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Endobacteria affect the metabolic profile of their host *Gigaspora margarita*, an arbuscular mycorrhizal fungus

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Summary

The aim of this paper was to understand whether the endobacterium identified as *Candidatus* Glomeribacter gigasporarum has an effect on the biology of its host, the arbuscular mycorrhizal fungus *Gigaspora margarita*, through the study of the modifications induced on the fungal proteome and lipid profile. The availability of *G. margarita* cured spores (i.e. spores that do not contain bacteria), represented a crucial tool to enable the comparison between two fungal homogeneous populations in the presence and the absence of the bacterial components. Our results demonstrate that the endobacterial presence leads to a modulation of fungal protein expression in all the different conditions we tested (quiescent, germinating and strigolactone-elicited germinating spores), and in particular after treatment with a strigolactone analogue. The fungal fatty acid profile resulted to be modified both quantitatively and qualitatively in the absence of endobacteria, being fatty acids less abundant in the cured spores. The results offer one of the first comparative metabolic studies of an AM fungus investigated under different physiological conditions, reveal that endobacteria have an important impact on the host fungal activity, influencing both protein expression and lipid profile, and suggest that the bacterial absence is perceived by *G. margarita* as a stimulus which activates stress-responsive proteins.

Introduction

The arbuscular mycorrhizal (AM) symbiosis is the most widespread mycorrhizal type, occurring in more than 80% of the land plants, and involving as symbiotic fungi the *Glomeromycota*, an ancient phylum that has coevolved with plants for at least 400 million years (Bonfante and Genre, 2008). The AM fungi contribute significantly to soil nutrient uptake in plants, increasing their productivity

and conferring resistance to stresses. At the same time, as obligate biotrophs, they depend on the plant for carbohydrates, being so far unculturable in the absence of their host. Their uniqueness is also mirrored by other biological traits: they possess thousands of syncytial nuclei in theirs spores and hyphae (Parniske, 2008) and are considered asexual microbes, even if genetically distinct AM fungi anastomose and perform genetic crosses (Croll *et al*., 2009). Lastly, they are known to contain endobacteria in their cytoplasm, which represent therefore the third component of mycorrhizal associations (Bonfante and Anca, 2009).

As for insect endosymbionts, the presence of endobacteria inside *Glomeromycota* cytoplasm has long been documented by electron microscopy, which has distinguished two bacterial morphotypes. The first has been detected inside AMF spores and hyphae colonizing plant roots sampled in the field. It is coccoid in shape, and has been labelled 'bacterium-like organism' (BLO) for long time, since its identity has been only recently solved as related to *Mollicutes* (Naumann *et al*., 2010). The other bacterial type, rod-shaped and restricted to a single AMF family (*Gigasporaceae*), has been studied in more detail. Use of a combination of microscopy and molecular analysis of 16S rRNA has led to the description of these latter organisms as bacteria related to *Burkholderia* (Bianciotto *et al*., 1996). They were placed in a new taxon named '*Candidatus* Glomeribacter gigasporarum' because of their unculturability (Bianciotto *et al*., 2003). Isolate BEG34 of *Gigaspora margarita* and its endobacterium *Candidatus* G. gigasporarum are currently used as a model system to investigate endobacteria–AM fungi interactions. These endocellular bacteria represent a stable and homogeneous population inside the *G. margarita* cytoplasm and are vertically transmitted (Bianciotto *et al*., 2004). Theypossess a Gram-negative cell wall, are rod-shaped with an approximate size of 0.8–1.2 × 1.5–2.0 µm, and occur singly or in groups inside fungal vacuole-like structures, being surrounded by a fungal membrane.

Our knowledge on the interaction between AM fungi and their endobacteria is still limited and fragmentary, mainly because of the physiological features of both the partners (both of them are obligate endosymbionts). The recent achievement of a *G. margarita* sporal line, which is devoid of bacteria ('cured' spores), helped us to shed some light on the effects of the endobacterium on its host (Lumini *et al*., 2007). Spores of the cured line have cells distinct in vacuole morphology, cell wall organization, lipid bodies and pigment granules. The absence of bacteria seems not to affect the symbiotic capacities of the fungal host, while it influences its presymbiotic growth to a great extent in terms of hyphal elongation and branching in response to a root exudates treatment (Lumini *et al*., 2007). The active fraction of root plant exudates, which is responsible for AM hyphal branching in the vicinity of the host root, was isolated and described as a molecule belonging to the strigolactone family (Akiyama *et al*., 2005). In addition to their effect in stimulating fungal presymbiotic development (Tamasloukht *et al*., 2003; Besserer *et al*., 2006; Besserer *et al*., 2008), strigolactones also seem to be perceived by the endobacterium, which responds to this stimulus with an increase in bacterial division mirrored by the upregulation of the FTz gene (Anca *et al*., 2009).

In this paper we investigate the impact of the endobacterium *Candidatus* G. gigasporarum on the proteome and lipid profile of its fungal host *G. margarita*. The availability of *G. margarita* cured spores (Lumini *et al*., 2007) allowed us to compare fungal populations containing or not the bacterial symbiont.

Since recent findings suggested that the endobacteria may modulate their life cycle according to the stages of the fungal host development (Anca *et al*., 2009), the proteome profile was investigated considering different physiological conditions, i.e quiescent, germinating spores and spores treated with a synthetic analogue of natural strigolactones.

Current knowledge on AM fungal proteome is limited to a few reports, and one in particular lists proteins detected in *Glomus intraradices* mycelium (Recorbet *et al*., 2009). In this context, our findings show that endobacteria have important impacts on the fungal proteome and lipid profile, and also represent one of the first comparative metabolic studies of an AM fungus, investigating two fungal lines under three physiological conditions.

Results

No evident phenotypic differences were found between germinating spores from the wild-type (wt) and cured lines after 10 days of germination. In independent preliminary tests, a more intense branching was detected in the wt line after treatment with the strigolactone analogue GR 24 after 15 days of incubation (data not shown). All these results are in agreement with what shown in Lumini and colleagues (2007).

Protein profiles of wt and cured spores

Two-dimensional (2D) protein maps were obtained for the wt and cured fungus under three physiological conditions, i.e, quiescent, germinating and GR24-treated germinating spores. On the whole, a total of 320 individual spots were detected, that were reproducibly displayed within the window of pH 4–7 and molecular mass 5–200 kDa. Among the spots, 159 were present in each considered condition (quiescent, germinating or GR4-treated germinating spores). Gels from wt and cured spores were compared pairwise in the three conditions leading to the detection of spots which were differentially expressed in a qualitative (presence versus absence) or quantitative (different level of expression) way. All the data are schematically presented in Fig. 1.

