RiPEIP1, a gene from the arbuscular mycorrhizal fungus Rhizophagus irregularis, is preferentially expressed in planta and may be involved in root colonization

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Mycorrhiza

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Corresponding Author:	Valentina Fiorilli, PhD University of Turin Torino, ITALY
Corresponding Author Secondary Information:	
Corresponding Author's Institution:	University of Turin
Corresponding Author's Secondary Institution:	
First Author:	Valentina Fiorilli, PhD
First Author Secondary Information:	
Order of Authors:	Valentina Fiorilli, PhD
	Simone Belmondo
	Hassine Radhouane Khouja
	Simona Abbà, PhD
	Antonella Faccio
	Stefania Daghino, PhD
	Luisa Lanfranco
Order of Authors Secondary Information:	

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Abstract:

Transcriptomics and genomics data recently obtained from the arbuscular mycorrhizal (AM) fungus Rhizophagus irregularis have offered new opportunities to decipher the contribution of the fungal partner to the establishment of the symbiotic association. The large number of genes which do not show similarity to known proteins, witnesses the uniqueness of this group of plant-associated fungi. In this work we characterize a gene that was called RiPEIP1 (Preferentially Expressed In Planta). Its expression is strongly induced in the intraradical phase, including arbuscules, and follows the expression profile of the Medicago truncatula phosphate transporter MtPT4, a molecular marker of a functional symbiosis. Indeed, mtpt4 mutant plants, which exhibit low mycorrhizal colonization and an accelerated arbuscule turnover, also show a reduced RiPEIP1 mRNA abundance. To further characterize RiPEIP1, in the absence of genetic transformation protocols for AM fungi, we took advantage of two different fungal heterologous systems. When expressed as a GFP fusion in yeast cells, RiPEIP1 localizes in the endomembrane system, in particular to the endoplasmic reticulum, which is consistent with the in silico prediction of four transmembrane domains. We then generated RiPEIP1-expressing strains of the fungus Oidiodendron maius, ericoid

	endomycorrhizal fungus for which transformation protocols are available. Roots of Vaccinium myrtillus colonized by RiPEIP1-expressing transgenic strains showed a higher mycorrhization level compared to roots colonized by the O. maius wild type strain, suggesting that RiPEIP1 may regulate the root colonization process.

Dear Editor,

Please here enclosed you will find the revised version of the manuscript "*RiPEIP1,* a gene from the arbuscular mycorrhizal fungus *Rhizophagus irregularis,* is preferentially expressed *in planta* and may be involved in root colonization". This new version has taken in account the editor and referee's suggestions and requests (text highlighted in yellow). All the changes have been listed and commented in the enclosed rebuttal letter.

We really hope that this new version may satisfy the high standards of Mycorrhiza.

With my best wishes

Valentina Fiorilli

Editor's comments:

Please note that _Mycorrhiza_ consistently organizes articles as: Introduction, Materials and Methods, Results, and Discussion. (Methods are not placed at the end as in your manuscript.) Please reorganize your manuscript revision.

Reply: the manuscript has been organized according to the Mycorrhiza's rules.

Lines 98-100: Instead of saying "we identified" which made me confused as to whether you are describing previous work or the current work, could you say something like "four genes were identified?" Although I recognize that you were involved in the 2012 work, the "we" might be misinterpreted as signaling "this study."

Reply: the text has been changed.

Line 106: I share the reviewers' concerns about calling this an "endomycorrhizal" fungus which imprecisely implies some relationship among ericoid, orchid, and glomeromycotan mycorrhizas. I strongly urge you to replace "endomycorrhizal" with "ericoid mycorrhizal." (Note that Reviewer #2 incorrectly thought this was an orchid mycorrhiza)

Reply: the text has been changed: we have used ericoid mycorrhizal fungus.

Lines 278-279: I suggest rewording this as: "…as it shares with AM fungi the capability to intracellularly colonize root cells." That is precisely what you intend by calling both "endomycorrhizal."

Reply: the text has been changed according to the suggestion (Line 446).

Line 393: "Table 1" should be "Table S1."

Reply: the text has been corrected.

Lines 667 & 678: "Single" should perhaps be "individual."

Reply: the text has been corrected (line 683)

Line 669: Three asterisks often are used to represent P<0.001 (not "0.01" as you have done).

Reply: we have checked again the statistical data: in this case the p-value was 0.0002228, so three asterisks are correct. The text has been corrected (line 685).

Lines 679-680 & 700-701: If you simultaneously compared all four times, then you likely used a post-hoc test procedure after ANOVA. That procedure should be indicated (e.g., Tukey's HSD, LSD, etc.).

Reply: the text has been corrected in the corresponding Figure legends.

Lines 687-690: Panel d could be omitted because the correlation already is apparent from Panels b and c. It seems inappropriate to use regression for these data in two distinct clusters because "two points determine a line;" a simple correlation coefficient could be stated in the text.
Reply: Panel d has been deleted from Fig. 4. The correlation coefficient (R^2: 0.8433) has been added in the text (line 369).

Lines 690-691: Some panels have only two asterisks and others have three; the different probabilities that they represent should be indicated explicitly.
Reply: the text has been modified (line 704).

Lines 692-695: Perhaps the right-hand lane of Panel a should be described.
Reply: the sample corresponds to a negative control; this information has been added in the Figure legend (line 707).

Lines 704-706: Panel c is entirely text, and so it should be a TABLE. "Plant" in Line 705 should be "plants."
Reply: Panel c has been transformed in Table 1. The word "plant" has been corrected (line 747).

Line 715: "Pars" should be "parts."
Reply: the word has been corrected (line 726).

Lines 721-725: It would be helpful to explain that the "A" lines are WT and the "B" are transgenic.
Reply: In both Panels (a and b) only the last lane on the right corresponds to the wild type (wt) sample. WT has been added on both pictures to make it clear.

Reviewer #1: Fiorilli et al. have characterized a presumably an orphan gene that is preferentially expressed in planta in the AM fungus R. irregularis. This gene was termed as RiPEIP1, whose role remains unclear but the authors showed that it may be involved in root colonization. The manuscript is well written, clear and concise. The experimental setup is appropriate and I found the use of the ericoïd mycorrhizal fungus O. maius as an alternative system for heterologous expression to characterize AM fungal gene very interesting and novel. However, my major criticism concerns the expression of RiPEIP1 in M. truncatula mycorrhizal roots. The authors collected arbuscule-colonized cortical cells and non-colonized cortical cells from paraffin root sections. It would be interesting to collect some vesicles and/or intraradical spores as well as intraradical hyphae to see whether RiPEIP1 is also expressed in these fungal parts or restricted to arbuscules. I know that this is a challenging task but it will strengthen the manuscript.

Reply: Although we do have a long experience in the analysis of laser microdissected cells from mycorrhizal samples, we never tried to collect vesicles or intraradical spores due to the difficulty of sampling (and, to the best of our knowledge, nobody has published data on such material). The non colonized cells from mycorrhizal roots (MNM) is the sample that we consider to contain intercellular hyphae; even if at a morphological inspection hyphae are not evident in the LMD sections, this sample shows fungal transcripts (i.e. RiTef) but never transcripts of MtPT4 which is a marker of arbusculated cells.

Minor comments
Page 2: L29: Please add ericoid endomycorrhizal fungus... to avoid misleading for readers.
Reply: the text has been corrected.

Page 3, L42-45: Please site the appropriate references.
Reply: the reference Schüßler et al., 2001 has been added.

Page 3, L64-65: No sexual cycle has ever been described although meiosis-related genes were found in the genome. The appropriate reference is: Halary et al. (2011). Conserved meiotic machinery in Glomus spp., a putatively ancient asexual fungal lineage. Genome Biology and Evolution 3: 950-958.
However, Halary et al. (2013) [PLoS One 8(11) e80729] have reported a complete Putative Sex Pheromone-Sensing Pathway in AM Fungi, meaning that AM fungi are able to undergo sex. An analogy can be done with Aspergillus and Candida albicans.
Reply: the text has been modified and the references have been added.

Page 6: L124-127: Why Lin et al. 2014 sequence was annotated with four additional amino acids at the N-terminus? Please discuss.
Reply: We initially characterized the gene sequence through RACE experiments and in several independent 5'-RACE-PCR assays we could never report the presence of these four amino acids at the N terminus. The discrepancy may be based on the fact that probably Lin et al. performed an automatic annotation of the genome sequence. Since this is our interpretation, we did not add further comments in the text.

Reviewer #2: Report on the Mycorrhiza Manuscript: MCOR-D-16-00008

"RiPEIP1, an orphan gene from the arbuscular mycorrhizal fungus Rhizophagus irregularis, is preferentially expressed in planta and may be involved in root colonization" by Fiorilli and collaborators.

This is a potentially very interesting manuscript describing the functional analysis of a novel AM fungal gene from Rhizophagus irregularis. The authors identified the gene on the basis of its induction in planta during symbiosis and carried out a series of experiments to determine its role in symbiosis. I have a series of comments and suggested controls that might help to shape the manuscript at places where, in my opinion, the statements or conclusions from the authors are not fully justified.

ABSTRACT AND INTRODUCTION
1. I am not fully happy with the use of the word orphan gene. I think the authors should precise it better. To my knowledge, an orphan gene is such without homologues in other lineages, and therefore, the sentence in line 18 of the abstract "…genes identified as orphan, and often lineage-specific,…" is confusing. I would rather say PEIP1 is probably an arbuscular mycorrhiza specific gene, which is what the authors intend to say, not present in any other organism, besides R. irregularis and Gigaspora sp.
Reply: since the definition of "orphan gene" may be ambiguous we decided not to use it. The text has been changed accordingly; in most cases we used the concept of "genes that do not show similarity to known proteins".

2. I find misleading the statement that Oiodendron maius is the only endomycorrhizal fungus for which an established transformation protocol exist. First of all, there are other endomycorrhizal fungi, ie. Piriformospora indica, for which a transformation protocols exist. But in addition, it is only in page 9 at the end of the result section where the authors for the first time indicate which type of an endomycorrhizal symbiont Oiodendrum is: an orchid mycorrhiza! I fully agree with the authors that morphologically seen, this infection is more similar to the AM fungi, but the fungi itself do not have to do with each other much, nor the plant is a host for AM fungi, as the authors in the discussion state. I think it would be fair to state from the beginning which fungus Oiodendrum is and why it might be a good heterologous host for AM fungi.
Reply: We have introduced the detail of the ericoid mycorrhizal fungus already in the abstract and in the Introduction section (line 116-117).
We would like only to highlight that *Piriformospora indica*, although it exerts several beneficial effects on the host plant, is not considered a mycorrhizal fungus but a plant-growth promoting endophyte (Zuccaro et al. 2011. Endophytic life strategies decoded by genome and transcriptome analyses of the mutualistic root symbiont *Piriformospora indica*. PLoS Pathog. 2011 Oct;7(10):e1002290. doi: 10.1371/journal.ppat.1002290).

3. Page 3, line 67, I think this sentence reads not very precise. The AM fungus or the fungal cell is homo- or heterokaryotic, but the nuclei are not. The whole paragraph needs rephrasing.
Reply: we agree with the comment. We have changed the sentence into "…. has been questioned whether AM fungi are in a heterokaryotic (Hijri and Sanders 2005; Ehinger et al. 2012) or in a homokaryotic condition.."

RESULTS
4. It is not clear, not in the text (page 5, line 118) nor in M&M, not in the figure legend how this experiment was carried out: How old were the plants? How old was the ERM? How was it collected, from liquid? From solid medium? How was the level of root colonization?
Reply: We have added more details in the Results section "… the expression profile of Glomus_c13083 was monitored by quantitative RT-PCR (qRT-PCR) in intraradical (IRM) and extraradical (ERM) mycelium obtained from *M. truncatula* mycorrhizal roots 60 days post-inoculation (dpi) in a sandwich system (lines 279-282).
We also added the percentage of AM colonization: "The mycorrhizal status of *M. truncatula* roots was confirmed by the calculation of the total root length colonization (62.8%±2.7)."(Lines 284-285)

5. The expression profiles from figure 1 and 3, are somehow redundant. This information could be collated in one figure and the manuscript will gain in clarity.
In this sense, it is not clear why the expression level in spores is not shown. The authors say they were not detectable but actually in Figure 4, much lower expression levels for PEIP are detected (10 times lower, see later). It would be good to know if there is a basal level of expression in spores or in germinated spores.
Reply: on our opinion the two experiments are rather different: Fig. 1 points to the comparison of ERM and IRM while Fig. 3 presents data of a time course of AM colonization; we would therefore prefer to keep the two figures separated.
Dealing with the RiPEIP1 expression in spores, based on a semi-quantitative RT-PCR assays, Tisserant et al. 2012 (Table S5) (we added the reference in line 286) already showed that the gene is not expressed in spores. Also when we analysed the transcript levels by the more sensitive quantitative RT-PCR assay we could only register very high Ct values (40-41).

6. line 174, the authors write the expression levels in ERM are negligible… I think this is not correct. They show ca. 0.01, which is the level of expression from plants at 7 and 14 days after colonization in Figure 3.
Reply: We agree with this comment: it is true that at 7 and 14 dpi the intraradical colonization is very low; the RiPEIP1 mRNAs we detect thus clearly derive from the ERM. These expression values (referred to the RiTef) are in line with those obtained in the experiment shown in Fig. 1: in the ERM RiPEIP1 transcript levels are always lower than those of the IRM.
We have modified the text into "Since *RiPEIP1* was shown by qRT-PCR to be expressed at low levels in the ERM, gene expression was evaluated in whole mycorrhizal roots…". (Lines 340-341)

My main criticism is that I am not convinced about the correlation between arbuscules and expression of PEIP. Here below some of the reasons (7,8,9)

7. The statement in line 178 is not totally correct, because while the expression of PT4 is still increasing, the PEIP expression is decreasing, even if it is not statistically significant. Furthermore, PEIP is expressed before PT4, indicating that it might be expressed if not in intercellular hyphae, at earlier stages of arbuscule formation where PT4 is not yet expressed, but I think it would be difficult to have such a precise time-point to detect this.
Reply: We agree with the comment about the 60 dpi time point: there is a trend but this is actually not supported by the statistical analysis. It is clear that there is a limitation coming from the experimental system, where a rather high variability in the colonization process among the different plants occurs; this does not allow such a precise level of detail.

