The *ftsZ* Gene of the Endocellular Bacterium ‘*Candidatus Glomeribacter gigasporarum*’ Is Preferentially Expressed During the Symbiotic Phases of Its Host Mycorrhizal Fungus

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The arbuscular mycorrhizal fungus (AM) *Gigaspora margarita* consistently hosts bacteria, named ‘*Candidatus Glomeribacter gigasporarum*,’ inside its cytoplasm. Endobacteria have a positive impact on fungal fitness during the presymbiotic phase, prior to plant roots colonization. We tested the hypothesis that the endobacterium and its cell divisions depend on fungal metabolism, mirroring also the events of the fungal life cycle which are influenced by plant signals. We first cloned a fragment of *ftsZ*, a marker gene for bacterial division, and then analyzed its expression along the different stages of fungus development. The bacterial gene transcripts showed the highest values when the fungus was associated to the plant, and peaked in the extraradical mycelium. Strigolactones, which are known to stimulate the AM fungal growth, caused a significant transcript increase in the germinated spores in the absence of the plant. The quantitative real-time reverse-transcription polymerase chain reaction data were strengthened by the quantification of the dividing bacteria, which were increasing in number in germinating spores after the strigolactone treatment. The bioactive molecule alone did not cause any change in the number of bacteria after their isolation from the fungus, thus showing that the strigolactone alone cannot confer free-living capacities to the bacterium.

Land plants share an ancient, 450-million-year-old co-evolutionary history with soil fungi with which they have established a mutualistic association, called arbuscular mycorrhiza (AM). Both partners benefit from nutrient transfers, because the AM fungi facilitate the uptake of nutrients such as phosphate from plants, while the plants provide them with carbon sources which are required to complete their life cycle (Bonfante and Genre 2008). AM are present in approximately 80% of land plants, from liverworts to ferns and from ancient trees such as *Ginkgo biloba* to domesticated plants such as rice and grapevine. Their widespread distribution, together with their constant structural and functional features, clearly suggests the existence of common genetic determinants across different

plant taxa. Regardless of their taxonomic position inside the phylum *Glomeromycota* (Schüßler et al. 2001), AM fungi show some constant features, because they are asexual, obligate biotrophic, multinucleate, and unculturable microbes. A poorly investigated aspect of their biology is the consistent presence of bacteria-like organelles (BLO) in their cytoplasm. These were first observed in 1970 (Mosse 1970), and since then, the presence of BLO has been cytologically documented inside the AM fungi which colonize liverwort gametophytes (Carafa et al. 2003; Ligrone et al. 2007), fern gametophytes, and sporophytes (Schmid and Oberwinkler 1995) as well as roots of *Ginkgoales*, *Taxaceae*, and *Liliaceae* spp. (Scannerini and Bonfante 1991). However, and in spite of their widespread distribution, the nature of these BLO in AM fungi is still elusive. A taxonomic identification has never been accomplished, with only one exception: that of the *Gigaspora margarita* isolate BEG 34, the BLO of which have been identified as true endobacteria. On the basis of their 16S rRNA sequence, they were grouped into a new taxon, which—due to their current unculturable status—was named ‘*Candidatus Glomeribacter gigasporarum*’ (Bianciotto et al. 2003). They cluster with the large group of β -proteobacteria, because they are related to the *Burkholderia* genus and their genome seems to be reduced to a great extent: 1.4 Mb consisting of one chromosome and one plasmid (Jargeat et al. 2004). The uncultivability outside fungal cells, the vertical transmission through fungal generations (Bianciotto et al. 2004), and the small genome clearly suggest that *Ca. Glomeribacter gigasporarum* is an obligate endosymbiont, like other known endobacteria that live in aphids (Moran 2003). This accounts for many fundamental questions regarding the nature of a fungus–bacterium symbiotic relationship.

The impact of *Ca. Glomeribacter gigasporarum* on its host is still not fully understood. Its absence has recently been shown to not affect the symbiotic capacities of the fungal host, whereas it influences presymbiotic growth to a great extent in terms of hyphal elongation and branching in response to a root exudate treatment, two prerequisites for colonization (Lumini et al. 2007). These findings would seem to suggest that *Ca. Glomeribacter gigasporarum* grants the optimal development of its fungal host in a fundamental moment of its life cycle (i.e., during presymbiotic growth). During this phase, a molecular dialogue has been demonstrated to take place between the plant root and the fungus, during which both partners are kept informed in a timely fashion about their reciprocal proximity. Using a simple bioassay, Akiyama and associates (2005) iden-

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tified a plant molecule (a strigolactone) that is present in root exudates and which is capable of eliciting AM fungus branching. Besserer and associates (2006) found that strigolactones strongly and rapidly stimulate the proliferation of the AM fungus *Gigaspora rosea* at concentrations as low as 10^{-13} M. The strigolactones are also able to increase the respiration and the density of mitochondria in the hyphae (Tamasloukht et al. 2003) as well as to increment the fungal nuclear divisions (Besserer et al. 2008). Recent findings have demonstrated the inhibitory effect of strigolactones on plant shoot branching, suggesting a more general role as a plant hormone (Gomez-Roldan et al. 2008).