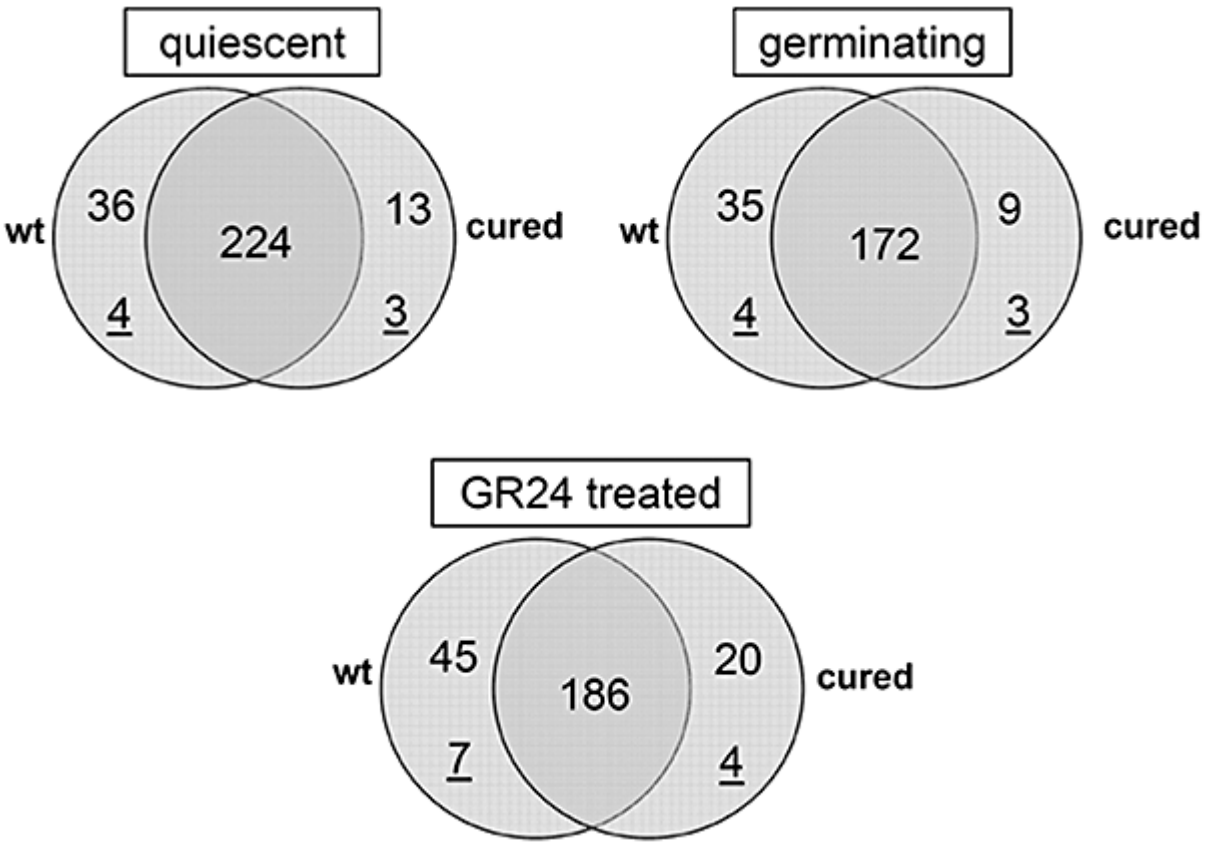

Figure 1. Schematic representation of the proteins detected in each 2-DE experiment; numbers outside the common area indicate qualitatively (not underlined) and quantitatively (underlined) differentially expressed proteins.

A total of 87 proteins were cut from the 2D gels and sent for sequencing; 17 out of them were chosen among the most highly and constitutively present spots (that is, they were common to all the considered conditions), while the other 70 corresponded to differentially expressed proteins. Out of these 87, 43 proteins were identified, 10 constitutively and 33 differentially expressed. The results of protein identification are listed in Table 1.

Table 1. List of the proteins identified from *G. margarita* 2D-E maps.

Spot	Description	Organism	Peptides	Score	Theoretical pI/Mw	Observed pI/Mw	Method	Differential expression
1. Last column indicates the fungal line (wt or cured) in which the differential expression is observed. Proteins in bold represent spots identified with a quantitative analysis. Theoretical and observed isoelectrical points and mass weights (pI/Mw) are given for each protein. In some cases the pI/Mw values for the potentially phosphorilated form of the protein is also reported (in brackets).								
Constitutively expressed proteins								
1	gi|37910032 NADH dehydrogenase subunit 2	*Paratomella rubra mitochondrion*	FLLMLM FLLFNVG NMIIMIM LMLLM	46 44 44 37	8.3/38581.19 (5.7/39204.9)	5.4/28000	*de novo*	–
2	gi|85374405 predicted ATP-dependent endonuclease, OLD family protein	*Erythrobacter litoralis* HTCC2594	LMGESNPEEVR	55	5.10/72402.57	5.50/28000	Mascot	–
3	gi|29345661 dolichol-phosphate mannosyltransferase	*Bacteroides thetaiotaomicron*	EGGDVSVGSRS WTAVSSRR	51 45	8/28163.64 (5.50/28795.1)	6/28000	Mascot	–
4	gi|45935132 putative phosphatidylcholine-sterol acyltransferase	*Ipomoea trifida*	YWSNPLE IMIIMNVGP	58 49	7/74207.08	6.2/28000	*de novo*	–
5	gi|16080935 YxkD protein (efflux transporter)	*Bacillus subtilis*	TVISENKTEI	67	8.48/30732.46	5.5/20000	*de novo*	–
6			TLNPTYNETF NTLNPTYNET	77 76	8.55/31566.21	6.2/12000	*de novo*	–

Spot	Description	Organism	Peptides	Score	Theoretical pI/Mw	Observed pI/Mw	Method	Differential expression
	gi|73912806 synaptotagmin protein 2	*Caenorhabditis elegans*	SDPYLELY	43				
			IGEVT	36				
7	gi|20977839 acyl-CoA desaturase CpomMPTQ	*Cydia pomonella*	LRVLAVWHA	66	9.55/21234.25	6/22000	*de novo*	–
8	gi|4103081 cytochrome c oxidase	*Cephalotaxus fortunei*	HSHYTPPMG	64	8.06/49988.14	6.2/45000	*de novo*	–
9	gi|46395888 glutamine synthetase	*Suillus bovinus*	HAEHIAVYGEDNDLR	64	6.11/39449	5.8/50000	Mascot	–
			RPASNIDPYR	48				
10	gi|14210102 isocitrate lyase gene	*Pichia angusta*	MRPLLADAD	65	8.2/54909.34 (6.38/55555.63)	6.5/50000	*de novo*	–
Quiescent spores								
11	gi|58737212 superoxide dismutase [Cu-Zn]	*Gigaspora margarita*	EITDAIITIEDAYSIIGR	106	5.79/16756	6.2/17000	Mascot	wt
			TVVVHEGLDDLGK	87				
			SITTGNAGGR	64				
			HVGDIGNVK	62				
			FPMTTGNAGGR	61				
			GVIGYIK	48				
			HTFPMTTGNA	44				
12	gi|58737212 superoxide dismutase [Cu-Zn]	*Gigaspora margarita*	EQITDAIITLEGEYSIIGR	173	5.79/16756	5.7/17000	Mascot	cured
			GHEFSLTTGNAGGR	69				
			LACGVIGYLK	65				
			AIAVLRPDKPDGTVDGTIVFTQEVGK	45				
13	gi|115620353 similar to MGC81930 protein (Flavin containing monooxygenase)	*Strongylocentrotus purpuratus*	FGTIVAIKHGHPNK	74	8.81/62813.22	4.6/30000	*de novo*	wt
			EKLVSFNN	34				
14	**gi|110636998 ribosome recycling factor**	***Cytophaga hutchinsonii***	**MEELNVYLDD**	**68**	**5.46/20793.73**	**5.2/20000**	***de novo***	**wt**
15	gi|72012618 similar to		ASAVDIMIIRISSR	72	8.53/32652.72	5.4/38000	*de novo*	wt
			INKVTR	45				