8. Then also results in Figure 3c: While there are two samples for arbuscules, the sample of MNM is single, and no other gene besides RiTEF has been analysed. At least another sample would help to clarify this, but in addition, expression of a mycorrhiza-induced plant gene expressed not only in arbuscules (there are plenty of them, maybe some induced in adjacent cells), as well as of a fungal gene induced in colonized roots but not only in arbuscules (maybe MST2) should be measured.
Reply: This MNM cell population is actually the most difficult to collect. It is important to stress that one biological replicate corresponds to a minimum of 1500-2000 collected cells that were derived from independent LMD sections and mycorrhizal roots; this sample can therefore be intrinsically considered a pool of biological replicates. The RiTef gene was selected on purpose as a defined marker for the presence of the fungus in the MNM sample (possibly as intercellular hyphae as the fungus is not morphologically evident in the LMD sections). We do not think that the analysis of the MST2 gene will provide more information.
On the plant side, we looked in the literature for works where gene expression in mycorrhizal roots was analysed by the laser microdissection technique. In most cases only arbusculated cells (Gomez et al., 2009 BMC Plant biology; Tisserant et al., 2012 New Phytologist) were considered. When also MNM cells were collected the analysed genes were specific of arbusculated cells or they were expressed in both cell types (Hogekamp et al., 2011 Plant Physiologist; Gaude et al., 2015 BMC Plant biology). As far as we know, there are not very well described plant markers for MNM that may be used to further dissect the AM colonization steps.

9. The level of expression of PEIP1 in Figure 4 for wild type plants is extremely low as compared with the other experiments and not really explainable looking at the pictures depicting colonization.
Reply: The explanation is simple: in this case, to provide a more correct comparison of the colonization level in wt and mutant plants, we decided to normalize the RiPEIP1 expression level to the MtTef plant housekeeping gene, while in the other Figures the RiPEIP1 expression values were given referring to the fungal RiTef housekeeping gene.

Considering overall gene expression data, including those from the mtpt4 mutants, we think that a kind of correlation between RiPEIP1 expression and arbuscule formation does occur. However, we are aware of limits of the experimental techniques and the biological system; for this reason we modified the text by softening the idea of a correlation; in particular, in the Discussion section at line 438 we have modified the sentence “Overall the data suggest (instead of support) a relationship between *RiPEIP1* and arbuscule differentiation.”

Finally, it would be interesting if the authors would speculate on the possible cellular function that PEIP could have and on the experiments that, as they say at the end, will be required to elucidate the function of this gene in the AM symbiosis.
Reply: at this point of the investigations it is rather difficult to speculate about possible cellular functions.
As we hypothesized that RiPEIP1 could be delivered to the plant cell, RiPEIP1-expressing transgenic lines of rice have been recently obtained as a tool to look for altered plants phenotypes including susceptibility to AM colonization and to dissect the metabolic pathway affected by RiPEIP1 and possibly its mechanisms of action. A sentence has been included at the and of the Discussion section (lines 454-457).

RiPEIP1, a gene from the arbuscular mycorrhizal fungus *Rhizophagus irregularis*, is preferentially expressed *in planta* and may be involved in root colonization

Valentina Fiorilli[1], Simone Belmondo[1], Hassine Radhouane Khouja[1], Simona Abbà[2], Antonella Faccio[2], Stefania Daghino[1] and Luisa Lanfranco[1]

[1]Department of Life Science and Systems Biology, University of Torino, via Accademia Albertina 13, 10123 Torino, Italy

[2]Institute for Sustainable Plant Protection (IPSP), CNR, Strada delle Cacce 73, 10135 Torino, Italy

Corresponding author: Valentina Fiorilli

e-mail: valentina.fiorilli@unito.it

ABSTRACT

Transcriptomics and genomics data recently obtained from the arbuscular mycorrhizal (AM) fungus *Rhizophagus irregularis* have offered new opportunities to decipher the contribution of the fungal partner to the establishment of the symbiotic association. The large number of genes which do not show similarity to known proteins, witnesses the uniqueness of this group of plant-associated fungi. In this work we characterize a gene that was called *RiPEIP1* (*P*referentially *E*xpressed *I*n *P*lanta). Its expression is strongly induced in the intraradical phase, including arbuscules, and follows the expression profile of the *Medicago truncatula* phosphate transporter *MtPT4*, a molecular marker of a functional symbiosis. Indeed, *mtpt4* mutant plants, which exhibit low mycorrhizal colonization and an accelerated arbuscule turnover, also show a reduced *RiPEIP1* mRNA abundance. To further characterize *RiPEIP1*, in the absence of genetic transformation protocols for AM fungi, we took advantage of two different fungal heterologous systems. When expressed as a GFP fusion in yeast cells, RiPEIP1 localizes in the endomembrane system, in particular to the endoplasmic reticulum,

which is consistent with the *in silico* prediction of four transmembrane domains. We then generated *RiPEIP1*-expressing strains of the fungus *Oidiodendron maius*, ericoid endomycorrhizal fungus for which transformation protocols are available. Roots of *Vaccinium myrtillus* colonized by *RiPEIP1*-expressing transgenic strains showed a higher mycorrhization level compared to roots colonized by the *O. maius* wild type strain, suggesting that *RiPEIP1* may regulate the root colonization process.

Keywords: Arbuscular Mycorrhizal Symbiosis, *Rhizophagus irregularis*, *Oidiodendron maius*, Heterologous expression system.

INTRODUCTION

The arbuscular mycorrhiza (AM), one of the most widespread symbiosis on earth, occurs between about 80% of land plants and soil fungi belonging to the ancient *Glomeromycota* Phylum (Schüßler et al. 2001; Redecker et al. 2013). This intimate mutualistic association allows the host plant to gain mineral nutrients and water from the soil *via* the activity of the large network of extraradical mycelium (Javot et al. 2007; Govindarajulu et al. 2005; Allen and Shachar-Hill 2009) while, in turn, the fungus acquires plant photoassimilates (up to 20%) that are essential to progress into the different developmental stages (Pfeffer et al. 1999). AM fungi are important members of the plant microbiome and provide important ecosystem services; they are therefore of great interest for the development of a sustainable and low-input agriculture (Gianinazzi et al. 2010).

The root colonization process comprises three main stages: i) a presymbiotic phase where the partners recognize each other through the exchanges of chemical compounds (Bonfante and Requena 2011; Bonfante and Genre 2015); ii) the root penetration where, after the formation of a hyphopodium on the root surface, the epidermal cell develops the pre-penetration apparatus to guide the entrance/accommodation of the fungal hypha (Genre et al. 2005, 2008); iii) the intraradical fungal growth, which culminates with the development of highly branched intracellular structures, the arbuscules, where nutrient exchanges between the two partners are thought to occur (Gutjahr and Parniske 2013). Arbuscule developmental dynamics was elegantly described by live cell imaging in rice roots: arbuscules were confirmed to be ephemeral structures with a lifetime at maturity of approximately two to three days (Kobae and Hata 2010).

AM fungi display many unusual biological features beside the obligate biotrophism, spores and hyphae contain multiple nuclei, making classic genetic approaches challenging (Lanfranco and Young 2012; Young 2015). No sexual cycle has ever been described although meiosis-related genes and conserved putative sex pheromone-sensing pathway were found in the genome (Halary et al. 2011; 2013; Tisserant et al. 2013; Riley et al. 2014). Moreover, genomic structure was for long time obscure, and it has been questioned whether AM fungi are in a heterokaryotic (Hijri and Sanders

2005; Ehinger et al. 2012) or in a homokaryotic condition (Pawlowska and Taylor 2004). Recent data from the genome sequence of the model AM fungus *Rhizophagus irregularis* (isolate DAOM 197198; Tisserant et al. 2013), also at the level of a single fungal nucleus (Lin et al. 2014), strongly support the homokaryotic status.

Several transcriptomic studies, mainly based on large-scale gene expression analysis, have been applied in the last decade to decipher the molecular mechanisms that accompany the formation of arbuscular mycorrhizas. They focused almost exclusively on the host plant (Salvioli and Bonfante 2013 and references within), whereas only a few studies addressed the fungal partner (Requena et al. 2002; Breuninger and Requena 2004; Cappellazzo et al. 2007; Kikuchi et al. 2014; Salvioli et al. 2016). A major advance has been obtained with transcriptomics and genomics data of *R. irregularis* (Tisserant et al. 2012; 2013; Ruzicka et al. 2013; Lin et al. 2014). The genome of *R. irregularis* is one of the largest (153 Mb) fungal genome sequenced to date, along with those of obligate biotrophic powdery mildews (Spanu et al. 2010) and the ectomycorrhizal symbiont *Tuber melanosporum* (Martin et al. 2010). The obligate biotrophy of AM fungi is not explained by genome erosion or any related loss of metabolic complexity in central metabolism. One striking genomic feature is the lack of genes encoding plant cell wall degrading enzymes in analogy to other obligate biotrophic pathogens (Spanu et al. 2010) and ectomycorrhizal symbionts (Martin et al. 2010).

Tisserant et al. (2012) provided the first genome-wide overview of the transcriptional changes that occur in the different fungal life stages. In particular, the abundance of c. 18,500 fungal non-redundant expressed transcripts was analyzed in spores, extra- and intraradical mycelium, and arbuscules. Interestingly, several transcripts coding for Small Secreted Proteins (SSPs) were identified as being induced in the intraradical mycelium (IRM) and in arbuscule-containing cells (ARB), as compared to the extraradical mycelium (ERM). This SSPs list included the recently described secreted effector protein SP7, which is the first gene described so far to play a crucial role in the accommodation of the fungus within the plant root. In particular, SP7 counteracts the plant

93 immune response by interacting with the pathogenesis-related transcription factor Ethylene
94 Response Factor (*ERF19*) in the host nucleus (Kloppholz et al. 2011).
95 This transcriptomic dataset has been instrumental for the characterization of several fungal genes
96 (Li et al. 2013; Belmondo et al. 2014; Tamayo et al. 2014), providing new insights into the genetic
97 program activated during the AM symbiosis and into the genetic characteristics, similarities and
98 uniqueness of AM organisms. Taking advantage of laser microdissection, four genes which were
99 preferentially expressed in the intraradical phase, including arbuscules, were identified: three did
100 not show similarity to known proteins and one showed similarities with ABC transporters (Tisserant
101 et al. 2012).
102 In this study, we characterize one of these genes, that we called *RiPEIP1* (*P*referentially *E*xpressed
103 *I*n *P*lanta) because its expression was strongly induced in the intraradical phase. Since stable
104 transformation protocols are not available for AM fungi (Helber and Requena 2008; Helber et al.,
105 2011), to characterize *RiPEIP1* we used two heterologous expression systems: *Saccharomyces*
106 *cerevisiae*, a classical fungal model system and, for the first time, *Oidodendron maius*, an ericoid
107 mycorrhizal fungus that, in analogy to AM fungi, intracellularly colonizes root cells, and for which
108 a stable transformation protocol is available (Martino et al. 2007).
109
110 **MATERIALS AND METHODS**
111 **Plant and fungal material**
112 Seeds of *Medicago truncatula* Gaertn cv Jemalong and the *mtpt4-2* TILLING mutant (Javot et al.,
113 2011) were treated as described in Fiorilli et al. (2013). *Rhizophagus irregularis* (Syn. *Glomus*
114 *intraradices*, DAOM 197198) inoculum was obtained from *in vitro* monoxenic cultures of
115 *Agrobacterium rhizogenes*-transformed chicory roots (Bécard and Fortin 1988) in two-compartment
116 Petri plates, as described in Belmondo et al. (2014). Plates were incubated in the dark at 24°C until
117 the fungal compartment, containing a solid M medium without sucrose (M-C medium), was
118 profusely colonized by the fungus (approximately 6 weeks).

M. truncatula wilde type (WT) and *mtpt4-2* mycorrhizal plants were obtained in the sandwich system (Giovannetti et al. 1993), inoculating seedlings with *R. irregularis* extraradical mycelium (ERM) between two sterile nitrocellulose membranes. Plants were fertilized with Long-Ashton nutrient solution containing 32 µM KH_2PO_4 and grown in climate-controlled rooms at 22°C with a photoperiod of 14-h light and 10-h dark. Plants were harvested 60 days post-inoculation (dpi).

Oidiodendron maius (CLM1381.98 strain; Martino et al. 2000) was grown in Czapek-Dox medium (supplemented with 1% agar for the solid medium). Petri dishes were kept in the dark at 25°C in a dark room at 25°C for 2 months. Flasks were kept under the same conditions on an orbital shaker.

In vitro endomycorrhizas were synthesized as described by Abbà et al. (2009) with some modifications. Axenic *V. myrtillus* seedlings (Les Semences du Puy, Le Puy-En-Velay, France) were inoculated on modified Ingestad's medium (Ingestad, 1971), where four mycelium plug of wt or transformants strains were previously grown for two months. Plates were placed in a growth chamber (16 h photoperiod, light at 170 µmol m^{-2} s^{-1}, temperatures at 23°C day and 21°C night), and roots were observed after 2 months of incubation. Four Petri dishes, each containing three seedlings (for a total of twelve replicates), were used for each fungal genotype.

Quantification of mycorrhizal colonization

M. truncatula WT and *mtpt4-2* mycorrhizal roots were stained after two months of inoculation with 0.1% cotton blue in lactic acid and the estimation of mycorrhizal parameters was performed as described by Trouvelot et al. (1986) using MYCOCALC (http:// www2.dijon.inra.fr/mychintec/Mycocalc-prg/download.html).

The degree of *V. myrtillus* mycorrhization was recorded after two months of inoculation by *O. maius* Zn WT or *RiPEIP1*-expressing strains. The magnified intersections method (Villarreal-Ruiz et al. 2004) was adapted to quantify the percentage of infection of *V. myrtillus* roots after staining with acid fuchsin. The root system was examined under the microscope using the rectangle around the cross-hair as intersection area at 200X magnification. A total of 100 intersections per seedling

root system were scored. Counts were recorded as percentage of root colonized (RC) by the fungus using the formula: RC% = 100 × Σ of coils counted for all the intersections, where Σ is the number of epidermal cells for all the intersections.

Biomass analysis

Conidia were harvested from two month-agar cultures of both WT and transformants by gently scraping cultures in 9 cm Petri dishes flooded with 1 ml of sterilized water. Conidia were counted in a Bürker counting chamber (Marienfeld, Germany) and, for each fungus, an aliquot containing a comparable number of conidia was transferred into liquid medium. After one month, fungal mycelia were harvested and the biomass weight evaluated. Analyses were carried out on three technical replicates for each biological condition.