The role of *Ca. Glomeribacter gigasporarum* seems to be crucial during this phase of intense chemical dialogue (Lumini et al. 2007); therefore, our main goal was to understand whether there was a relationship between the bacterial cell division and the host fungus life cycle, also considering the fungal steps which are influenced by plant signals. Bacterial cell multiplication is a complex mechanism where a central role has been widely attributed to FtsZ, a structural and functional homologue of eukaryotic tubulins (Addinall and Holland 2002) and, therefore, *ftsZ* is used as a marker of bacterial division (Holtzendorff et al. 2002). The characterization and expression analysis of a putative *ftsZ* gene in *Ca. Glomeribacter gigasporarum* has allowed us to identify the symbiotic mycelium as the phase of the fungal life cycle where the transcripts are more abundant. Using synthetic analogues of natural strigolactones (GR7 and GR24), which were shown to be both active in stimulating presymbiotic development (Besserer et al. 2006), we have demonstrated an upregulation of *ftsZ* which was quantitatively mirrored by the increased number of dividing bacteria. The results demonstrate that the endobacteria modulate their population number according to the different stages of the host fungus development, with a proliferation peak during the extraradical mycelium (symbiotic phase). The bioactive molecules alone did not cause any change in the number of bacteria after their isolation from the fungus; therefore, we suggest that the fungus could play a crucial role in modulating bacterial proliferation.

RESULTS

Identification of putative *ftsZ* in *Ca. Glomeribacter gigasporarum*.

Phylogenetic trees, constructed on the basis of 16S rRNA, located *Ca. Glomeribacter gigasporarum* as closely related to *Burkholderia* and *Ralstonia* spp. (Bianciotto et al. 2003; Lumini et al. 2006); therefore, the alignment of the FtsZ protein from

Fig. 1. Polymerase chain reaction (PCR) amplification of a 1,031-bp *ftsZ* fragment of *Candidatus Glomeribacter gigasporarum* with FNF3/FNR2 degenerated primer pair. Lane 1, water negative control; lane 2, negative control with DNA template of *Gigaspora margarita* BEG 34 'cured'; lane 3, PCR amplification with DNA template of *G. margarita* BEG 34 wild-type quiescent spores; lane 4, PCR amplification with DNA template of *G. margarita* BEG 34 wild-type germinated spores; L, Low Mass Ladder (Invitrogen).

these two bacterial groups (Supplementary Fig. 1) led to the identification of the protein-conserved regions. Several pairs of degenerated primers were designed and tested in polymerase chain reaction (PCR) using as a template DNA extracted from spores of *Gigaspora margarita* BEG 34 (data not shown). After PCR amplification, a single band of the expected size was detected with the degenerated primers FNF3 and FNR2 designed on the conserved protein regions –GTIIKV and –DTPAVWR (Fig. 1). The bacterial origin of the PCR-amplified sequence was confirmed by performing a control PCR with a DNA template from the cured *G. margarita* spores: no amplification was obtained (Fig. 1), demonstrating that the cured spores could represent a consistent negative control for all the PCR experiments, and confirming the absence of the *ftsZ* gene from the fungal genomes (Margolin 2004; Osteryoung and Nunari 2003). The 1,031-bp PCR-amplified fragment of the putative *ftsZ* of *Ca. Glomeribacter gigasporarum* was cloned and sequenced.

As a second step, the full sequence of the *ftsZ* gene was obtained from the sequencing of the Fosmid_10 that originates from the metagenomic library of the host fungus *Gigaspora margarita* (S. Ghignone, personal communication). Identity between the two independently obtained sequences was confirmed through nucleotide alignment (Supplementary Fig. 2). The sequence of the putative *ftsZ* gene of *Ca. Glomeribacter gigasporarum* was deposited in the EMBL/GenBank data libraries with accession number AM086172. The full-length sequence was subjected to Blastx analysis. This revealed 82% similarity of the putatively encoded protein with the FtsZ from *Burkholderia cenocepacia* J2315, *Burkholderia* sp. 383, *B. ambifaria* AMMD, and *B. cenocepacia* AU 1054.

Similarly, the GC content of the sequence was 60% and was similar to the content of other β -proteobacteria. The protein was estimated to be composed of 387 amino acids, with a calculated molecular weight of 40.27 kDa; the first domain corre-

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1 atggaactggactttgaaatactagaacaggaaaccagcgccacatcatcaaaagtcgtc 60
1 M E L D F E I L E T E T S G T I I K V V 20
61 agcgctgcggcgcggaatgcggtgcagcatatgcttaaccgcgcgctgcagggc 120
21 G V G G A G G N A V Q H M L N R G V Q G 40
121 gtggatttctgtgcatgaataccgatgcgaagcgcttgacgctcaaaagccgcaatg 180
41 V D F L C M N T D A Q A L G R S K A A M 60
181 ctgattcaattggggcaaacgggtttggcgcgcgcccaactgaaatggggcgcgcg 240
61 L I Q L L G Q T G L G A G A K P E M G R A 80
241 gcggcggaagaagcgcgcggaatcagcgatgcgctgagcggaagcgcatatgcttt 300
81 A A E E A R G R I S D A L S G A H M V F 100
301 attgcggcggaatggcgcgcgcaactggcggcgcgcgcgctgcgctgcagatt 360
101 I A A G M G G G T G T G A A P V V A Q I 120
361 gcgaagaagaatggcgattctgacgctggcggtctcaaaagcgcttggatttgagggc 420
121 A K E M G I L T V G V V S K P F E F E G 140
421 ggcaaacggatgcggattgcgaacggcgcgcgcgagcttgaggagcatgtggattgc 480
141 G K R M R I A E T G A A Q L E E H V D S 160
481 ctgattgtggtgttgaaacgaacggctcttctcagtgatgggtgacgacgctgaatggag 540
161 L I V V L N E R L F S V M G D D A E M E 180
541 aaatgcttcagtgcgcgacgatgtattgcataacggtcgcgcgcttcggaatttgcggaatt 600
181 K C F Q C A D D V L H N A V A G I A E I 200
601 attaatgttgaggcgctggtgaatgtggtatttgaagatgtcaaacggtgtagggcgaa 660
201 I N V E G L V N V D F E D V K T V M G E 220
661 caggcgaaagcgatgatggcgacggcgacgctgtccggcatcgacgcgcgctgtggct 720
221 Q G K A M M G T A T V S G I D R A R L A 240
721 gccgaacagcggtggcgacggctgttcgaagcgctgatttgcggcgcgcgcgcg 780
241 A E Q A V A S P L L E G V D L S G A R G 260
781 gtgctggtcaattaccgcgagcgccacattgcgctggcgagacgcgcaagtgatg 840
261 V L V N I T A S R T L R L A E T R E V M 280
841 aatgcgatcaagcactacgcgcggaacgacgacgcaatctctggcgctgtctacgat 900
281 N A I K H Y A A N D A T V I L G A V Y D 300
901 gatcgatggcgatggaactgcgctgacgctggcgacgacgctggcgctggcgcg 960
301 D A M G D G L R V T V V A T G L G R S A 320
961 aaaaaaacgcatcaggtgtccatgaccttgcctccacacagggacgcatcattcaacggcg 1020
321 K K T H Q V S M T L L H T G T D H S T G 340
1021 gctggactggaactacggcgctggatccccggcgctgtggcgacgcgcgcgaaact 1080
341 A G L D Y G A L D T P A V W R S A R E T 360
1081 gctcggcgacgctcagcgtgttcaggagaaaggcggtggaactacgcatatcccggtc 1140
361 A S A H V S V L Q E K G V D T Y D I P A 380
1141 ttttgcgcaaacaggcggttga 1164
381 F L R K Q A D * 387