Spot	Description	Organism	Peptides	Score	Theoretical pI/Mw	Observed pI/Mw	Method	Differential expression
	Retinoic acid receptor beta	*Strongylocentrotus purpuratus*	SSRAI	34				
			ASSIRIMIID	32				
16	gi|50904194 histidine ammonia-lyase	*Streptococcus pyogenes*	DATRDIMIIRISSL AVDVNKKIVD	72 43	7.57/53333.69	5.4/38000	*de novo*	wt
17	gi|30181199 putative transmembrane sensor	*Nitrosomonas europaea*	TGWRARENTPW	79	6.07/38830.84	5.8/30000	*de novo*	wt
18	gi|41582887 leucyl-tRNA synthetase	*Lactobacillus johnsonii* NCC 533	MLEWLEDHN IEDHNV	72 44	5.41/92335.58	5.8/80000	*de novo*	wt
19	gi|50960580 protein phosphatase 1, regulatory subunit (inhibitor) 3D	*Homo sapiens*	KAFNVGDDPSVP	71	8.44/32559.11	5.5/11000	*de novo*	cured
20	gi|82998430 PREDICTED: proprotein convertase subtilisin/kexin type 5	*Mus musculus*	CMPCEEGC CNANH	71 33	6.78/101488.9	6/40000	*de novo*	cured
21	gi|1653116 slr1520 (putative aldo/keto reductase)	*Synechocystis sp.* PCC 6803	ATHDLNTEEHWNWL	68	6.27/39831.30	5.4/80000	*de novo*	cured
22	gi|34979817 Voltage-gated sodium channel cardiac isoform Nav1.5	*Equus caballus*	PHTEKAPPLRKET	71	4.24/17448.57	6.5/28000	*de novo*	cured
Germinating spores								
23	gi|15026062 adenosine deaminase	*Clostridium acetobutylicum* ATCC	DVVEAALEAL	66	5.10/38023.29	5.2/35000	*de novo*	wt
24	**gi|33859482 eukaryotic translation elongation factor 2**	***Mus musculus***	**SDGHIMLTDFDLSLKFDVR**	**70**	**6.41/453114.04**	**6.2/40000**	**Mascot**	**wt**

Spot	Description	Organism	Peptides	Score	Theoretical pI/Mw	Observed pI/Mw	Method	Differential expression
25	gi|74662366 Enolase (2-phospoglycerate dehydratase)	*Penicillium chrysogenum*	SGETEDVTIADIAVGLR	77	5.14/47236.46	5.4/50000	Mascot	wt
			TSDFQIVGDDLTVTNPLR	77				
			LAFQEFMIVPDTAPTFSEGLR	50				
26	gi|46108552 hypothetical protein FG01158.1 (heat shock protein 30)	*Gibberella zeae* PH-1	LLDDFDSYSR	54	5.40/24247.46	5.6/25000	Mascot	cured
			NFYNSDASFTPLFR	33				
GR 24-treated germinating spores								
27	gi|56696710 acetamidase/formamidase family protein	*Ruegeria pomeroyi* DSS-3	LWAYDKVALR	64	4.76/32865.23	5/32000	*de novo*	wt
28	gi|41815769 glycine reductase complex protein GrdC	*Treponema denticola*	GNLPETNAKMVAVVTV	66	6.07/54831.02	6.2/50000	*de novo*	wt
29	gi|268558040 CBR-PQN-67 protein	*Caenorhabditis briggsae*	SGNGNG	40	7.33/75640.96	6.9/75000	*de novo*	wt
			NSSNN	36				
			NSGSGN	34				
			NSGSGNG	34				
			GNNGGR	33				
30	gi|154247161 ABC transporter related	*Xanthobacter autotrophicus*	VGGVLADAVGCNAVVKV	50	10.09/65101.45 (7/65754.66)	6/65000	Mascot	wt
31	gi|3420603 Thiol-specific antioxidant proteinLsfA	*Pseudomonas putida*	LTITYPASTGR	67	5.68/24073	6/26000	Mascot	wt
32	gi|114799774 ATP-dependent chaperone protein ClpB	*Hyphomonas neptunium* ATCC 15444	MKLDLSLDAR	59	5.47/94917	6/90000	Mascot	wt
33	gi|254528436 0 kDa chaperonin (groEL protein)	*Candidatus Tremblaya princeps*	AAVEEGIVPGGGVALIR	78	5.76/57518	5.5/60000	Mascot	wt
			VEDALHATR	21				
34			SYPLDIHNVQDHLK	65				wt

Spot	Description	Organism	Peptides	Score	Theoretical pI/Mw	Observed pI/Mw	Method	Differential expression
	gi|384221 starvation-inducible DNA-binding protein	*Escherichia coli*	GANFIAVHEMLDGFR TALIDHLDTMAER YAIVANDVR	55 51 49	6.21/18697	6.2/17500	Mascot	
35	**gi|16130152 outer membrane porin protein C**	***Escherichia coli***	**NGNPSGEGFTSGVTNNGR** **FQDVGSFDYGR** **INLLDDNQFTR** **GNGFATYR**	**65** **63** **58** **17**	**4.58/40343**	**4.5/40000**	**Mascot**	**wt**
36	gi|129137 outer membrane protein A	*Escherichia fergusonii*	DGSVVVLGYTDR IGSDAYNQGLSER LGYPITDDLDIYTR	39 39 36	5.14/26242	5.4/30000	Mascot	wt
37	gi|7532784 outer membrane protein A	*Acinetobacter sp.*	IEGHTDNTGPR LVEYPNATAR	63 24	4.83/40101	5/41000	Mascot	wt
38	**gi|40743120 HS70_TRIRU Heat shock 70 kDa protein**	***Aspergillus nidulans***	**SSVHELVLVG** **SDYFHK**	**66** **40**	**5.03/6991 6.51**	**5.2/70000**	***de novo***	**cured**
39	gi|169610155 hypothetical protein SNOG_08173	*Phaeosphaeria nodorum*	LGEHNIDVLEGNEQFINAAK	59	8.47/78009.07	5.8/60000	Mascot	cured
40	gi|46114675 hypothetical protein FG03180.1	*Gibberella zeae* PH-1	QITVNDLPVGR	44	4.09/22759	4.5/24000	Mascot	cured
41	gi|167751914 hypothetical protein ALIPUT_00156	*Alistipes putredinis*	EFNGLIEAR	53	5.25/169914	5.4/17000	Mascot	cured
42	gi|4097891 heat shock protein 70	*Pneumocystis carinii*	SSVHEIVLVGGSTR TTPSYVAFSDTER	32 10	5.23/71474	5.4/70000	Mascot	cured
43	gi|114286101 tyrosine-protein phosphatase corkscrew	*Drosophila melanogaster*	PTTERWFH	69	8.67/924300.53	5.8/85000	*de novo*	only present in GR24-treated spores

Constitutively expressed proteins

Since data concerning *G. margarita* protein expression are so far quite scanty, we selected some of the more intense spots among the 159 that were common to all the considered conditions. A reliable result was obtained for 10 out of 17 spots originally sent for sequencing.