Agrobacterium tumefaciens*-mediated transformation of *Oidiodendron maius

The pCAMBIA0380 (CAMBIA) was used as a backbone to construct the p*RiPEIP1* expression vector. The hygromycin resistance cassette (containing the *A. nidulans gpdA* promoter, the *hph* gene encoding resistance to hygromycin, and the *trpC* terminator from *A. nidulans*) was excised from pAN7-1 (Punt et al. 1987) with *Hind*III and BglII and inserted into the pCAMBIA030 at the HindIII/BglII sites to create the pCAMBIA0380_HYG. The insertion of the Hygromycin resistance cassette introduced at the end of the trpC terminator a XbaI restriction site that was not originally present in the pCAMBIA0380 vector. The vector was then *XbaI-XmaI* digested to insert another copy of the *A. nidulans gpdA* constitutive promoter, which was modified at the 3' end to carry also a *KpnI* restriction site before the *XmaI* site. *RiPEIP1* full length cDNA was then amplified using a forward primer (*RiPEIP1-K)* containing a *Kpn*I site and a reverse primer (*RiPEIP1-X)* containing a *Xma*I site (Table S1). The PCR product was *Kpn*I and *Xma*I digested and inserted into the *Kpn*I-*Xma*I-digested pCAMBIA0380_HYG plasmid under the A. *nidulans gpdA* constitutive promoter. The resulting recombinant plasmid was introduced into *A. tumefaciens* LBA1100 strain, which was

used to transform *O. maius* ungerminated conidia according to the protocol described by Abbà et al. (2009). Transformants were isolated and transferred into 24-well plates with Czapek-Dox agar medium supplemented with 100 µg/ml hygromycin B. Transgenic strains were confirmed by PCR assays and Southern blot hybridization.

Genomic DNA of transformants strains was extracted using the CTAB protocol from mycelium grown for 30 days in liquid Czapek-2% DOX medium. For Southern blot, 10 micrograms of genomic DNA was digested with *Bgl*II restriction enzyme, size-fractionated on 1% (w/v) agarose gel and blotted onto nylon membranes following standard procedures (Sambrook and Russel 2001). Hybridization with a chemiluminescent detection system (ELC Direct DNA labelling and Detection System, Amersham) was performed according to the manufacturer's recommendation using a probe corresponding to the full length *RiPEIP1* cDNA sequence using *RiPEIP1* full length (RiPEIP1fl) forward/reverse primer (Table S1). Probe labelling and high stringency hybridization were carried out using ECL protocol (GE Healthcare, Chalfont St. Giles, U.K.).

5′- and 3′ RACE

Both 5′- and 3′ RACE were performed on total RNA extracted from the mycorrhizal roots with the SMART RACE cDNA amplification kit (Clontech). The PCR product was obtained using the primers *RiPEIP1*-race-forward/reverse (Table S1). PCR was performed according to the Clontech protocol using the Advantage 2 PCR enzyme system and 35 cycles of 95°C for 30 sec, 60°C for 30 sec and 72°C for 2 min, with a final extension at 72°C for 10 min. The RACE products were cloned into pCRII vector (TOPO cloning kit; Invitrogen) and sequenced.

Nucleic acid extraction, cDNA synthesis, RT-PCR assay

Total genomic DNA was extracted from *R. irregularis* ERM and *M. truncatula* shoot using the DNeasy Plant Mini Kit (Qiagen), according to the manufacturer's instructions. Each primer pair

was first tested on plant or fungal genomic DNA as a positive control and to exclude cross hybridization.

Total RNA was extracted from *R. irregularis* ERM and mycorrhizal *M. truncatula* roots using the Plant RNeasy Kit (Qiagen), according to the manufacturer's instructions.

Total RNA was extracted from *O. maius* transformats and WT mycelia using a Tris-HCl extraction buffer (Tris-HCl 100 mM pH 8, NaCl 100 mM, Na-EDTA 20 mM, PVP 0.1 %, Na-laurylsarcosine 1 % in DEPC-treated H_2O), followed by phenol (Roti-Phenol, Roth) extraction, phenol:chloroform:isoamyl alcool (25:24:1) extraction, chloroform extraction and isopropanol precipitation (30 min at -80°C). The pellet was then resuspended in DEPC-treated water and precipitated in 6M LiCl (12 hours at 4°C). Finally, RNA was pelleted by centrifugation, rinsed with 70 % ethanol and resuspended in DEPC-treated H_2O.

Samples were treated with TURBO™ DNase (Ambion) according to the manifacturer's instructions. RNA samples were routinely checked for DNA contamination by means of RT-PCR (One-RT-PCR, Qiagen) analysis, using for *R. irregularis RiEF1α*, *M. trucatula MtTef*, and *O. maius OmTubulin* primers (Table S1).

For conventional RT-PCR analyses, RNAs were amplified with *RiPEIP1-qpcr* forward/reverse primers and for the *O. maius* tubulin housekeeping gene (Table S1) using the One Step RT-PCR kit (Qiagen). RNA samples were incubated for 30 min at 50°C, followed by 15 min of incubation at 95°C. Amplification reactions were run for 40 cycles at 94°C for 30 s, 60°C for 30 s, and 72°C for 40 s.

cDNA synthesis was carried out on about 700 ng of total RNA which was denatured at 65°C for 5 min and then reverse-transcribed at 25°C for 10 min, 42°C for 50 min and 70° for 15 min in a final volume of 20 µl containing 10 µM random primers, 0.5 mM dNTPs, 4 µl 5X buffer, 2 µl 0.1 M DTT, and 1 µl Super-ScriptII (Invitrogen).

Quantitative RT-PCR

Quantitative RT-PCR (qRT-PCR) assays were performed using an iCycler apparatus (Bio-Rad) as described in Belmondo et al. (2014) with primers pairs listed in Table S1. All reactions were performed on three technical replicates and on at least three biological replicates. Baseline range and Ct values were automatically calculated using iCycler software. Transcript levels were normalized to Ct values registered for the *RiEF1α* (Gonzàlez-Guerrero et al. 2010) fungal gene and the *MtTef* (Hohnjec et al. 2005) plant gene. Only Ct values leading to a Ct mean with a standard deviation below 0.5 were considered.

Laser microdissection (LMD)

M. truncatula mycorrhizal roots, obtained using the sandwich method, were dissected, fixed and embedded in paraffin according to the method described in Perez-Tienda et al. (2011). A Leica AS LMD system (Leica Microsystem, Inc.) was used to collect arbuscule-colonized cortical cells (ARB) and non-colonized cortical cells (MNM) from paraffin root sections, as described by Balestrini et al. (2007). Two thousand ARB and MNM cells (for each biological replicate) from *M. truncatula* roots were collected. RNA was extracted following the Pico Pure kit (Arcturus Engineering) protocol. A DNAse treatment was performed using an RNA-free DNase Set (Qiagen) in a Pico Pure column, according to the manufacturer's instructions. RNA was then quantified using a NanoDrop 1000 spectrophotometer. DNA contamination in RNA samples was evaluated using *RiEF1α* (Table S1) by means of RT-PCR assays carried out using One Step RT-PCR kit (Qiagen).

Construction of GFP fusion proteins for expression in yeast

The full length cDNA of *RiPEIP1* was amplified from *R. irregularis* cDNA by PCR using the Phusion DNA-Polymerase (Finnzymes, Espoo, Finland). cDNAs were amplified using a forward primer containing the *Kpn*I site (*RiPEIP1-K*) and a reverse primer containing the *Not*I site (*RiPEIP1-N)* (Table S1). The PCR products were *Kpn*I and *Not*I -digested and inserted into the *Kpn*I-*Not*I-digested pYES2-GFP plasmid (Blaudez et al. 2003) under the control of the GAL1

promoter and allowing a 3' fusion with the enhanced GFP reporter gene. *Saccharomyces cerevisiae* (BY4742 strain) transformation was performed using the lithium acetate based method described by Gietz et al. (1992). As a control of subcellular localization, two yeast strains constitutively expressing the red fluorescent protein fused to Sec13 (marker of both the endoplasmic reticulum and Golgi stacks) or Cop1 (marker of early Golgi) were analysed in parallel (http://yeastgfp.yeastgenome.org/info.php).

Microscopy

Fluorescence emission from yeast cells expressing RiPEIP1::GFP, Sec13::RFP or Cop1::RFP was examined with a Leica TCS-SP2 confocal laser-scanning microscope equipped with a 63x water immersion objective. GFP was excited at 488 nm (Ar laser) and fluorescence was detected at 515-530 nm. RFP was excited at 546 nm and fluorescence was detected at 570-650 nm.

For acid fuchsin staining mycorrhizal roots were stained with 0.01% (w/v) acid fuchsin in lactoglycerol (lactic acid-glycerol-water, 14:1:1; Kormanik and McGraw 1982). Confocal microscopy observations were done using a Leica TCS-SP2 microscope equipped with a 40x long-distance objective. Acid fuchsin fluorescence was excited at 488nm and detected using a 560-680 nm emission window.

For calcofluor white (CFW; Fluorescent Brightener 28, F3543; Sigma-Aldrich) staining *O. maius* mycelia were let grown on a coverslip. The CFW solution was dropped onto the coverslips immediately before observation. CFW fluorescence was visualized with a Leica TCS-SP2 confocal laser-scanning microscope using a 405nm diode and an emission window at 410-460nm.

Statistical analyses

Statistical analyses were performed through one-way ANOVA and Tukey's post hoc test, using a probability level of $p<0.05$. All statistical elaborations were performed using PAST statistical package (version 2.16; Hammer et al. 2001).

RESULTS

RiPEIP1 gene isolation

With the aim to identify fungal genes involved in the functioning of arbuscules, the key structures of the AM symbiosis, we exploited transcriptomics data (Tisserant et al. 2012) generated for the AM fungus *Rhizophagus irregularis*. We focused our attention on an EST (contig Glomus_c13083 - v1 assembly) that turned out to be up-regulated in the intraradical phase in microarray experiments (Tisserant et al. 2012). In order to confirm the microarray data, the expression profile of Glomus_c13083 was monitored by quantitative RT-PCR (qRT-PCR) in intraradical (IRM) and extraradical (ERM) mycelium obtained from *M. truncatula* mycorrhizal roots 60 days post-inoculation (dpi) in a sandwich system. In particular, RNAs were extracted from the ERM and from *M. truncatula* roots fragments from which ERM was carefully removed to generate the IRM sample. The mycorrhizal status of *M. truncatula* roots was confirmed by the calculation of the total root length colonization (62.8%±2.7). Glomus_c13083 transcripts were highly abundant in the IRM compared to ERM (Fig. 1), and they were barely detected in spores (Tisserant et al., 2012; data not shown). From this expression profile, we called this gene *RiPEIP1* for *P*referentially *E*xpressed *I*n *P*lanta.

The 786 bp full length cDNA sequence of *RiPEIP1* was obtained by RACE assays. The corresponding genomic sequence (1267 bp), which comprises five introns (Supplementary Fig. S1A), was also obtained by conventional PCR. The recent release of the complete genome sequence of *R. irregularis* (Lin et al. 2014) confirmed the presence of this gene in the genome assembly (RirG_002110 - GenBank EXX79805.1), although the sequence was annotated with four additional amino acids at the N-terminus. Considering that the coding sequence isolated through several independent 5'-RACE-PCR assays never reported the presence of these four amino acids we considered the shorter cDNA sequence for the further analysis.

The predicted protein sequence (261 amino acids) showed no significant similarity (all E-values > 0.2) with proteins deposited in the NCBI "nr" database. The only hit found by a BlastP analysis,

spanning more than 50% of the sequence length, was another *R. irregularis* protein with unknown function (GenBank ESA21063.1, percentage of identity 22%; E-value 0.38). The second round of a Psi-Blast analysis, including this protein, identified three additional *R. irregularis* proteins (GenBank EXX76561.1, ESA00383.1, EXX59269.1) of unknown function slightly shorter (less than 200 amino acids) than RiPEIP1. Interestingly, a TBlastN search within an extensive EST dataset recently published for another AM fungus, *Gigaspora margarita* (Salvioli et al. 2016), led to the identification of a cDNA sequence (GenBank: GBYF01016486.1; percentage of identity 26%; E-value 0.036) coding for a 182 amino acids polypeptide, which is probably a partial sequence lacking the C-terminus.

TMHMM analysis of the RiPEIP1 amino acid sequence revealed the presence of four transmembrane-helix domains with cytoplasmic N- and C-terminus regions (Supplementary Fig. S1B). Additionally, the WoLFPSORT subcellular location predictor identified RiPEIP1 as a putative integral membrane protein. Remarkably, the sequence from *G. margarita*, notwithstanding the rather low similarity percentage, presents the same four transmembrane domains topology.
The C-terminus region, just after the fourth transmembrane domain, is highly hydrophilic, with a high percentage of charged (lysine, arginine, aspartate and glutamate) and polar (especially serines) amino acids. Some serines were also predicted (score > 0.99) by the NetPhos 2.0 Server to be potentially phosphorylated (Supplementary Fig. S1B). Notably, this region contains three typical ER-retention/retrieval C-terminus motifs: two KKXX motifs (starting from amino acid 188 and amino acid 200) and one KXKXX motif (starting from amino acid 246) (Jackson et al. 1990; Supplementary Fig. S1B).

RiPEIP1 is localized in the endomembrane system

In order to investigate the sub-cellular localization of RiPEIP1, we expressed a RiPEIP1::GFP fusion construct in *Saccharomyces cerevisiae* yeast cells. The fluorescent signal was observed

outlining the nucleus and extending into a network-like pattern in the cytoplasm (Fig. 2A). To better characterize this protein localization, we compared the RiPEIP1::GFP fluorescence pattern with that of two strains constitutively expressing the red fluorescent protein (RFP) fused to Sec13, which is a marker of the endoplasmic reticulum and Golgi stacks, or Cop1 which is localized in the early Golgi. Indeed, Sec13::RFP showed a very similar pattern to RiPEIP1::GFP, with the addition of several bright spots in the cytoplasm (Fig. 2C). Since analogous spots were observed in the Cop1::RFP line (Fig. 2D), we concluded that the RiPEIP1::GFP fluorescence pattern was compatible with protein localization in the endoplasmic reticulum and nuclear envelope. These results are in line with *in silico* predictions.

RiPEIP1 expression profiles

To monitor the dynamics of *RiPEIP1* expression pattern along the colonization process, we set up a time course experiment of *M. truncatula* plants inoculated with *R. irregularis* in the sandwich system and sampled 7, 14, 28 and 60 days post-inoculation (dpi). Morphological analyses of roots revealed almost no intraradical fungal structures at 7 or 14 dpi. Mycorrhization frequency increased from 28 to 60 dpi, although arbuscules, detected starting from 28 dpi, decreased at 60 dpi (Supplementary Fig. S2). Since *RiPEIP1* was shown by qRT-PCR to be expressed at low levels in the ERM, gene expression was evaluated in whole mycorrhizal roots, without a distinction between IRM and ERM. *RiPEIP1* mRNA abundance increased in parallel to the development of the intraradical phase, and in particular to arbuscules formation, as demonstrated by morphological data and by the parallel mRNA accumulation of *MtPT4*, the *M. truncatula* phosphate transporter-encoding gene which is considered a molecular marker of arbuscule-containing cells (Harrison et al. 2002; Fig. 3 A,B).