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Fig. 2. *FtsZ* translation. Nucleotide and deduced amino acid sequence of the putative *ftsZ* of *Candidatus Glomeribacter gigasporarum*. In the rectangle is shown the GGGTGTGA essential FtsZ protein motif involved in GTP hydrolysis.

sponded to 15 to 322 amino acids and was highly conserved between the species. The N-terminal contains the GGGTGTGA motif involved in the GTP hydrolysis (Fig. 2).

A Bayesian inference was carried out using MrBayes v3.1.2 (Huelsenbeck et al. 2001; Ronquist and Huelsenbeck 2003) to analyze the phylogenetic relationships of the *ftsZ* gene sequence. The *ftsZ* sequence of *Ca. Glomeribacter gigasporarum* clustered together with all the *ftsZ* sequences from other *Burkholderiaceae* (*Burkholderia* spp. and *Ralstonia* spp.; posterior probability of 0.95). Within this cluster, the *Ca. Glomeribacter gigasporarum* sequence alone formed a sister group with the *Burkholderia* spp. sequences group (supported by 0.95 posterior probability) (Fig. 3).

Analysis of the *ftsZ* expression during the presymbiotic and symbiotic phase of the fungal host.

More than 20,000 cells of *Ca. Glomeribacter gigasporarum* are present in a single spore of *Gigaspora margarita*, but they

seem to be more diluted along the germinating hyphae and inside the intraradical fungal structures (Bianciotto et al. 2004). To understand whether *ftsZ* can be considered as a marker of bacterial activity, *ftsZ* mRNA expression levels were analyzed throughout the fungal life cycle by real-time reverse-transcription (RT)-PCR.

cDNA samples were prepared from quiescent and 10-day germinated spores as well as from mycorrhizal root pieces of *Lotus japonicus*, from which the external hyphae were removed and collected separately. The quiescent and germinated spores represented the presymbiotic phases while the extraradical mycelium and roots represented the symbiotic phases. Samples were calibrated using the bacterial 23S rRNA transcript and the quiescent spores were taken as a reference sample to calculate the relative expression.

The *ftsZ* expression increased from the quiescent to the 10-day germinated spores but the highest values were found in the two symbiotic stages (i.e., when the fungus is associated to the

Fig. 3. Bayesian inference based phylogeny of the *ftsZ* gene of *Candidatus Glomeribacter gigasporarum* among β -proteobacteria. Bayesian posterior probabilities are shown at the branch nodes. *Wolbachia endosymbiont of Drosophila ananassae* AAS14415 was used as the outgroup to root the tree. Bar = posterior probability. In bold: *Ca. G. gigasporarum* *ftsZ* product position in the phylogenetic tree.

root as extraradical mycelium with a 164.24-fold expression and when the fungus colonizes the mycorrhizal roots with a 4.10-fold expression) (Fig. 4A).

Accordingly, the statistic analysis showed significant differences in the extraradical mycelium ($P = 0$) compared with the quiescent spores, 10-day germinated spores, and mycorrhizal roots.

No specific amplification evaluated on the basis of the melting curve (Ririe et al. 1997) was found in the cured variant of the host fungus or in the nonmycorrhizal *L. japonicus* with real-time RT-PCR (not shown), confirming the endobacterial specificity of the amplified *ftsZ* and of the 23S rRNA fragments.

Analysis of the *ftsZ* expression in the presence of strigolactone analogues.

The highest expression level of the bacterial *ftsZ* gene associated with the hyphae, which are involved in a symbiotic relationship with the host plant, suggested that the plant presence might directly or indirectly influence the expression of the bacterial gene. Strigolactones present in plant exudates are known to strongly stimulate the presymbiotic growth of AM fungi by increasing their hyphal branching (Akiyama et al. 2005) and we have already demonstrated that cured fungi are less sensitive to GR7, a strigolactone analogue (Lumini et al. 2007); therefore, we considered the possibility that strigolactones could have an effect on the *ftsZ* bacterial gene expression.