Consistently with their constant detection in both wt and cured two-dimensional gel electrophoresis (2D-E) maps at high level of intensity (data not shown), the spots from 1 to 10 were identified as proteins mainly involved in some central metabolic pathways (i.e. respiration, energy production, fatty acid (FA) and sterol ester biosynthesis). Protein identification by database search retrieved best hits belonging to a wide range of organisms, from plants to bacteria. However, the constant observation of the corresponding proteins in all the analysed maps strongly supports the hypothesis that they belong to the fungal proteome.

Spots 1, 2, 3 and 4 share the same Mw but have different pI; taken together, they account for a considerable part of the total amount of protein detected on the2D-E maps. They seem to be related to proteins with different functions, and belonging to a wide range of organisms.

Spot 5 was identified as an uncharacterized conserved protein of unknown function, while spot 6 showed highest similarity with a synaptotagmin protein 2 from *Caenorhabditis elegans*. Synaptotagmins are globally known as membrane proteins involved in vesicle-mediated transport and exocytosis. *De novo* search for spot 7 led to the identification of an acyl-CoA desaturase; delta-9 FA desaturases, found in various eukaryotes and bacteria, play essential roles in FA metabolism, being involved in the CoA-bound desaturation of FAs.

For spot 8, the best hit was represented by a cytochrome C oxidase from the plant *Cephalotaxus fortunei*, the enzyme responsible for energy production via the mitochondrial respiratory chain.

Mascot search for spot 9 led to the identification of a glutamine synthetase (GS); although a sequence of a GS from the AM fungus *G. intraradices* is already present in public databases (gi|161406807), the peptides retrieved for spot 9 showed the best similarity with a GS from the ectomycorrhizal fungus *Suillus bovinus*. In any case, the GS from *G. intraradices* (gi|161406807) shows a calculated Mass/pI of 42.179/5.46, which is highly consistent with what observed on our 2D-E map. This protein represents a central enzyme of nitrogen metabolism since it allows assimilation of nitrogen and biosynthesis of glutamine (Breuninger *et al*., 2004). It has been described as crucial for nitrogen metabolism in germinating spores, and its mRNA has been reported not to be differentially expressed in AM fungi exposed to root exudates and different N sources (Breuninger *et al*., 2004; Gachomo *et al*., 2009). A possible functional regulation driven by phosphorylation has been reported for this enzyme in plants (Riedel *et al*., 2001). Spot 10 shared the highest similarity with the protein isocitrate lyase from *Pichia angusta.* This protein represents a key enzyme of the glyoxylate cycle, which was suggested to play a central role in the flow of carbon during AM symbiosis (Bago *et al*., 2002). A high expression of the ICL gene transcript was observed in the AM fungus *G. intraradices* germinating spores and extraradical mycelium (Lammers *et al*., 2001).

Wild-type and cured spores: differentially expressed proteins

A number of spots differentially expressed between wt and cured spores was identified under the three physiological conditions considered, showing both qualitative (presence versus absence) or quantitative (different level of expression) differences (Fig. 1 and Table 2).

Table 2. Differentially expressed spots identified for each physiological condition by Mascot or *de novo* search.

Spore status	Wild-type	Cured
Quiescent	7 (1)	5
Germinating	3 (1)	1
GR24-treated	11 (1)	5 (1)

1. The numbers in brackets indicate spots for which a quantitative analysis was done.

Quiescent spores. For the quiescent spores condition, 23 spots were selected for sequencing, and good results were obtained for 12.

Spots 11 and 12, from the quiescent wt and cured spore map, turned out to represent the same protein, a superoxide dismutase (SOD) from *G. margarita*. This protein represents one of the few described so far for *G. margarita* and the transcript of the corresponding gene was already demonstrated to be differentially expressed during the fungal life cycle, with the highest mRNA level in the symbiotic phase (Lanfranco *et al*., 2005). On the map, spots 11 and 12 shared the same mass, but possessed slightly different isoelectric points. The observation of a shift in the isoelectric point is often due to post-translational modifications such as glycosylation or phosphorylation. *In silico* analysis of the *G. margarita* SOD sequence shows the presence of 5 and 10 predicted sites for phosphorylation and glycosylation respectively. Some SOD from animals are known to be glycosylated (Oda *et al*., 1994; Tang *et al*., 1994) or to possess putative glycosylation sites (Cheng *et al*., 2006a,b). It has also been shown that the cytoplasmic *Listeria monocytogenes* MnSOD is phosphorylated on serine and threonine residues and less active when bacteria reach the stationary phase (Archambaud *et al*., 2006).

The sequence obtained from spot 13, isolated from quiescent wt spores, showed the highest similarity with flavin containing monoxygenases (FMOs) from the purple sea urchin *Strongylocentrotus purpuratus*. This protein belongs to a group of microsomal proteins involved in the process of non-nutritional foreign compounds metabolism known as xenobiotics. Generally, FMO converts lipophilic nucleophile xenobiotics to more polar, readily excreted metabolites, decreasing their pharmacological activity. The FMOs oxygenate nucleophilic O, N, S and Se atoms of a wide range of substrates, such as amines, amides, thiols and sulfides. Fungi are known to possess FMOs; for example, an FMO from the yeast *Schizosaccharomyces pombe* was identified, and its mechanism of action was described (Eswaramoorthy *et al*., 2006).

Spot number 20 was identified in the quiescent cured condition as a subtilisin-kexin convertase. Subtilisin-like proteases (SLPs) form a superfamily of enzymes that act to degrade protein substrates. In fungi, SLPs can play either a general nutritive role, or may play specific roles in cell metabolism; in addition, they have been shown to act as pathogenicity or virulence factors (Bryant *et al*., 2009). These proteins have been recently characterized in fungi, which interact with other eukaryotes, like the endophytic fungus *Epichloë festucae* and some entomopathogenic fungi (Bryant *et al*., 2009; Fang *et al*., 2009).

Germinating spores. The number of differentially expressed proteins identified from germinating spores was very low: only 4 proteins out of the 20 selected could be reliably identified. Two proteins (Spots 24 and 25), which can be related to a metabolically active condition (elongation factor and enolase), were identified in wt spores. By contrast spot 26, which was specifically detected in the cured condition, showed the highest similarity with a protein from *Gibberella zeae*, which represents a putative stress-inducible heat shock protein HSP30 (Seymour and Piper, 1999).

GR24-treated spores. The third considered condition was represented by spores germinating under treatment with the strigolactone analogue GR24. Strigolactones are carotenoid-derived molecules which are naturally present in plant root exudates; they are known to stimulate the growth and branching of AM germinating hyphae, as well as the activity of fungal mitochondria. Cured spores display a reduced response to such a treatment, in terms of hyphal branching and growth (Lumini *et al*., 2007).