Using the laser microdissection, we investigated in more detail the *RiPEIP1* expression profile during the intraradical phase by comparing gene expression in arbusculated cells (ARB) and in non-colonized cortical cells from mycorrhizal roots (MNM). We used primers to the fungal

housekeeping gene *RiTEFα* to monitor the presence of fungal structures in the two cell types, and primers to *MtPT4* to confirm the presence of arbuscules (Fig. 3C). Since the transcript for the fungal housekeeping gene *RiTEFα*, but not *MtPT4*, was also detected in the MNM cell population, we considered this sample representative of intercellular hyphae. As witnessed by the detection of *MtPT4* mRNA, *RiPEIP1* was only expressed in the ARB cell population, indicating that, in the intraradical phase, *RiPEIP1* expression occurred mainly in arbuscules and presumably not in intercellular hyphae (Fig. 3C).

To gather information on the relationship between *RiPEIP1* expression and arbuscule functionality, we analysed *RiPEIP1* expression profile in the *M. truncatula mtpt4-2* mutant line, which is defective of MtPT4. Inactivation of MtPT4 causes low mycorrhizal colonization and an increased number of stunted arbuscules as a result of accelerated arbuscules turnover (Javot et al. 2011; Fig S3). As expected, a lower colonization level, based on relative abundance of fungal to plant *Tef* transcripts, was observed for *mtpt4-2* compared to wt plants (Fig. 4A). To have an overview of arbuscule abundance in the wt and *mtpt4-2* plants, we checked the expression level of the blue copper-binding protein1 (*MtBCP1*), a protein localized in the plasma membrane of cortical cells before and during the growth arbuscules and in the periarbuscular membrane surrounding arbuscule trunks (Pumplin and Harrison 2009). *mtpt4-2* mycorrhizal plants showed a lower level of *MtBCP1* transcripts compared to wt plants (Fig. 4B). Similarly, *RiPEIP1* mRNA abundance was lower in the *mtpt4-2* genotype (Fig. 4C). *MtBCP1* and *RiPEIP1* expression levels showed a positive correlation in wt and *mtpt4-2* genotypes (R^2:0.8433). These data clearly indicate that *RiPEIP1* expression is therefore associated to arbuscule development.

Heterologous expression of *RiPEIP1* in *Oidiodendron maius*

To gain further information on RiPEIP1 function a novel fungal heterologous expression system has been exploited. In particular, we expressed RiPEIP1 in the ericoid mycorrhizal fungus *Oidiodendron maius*, for which a protocol of genetic transformation is available (Martino et al.

2007). By means of *A. tumefaciens*-mediated genetic transformation, transgenic *O. maius* strains expressing *RiPEIP1* under a constitutive promoter were obtained. We confirmed the presence of *RiPEIP1* in *O. maius* genome by PCR and Southern blot analyses (Supplementary Fig. S4). Three transformants (BA2, BA4 and BC6) with a single genomic insertion were selected for further analyses (Supplementary Fig. S4).

RiPEIP1 expression in free-living mycelia of *O. maius* transformants was confirmed by RT-PCR assays (Fig. 5A). As shown in Fig 5B the growth rate of *RiPEIP1*-expressing mycelia was similar to that of the wt strain. A phenotypic analysis carried out on free-living mycelia stained with calcoflour white showed no difference in hyphal morphology (Supplementary Fig. S5).

To investigate the impact of *RiPEIP1* expression on the establishment of the mycorrhizal symbiosis, seedlings of *Vaccinium myrtillus*, a common host plant for ericoid fungi, were colonized *in vitro* with wt or *RiPEIP1*-expressing strains. Plates with *V. myrtillus* seedlings without fungal inoculation were also set up. As expected, after 2 months uninoculated seedlings were dead (Fig. 6A). Root colonization level of inoculated plants was analysed through the morphological evaluation of the numbers of coils/root intersections. Interestingly, roots colonized by *O. maius RiPEIP1*-expressing strains showed a statistically significant higher mycorrhization degree compared to roots colonized by the wt strain (Fig. 6B: Fig S6). Moreover, morphological changes were also observed in the root apparatus colonized by *RiPEIP1*-expressing strains (Fig 7): in particular, a stimulation of root branching was observed. Roots colonized by the transgenic strains formed lateral roots up to the 6th order while the roots colonized by the wt strain only developed up to 4th order lateral roots (Table 1).

DISCUSSION

Among plant-associated microbes, AM fungi form the most ecologically and agriculturally important mutualistic association with plant roots in terrestrial ecosystems. Yet, the genetic determinants that control the fungal development *in planta*, which are necessary to support a long-

lasting interaction between the partners, are still largely unknown. Transcriptomic (Tisserant et al. 2012) and genomic (Tisserant et al. 2013; Lin et al. 2014) data recently obtained for *R. irregularis* have offered new opportunities to decipher the contribution of the fungal partner to the establishment of the symbiotic association. A large number of genes expressed in the intraradical and the extraradical phases do not show similarity with proteins listed in databanks and may therefore be AM-specific. This may be an indicator of the uniqueness of Glomeromycota. In this work, we have focused on the characterization of one of these genes that was called *RiPEIP1* based on its expression profiles.

Based on bioinformatics analyses, RiPEIP1 is a protein with no similarity to known sequences; consistently with *in silico* predictions, RiPEIP1 seems to be a transmembrane protein localized in particular in the endoplasmic reticulum and nuclear envelope when expressed as a GFP fusion in yeast cells. It is worth to note that a similar sequence was found in the recently published transcriptome (Salvioli et al. 2016) of another AM fungus, *G. margarita*. Even if the sequence is only partial, lacking the C-terminus portion, and the percentage of identity is relatively low (22%), the two proteins share the same 4 transmembrane domains topology. Moreover, RNA-seq data showed an up-regulation of about 4 folds in mycorrhizal roots, as compared to germinating spores (Salvioli et al. 2016). Only the search within genomic and transcriptomic data from other AM fungi, once available, will clarify whether *RiPEIP1*-related sequences are a specific feature of Glomeromycota and whether they may have a general role in the *in planta* phase.

RiPEIP1 expression is strongly induced in the intraradical phase, and the time course experiment showed that the highest expression levels were reached in mature mycorrhizal roots; *RiPEIP1* mRNA abundance perfectly matches the expression profile of *MtPT4* (at 28 and 60 dpi), a phosphate transporter essential for the acquisition of Pi delivered by the AM fungus (Javot et al. 2007) and thus considered a marker of a functional symbiosis. We therefore suggest that *RiPEIP1* expression is, to some extent, related to arbuscules formation. This hypothesis is supported by the fact that *RiPEIP1* transcripts were detected in laser microdissected arbuscule-containing cells, while

428 they were absent in adjacent cortical cells that likely contained only intercellular hyphae. To better
429 understand the relationship with arbuscule development, we analysed *RiPEIP1* expression in the *M.*
430 *truncatula mtpt4-2* mutant line that is defective of MtPT4. MtPT4 function was shown to be critical
431 for the AM symbiosis since its inactivation led to an altered arbuscules morphology, with premature
432 senescence (Javot et al. 2007). As expected, we observed a reduced level of fungal colonization in
433 *mtpt4-2* mutants, as monitored by *RiTEFα* transcripts abundance, in comparison with colonized
434 roots of wt plants. Interestingly, *mtpt4-2* mycorrhizal roots showed lower levels of *RiPEIP1*
435 mRNAs. Transcripts for *MtBCP1*, coding for a protein localized in the periarbuscular membrane
436 and considered a molecular marker of arbuscule development and of colonization of the root system
437 (Pumplin and Harrison 2009), were also found expressed at lower levels in the *mtpt4-2* genotype.
438 Overall the data suggest a relationship between *RiPEIP1* and arbuscule differentiation.
439 The involvement of *RiPEIP1* in the intraradical phase of the colonization process was also
440 supported by the heterologous expression of *RiPEIP1* in the ericoid mycorrhizal fungus *O. maius*.
441 In the absence of genetic transformation protocols for AM fungi, AM fungal genes have been
442 characterized by heterologous expression in filamentous fungi in few studies. To our knowledge,
443 this approach was limited to pathogenic systems such as *Magnaporthe oryzae* (Kloppholz et al.
444 2011) or *Colletotrichum lindemuthianum* (Tollot et al. 2009). We suggest that *O. maius* could
445 represent an additional, possibly more suitable biological system for the characterization of AM
446 fungal genes, as it shares with AM fungi the capability to intracellularly colonize root cells.
447 Roots colonized by *O. maius RiPEIP1*-expressing strains showed a higher mycorrhization degree
448 compared to roots colonized by the wt strain. Although no *RiPEIP1* homolog has been found in the
449 complete *O. maius* genome sequence (Kohler et al. 2015) and *V. myrtillus* is not a host for AM
450 fungi, the stimulation of mycorrhization observed in this heterologous mycorrhizal system suggests
451 a general role for *RiPEIP1* in endosymbiosis establishment or functioning. Remarkably, *O. maius*
452 transgenic strains induced changes on *V. myrtillus* root morphology with the stimulation of lateral
453 roots formation up to the 6th order. The molecular basis of this phenomenon are unknown and

deserve further investigations. To this purpose, RiPEIP1-expressing transgenic lines of rice have been recently obtained as a tool to look for altered plants phenotypes including susceptibility to AM colonization and to dissect the metabolic pathway affected by RiPEIP1 and possibly its mechanisms of action.

In summary, although the mechanism of action still remain obscure, we showed that *RiPEIP1*, a gene from the AM fungus *R. irregularis*, is preferentially expressed *in planta* and may play a role in the root accommodation of fungal structures. Our data also underlies the potential of the endomycorrhizal fungus *O. maius* to characterize genes from AM fungi.

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FIGURE LEGENDS

Figure 1 *RiPEIP1* is up-regulated in the intraradical mycelium. Expression of *RiPEIP1* (relative to *RiTef*) assessed by qRT-PCR in intraradical mycelium (IRM) and extraradical mycelium (ERM) from mycorrhizal roots of *M. truncatula* grown in the sandwich system (n= 5). Individual data for each condition are shown as dots and the median as black bars. Asterisks indicate a statistically significant difference ($p < 0.001$, ANOVA, Tukey's post-hoc test)

Figure 2 Confocal imaging of RiPEIP1::GFP localization in yeast cells. The observed fluorescence pattern (**a**) is compatible with GFP localization in the endoplasmic reticulum and nuclear (n) envelope. A brightfield image of the same cells is shown in panel **b**. RiPEIP1::GFP localization is confirmed by a comparison with the fluorescence pattern from constitutively expressed Sec13::RFP (**c**), which labels the endoplasmic reticulum/nuclear envelope (n) and Golgi stacks, and Cop1::GFP (**d**), which targets the early Golgi stacks (arrowheads). Bars: 5 µm

Figure 3 *RiPEIP1* gene expression along the AM colonization process. Relative expression of *MtPT4* (**a**) and *RiPEIP1* (**b**) assessed by qRT-PCR in a time course experiment of root colonization at 7, 14, 28 and 60 days post-inoculation (dpi). Single data for each condition are shown as dots and the median as black bars. Different letters indicate statistically significant difference ($p < 0.05$, ANOVA, Tukey's post-hoc test). (**c**) Gel electrophoresis of RT-PCR products obtained from two independent samples of RNA from laser-microdissected arbuscule-containing cells (ARB) and one sample of not colonized cortical cells from mycorrhizal roots (MNM) using primers specific for *MtPT4*, *RiTef* or *RiPEIP1*. No RNA sample (-)

Figure 4 *RiPEIP1* gene expression is affected in the *mtpt4-2* mutant line. The AM colonization of wt and *mtpt4-2* genotypes was evaluated through the assessment of fungal *RiTef* mRNA abundance (**a**); *MtBCP* expression was used as a marker of AM intraradical phase (**b**). The relative expression of *RiPEIP1* in wt and *mtpt4-2* roots is shown in panel (**c**). Asterisks indicate a statistically significant difference (**$p < 0.01$, *** $p<0.001$, ANOVA, Tukey's post-hoc test)

Figure 5 Expression of *RiPEIP1* in *Oidiodendron maius* does not affect the growth of free-living mycelia. Gel electrophoresis of RT-PCR products obtained from wt and three *RiPEIP1*-expressing (BA2, BA4, BC6) free-living strains using *RiPEIP1* specific primers; - : negative control (**a**). Dry weight of free-living mycelia of wt and transgenic strains grown in liquid cultures (**b**)

Figure 6 *RiPEIP1*-expressing strains led to a higher *Vaccinum myrtillus* root colonization level compared to the WT strain. (**a**) *In vitro* mycorrhization system between *V. myrtillus* seedlings and *O. maius.* Non inoculated *V. myrtillus* seedlings were unable to correctly develop (right panel) (**b**) The percentage of root colonization of *V. myrtillus* seedlings colonized with the *O. maius* wt or the three *RiPEIP1*-expressing strains was quantified after staining with acid fuchsin. Different letters indicate statistically significant difference ($p < 0.05$, ANOVA, Tukey's post-hoc test)

Figure 7 Representative details of the *V. myrtillus* root system colonized by *O. maius* wt (**a**) or BC6 RiPEIP1-expressing (**b**) strains after two months of inoculation. Note the stimulation of root branching in roots colonized by the RiPEIP1-expressing strain. Bar = 1 cm.

Supplementary material

Figure S1 Nucleotide and deduced amino acid sequences of *RiPEIP1*. **a**) *RiPEIP1* genomic DNA sequence showing the presence of five introns. **b**) RiPEIP1 protein sequence showing the 4 transmembrane domains (underlined), the typical ER-retention/retrieval motifs (bold) and the predicted phosphorylation sites (red). See text for details

Figure S2 Colonization level of *M. truncatula* roots at 28 and 60 days post inoculation (dpi) assessed accordingly to Trouvelot et al (1986). F%: frequency of mycorrhization in the root system (**a**), a%: arbuscules abundance in mycorrhizal parts of root fragments (**b**). Different letters indicate statistically significant difference ($p < 0.05$ ANOVA, Tukey's post-hoc test)

Figure S3 Mycorrhizal phenotype of *M. truncatula* wt (**a, b**) and *mtpt4-2* (**c, d**) roots colonized by *R. irregularis*. Roots were harvested 60 dpi, stained with acid fuchsin and observed with a confocal microscope. Arbuscules in *mtpt4-2* are degenerated as described in Javot et al. (2011). Bars = 25 µm

Figure S4 Molecular analyses of *O. maius* transgenic strains expressing *RiPEIP1*. (**a**) Gel electrophoresis of PCR products obtained from genomic DNA of wt and transgenic strains using *RiPEIP1* specific primers (**b**) Southern blot of genomic DNA from wt and transgenic strains restricted with *Bgl*II enzyme and hybridized with the *RiPEIP1* probe. Lanes corresponding to BC6, BA2, BA4 samples exhibited a single genomic insertion.