Spores germinated for 10 days were treated with GR7, a synthetic strigolactone molecule that has been shown to have an effect similar to root exudates and to induce hyphal branching in *G. rosea* (Bessener et al. 2006). A statistically significant ($P = 0.034$) induction of the *ftsZ* gene transcript was observed

Fig. 4. Relative expression of *ftsZ* gene over different stages of fungal life cycle. **A**, Values are shown as means of the *ftsZ* transcript level calibrated with 23S rRNA level from three biological replicates. The dashed line defines the scale change in chart representation (first scale, 0- to 10-fold expression; second scale, 100- to 250-fold expression). Different letters indicate significantly different values according to two-way analysis of variance with Tukey as the post hoc test ($P < 0.05$). **B**, Arbuscular mycorrhizal (AM) host fungus life cycle mirroring the phases investigated in A. Presymbiotic phase: a = quiescent spore and b = germinated spore; symbiotic phase: c = spore with extraradical mycelium continuously connected to the plant root with the intraradical phase and d = intraradical mycelium where the inter-intracellular hyphae and arbuscules are depicted.

in the germinated spores exposed to the bioactive molecule compared with the control (Fig. 5).

Quantification of the dividing bacteria from the spores in response to spore germination and treatment with GR24.

To understand whether the *ftsZ* activation could correspond to an increase in the number of bacteria in division, a quantitative estimation was carried out with the confocal microscope considering the conditions selected for the *ftsZ* expression analysis after treatment with GR7 and the quiescent spore condition. However, in this case, synthetic strigolactone GR24 was used to treat the spores instead of GR7, because it has been demonstrated to have higher efficiency than GR7 on *G. rosea* hyphal branching (Bessener et al. 2006). GR24 has also induced a significant increase in hyphal branching in *G. margarita* BEG 34 under our experimental laboratory conditions (M. Novero, *personal communication*).

Fig. 5. Effect of the strigolactone analogue GR7 on the *ftsZ* gene transcript levels from germinated spores. The values are shown as means of the transcript from four biological replicates, Germinated = 10-day germinated spores, GR treated = 10-day germinated spores with GR7. Different letters indicate significantly different values according to the Kruskal-Wallis test of variance ($P < 0.05$).

Fig. 6. Effect of the strigolactone analogue GR24 on the percentage of bacteria in division in quiescent and germinated spores. **A**, Morphology of bacteria cells beginning to divide (from left, nondividing cell, to right, separated cells after division) as used for counting in **B**, the bacterial dividing cells. **B**, Values are shown as the mean of the percentage of dividing bacteria from quiescent, germinating, and GR24-treated germinating spores, from four biological replicates. Different letters indicate significantly different values according to two-way analysis of variance with Tukey as the post hoc test ($P < 0.05$).

After staining with Syto BC (Invitrogen, Molecular Probes, Eugene, OR, U.S.A.), the spores were inspected with the confocal microscope and the frequency of bacterial division was estimated as a percentage, as described below (Supplementary Table 1). The Syto BC stain was already used to differentiate between the *G. margarita* BEG 34 spores containing endocellular bacteria and its cured variant, that presented only cellular components of exclusive fungal origin (Lumini et al. 2007). The dividing bacteria were identified and counted whenever two bacterial cells were in close association and drifted together in the suspension. Therefore, different stages of cell division were noted during the observation period (Fig. 6A).

The lowest percentage of dividing bacteria was obtained in the quiescent spores, thus mirroring the lowest *ftsZ* gene transcript activity (Fig. 5). The value was slightly increased during germination ($P > 0.05$) compared with the quiescent spores; finally, the percentage of dividing bacteria was significantly higher for the spores germinated in the presence of GR24 compared with both the quiescent ($P = 0.000$) and control germinated spores ($P = 0.005$) (Fig. 6B). This result, to our knowledge, represents the first direct correlation between *ftsZ* transcript activation and cell division in endobacteria and it confirms data so far only available for free-living microbes (Holtzendorff et al. 2002).

Quantification of isolated bacteria after GR24 treatment in a time-course experiment.

In order to understand whether the plant exudates could play a direct role on the bacterial division and survival outside the fungal host, an absolute quantification of bacteria was performed on isolated bacteria after the GR24 treatment.

No significant difference was observed between the growth of the treated and control bacteria over a time-course experiment (Fig. 7). The time intervals and the mean values obtained from the absolute counting of the bacteria in two biological replicates are shown in Supplementary Table 2.

Fig. 7. Determination of endobacteria numbers in the absence of the fungus after treatment with the GR24 strigolactone analogue. Absolute quantification of the endobacterial cells isolated from the host fungus spores and stained with the cell stain Syto BC (Invitrogen). The cells were maintained in TY medium and aliquots of 10 μ l were used for the quantification performed at six time points (0 h, 1 h, 3 h 30 min, 1 day, 9 days, and 16 days) with the confocal microscope. Values are shown as means of the absolute quantification from two biological replicates: light-gray bars, absolute quantification of the bacterial cells from the control (nontreated bacterial cell suspension); dark-gray bars, absolute quantification of the bacterial cells from the GR24-treated bacterial cell suspension. The starting point (0 h) represents the absolute quantification of bacterial cells performed immediately after the endobacterial separation from the host fungus spores. No statistically significant differences were detected between the GR24-treated and the control in the time course experiment when using two-way analysis of variance with Tukey as the post hoc test ($P > 0.05$).

Interestingly, some bacteria were viable, as could be deduced from their staining reactivity (not shown), even up to 16 days, and some individual cells were seen to be in division, confirming observations that claim the capacity of *Ca. Glomeribacter gigasporarum* to survive and possibly to duplicate out of its host for up to 4 weeks (Jargeat et al. 2004). Notwithstanding these sporadic observations, the general trend was a decrease in the total number of bacteria during the observations from the first to the last analyzed timepoint, confirming the unculturable status of *Ca. Glomeribacter gigasporarum* (Jargeat et al. 2004).