The GR24-treated condition detected the highest number of differentially expressed proteins (a total of 16 out of 27 sent for sequencing). Some interesting spots of putatively endobacterial origin were identified in the wt condition (see 'Spots of potentially bacterial origin' paragraph below). On the fungal side, a protein that was detected in cured GR24-treated condition and described as a hypothetical protein from *G. zeae* was identified as a putative peroxyredoxin (spot 40). This group of proteins are possibly involved in redox-regulated antioxidant defence, a mechanism well described also in fungi (Belozerskaya and Gessler, 2007). Similarly to what observed for germinating spores, two heat-shock proteins seem to be more expressed in cured than wt spores after strigolactone analogue treatment (spots 38 and 42).

Cross-comparison between conditions

As a further step, a global statistical analysis was applied to all the 2D maps obtained, in order to identify spots specifically expressed in one of the considered physiological conditions. Following this comparative analysis, spot 43 was identified to be specifically expressed in GR24-treated spores, in wt as well as in cured condition. This protein, which shares higher similarity with a tyrosine-protein phosphatase from *Drosophila melanogaster*, seems not to be expressed in quiescent and germinating spores.

Spot 35, which was originally identified as more expressed in wt versus cured spores in GR24-treated condition, and identified as an outer membrane porin protein C (ompC), also revealed to be specifically expressed during strigolactone treatment, as it was absent in quiescent and germinating maps. Although the Mascot search suggested a possible bacterial origin for this spot, the Blast analysis against the *Candidatus* Glomeribacter sequence database (S. Ghignone, I.A. Anca, A. Salvioli, L. Lanfranco and P. Bonfante, in preparation) did not produce any significant hit, suggesting that this protein might not belong to the endobacterium. This is consistent with its detection in GR24-treated cured spores, even though at a lower concentration. We can thus postulate that this protein belongs to the fungal proteome; indeed in eukaryotic cells beta-barrel proteins similar to bacterial ompC are known to be involved in mitochondrial outer membrane synthesis (Becker *et al*., 2009; Walther *et al*., 2009).

Spots of potentially bacterial origin

The database search for protein identification retrieved a bacterial entry as best hit for the 39% of the identified proteins (17 spots out of 43). To check the possibility that these proteins belong indeed to the endobacterium, the corresponding sequences were blasted against the provisional database constructed on the basis of the *Candidatus* G. gigasporarum genome sequencing (S. Ghignone, I.A. Anca, A. Salvioli, L. Lanfranco and P. Bonfante, in preparation). For six of the analysed proteins (here identified in Table 1 as spot 18, 30, 31, 32, 33 and 36), a result with *E* value < e^{-50} was obtained, suggesting that in these cases a protein belonging to the endobacterium proteome was sequenced. Consistently with their nature, these six putative endobacterial proteins were uniquely detected in maps coming from the WT condition, and four out of them from the spores treated with GR24. Spot 18, 30 and 36 show the highest similarity with proteins involved in the general bacterial cell functioning (protein synthesis, membrane transport and structural

component). Spots 32 and 33 were identified as an ATP-dependent chaperone protein ClpB and a 60 kDa chaperonin groEL respectively. They are related to the chaperonin pathway. Similarly, spot 31 was identified as a bacterial peroxyredoxin, a thiol-specific antioxidant protein, which is considered to act as redox-regulated chaperone involved in bacterial antioxidant defence (Kumsta and Jakob, 2009). As demonstrated in early studies, the universal heat shock chaperonin groEL is constitutively highly expressed in *Buchnera* and in other endosymbionts and host-restricted organisms (Aksoy, 2000). An increased investment in mechanisms for protein stabilization has been postulated to have evolved as a compensation for accumulated mutations that reduce protein stability (Wernegreen and Moran, 2000; Van Ham *et al*., 2003).

Lipid profile

The FA composition of wt and cured spores of *G. margarita* was established following a chromatographic methodology (Table 3 and Fig. 2), and the results are consistent with what previously observed for this fungus (Bentivenga and Morton, 1996). The FA composition of wt spores ranged from C12:0 to C22:1. The predominant FA compounds were C16:0 (palmitic acid) and C18:1ω9 (oleic acid); they constituted more than 69% of the total FA with 35.8% of C18:1ω9 and 33.7% of C16:0. In cured spores a significant reduction of C16:0, C18:1ω5, C20:3 and C20:4 proportions was observed. The content of major FAs C16:0 and C18:1ω9 decreased of three- and twofold, respectively, in comparison with wt spores values. A reduced content of other minor FAs: C16:1ω9, C16:1ω5, C18:1ω7, C18:3 and C20:1 was also observed. Moreover, the total FA amount decreased more than twofold in cured spores.

Table 3. Fatty acid composition of wt and cured spores of *G. margarita*.

Fatty acids	**Wild-type spores**		**Cured spores**	
	µg per 200 spores	**%**	**µg per 200 spores**	**%**
C12:0	1.8 ± 1.9	Tr	0.9 ± 0.6	Tr
C 14:0	1.8 ± 1.9	Tr	1.1 ± 0.6	Tr
C 16:0	581.7 ± 124.5	33.7	196.9 ± 99.8*	25.1*
C 16:1ω9	6.5 ± 5.0	Tr	2.4 ± 0.8*	Tr
C 16:1ω5	73.0 ± 23.9	4.2	38.4 ± 20.5*	4.9
C 18:0	47.1 ± 48.2	2.7	16.5 ± 7.8	2.1
C 18:1ω9	617.1 ± 79.6	35.8	295.1 ± 42.2*	37.6
C 18:1ω5	76.6 ± 11.2	4.4	52.2 ± 21.8	6.7*
C 18:1ω7	14.7 ± 2.4	0.9	9.1 ± 1.8*	1.2
C 18:2	14.3 ± 2.5	0.8	10.7 ± 5.3	1.4
C 18:3	9.5 ± 4.9	0.6	4.6 ± 1.3*	0.6
C 20:1	135.7 ± 53.7	7.9	56.0 ± 9.2*	7.1
C 20:2	43.7 ± 20.0	2.5	23.6 ± 3.7	3.0
C 20:3	33.8 ± 9.7	2.0	28.8 ± 4.3	3.7*
C 20:4	26.9 ± 10.1	1.6	25.7 ± 3.0	3.3*
C 22:0	23.6 ± 7.2	1.4	15.8 ± 2.3	2.0
C 22:1	15.7 ± 7.5	0.9	7.1 ± 1.0	0.9

1. Data are presented as means ± standard error (n = 3). The asterisk indicates significantly differences between spores type according to non-parametric permutation test ($P < 0.05$). Tr (traces): amounts < 0.5% or 1 µg per 200 spores.