Figure S5 Phenotype of *O. maius* free living mycelia from wt and *RiPEIP1*-expressing strain BA2 as revealed by calcoflour white staining. Laser-scanning microscope observation of one-month-old *O. maius* wt and transgenic strains stained with calcofluor white. Transmitted light images are shown on the right (**b, d**) and the corresponding fluorescence images on the left (**a, c**). Bars: 10 µm

Figure S6 Mycorrhizal phenotype of *O. maius* wt and *RiPEIP1*-expressing strain BC6. Mycorrhized *V. myrtillus* roots were stained with acid fuchsin and observed 2 months after fungal inoculation. *O. maius* transgenic strains showed an increased number of coils (asterisk) in epidermal cells compared to the wt.

Table 1. Average values and standard deviation of the number of lateral roots of the different orders in plants colonized by the wt or the transgenic strains (BA2, BA4, BC6)

	Root orders	samples			
		BA2	BA4	BC6	WT
Lateral roots average values	1°	14 ± 7	14.6 ± 7.2	16 ± 10.3	9.6 ± 2
	2°	33.3 ± 25	39 ± 14.9	51.6 ± 38.4	17.3 ± 4
	3°	23 ± 24	31 ± 18-1	52 ± 28.7	10.3 ± 1.1
	4°	8.6 ± 9.8	15 ± 14	27 ± 18.5	2.3 ± 4
	5°	0.7 ± 1.1	6.3 ± 7	13.3 ± 8.7	0
	6°	0.3 ± 0.5	1.7 ± 1.5	2.3 ± 1.5	0

751 **Table S1.** List of primers used in this study. Sites for GATEWAY recombination or restriction

752 enzymes are underlined.

Primer ID	Primer sequences [5'-3']
RiEFαf	GCTATTTTGATCATTGCCGCC
RiEFαr	TCATTAAAACGTTCTTCCGACC
MtTEFf	AAGCTAGGAGGTATTGACAAG
MtTEFr	ACTGTGCAGTAGTACTTGGTG
MtPT4 f	TCGCGCGCCATGTTTGTTGT
MtPT4r	CGCAAGAAGAATGTTAGCCC
RiPEIP1-attB-forward	GGGGACAAGTTTGTACAAAAAAGCAGGCTATGTCAGCTAAATTTATCAAGC
RiPEIP1-attB-reverse	GGGGACCACTTTGTACAAGAAAGCTGGGTCTTTAAACATTTTCATTAACACTC
RiPEIP1flF:	ATGTCAGCTAAATTTATCAAGC
RiPEIP1flR:	CTTTAAACATTTTCATTAACACTCATCTCG
RiPEIP1-N	GTCAGCGGCCGCCTTTAAACATTTTCATTAAC
RiPEIP1-K	GATCGGTACCATGTCAGCTAAATTTATC
RiPEIP1-X	GCATCCCGGGCTTTAAACATTTTCATTAAC
RiPEIP1-race-forward	AGTAGAAGCACTAAAGGTGCCAAGAAAAGT
RiPEIP1-race-reverse	TAACACTCATCTCGGGACTGACTTCATTCT
RiPEIP1-qpcrF	AAGAAAGTAAACGTGTGGCT
RiPEIP1-qpcrR	TAACACTCATCTCGGGACTG
OmTubulin-forward	GTTTCCATGAAGGAGGTTGAGG
OmTubulin-reverse	CAGAGAGCAGTCTGGACGTTGT

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Figure 1

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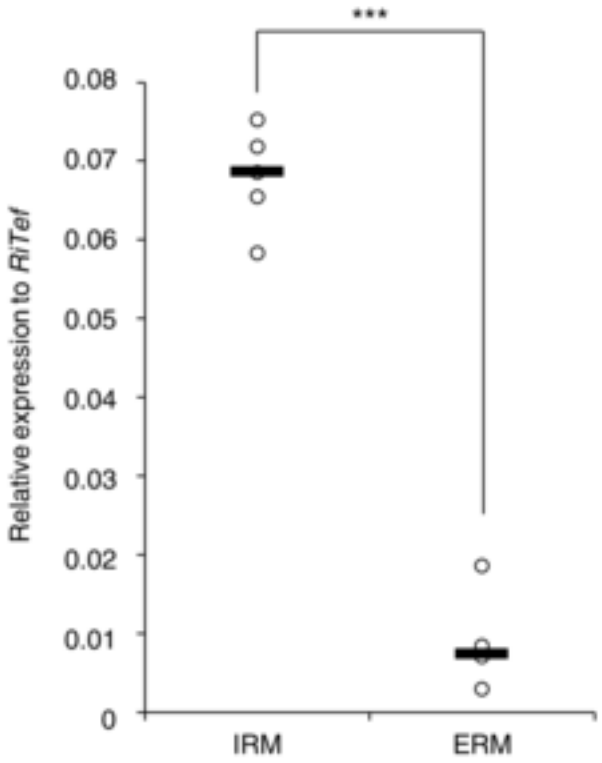

Figure 1

Figure 2

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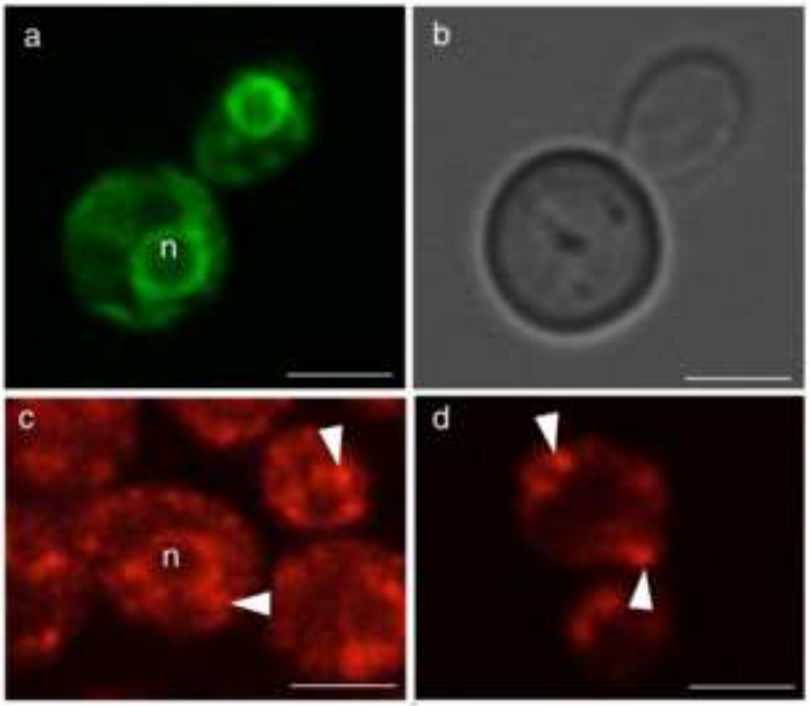

Figure 2

Figure 3

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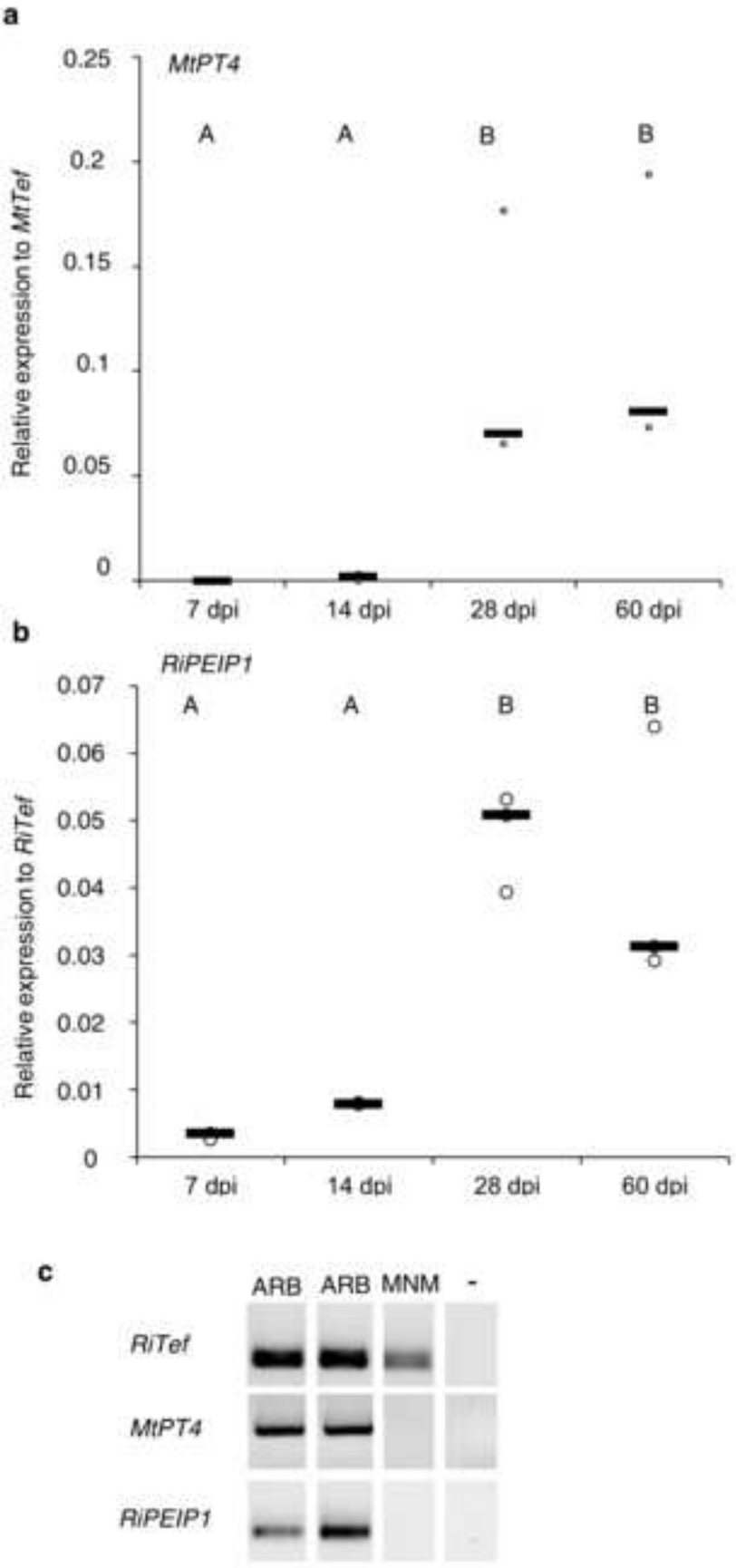

Figure 3

Figure 4

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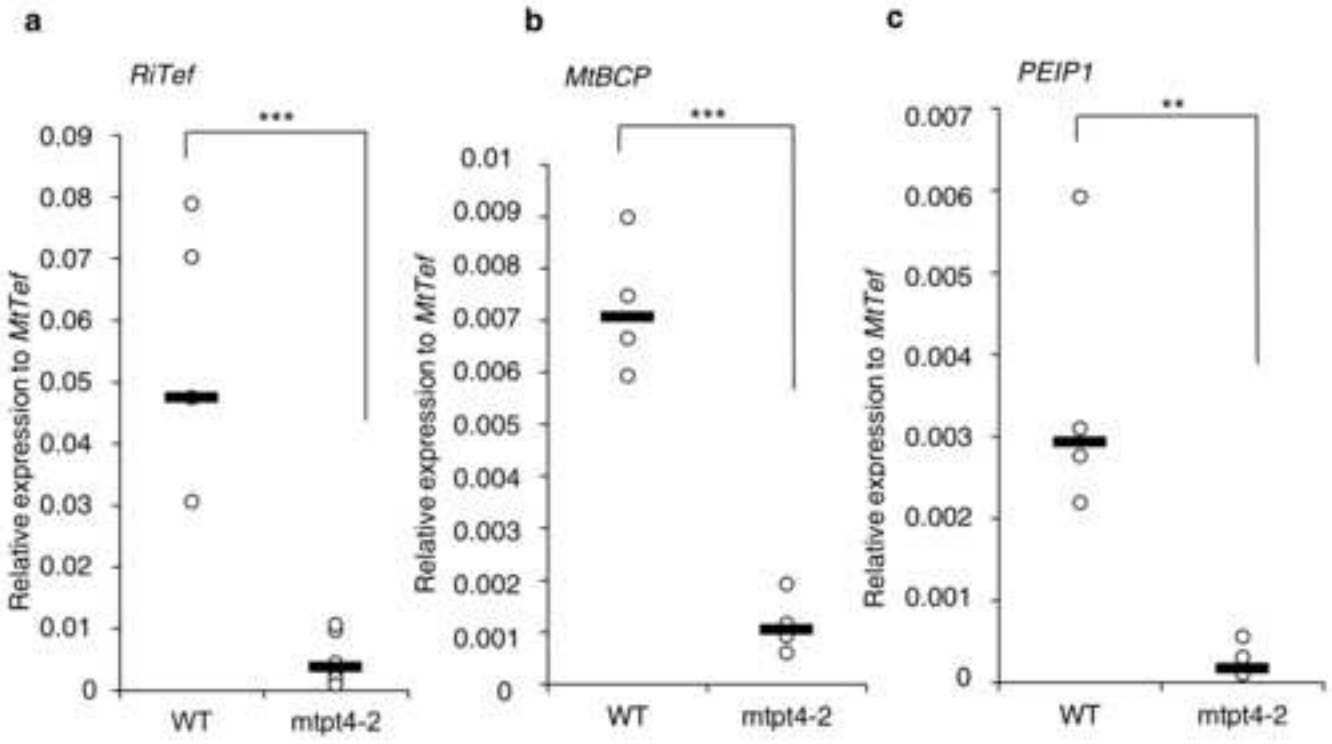

Figure 4

Figure 5

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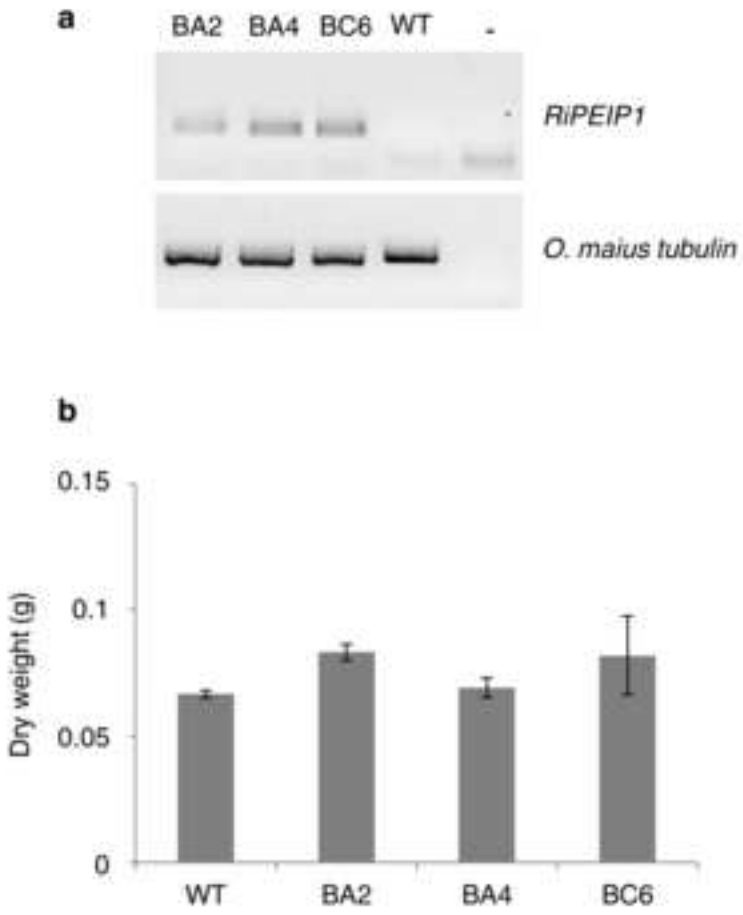