DISCUSSION

Gigaspora margarita BEG 34 and *Ca. Glomeribacter gigasporarum* are, at present, the only AM fungus–endobacterium couple that have been clearly identified from the several AM fungi that host prokaryotes (Lumini et al. 2006). Although the biological significance of such a close association is not yet clear, recent data would seem to suggest the possible role of *Ca. Glomeribacter gigasporarum* on the morphology and general fitness of the fungus, at least during its presymbiotic stage (Lumini et al. 2007). This stage corresponds to a phase of intensive exchange of signal molecules between the plant and fungus prior to root colonization. The plant releases root-branching factors, identified as strigolactones, and responds to fungus-derived signals by activating specific genes and reorganizing epidermal cells (Genre and Bonfante 2007; Paszkowski 2006). Here, we have demonstrated that this complex molecular dialogue is also directly or indirectly perceived by the fungal endobacterium *Ca. Glomeribacter gigasporarum* that modulates its transcription and cell proliferation activity according to the fungal life cycle. As a consequence, when the fungus responds to the presence of the plant, as well as to synthetic analogues of the plant exudate strigolactone (Bessener et al. 2006, 2008), the expression level of *ftsZ*, a bacterial division marker, is enhanced and the percentage of bacteria in division, which results from the counting of the dividing cells, is increased. The fact that the endobacteria that are isolated from their fungal host do not appear to be stimulated by the presence of the strigolactone demonstrates that the molecule alone is not sufficient to stimulate free-living capacity in *Ca. Glomeribacter gigasporarum*. On the other hand, it could also be suggested that these events do not result from the direct bacterial perception of plant signals but, instead, are most likely mediated by the fungus.

The *ftsZ* gene is a good marker of bacterial division and phylogenesis.

The FtsZ protein is responsible for the formation of the cytokinetic ring at the midcell, thus giving the first visible evidence of prokaryotic septation (Vincente and Löwe 2003). Recent findings have demonstrated that FtsZ is able to activate constriction events in artificial systems such as liposomes (Osawa et al. 2008).

FtsZ is found in prokaryotes, chloroplasts, and the mitochondria of primitive eukaryotes such as protists. It is absent in the mitochondria of higher eukaryotes such as fungi, plants, and animals, where it has been replaced by dynamins (Margolin 2005; Osteryoung and Nunari 2003).

In the context of the very limited information available about the gene content in *Ca. Glomeribacter gigasporarum*, one of the first steps of our study was to identify whether the endosymbiont possesses the essential *ftsZ* gene. The degenerated primer strategy was successfully applied to obtain a sequence of more than 1,000 bp of the gene and the initial gene fragment was confirmed and completed to a total length of

1,164 bp by full-length sequencing of the Fosmid_10 from the genomic library of the host fungus. The presence of the *ftsZ* gene inside the *Ca. Glomeribacter gigasporarum* genome, with more than 80% homology with the *Burkholderia* group, demonstrates that this endobacterium has likely adopted the same division strategy as the majority of prokaryotes (Osteryoung and Nunari 2003; Vincente and Lowe 2003). The *ftsZ* gene has already been found in endosymbionts such as *Buchnera* spp. (Baumann and Bauman 1998), *Wolbachia* spp. (Von der Schulenburg et al. 2000), and *Sodalis glossinidius* (Akman et al. 2001). Instead, it has not been found in several species of intracellular microorganisms with a completely sequenced genome; for example, 2 species of mycoplasmas (*Ureaplasma parvum* and *Mycoplasma mobile*), *Prostecobacter dejongei*, 10 species of *Chlamydia*, and 5 species of archaea (Vishnyakov and Borchsenius 2007). Considering the high number of open reading frames, which encode proteins with unknown functions in *U. parvum* and *M. mobile* (Vishnyakov and Borchsenius 2007), it is highly likely that alternative cell division mechanisms exist in these microorganisms.

The phylogenetic tree constructed on the basis of the *ftsZ* homology clearly shows the distinct distribution of *Ca. Glomeribacter gigasporarum* among other β -proteobacteria. This distribution is similar to the one obtained by 16S rRNA (Lumini et al. 2006) and suggests the *ftsZ* gene as a strong candidate for further phylogenetical studies, because it was successfully used by Von der Schulenburg and associates (2000) in the case of *Wolbachia* spp.

The potential of *ftsZ* as a cell division marker has already been demonstrated in studies on free-living microbes; in other words, in the relationships between midcell Z-ring assembly and the round of chromosome replication in *Bacillus subtilis* outgrowing spores (Harry 2001) and between *ftsZ* gene expression, DNA replication, and the increase in cell numbers in *Prochlorococcus* marine populations (Hotlendorff et al. 2002). In spite of the constraints given by the status of *Ca. Glomeribacter gigasporarum*, which is an obligate endobacterium living inside an obligate endomycorrhizal fungus, we detected a clear correlation between the gene transcript activation and bacterial division events that were observed with the confocal microscope. Interestingly, the morphological observations nicely mirrored the real-time RT-PCR results, showing the lowest percentage of bacteria in division in the quiescent spores, followed by an increase in the 10-day germinated spores and with the highest increase in the 10-day germinated spores in the presence of GR24. The result consistently suggests an active role of the *ftsZ* gene in the modulation of the endobacterial population as a response to the fungal host metabolism and its capacity to respond to the presence of the plant analogue bioactive molecules.

The expression of bacterial genes from both fungal structures and plant tissues.

