discarded. Sequencing was performed by Macrogen (South Korea).

All the libraries used in this study were pre-processed with BBmap (Bushnell, 2014) for contaminants removal and quality trimming. Each library was mapped to the set of transcripts with salmon v.0.10.2 (Patro et al., 2017). Differential gene expression was calculated as detailed in Supplementary Text.

643

# 644 Gene prediction and annotation

Protein coding genes were predicted using the BRAKER2 pipeline (Hoff et al., 2016; Stanke et al.,
2008; Stanke et al., 2006; Lomsadze et al., 2014). The training process was performed with the aid
of the GOU13/14/15, BUNYW/X/Y and BUOAC/G/H RNA-seq libraries (Supporting information
5), the UNIPROT Mucoromycota proteins, and the coordinates of repeated sequences. The
annotation process is detailed in Supplementary Text.

The predicted gene models were functionally annotated with a blastx search against the nr protein database. Blast2GO v.4.1 (Götz et al., 2008) was used for the attribution of best blast hit, Gene Ontology Terms and E.C. numbers.

### 654 Identification and analysis of repetitive elements

655 The REPET v.2.5 pipeline (Flutre et al., 2011) was used for the detection, classification (TEdenovo) and annotation (TEannot) of Transposable Elements (TE) and other repetitive DNA sequences. We 656 657 used the same analytic strategy already used elsewhere (Plomion et al., 2018). The library of classified consensus sequences provided by the TEdenovo pipeline was used to annotate the TE 658 copies with the TEannot pipeline. After manual curation, we obtained a final set of TEs 659 660 consensuses, with at least one full-length fragment (FLF), representing the 63.8% of the genome. OcculterCut v.1.1 (Testa et al., 2016) was used to detect the eventually occurring GC-bias in the G. 661 margarita genome. Ripcal v.2.0 (Hane and Oliver, 2008) was used to detect the occurrence of RIP 662 663 in G. margarita. A more extensive explanations on TEs discovery are reported in Supplementary Text. 664

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### 666 Additional analyses

All the other downstream analyses performed on the *G. margarita* genome (including gene family
evolution, detection of putatively horizontally transferred genes, classification chitin synthases,
identification of secreted proteins and CAZymes, etc...) are reported in Supplementary Text.

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684	Competing interest: the authors declare there are no competing financial interests in relation to the
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1033 Figure 1: G. margarita BEG34 as a metaorganism A) a G. margarita quiescent spore crushed to 1034 allow the spreading of nuclei and cytoplasm; B) a section of the fungal spore seen under an electron microscope: mitochondria (asterisks) and fungal nuclei (N) are highlighted; C) CaGg (arrowheads) 1035 1036 cells are situated inside the fungal vacuole (Fv). Below, the same components are represented as 1037 metagenome scaffolds as a result of the Blobtools analysis, which integrates coverage, G/C content 1038 and sequence homology information as a method to identify and separate the different biological 1039 entities of the metagenome. Nuclear, mitochondrial and bacterial scaffolds are visualized as circles 1040 (blobs) with a diameter corresponding to their size in base pairs. The blobs are distributed on the basis of their G/C content (horizontal axis) and their coverage in terms of genomic reads (vertical 1041 1042 axis), and colored according to their taxonomic affiliation as determined by sequence homology. 1043 The higher G/C content in mitochondrial and bacterial genomes is evident from the separation on 1044 the horizontal axis. The sequence homology search also confirmed the classification based on the 1045 other parameters.

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Figure 2: Distribution of the *G. margarita* TE families according to their main order or superfamily
(Gypsy/Copia) in the consensus Library (4/264 consensus; A) and according to their genome
coverage (602/060 copies/ 64% of the genome; B). PHG stands for "Potential host genes".

**Figure 3:** a) Comparison of the genomes of *G. margarita* and related fungi. The comparison includes the other sequenced AMF (red circles), free living Mucoromycota (blue circles), and mycorrhizal fungi from distant clades (orange circles). The size of the circles and their position on the horizontal axis is proportional to the genome sizes and the coding space (the space occupied by protein coding genes in each genome) is plotted on the vertical axis; b) a network showing the correlation of *G. margarita* with some of its relatives. In this analysis the correlation was measured

as the number of orthologs between the considered species. The size of the nodes is proportional to
the number of proteins in each species and the size/colour of the lines depend on the number of
orthologs (the thicker/darker, the higher the number of orthologs).

1060

Figure 4: genome-scale phylogeny as reconstructed by orthogroups-based data. The ultrametric tree 1061 1062 shows the strong phylogenetic relationship between the two *Gigaspora* species, which diverged in 1063 recent evolutionary time (the divergence date are shown next to the branches). The divergence 1064 between the Gigaspora species and the other sequenced AMF groups (Diversisporaceae and 1065 Glomerales) is also shown. The leftmost pie chart displays the number of gene families that 1066 underwent rapid evolution (red for expansions and blue for contractions) after the divergence between the Gigaspora and Diversispora epigaea (Diversisporaceae). The pie charts on the right of 1067 1068 the tree display the families that rapidly evolved in G. margarita or G. rosea after the two species 1069 diverged.

1070

1071 Figure 5: evolutionary relationships of phosphate transporters among fungal species. the 1072 evolutionary history was inferred using the Neighbor-Joining method 149. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the 1073 1074 phylogenetic tree. The evolutionary distances were computed using the Poisson correction method 1075 and are in the units of number of amino acid substitutions per site. The analysis involved 82 amino 1076 acid sequences. All positions containing gaps and missing data were eliminated. There was a total 1077 of 58 positions in the final dataset. Evolutionary analyses were conducted in MEGA7. The marked 1078 red circles shown in the figure indicate the multiple isoforms of phosphate transporters from G. 1079 margarita.

1080

Figure 6: By crossing genomics and transcriptomic data the scheme illustrates an hypothetical
 model of how *G. margarita* handles Pi. PT1-2-4-9, supported by ATPases, may take up phosphate

1083 from the soil; the phosphate is aggregated in polyphosphate granules and released at the interfaces 1084 with the plant and with the bacterium. The insets reveal the differences between the two contact 1085 areas: at the plant interface, the fungus is surrounded by the perifungal membrane, which is 1086 continuous with the plant plasma membrane. Here the fungal Pho1 could mediate Pi export from the intraradical mycelium into the periarbuscular space. Due to the plant PT4 activity, Pi is loaded into 1087 1088 plant cells. Pi diffusion may also occur across the perifungal membrane. In addition, PT, PT3 and 1089 PT6 could also contribute to Pi re-uptake in the intraradical mycelium under low Pi levels. The 1090 bacterium is located inside the fungal vacuole: several fungal vacuolar export chaperones are 1091 actively expressed, and the one that mostly seemed to respond to the CaGg presence is VTC1-2. 1092 The endobacterium in turn has two PTs located at the outer membrane (PstA, PstC), and one located 1093 in the periplasmic space (PtsS). Furthermore, the bacterium has cytoplasmic components which may respond to intracellular Pi (PstB and PhoU). All the membrane localisations of the fungal P 1094 1095 transporters are hypothetical.

1096

**Figure 7:** *G. margarita* has two pathways for chitin breakdown. On one side, the activity of chitinases leads to the formation of chitoligosaccharides, which are recognized by plant chitin receptors.

1100

**Table 1**: assembly and annotation statistics of the *G. margarita* BEG34 genome.

1102

1103 In addition to these files we provide:

1104 16 supplemental tables (individual excel workbooks)

1105 16 supplemental figures (embedded as separate pages in a single PDF file)

1106 1 Supplementary text file (a word document containing supplementary Materials and Methods and

1107 Results)