Figure 5

Figure 6

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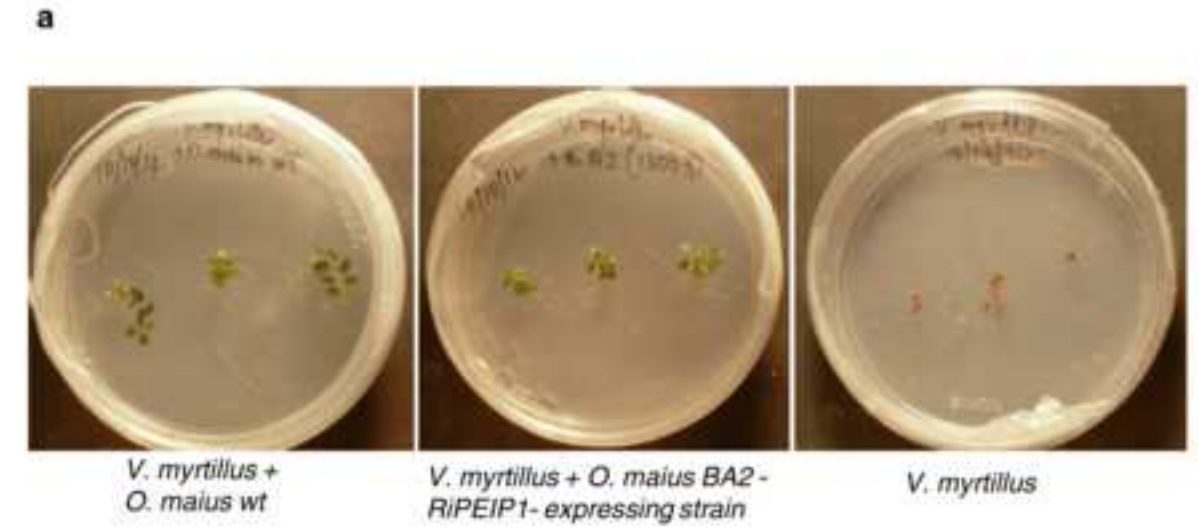

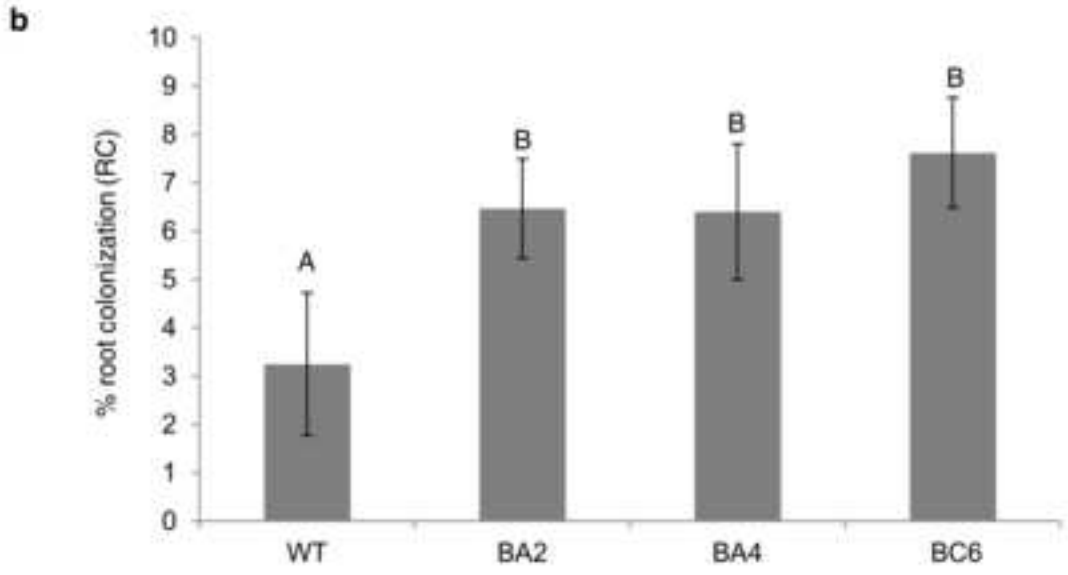

Figure 6

Figure 7

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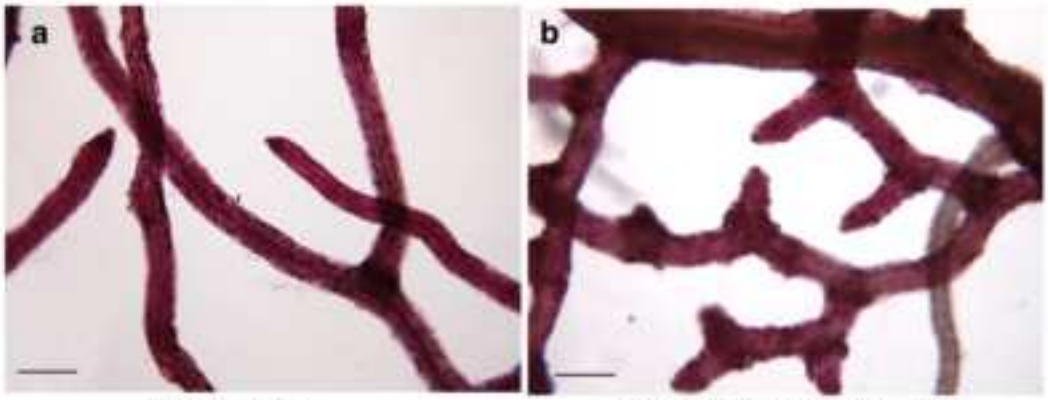

Figure 7

Fig S1

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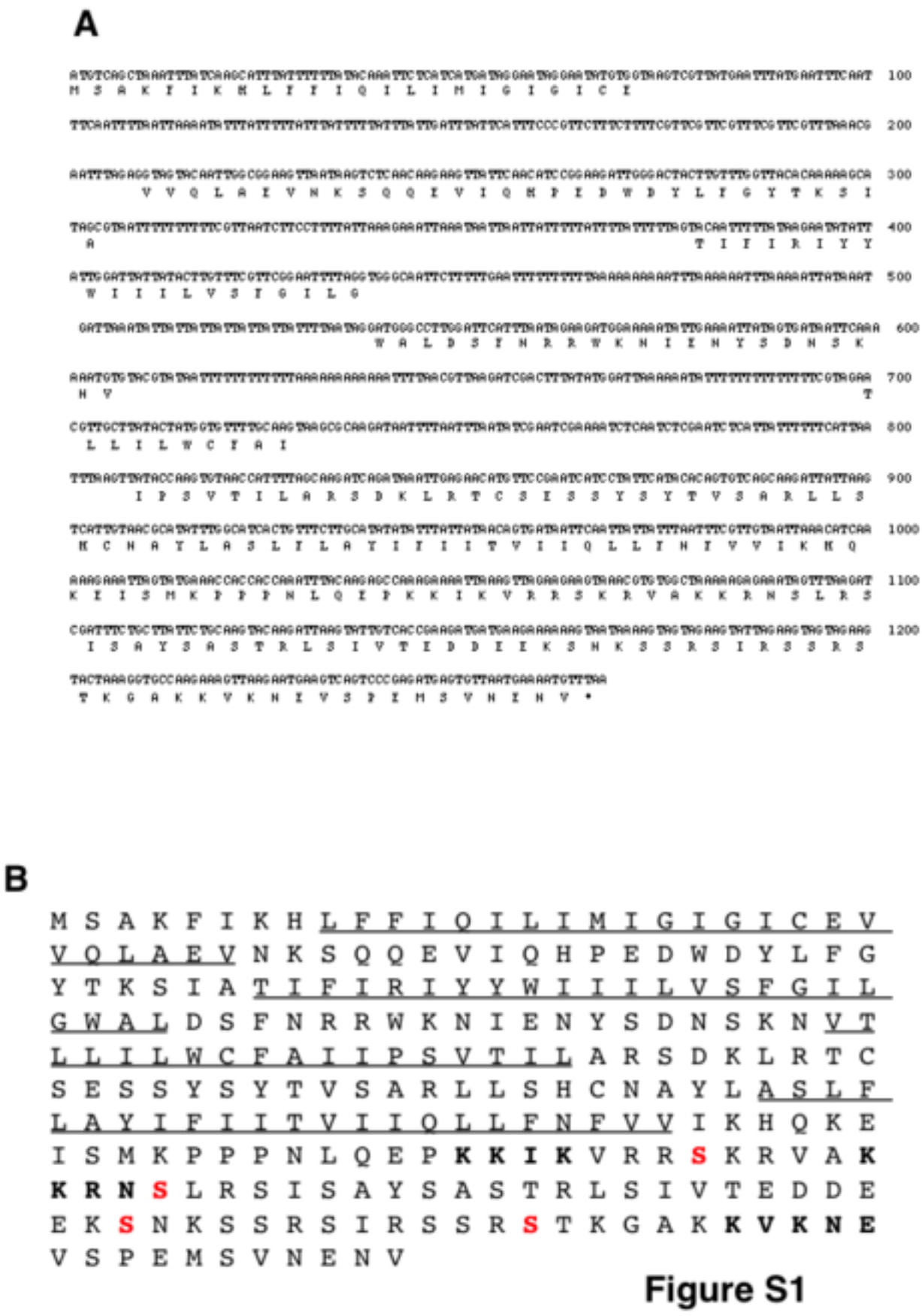

Fig S2

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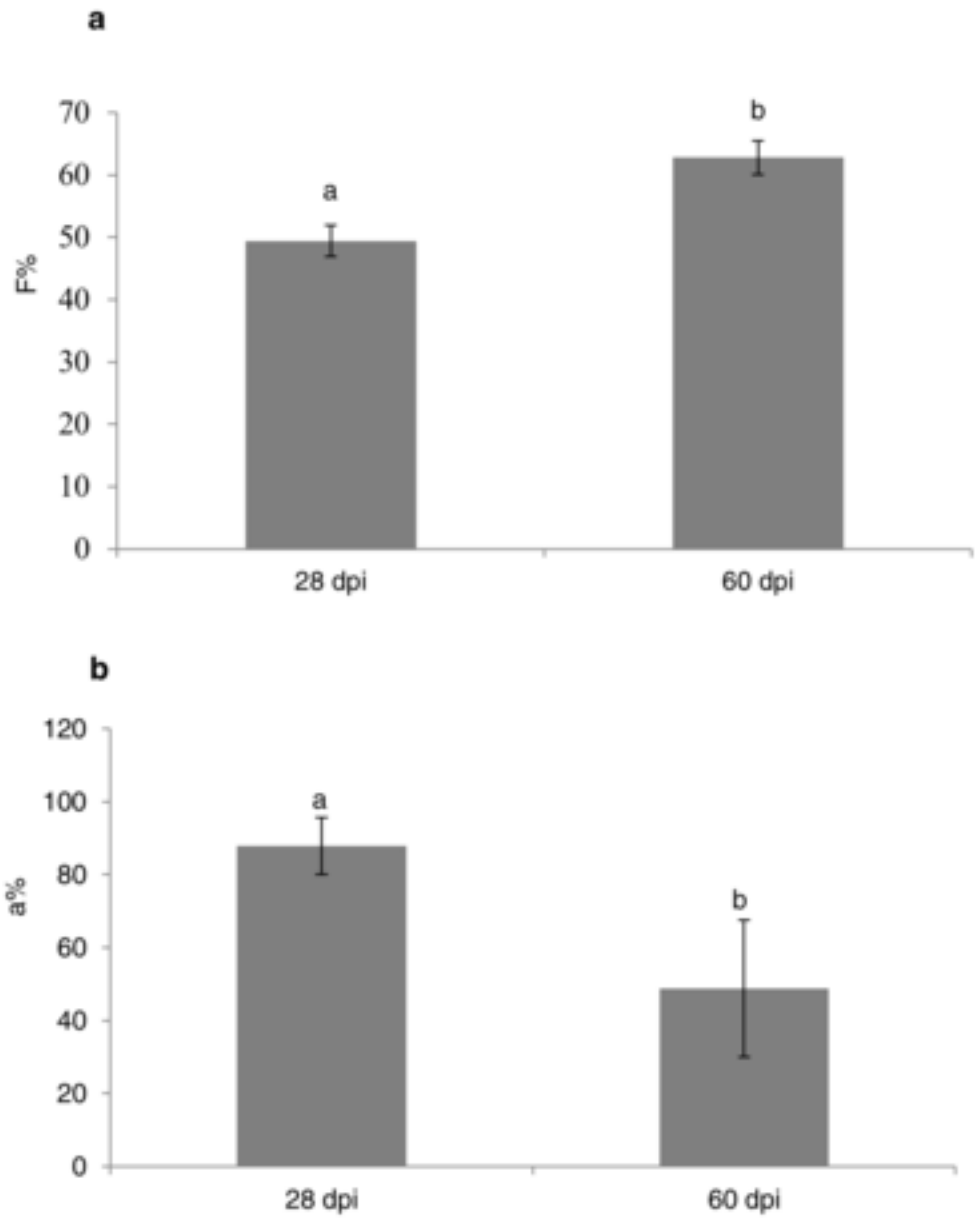