The second important outcome of our experiments was the detection of bacterial transcripts not only inside the fungal spores or germinating mycelia but also from mycorrhizal plant tissues. As far as the authors know, this is the first time that a differential expression for a specific gene of a fungal endobacterium has been followed along the different steps of its host life cycle.

The real-time RT-PCR investigation of the *ftsZ* gene at the transcript level gave the first indications of the gene functionality. Using the quiescent spores as the reference sample, we observed a slight increase in expression in the 10-day germinated spores and a huge peak (more than 164-fold) in the extraradical mycelium, followed by a decrease in the intraradical mycelium. The expression data nicely fit the bacterial ge-

nome copy number quantification assay, which demonstrates a higher bacterial/fungal nuclei ratio in the extraradical mycelium (Salvioli et al. 2008). The trend of the *ftsZ* expression showing the highest peak when the fungus was in the vicinity of plant roots encouraged us to test the *ftsZ* gene expression as a response to the presence of GR7, a strigolactone with a stimulating effect on AM fungi growth (Besserer et al. 2006, 2008). The bioactive molecule caused activation of the bacterial gene in the 10-day germinated spores compared with the control. The experiment confirmed our hypothesis concerning the correlation between bacterial divisions, fungal life cycle, and plant presence but failed to demonstrate whether the bacteria directly perceive the plant stimuli. However, the way in which the mitochondria of *Gigaspora rosea*, which does not contain endobacteria, perceive the plant bioactive molecules is still unknown (Besserer et al. 2008).

Strigolactones may influence both mitochondria and endobacteria in AM fungi.

Strigolactones have a stimulating action on AM fungal mitochondria, which increase in number when the germinating spore responds to plant signals, probably due to the high fungal energetic requirements for plant colonization (Besserer et al. 2006, 2008; Tamasloukht et al. 2003). However, once the mycorrhization is established, the host plant decreases its strigolactone production (Bouwmeester et al. 2007). In our system, the strigolactones may play a role in the activation of the transcriptional activity and the cell division of the endobacteria living in AM fungi prior to fungal colonization, as shown by expression and morphological analysis, in a similar way to fungal nuclei and mitochondria. The increased activity of the bacteria in the symbiotic phase of the fungus (extraradical and intraradical mycelium) could instead be attributed to different molecular mechanisms, such as an increased nutrient source occurring in the fungus and available for '*Candidatus Glomeribacter gigasporarum*.'

In conclusion, we have demonstrated that the bacterial *ftsZ* gene is preferentially expressed in the symbiotic mycelia and in particular extraradical hyphae. Because cell division is an energy-requiring process, it seems reasonable to assume that *Ca. Glomeribacter gigasporarum* relies on its fungal host in the phases in which sugars flow from the plant toward the fungus (Bago et al. 1999).

The complex level of tripartite organism communication we have detected seems, so far, to be unique in the symbiotic world; however, because there is very little information available at a genomic level about both fungus and bacterial partners, it is still difficult to explain the molecular mechanisms that are behind such complex networks.

MATERIALS AND METHODS

Biological material preparation.

Fungal spores. The *Gigaspora margarita* (BEG 34) wild-type spores were purchased from Biorize (Dijon, France), whereas the spores devoid of endobacteria were derived from a cured line obtained from *G. margarita* BEG 34 strain (Lumini et al. 2007); both spore populations were collected and surface sterilized with 3% (wt/vol) chloramine T and 0.03% (wt/vol) streptomycin, plus one round of sonication (Lanfranco et al. 2005). Germination was induced by incubating the spores in sterile water at 28°C for 10 days.

Plant material and extraradical mycelium. The mycorrhizal roots and extraradical mycelium were obtained from *L. japonicus* (Regel) Larsen wild-type culture in a 'sandwich' system, as described by Lanfranco and associates (2005). The plants were kept in a growth chamber; the roots were harvested after

1 month. After inspection with the stereomicroscope, the extraradical mycelium was collected from the surface of the mycorrhizal roots, whereas the mycorrhizal roots of *L. japonicus* were cut into 1-cm-long segments. The extraradical mycelium and the mycorrhizal roots were frozen in liquid nitrogen and stored at -80°C for further RNA extractions.

The plant material and the extraradical mycelium were produced in three biological repetitions. Samples were collected from approximately 50 sandwiches with *L. japonicus* per repetition, while 10 sandwiches per repetition with nonmycorrhizal plants were used as controls.

GR7 strigolactone treatment. The strigolactone GR7 was generously provided by G. Bédard (Université Paul Sabatier, Castanet-Tolosan, France) and dissolved in 50:50 methanol/water up to a concentration 10^{-4} M. Samples were prepared in three biological repetitions. Approximately 100 spores of *G. margarita* BEG 34 per repetition were sterilized and germinated for 10 days in M medium (Bédard and Fortin 1988) and treated with 10^{-7} M GR7 (Besserer et al. 2006) or 0.1% methanol: water. After the germination period, the spores were frozen in liquid nitrogen and stored at -80°C for subsequent RNA extraction.

GR24 strigolactone treatment. The highly efficient strigolactone GR24 (Besserer et al. 2006), kindly provided by P. Karlowsky (Göttingen University, Germany), was diluted in water and 1% acetone to obtain a 10^{-6} M stock solution. The *G. margarita* BEG 34 spores were germinated for 10 days in M medium in Nunclon Δ Surface microwell plates (Nunc, Roskilde, Denmark) and treated with GR24 at 10^{-8} M final concentration or with acetone 0.001% for the controls. The treated and control spores were germinated in different plates to exclude any volatile effects of GR24. The prepared spores were used for the estimation of bacteria in division with the confocal microscope.

Putative *ftsZ* PCR amplifications on genomic DNA.