Fatty acids	Wild-type spores		Cured spores	
	µg per 200 spores	%	µg per 200 spores	%
Total fatty acids (µg per 200 spores)	1723.6 ± 141.5		784.8 ± 212.6*	

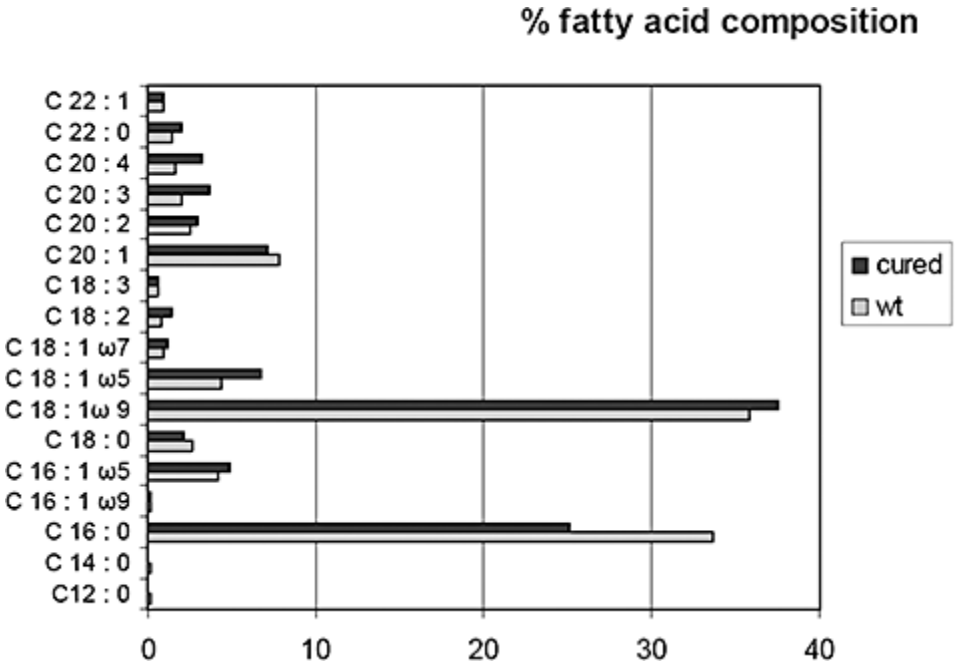

Figure 2. Histogram representing the percentage FA composition of wild-type and cured *G. margarita* spores.

While sterol composition has been already assessed for AM fungi belonging to the order Glomales (Grandmougin-Ferjani *et al*., 1999; Declerck *et al*., 2000; Fontaine *et al*., 2001a), no information was so far available for *G. margarita* grown *in vitro*. Here, sterol composition of both wt and cured spores was determined (Table 4 and Fig. 3). Major sterols of *G. margarita* (wt) are 24-methylcholesterol (43%) and 24-ethylcholesterol (38%), and they together represent 81% of the total sterols. Unlike what reported in literature for *G. intraradices* grown *in vitro*, the most abundant sterol is thus 24-methylcholesterol instead of 24-ethylcholesterol (Grandmougin-Ferjani *et al*., 2005). Other sterols were detected: desmosterol, cholesterol, 24-ethylcholesta-5.22-dienol, 24-ethyl-25(27)-methylene cholesterol, 24-ethylidene cholesterol and one unidentified sterol. Ergosterol, the fungal specific sterol commonly used as indicator of fungal biomass in soil (Bossio *et al*., 1998; Bardgett and McAlister, 1999; Montgomery *et al*., 2000), was not detected in both wt and cured *G. margarita* spores. This finding is in agreement with the data available in literature, showing that ergosterol is absent in AM fungi (Grandmougin-Ferjani *et al*., 1999; Olsson *et al*., 2003; Fontaine *et al*., 2004) and not synthesized in *G. intraradices* (Fontaine *et al*., 2001b). A similar sterol profile was found in cured spores.

Table 4. Sterol composition of wt and cured spores of *G. margarita.*

Sterols	WT spores		Cured spores	
	µg per 200 spores	%	µg per 200 spores	%
Desmosterol	Tr	1	Tr	1
Cholesterol	2.3 ± 0.2	12	2.3 ± 0.2	12
24-methyl cholesterol	8.4 ± 1.0	43	8.2 ± 0.7	42
24-ethylcholesta-5.22-dienol	Tr	1	Tr	1
24-ethyl-25(27)-methylene cholesterol	Tr	1	Tr	1
24-ethyl cholesterol	7.3 ± 0.9	37	6.6 ± 0.1	35
24-ethylidene cholesterol	0.5 ± 0.2	3	Tr	2
Unidentified sterol	Tr	2	1.2 ± 0.3	6
Total sterols (µg per 200 spores)	19.6 ± 2.1		19.1 ± 1.5	

1. Data are presented as means ± standard error (n = 4). Tr (traces): amounts < 0.5% or 1 µg per 200 spores.

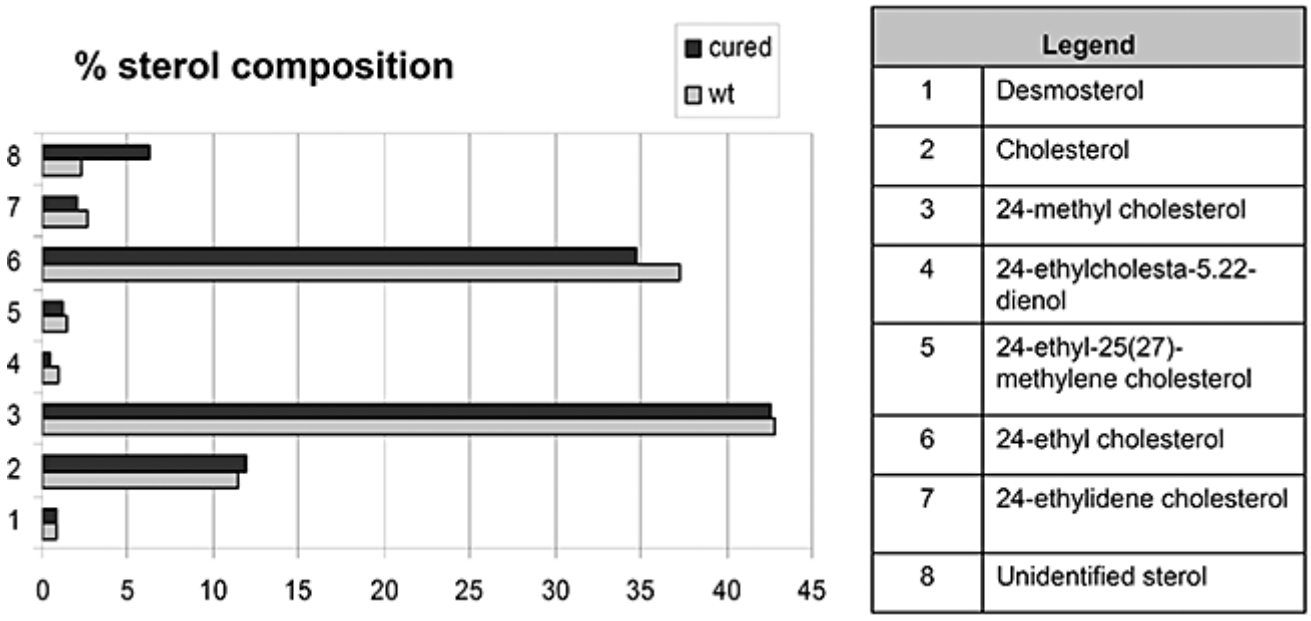

Figure 3. Histogram representing the percentage sterol composition of wild-type and cured *G. margarita* spores.