Figure S2

Fig S3

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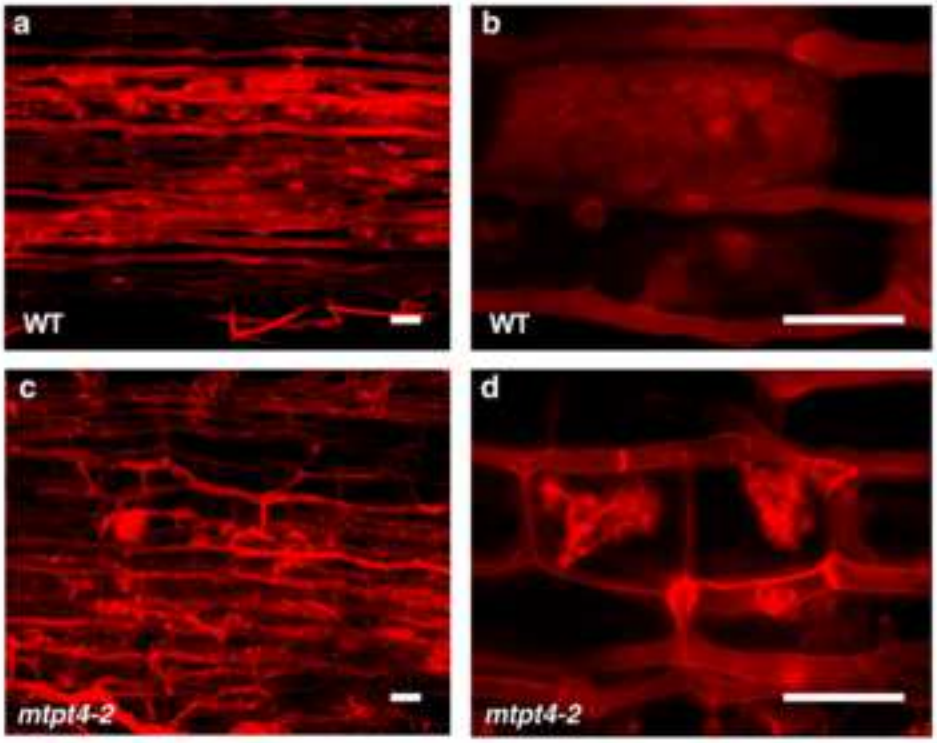

Figure S3

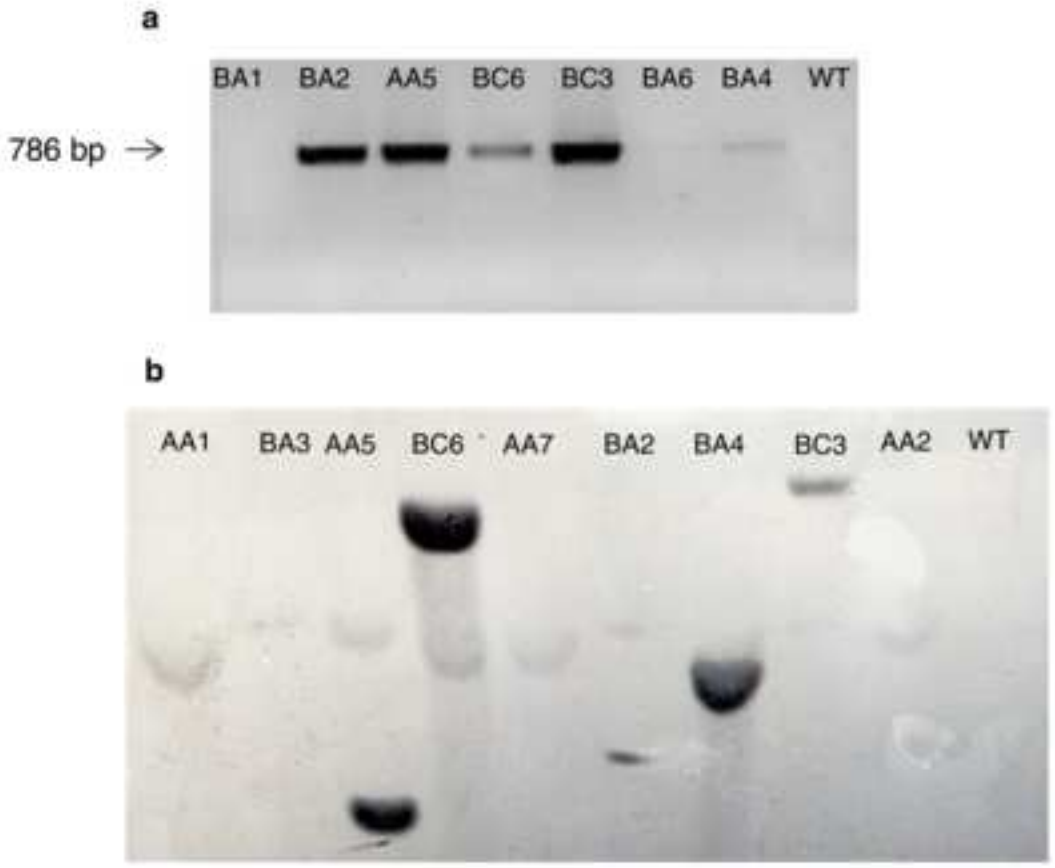

Figure S4

Fig S5

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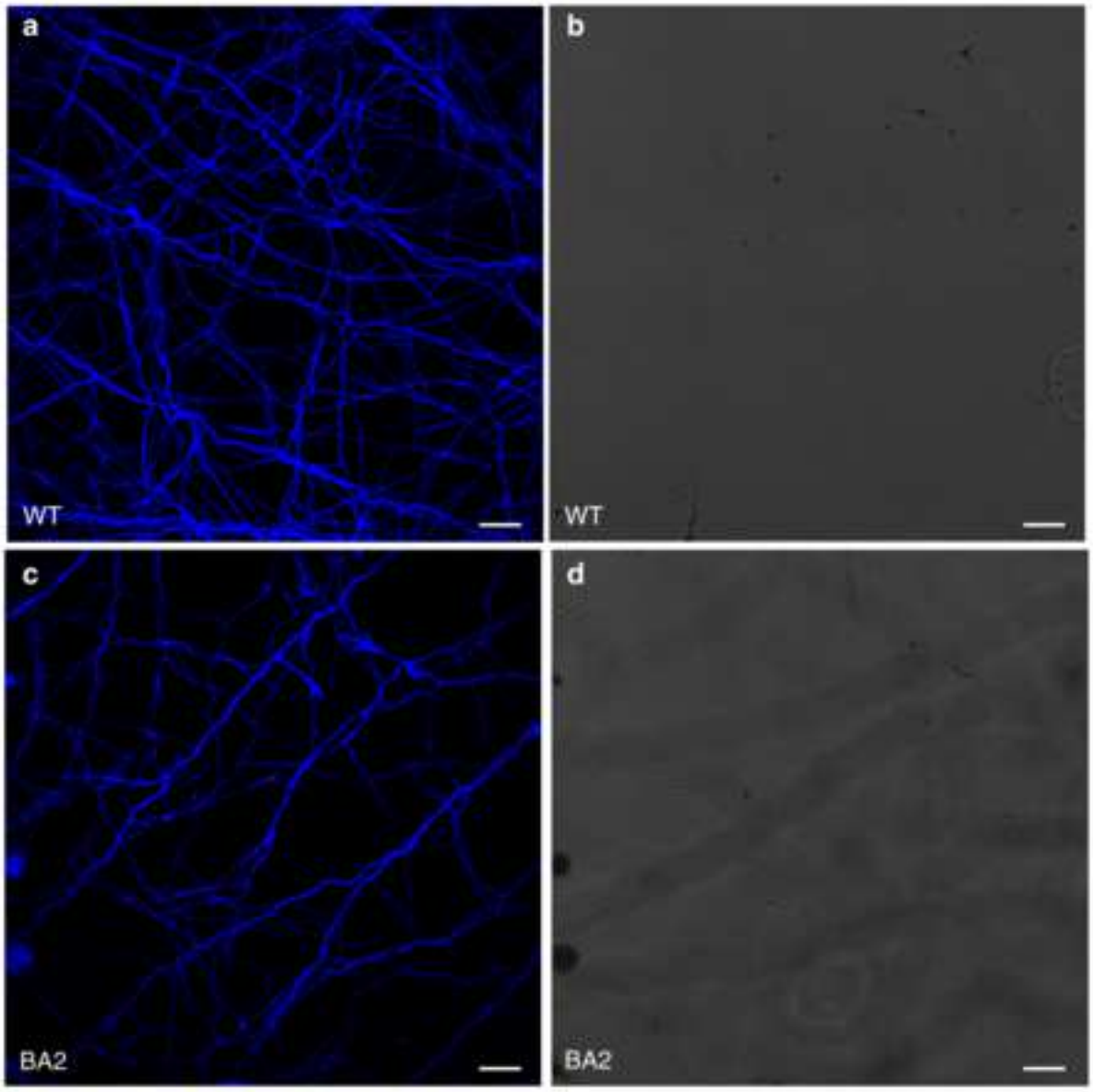

Figure S5

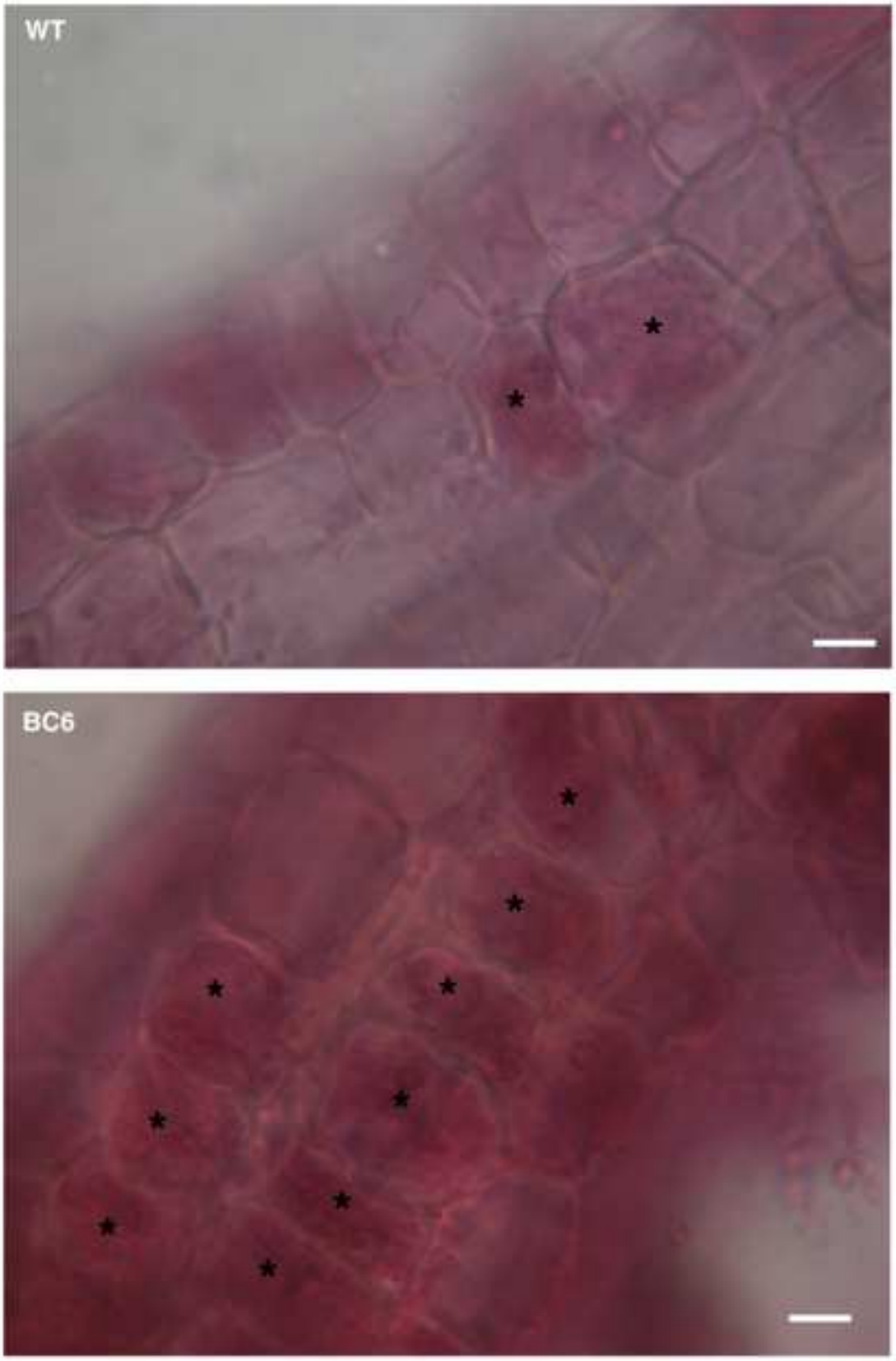

Figure S6

RiPEIP1, an orphan gene from the arbuscular mycorrhizal fungus *Rhizophagus irregularis*, is preferentially expressed *in planta* and may be involved in root colonization

Valentina Fiorilli[1], Simone Belmondo[1], Hassine Radhouane Khouja[1], Simona Abbà[2], Antonella Faccio[2], Stefania Daghino[1] and Luisa Lanfranco[1]

[1]Department of Life Science and Systems Biology, University of Torino, via Accademia Albertina 13, 10123 Torino, Italy

[2]Institute for Sustainable Plant Protection (IPSP), CNR, Strada delle Cacce 73, 10135 Torino, Italy

Corresponding author: Valentina Fiorilli

e-mail: valentina.fiorilli@unito.it

ABSTRACT

Transcriptomics and genomics data recently obtained from the arbuscular mycorrhizal (AM) fungus *Rhizophagus irregularis* have offered new opportunities to decipher the contribution of the fungal partner to the establishment of the symbiotic association. The large number of genes identified as orphan, and often lineage-specific, witnesses the uniqueness of this group of plant-associated fungi. In this work we characterize an orphan gene that was called *RiPEIP1* (*P*referentially *E*xpressed *I*n *P*lanta). Its expression is strongly induced in the intraradical phase, including arbuscules, and follows the expression profile of the *Medicago truncatula* phosphate transporter *MtPT4*, a molecular marker of a functional symbiosis. Indeed, *mtpt4* mutant plants, which exhibit low mycorrhizal colonization and an accelerated arbuscule turnover, also show a reduced *RiPEIP1* mRNA abundance. To further characterize *RiPEIP1*, in the absence of genetic transformation protocols for AM fungi, we took advantage of two different fungal heterologous systems. When expressed as a GFP fusion in yeast cells, RiPEIP1 localizes in the endomembrane system, in

particular to the endoplasmic reticulum, which is consistent with the *in silico* prediction of four transmembrane domains. We then generated *RiPEIP1*-expressing strains of the fungus *Oidiodendron maius*, the only endomycorrhizal fungus for which transformation protocols are available. Roots of *Vaccinium myrtillus* colonized by *RiPEIP1*-expressing transgenic strains showed a higher mycorrhization level compared to roots colonized by the *O. maius* wild type strain, suggesting that *RiPEIP1* may regulate the root colonization process.

Keywords: Arbuscular Mycorrhizal Symbiosis, *Rhizophagus irregularis*, *Oidiodendron maius*, Heterologous expression system.