Genomic DNA was extracted from the spores using the cetyltrimethylammonium bromide method (Henrion et al. 1994). The degenerated primers FNF3 (5'-ACGGCACCATCA TCAAGGTGGTC-3') and FNR2 (5'-TGCGCCACACSGCCG GSGTATC-3') were designed for the amplification of the potential *ftsZ* gene of *Ca. Glomeribacter gigasporarum* on the basis of protein and nucleotide alignment of the *ftsZ* present in *Burkholderia pseudomallei* K96243, *B. fungorum* LB400, *B. cepacia* R18194, *B. cepacia* R1808, *B. mallei* ATCC23344, *Ralstonia solanacearum* GMI, *R. eutropha* JMP, and *R. metalidurans* CH. The alignment was performed with the Multalin software.

PCR reactions were carried out in a final volume of 25 μl containing 2 μl of 10 $\times$ PCR buffer for HotStart Taq (Qiagen, Hilden, Germany), 200 μM dNTPs, 1 μM each primer, 1.5 units of HotStart Taq DNA polymerase (Qiagen), and 2 μl of DNA. The PCR products were separated on 1.2 to 2% (wt/vol) agarose gels and visualized by ethidium bromide staining. The negative controls for all the PCR experiments consisted of reaction mixtures from which template DNA was omitted and of reaction mixtures with template DNA from the cured variant of *Gigaspora margarita* BEG34. The PCR program was as follows: 95°C for 15 min (1 cycle); 94°C for 1 min, 58°C for 1 min 30 s, and 72°C for 2 min (40 cycles); and 72°C for 7 min (1 cycle).

The amplified PCR product was purified with the QIAquick kit (Qiagen) and directly cloned into the pGEM-T vector (Promega Corp., Madison, WI, U.S.A.). XL-2 Blue ultracompetent cells (Stratagene, La Jolla, CA, U.S.A.) were transformed and plated onto a selective medium according to the manufacturer's instructions. The plasmid DNAs were prepared with the

Qiagen plasmid mini kit (Qiagen). DNA sequencing was performed by GeneLab (Rome) using T7 and Sp6 primers.

The obtained sequences were assembled with the Sequencer 4.1.4 software (Gene Codes Corporation, Ann Arbor, MI, U.S.A.) and the ambiguous bases were manually corrected.

Sequencing of the complete *ftsZ* gene from a metagenomic library.

A fosmid metagenomic library of *G. margarita* BEG 34 and *Ca. Glomeribacter gigasporarum* was obtained from 8,000 spores (40 μg of DNA) of *Gigaspora margarita* BEG 34 using CopyControl pCC1FOS and pCC2FOS vectors (Epicentre Biotechnologies, Madison, WI, U.S.A.) (S. Ghignone, *personal communication*).

Fosmid_10 of bacterial origin was fully sequenced and analyzed (data not shown). Specific primers Fos10TF1 (5'-TTCACGCGACGCAAAAGGAC-3') and Fos10TR1 (5'-CAGTCGCCATGAACGATTTC-3') were designed to check the bacterial origin of the fosmid insert by PCR. PCR reaction was performed in a total volume of 25 μl that contained 2 μl of 10 $\times$ REDTaq Reaction Buffer (100 mM Tris-HCl, pH 8.3; 500 mM KCl; 11 mM MgCl_2 ; and 0.1% gelatine), 100 μM dNTPs, 0.5 μM each primer, and 0.2 μl of RedTaqDNA polymerase (Qiagen). The PCR program was 95°C for 5 min (1 cycle); 94°C for 1 min, 57°C for 1 min, and 72°C for 1 min (40 cycles); and 72°C for 7 min.

Real-time RT-PCR analysis.

RNA extractions were performed using quiescent, 10-day-germinated spores, as well as the extraradical mycelium and mycorrhizal root pieces which were collected using forceps under a stereomicroscope. All the biological material was immediately frozen in liquid nitrogen. The RNA was extracted using the SV Total RNA isolation system kit (Promega Corp.) starting from approximately 300 spores per biological replicate for each developmental stage, 500 mg of mycorrhizal root pieces, and the extraradical mycelium collected from the same mycorrhizal roots (Lanfranco et al. 2005). The RNA was precipitated by adding an equal volume of 6 M LiCl centrifuged at $14,000 \times g$ for 30 min and resuspended in 20 ml of diethyl pyrocarbonate-treated sterile water. All the RNA samples were routinely checked for DNA contamination through RT-PCR analyses conducted with a one-step RT-PCR kit (Qiagen). The reactions were carried out in a final volume of 25 μl containing 2 μl of 5 $\times$ buffer, 160 μM dNTPs, and 0.32 μM each bacterial GlomGIGF/GlomGIGR (Bianciotto et al. 2004) or fungal universal primers AM1/NS31 (Helgason et al. 1998), 0.5 μl of One-Step RT-PCR enzyme mix (Qiagen), and 0.5 μl of total RNA. The samples were incubated for 30 min at 50°C , followed by 15 min of incubation at 95°C . The samples corresponding to RT minus were kept on ice instead at 50°C . Amplification reactions (92°C for 45 s, 60°C for 45 s, and 72°C for 45 s) were run for 35 cycles.

To obtain cDNAs from the different samples, RT reactions were performed in a final volume of 20 μl , containing 4 μl of 5 $\times$ Buffer RT, 500 μM dNTPs, 1 μM Oligo-dT primer, 1 μl of Superscript II Reverse Transcriptase (Invitrogen), 10 μl of template, and RNase-free water up to the final volume. The samples were incubated at 25°C for 10 min and 42°C for 50 min.