Discussion

Analysis of the multiple interactions established by mycorrhizal fungi with plant and their associated bacteria offers new understanding of the complexity of mycorrhizas, which can be defined as tripartite associations, at least under natural conditions (Bonfante and Anca, 2009). Details of these interactions are still unclear since the limited availability of genome sequences for mycorrhizal fungi has only allowed a study of the impact of associated bacteria on the transcriptome profile of *Laccaria bicolor* (Deveau *et al.*, 2007). In this case, the helper bacterium *P. fluorescens* stimulated fungal growth and development as well as altered fungal gene expression, leading to activation of genes potentially involved in recognition processes, transcription regulation

and synthesis of primary metabolism proteins. Unlike ectomycorrhizal fungi, the genome of an AM fungus has not been completely analysed yet (http://mycor.nancy.inra.fr/IMGC/genomesequencing.html), making a transcriptomic investigation on AM fungal/bacterial interaction not currently feasible.

Information on the AM fungal proteome is quite scanty. The most exhaustive proteomic study is represented by the work of Recorbet and colleagues (2009), in which the authors isolated and identified 92 proteins from the extraradical mycelium of *G. intraradices* with the GeLC-MS/MS high-throughput technique.

Our results demonstrate that the endobacterial presence leads to a modulation of fungal protein expression in all the different conditions we tested and, in particular, after treatment with a strigolactone analogue. GR24-treatment allowed in fact the detection of the highest number of differentially expressed proteins between wt and cured condition, in agreement with Lumini and colleagues (2007), who showed that wt spores are more responsive to GR24 in terms of hyphal growth and branching. The current view suggests that strigolactone-treated spores might be in a more active status (Besserer *et al*., 2006; Besserer *et al*., 2008); very recently Bucking and colleagues (2008) strengthened this vision, showing that the expression of genes involved in primary metabolic pathways are induced by root exudates stimulation. The global statistical analysis revealed that two proteins are specific of this treatment. Interestingly, one of them was identified as a tyrosine-protein phosphatase, which belongs to a group of enzymes that, together with tyrosine kinases, regulate the phosphorylation state of many important signalling molecules. Such a protein could be a good candidate for the identification of components of the signal transduction cascades involved in the perception of the branching factor.

The second observation is that five out of these GR24-stimulated proteins resulted to be bacterial proteins. Under this condition, an increase of bacterial divisions was in fact observed (Anca *et al*., 2009), which could lead to an easier detection of bacterial proteins. Thus, the detection of bacterial protein in the GR24-treated wt spores map can be related to the fact that bacteria are more abundant in this condition, and/or can indicate that such proteins are part of the molecular response of the endobacterium to the strigolactone stimulus.

By contrast, cured line-specific proteins were relatively more limited (11) and four out of them were identified as stress-responsive proteins. Even if the cured line keeps its symbiotic capabilities, the germinating mycelium resulted to be less efficient in developing and contacting the host roots in axenic cultures (Lumini *et al*., 2007). It seems therefore that the *G. margarita* perceives the bacterium absence as an indirect or direct stimulus, which activates stress-responsive proteins.

The impact of the endobacterium on the metabolism of its host is also demonstrated by the strong differences in the FA profile. Lipids are crucial molecules for signalling and functioning of AM symbiosis (Bucher, 2010). In fact, AM fungi store predominantly the organic carbon acquired from the plant root as lipids. In addition, it was suggested that fungal FA metabolism may play a major role in the obligate biotrophism of AM fungi (Trepanier *et al*., 2005).

The FA profile here obtained for the wt spores is in good agreement with literature data for *G. margarita*, showing a predominance of the FA C18:1ω9 and a lower presence of C16:1ω5, which is, by contrast, highly represented in other AM fungi (for example, in *G. intraradices*, this FA represents the 50-70% of the total neutral lipids content) (Graham *et al*., 1995; Bentivenga and Morton, 1996; Olsson and Johansen, 2000). The comparison of FA composition from wt and cured spores highlighted important differences, showing that the total FA content decreases in the absence of the endobacteria and that the specific profile is affected in the cured spores. However, and

differently from the protein profile, bacteria-specific FAs were not detected in the wt spores. The relatively low number of bacteria per spores and/or some similarities with fungal FA could explain the absence of bacterial markers in the FA profile. Several β-*Proteobacteria* are in fact characterized by C16:0 and C18:1ω7 (Krejci and Kroppenstedt, 2006), which are also the major FA in fungi.

As a second point, our biochemical analyses are in good agreement with morphological observations of the cured *G. margarita* spores, which revealed a decrease of size and number of lipid masses (Lumini *et al*., 2007). The lower total FA content in the cured line could also point to the absence of the fungal membrane, which regularly surrounds the endobacteria as detected at ultrastructural level (Bonfante *et al*., 1994).

It is known that fungal storage lipids mainly consist of neutral lipid FAs (Olsson and Johansen, 2000), of which triacylglycerols are the dominant type in spores and vesicles. Triacylglycerols are specifically enriched in 16-carbon FA (Grandmougin-Ferjani *et al*., 2005). Our results show that FA C16:0 (palmitic acid) resulted to be less abundant in the cured spores. Since palmitic acid is the direct product of the fatty acid synthase (FAS) complex, prior to any subsequent modification (elongation, desaturation, etc.), the data may suggest that the cured spores are less efficient in FA biosynthesis and/or storage. Moreover, we cannot completely rule out that the oxidative stress highlighted by the stress-responsive proteins detected in cured spores has an impact on metabolic enzymes and on FA profile. Indeed, the reduction of the polyinsatured FA C18:3 could suggest an induction of lipid peroxydation in cured spores. Since lipids stored inside the spores are used to sustain germination and pre-symbiotic growth (Bago *et al*., 1999; Fontaine *et al*., 2001b), a decreased FA availability in spores could be proposed to explain the reduced pre-symbiotic growth observed in the cured fungus (Lumini *et al*., 2007).

Differently from the FA, sterol biosynthesis was not affected by endobacteria, since the peculiar profile found for wt *G. margarita*, was maintained also in the cured line.

In conclusion, the comparison between the proteic and lipidic profiles of *G. margarita* spores containing endobacteria versus its cured line has offered a first snapshot of the biological impact of the *Candidatus* G. gigasporarum on its fungal host: its absence does not affect the expression of crucial enzymes for the fungal metabolism like GS or isocitrate lyase. By contrast, heat shock proteins are unambiguously upregulated, suggesting that the fungus has to face a stress situation. In the mean time, FA profile changes, and the synthesis of palmitic acid decreases; this fact can be morphologically mirrored by the decreased lipid storage observed in cured spores and, on the physiological side, can offer an explanation to the reduced pre-symbiotic growth. This metabolic snapshot seems therefore to put forward a model in which *G. margarita* and *Candidatus* G. gigasporarum live together in a well-established balance; as a consequence, the absence of the endobacterium is mirrored by changes in physiological and molecular fungal features.

Experimental procedures

Fungal material

Spores of *G. margarita* Becker and Hall (BEG 34; deposited at the European Bank of *Glomeromycota*) containing *Candidatus* G. gigasporarum endobacteria were used for all experiments, together with the cured spores, which were obtained as described in Lumini and colleagues (2007). Spores are referred as wt (wild-type) spores, while the spores without bacteria are identified as ‘cured’. The cured status was routinely checked with PCR by using specific

primers (Lumini *et al*., 2007). To allow them to germinate, the spores were placed in 60 mm of diameter Petri dishes with 1 ml of sterile water, and let in the dark at 28–30°C for ten days.