At least two separate RT reactions were pooled for each RNA preparation to minimize the potentially differential efficiency of the enzyme. cDNAs, prior to real-time PCR experiments, were tested in conventional PCR experiments with *Ca. Glomeribacter gigasporarum* 23S rRNA primers (GlomGIGF/GlomGIGR) or fungal universal primers (AM1/NS31) as described above.

Real-time reactions were carried out in a final volume of 20 µl containing 12.5 µl of iQ SYBR Green Supermix 2X (100 mM KCl; 40 mM Tris-HCl, pH 8.4; 0.4 mM dNTPs; iTaq DNA polymerase at 50 units/ml; 6 mM MgCl₂; 20 nM SYBR Green I; and 20 nM fluorescein) (Bio-Rad Laboratories, Hercules, CA, U.S.A.), 0.45 µM each oligonucleotide GlomGIGf (Bianciotto et al. 2004) and GIGrA (Lumini et al. 2007) for amplification of 23S rRNA, and 0.25 µM FtsZF1 (5'-ATTCTGACCGTTGGCGTGGTC-3') and FtsZR1 (5'-CAGC GAATCCACATGCTCCTC-3') primers, designed to amplify the *ftsZ* gene fragment.

The appropriate amount of cDNAs was added for each reaction and the following program was run: 95°C for 3 min (1 cycle) and 95°C for 15 s and 60°C for 30 s (55 cycles) in an iCycler iQTM Real-Time PCR detection system (Bio-Rad Laboratories). The data were analyzed with the iCycler software. Single amplicons (106 and 108 bp long for the 23S rRNA and *ftsZ*, respectively) were produced by both primer sets. The *ftsZ* sequence amplified with the primers for real-time RT-PCR was tBlastx against the plants and invertebrates divisions and against expressed sequence tag databases. As expected, it showed similarity with corresponding FtsZ sequences from chloroplasts and mitochondria of primitive eukaryotes with a maximum *E* value of 6e-08. No similarity with mitochondrial proteins of completely sequenced fungi such as *Neurospora*, *Aspergillus*, and *Laccaria* spp. was detected because it is widely known that, in fungi, the FtsZ role was replaced by dynamins. However, a closer look to the databases present in the National Center for Biotechnology Information of the mentioned fungi did not reveal any FtsZ sequence. For the sequence amplified with the primers for 23S rRNA, no strong similarity (maximum *E* value = 0.23) was detected when using tBlastx against already sequenced fungi. A melting curve (55 to 95°C with a heating rate of 0.5°C per 10 s and continuous fluorescence measurement) was generated at the end of each run to ensure correct identity of the amplified product (Ririe et al. 1997). Standard curves were obtained using recombinant plasmids containing a portion of the *Ca. Glomeribacter gigasporarum* 23S ribosomal gene (Salvioli et al. 2008) or the *ftsZ* sequence.

RNA extractions were performed on at least three independent biological samples. Real-time PCR reactions were carried out in triplicate and only comparative threshold cycle (Ct) values, leading to a Ct mean with a standard deviation below 0.2, were considered. The Ct method was used to calculate the relative *ftsZ* expression levels with the 23S rRNA as a reference (Rasmussen 2001).

Quantification of the dividing bacteria from the spores.

Spores of *Gigaspora margarita* BEG 34 were stained with Syto BC (Invitrogen) (dilution 1:1000) and crushed while mounting the cover slip for the estimation of the dividing bacteria. Lumini and associates (2007) describe the use of Syto BC stain to differentiate between the spores of *G. margarita* BEG 34 containing endobacteria and the variant 'cured' that did not contain endocellular bacteria; thus, we were confident in the specific discrimination of the endobacteria of other fungal components such as nuclei or mitochondria. The spores were analyzed in three conditions: quiescent, 10-day germinated, and 10-day germinated after the GR24 treatment (discussed above). Four biological replicates were prepared for each of the three analyzed conditions. Bacteria were observed after 5 min of incubation in the stain with a Leica TCS SP2 confocal microscope with a ×40 water-immersion objective using the 488-nm line of an argon laser for excitation and an emission filter window set at 500 to 540 nm. At least three to six images (375/375 µm) were acquired for each slide and over

1,500 bacteria were manually counted for each spore. The ratio among the number of bacteria showing clear features of recent cell division and the total count was calculated as a percentage.

Quantification of the bacterial number after their isolation from the fungus.

Spores of *G. margarita* BEG 34 (in pools of 100) were used for bacterial isolation (I.-A. Anca, *personal communication*). The isolated cells were resuspended in 500 µl of tryptone yeast, were vortexed, and were split equally into two 1.5-ml tubes. GR24 10⁻⁸ M was added to one of them whereas the second aliquot was treated with 0.001% acetone and used as a control. The tubes were then wrapped in an aluminum foil and incubated at 28°C without shaking. Aliquots of 10 µl of cell suspension were taken for each of the six selected timepoints (0 h, 1 h, 3.30 h, 1 day, 9 days, and 16 days) and stained with 1 µl of Syto BC (Invitrogen) (dilution 1:1000) for the confocal microscopy observations, as described above. All the samples were prepared in two biological repetitions. Bacterial counting was made on a volume of 375 by 375 by 51 µm (modified from Bianciotto et al. 2004), with three repetitions per slide and per timepoint in the treated and control samples. Then, 17 serial optical sections were acquired at 3-µm intervals for a total thickness of 51 µm. The bacteria were then manually counted on each optical section.

Statistical analysis.

The statistic analysis was performed using two-way analysis of variance with Tukey as the post-hoc or Kruskal-Wallis test of variance.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

Multalin software: npsa-pbil
 DNA databank of Japan: blast.ddbj.nig.ac.jp