Wild-type and cured germinating spores were also treated with a chemically synthesized strigolactone, a molecule called GR24 that is analogue to the natural strigolactones contained in roots exudates (Buee *et al*., 2000; Akiyama *et al*., 2005). This molecule was kindly provided by Peter Karlowsky (Göttingen University, Germany), and was added to the germination water to the final concentration of 0.01 p.p.m.

Protein extraction

The protein extraction was performed on 400 quiescent, germinating and GR24-treated wt and cured spores for each experiment, according to the procedure described by Bestel-Corre and colleagues (2002). The pellet was dried and resuspended in 800 µl of solubilization buffer containing 9 M urea, 2% Triton X-100. Lipids and nucleic acids were removed by supercentrifuging at 25 krpm during 30 min (Beckman Coulter mod. Avanti j-301), and protein content of the supernatants was quantified by the method of Bradford, using bovine serum albumin (BSA) as a standard.

2D-Electrophoresis

The first dimension was performed with 17 cm IPG strips, pH 4–7 (Bio-Rad), followed by the second dimension on a 10% SDS-polyacrylamide gels (0.1 cm × 19 cm × 23 cm). Electrophoresis was run for 5 h at 10°C under constant mA (24 for each gel). Gels were then fixed in 10% methanol, 7% acetic acid solution for 30 min, and stained with the SYPRO Ruby fluorescent dye (Molecular Probes, Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions, and visualized under UV illumination at 365 nm using the Molecular Imager VersaDoc MP 4000 Imaging System (Bio-Rad). Three replicates were considered for each fungal sample.

2D-PAGE analysis

Digital images of the gels were analysed using PDQuest 2-DE Analysis Software (Bio-Rad). Protein spots were considered only when their intensity was at least 20 times the background intensity. The similarity index between two gels was calculated as the ratio between the common spots and the total detected spots. The correlation coefficient was calculated by the PDQuest software. In order to verify whether the same protein content was separated on the gels, a two-step statistical analysis was performed. First, normality tests were performed (Kolmogorov–Smirnov test and Shapiro–Wilk test). Then, as the samples were not normally distributed, the Wilcoxon–Mann–Whitney test was used to compare both the technical replicates and the biological replicates.

To identify spots of interest, gels from wild-type spores and cured spores were compared pairwise, in the three treatments (quiescent, germinating or GR4-treated spores).’

The PDQuest software allowed us to perform both a qualitative (presence versus absence) and a quantitative analysis was done. For the quantitative analysis, spots were considered differentially expressed if they were at least twice as intense as in the comparative gel.

Finally, a cross-comparison among the maps obtained in the different conditions (i.e. quiescent, germinating and GR24-treated spores) was done using PDQuest 2-DE Analysis Software (Bio-Rad) as already described, and considering two replicate gels for each condition. Relative spot volumes of the replicate gels were compared and were analysed according to the T-student test to verify

whether the changes were statistically significant ($P < 0.05$). Only spots showing at least a twofold change in their relative volumes were considered.

Protein identification

After image analysis of the 2DE gels, protein spots of interest were excised from illuminated SYPRO Ruby-stained gels for sequencing by mass spectrometry. Proteins were reduced, alkylated and digested with trypsin. The resulting peptide mixture was spotted on a MALDI plate: 0.5 µl of sample combined with 0.5 µl of matrix (alpha-cyano-4-hydrocinnamic acid). Positive ion MALDI mass spectra were obtained using an Applied Biosystems 4700 Proteomics Analyzer (Applied Biosystems, Foster City, CA, USA) in reflectron mode. MS spectra were acquired over a mass range of m/z 800–4000. Monoisotopic masses were obtained from centroids of raw, unsmoothed data. The 10 strongest peaks, with a signal to noise greater than 50, from each fraction were selected for CID-MS/MS analysis. The default calibration was used for MS/MS spectra, which were baseline-subtracted (peak width 50) and smoothed (Savitsky–Golay with 3 points across a peak and polynomial order 4); peak detection used a minimum S/N of 5, local noise window of 50 m/z, and minimum peak width of 2.9 bins. Mass spectral data obtained in batch mode were submitted to database searching using TS2Mascot (Matrix Science, version 1.0.0). A locally running copy of the Mascot program (Matrix Science, version 2.1) was used to perform the searches. MS/MS data were analysed with Mascot against the NCBInr database, allowing one missed cleavage of trypsin per peptide, accuracy level of 100 p.p.m. and a mass tolerance of 1. Fixed and variable modifications were carbamidomethylation of cysteine and oxidation of methionine respectively. Mass tolerance was set at 100 p.p.m. for peptide precursors and at 0.1 Da for fragment ions. Only matches with $P < 0.05$ for random occurrence were considered to be significant, and a minimum of 98% ion Ci. was required for a positive identification. Some MS/MS spectra were interpreted *de novo* using the DeNovo program within GPS version 3.6 (Applied Biosystems). The MS blast search was performed against ncbi95 protein database, and the hits were considered statistically confident according to the MS blast scoring scheme.

Theoretical molecular weight and isolelectric point for the non-modified and the phosphorilated form were calculated for each identified protein using the pI/Mw scan tool from ExPASY.

Lipids extraction and analysis

Lipids extraction was performed on freeze-dried aliquots of 200 wt and cured quiescent spores. The fungal material was saponified with 4 ml of 6% (w/v) in methanolic KOH at 85°C for 2 h. After addition of one volume of distilled water, the saponiable fraction was extracted three times with 5 volumes of hexane and submitted to acetylation in a toluene:Ac2O:pyridine mixture (1:2:1, v:v:v) for 16 h at room temperature. After evaporation of reagents, acetate derivatives were purified on silica gel thin-layer chromatography plates (60F254; Merck Darstadt, Germany) with dichloromethane as the solvent (one run). Steryl acetates migrated as a single band, which was scraped off and eluted 30 min with dichloromethane, and transferred to chromatography vials. Acetylated sterols were analysed and identified according to a technique described in Campagnac and colleagues (2009). The residual aqueous phase was adjusted to pH 1 with concentrated HCl. The saponifiable fraction were extracted three times with 5 volumes of hexane and evaporated under N2. Fatty acids were methylated using 3 ml of BF3/methanol (14%) at 70°C for 3 min, and reaction was stopped in ice. Fatty acid methyl esters were extracted three times with 5 volumes of hexane after the addition of 1 ml of distilled water. These extracts were evaporated under N2 and transferred to chromatography vials. Fatty acid methyl esters were analysed using a PerkinElmer Autosystem gas chromatograph (GC) equipped with a flame-ionization detector (Norwalk, CT, USA) and a ECTM-1000 (Alltech Associates, Deerfield, IL, USA) capillary column

(30 m × 0.53 mm inside diameter) with hydrogen as carrier gas (3.6 ml min^{-1}). The temperature program included a fast rise from 50°C to 150°C at 15°C min^{-1} and then a rise from 150°C to 220°C at 5°C min^{-1}. Fatty acids were quantified using heptadecanoic acid methyl ester (C17:0) as an internal standard and by introducing a defined amount of this compound into every sample just before running on GC. Their identification relied on the retention times of a wide range of standards (Sigma-Aldrich). All the experiments were done in 3 or 4 replicates.

Statistical analysis

The differences between WT spores and cured spores were analysed with the non-parametric permutation test with general scores for independent samples using the Stat Xact software ($P < 0.05$).

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