INTRODUCTION

The arbuscular mycorrhiza (AM), one of the most widespread symbiosis on earth, occurs between about 80% of land plants and soil fungi belonging to the ancient *Glomeromycota* Phylum (Redecker et al. 2013). This intimate mutualistic association allows the host plant to gain mineral nutrients and water from the soil *via* the activity of the large network of extraradical mycelium (Javot et al. 2007; Govindarajulu et al. 2005; Allen and Shachar-Hill 2009) while, in turn, the fungus acquires plant photoassimilates (up to 20%) that are essential to progress into the different developmental stages (Pfeffer et al. 1999). AM fungi are important members of the plant microbiome and provide important ecosystem services; they are therefore of great interest for the development of a sustainable and low-input agriculture (Gianinazzi et al. 2010).

The root colonization process comprises three main stages: i) a presymbiotic phase where the partners recognize each other through the exchanges of chemical compounds (Bonfante and Requena 2011; Bonfante and Genre 2015); ii) the root penetration where, after the formation of a hyphopodium on the root surface, the epidermal cell develops the pre-penetration apparatus to guide the entrance/accommodation of the fungal hypha (Genre et al. 2005, 2008); iii) the intraradical fungal growth, which culminates with the development of highly branched intracellular structures, the arbuscules, where nutrient exchanges between the two partners are thought to occur (Gutjahr and Parniske 2013). Arbuscule developmental dynamics was elegantly described by live cell imaging in rice roots: arbuscules were confirmed to be ephemeral structures with a lifetime at maturity of approximately two to three days (Kobae and Hata 2010).

AM fungi display many unusual biological features beside the obligate biotrophism, spores and hyphae contain multiple nuclei, making classic genetic approaches challenging (Lanfranco and Young 2012; Young 2015). No sexual cycle has ever been described although meiosis-related genes were found in the genome (Tisserant et al. 2013; Riley et al. 2014). Moreover, their genomic structure was for long time obscure, and it has been questioned whether nuclei were in a heterokaryotic (Hijri and Sanders 2005; Ehinger et al. 2012) or in a homokaryotic condition

(Pawlowska and Taylor 2004). Recent data from the genome sequence of the model AM fungus *Rhizophagus irregularis* (isolate DAOM 197198; Tisserant et al. 2013), also at the level of a single fungal nucleus (Lin et al. 2014), strongly support the homokaryotic status.

Several transcriptomic studies, mainly based on large-scale gene expression analysis, have been applied in the last decade to decipher the molecular mechanisms that accompany the formation of arbuscular mycorrhizas. They focused almost exclusively on the host plant (Salvioli and Bonfante 2013 and references within), whereas only a few studies addressed the fungal partner (Requena et al. 2002; Breuninger and Requena 2004; Cappellazzo et al. 2007; Kikuchi et al. 2014; Salvioli et al. 2016). A major advance has been obtained with transcriptomics and genomics data of *R. irregularis* (Tisserant et al. 2012; 2013; Ruzicka et al. 2013; Lin et al. 2014). The genome of *R. irregularis* is one of the largest (153 Mb) fungal genome sequenced to date, along with those of obligate biotrophic powdery mildews (Spanu et al. 2010) and the ectomycorrhizal symbiont *Tuber melanosporum* (Martin et al. 2010). The obligate biotrophy of AM fungi is not explained by genome erosion or any related loss of metabolic complexity in central metabolism. One striking genomic feature is the lack of genes encoding plant cell wall degrading enzymes in analogy to other obligate biotrophic pathogens (Spanu et al. 2010) and ectomycorrhizal symbionts (Martin et al. 2010).

Tisserant et al. (2012) provided the first genome-wide overview of the transcriptional changes that occur in the different fungal life stages. In particular, the abundance of c. 18,500 fungal non-redundant expressed transcripts was analyzed in spores, extra- and intraradical mycelium, and arbuscules. Interestingly, several transcripts coding for Small Secreted Proteins (SSPs) were identified as being induced in the intraradical mycelium (IRM) and in arbuscule-containing cells (ARB), as compared to the extraradical mycelium (ERM). This SSPs list included the recently described secreted effector protein SP7, which is the first gene described so far to play a crucial role in the accommodation of the fungus within the plant root. In particular, SP7 counteracts the plant

immune response by interacting with the pathogenesis-related transcription factor Ethylene Response Factor (*ERF19*) in the host nucleus (Kloppholz et al. 2011).

This transcriptomic dataset has been instrumental for the characterization of several fungal genes (Li et al. 2013; Belmondo et al. 2014; Tamayo et al. 2014), providing new insights into the genetic program activated during the AM symbiosis and into the genetic characteristics, similarities and uniqueness of AM organisms. Taking advantage of laser microdissection, we identified four genes which were preferentially expressed in the intraradical phase, including arbuscules: three were orphan genes and one showed similarities with ABC transporters (Tisserant et al. 2012).

In this study, we characterize one of these orphan genes, that we called *RiPEIP1* (*P*referentially *E*xpressed *I*n *P*lanta) because its expression was strongly induced in the intraradical phase. Since stable transformation protocols are not available for AM fungi (Helber and Requena 2008; Helber et al., 2011), to characterize *RiPEIP1* we used two heterologous expression systems: *Saccharomyces cerevisiae*, a classical fungal model system and, for the first time, *Oidodendron maius*, the only endomycorrhizal fungus for which a stable transformation protocol is available (Martino et al. 2007).

RESULTS

RiPEIP1 gene isolation

With the aim to identify fungal genes involved in the functioning of arbuscules, the key structures of the AM symbiosis, we exploited transcriptomics data (Tisserant et al. 2012) generated for the AM fungus *Rhizophagus irregularis*. We focused our attention on an EST (contig Glomus_c13083 - v1 assembly) that turned out to be up-regulated in the intraradical phase in microarray experiments (Tisserant et al. 2012). In order to confirm the microarray data, the expression profile of Glomus_c13083 was monitored in intraradical (IRM) and extraradical (ERM) mycelium by quantitative RT-PCR (qRT-PCR). In particular, RNAs were extracted from ERM and from *M. truncatula* roots fragments from which ERM was carefully removed to generate the IRM sample.

Glomus_c13083 transcripts were highly abundant in the IRM compared to ERM (Fig. 1), and they were barely detected in spores (data not shown). From this expression profile, we called this gene *RiPEIP1* for *P*referentially *E*xpressed *I*n *P*lanta.

The 786 bp full length cDNA sequence of *RiPEIP1* was obtained by RACE assays. The corresponding genomic sequence (1267 bp), which comprises five introns (Supplementary Fig. S1A), was also obtained by conventional PCR. The recent release of the complete genome sequence of *R. irregularis* (Lin et al. 2014) confirmed the presence of this gene in the genome assembly (RirG_002110 - GenBank EXX79805.1), although the sequence was annotated with four additional amino acids at the N-terminus. Considering that the coding sequence isolated through several independent 5'-RACE-PCR assays never reported the presence of these four amino acids we considered the shorter cDNA sequence for the further analysis.

The predicted protein sequence (261 amino acids) showed no significant similarity (all E-values > 0.2) with proteins deposited in the NCBI "nr" database. The only hit found by a BlastP analysis, spanning more than 50% of the sequence length, was another *R. irregularis* protein with unknown function (GenBank ESA21063.1, percentage of identity 22%; E-value 0.38). The second round of a Psi-Blast analysis, including this protein, identified three additional *R. irregularis* proteins (GenBank EXX76561.1, ESA00383.1, EXX59269.1) of unknown function slightly shorter (less than 200 amino acids) than RiPEIP1. Interestingly, a TBlastN search within an extensive EST dataset recently published for another AM fungus, *Gigaspora margarita* (Salvioli et al. 2016), led to the identification of a cDNA sequence (GenBank: GBYF01016486.1; percentage of identity 26%; E-value 0.036) coding for a 182 amino acids polypeptide, which is probably a partial sequence lacking the C-terminus.

TMHMM analysis of the RiPEIP1 amino acid sequence revealed the presence of four transmembrane-helix domains with cytoplasmic N- and C-terminus regions (Supplementary Fig. S1B). Additionally, the WoLFPSORT subcellular location predictor identified RiPEIP1 as a

putative integral membrane protein. Remarkably, the sequence from *G. margarita*, notwithstanding the rather low similarity percentage, presents the same four transmembrane domains topology.

The C-terminus region, just after the fourth transmembrane domain, is highly hydrophilic, with a high percentage of charged (lysine, arginine, aspartate and glutamate) and polar (especially serines) amino acids. Some serines were also predicted (score > 0.99) by the NetPhos 2.0 Server to be potentially phosphorylated (Supplementary Fig. S1B). Notably, this region contains three typical ER-retention/retrieval C-terminus motifs: two KKXX motifs (starting from amino acid 188 and amino acid 200) and one KXKXX motif (starting from amino acid 246) (Jackson et al. 1990; Supplementary Fig. S1B).

RiPEIP1 is localized in the endomembrane system

In order to investigate the sub-cellular localization of RiPEIP1, we expressed a RiPEIP1::GFP fusion construct in *Saccharomyces cerevisiae* yeast cells. The fluorescent signal was observed outlining the nucleus and extending into a network-like pattern in the cytoplasm (Fig. 2A). To better characterize this protein localization, we compared the RiPEIP1::GFP fluorescence pattern with that of two strains constitutively expressing the red fluorescent protein (RFP) fused to Sec13, which is a marker of the endoplasmic reticulum and Golgi stacks, or Cop1 which is localized in the early Golgi. Indeed, Sec13::RFP showed a very similar pattern to RiPEIP1::GFP, with the addition of several bright spots in the cytoplasm (Fig. 2C). Since analogous spots were observed in the Cop1::RFP line (Fig. 2D), we concluded that the RiPEIP1::GFP fluorescence pattern was compatible with protein localization in the endoplasmic reticulum and nuclear envelope. These results are in line with *in silico* predictions.

***RiPEIP1* expression profiles**

To monitor the dynamics of *RiPEIP1* expression pattern along the colonization process, we set up a time course experiment of *M. truncatula* plants inoculated with *R. irregularis* in the sandwich

system and sampled 7, 14, 28 and 60 days post-inoculation (dpi). Morphological analyses of roots revealed almost no intraradical fungal structures at 7 or 14 dpi. Mycorrhization frequency increased from 28 to 60 dpi, although arbuscules, detected starting from 28 dpi, decreased at 60 dpi (Supplementary Fig. S2). Since *RiPEIP1* was shown by qRT-PCR to be expressed at negligible levels in the ERM, gene expression was evaluated in whole mycorrhizal roots, without a distinction between IRM and ERM. *RiPEIP1* mRNA abundance increased in parallel to the development of the intraradical phase, and in particular to arbuscules formation, as demonstrated by morphological data and by the parallel mRNA accumulation of *MtPT4*, the *M. truncatula* phosphate transporter-encoding gene which is considered a molecular marker of arbuscule-containing cells (Harrison et al. 2002; Fig. 3 A,B).

Using the laser microdissection, we investigated in more detail the *RiPEIP1* expression profile during the intraradical phase by comparing gene expression in arbusculated cells (ARB) and in non-colonized cortical cells from mycorrhizal roots (MNM). We used primers to the fungal housekeeping gene *RiTEFα* to monitor the presence of fungal structures in the two cell types, and primers to *MtPT4* to confirm the presence of arbuscules (Fig. 3C). Since the transcript for the fungal housekeeping gene *RiTEFα*, but not *MtPT4*, was also detected in the MNM cell population, we considered this sample representative of intercellular hyphae. As witnessed by the detection of *MtPT4* mRNA, *RiPEIP1* was only expressed in the ARB cell population, indicating that, in the intraradical phase, *RiPEIP1* expression occurred mainly in arbuscules and presumably not in intercellular hyphae (Fig. 3C).

To gather information on the relationship between *RiPEIP1* expression and arbuscule functionality, we analysed *RiPEIP1* expression profile in the *M. truncatula mtpt4-2* mutant line, which is defective of MtPT4. Inactivation of MtPT4 causes low mycorrhizal colonization and an increased number of stunted arbuscules as a result of accelerated arbuscules turnover (Javot et al. 2011; Fig S3). As expected, a lower colonization level, based on relative abundance of fungal to plant *Tef* transcripts, was observed for *mtpt4-2* compared to wt plants (Fig. 4A). To have an overview of

196 arbuscule abundance in the wt and *mtpt4-2* plants, we checked the expression level of the blue
197 copper-binding protein1 (*MtBCP1*), a protein localized in the plasma membrane of cortical cells
198 before and during the growth arbuscules and in the periarbuscular membrane surrounding arbuscule
199 trunks (Pumplin and Harrison 2009). *mtpt4-2* mycorrhizal plants showed a lower level of *MtBCP1*
200 transcripts compared to wt plants (Fig. 4B). Similarly, *RiPEIP1* mRNA abundance was lower in the
201 *mtpt4-2* genotype (Fig. 4C). *MtBCP1* and *RiPEIP1* expression levels showed a positive correlation
202 in wt and *mtpt4-2* genotypes (Fig. 4D). These data clearly indicate that *RiPEIP1* expression is
203 therefore associated to arbuscule development.
204
205 **Heterologous expression of *RiPEIP1* in *Oidiodendron maius***
206 To gain further information on RiPEIP1 function a novel fungal heterologous expression system has
207 been exploited. In particular, we expressed RiPEIP1 in the ericoid mycorrhizal fungus
208 *Oidiodendron maius*, the only endomycorrhizal fungus for which a protocol of genetic
209 transformation is available (Martino et al. 2007). By means of *A. tumefaciens*-mediated genetic
210 transformation, transgenic *O. maius* strains expressing *RiPEIP1* under a constitutive promoter were
211 obtained. We confirmed the presence of *RiPEIP1* in *O. maius* genome by PCR and Southern blot
212 analyses (Supplementary Fig. S4). Three transformants (BA2, BA4 and BC6) with a single genomic
213 insertion were selected for further analyses (Supplementary Fig. S4).
214 *RiPEIP1* expression in free-living mycelia of *O. maius* transformants was confirmed by RT-PCR
215 assays (Fig. 5A). As shown in Fig 5B the growth rate of *RiPEIP1*-expressing mycelia was similar to
216 that of the wt strain. A phenotypic analysis carried out on free-living mycelia stained with
217 calcoflour white showed no difference in hyphal morphology (Supplementary Fig. S5).
218 To investigate the impact of *RiPEIP1* expression on the establishment of the mycorrhizal symbiosis,
219 seedlings of *Vaccinium myrtillus*, a common host plant for ericoid fungi, were colonized *in vitro*
220 with wt or *RiPEIP1*-expressing strains. Plates with *V. myrtillus* seedlings without fungal inoculation
221 were also set up. As expected, after 2 months uninoculated seedlings were dead (Fig. 6A). Root

colonization level of inoculated plants was analysed through the morphological evaluation of the numbers of coils/root intersections. Interestingly, roots colonized by *O. maius RiPEIP1*-expressing strains showed a statistically significant higher mycorrhization degree compared to roots colonized by the wt strain (Fig. 6B: Fig S6). Moreover, morphological changes were also observed in the root apparatus colonized by *RiPEIP1*-expressing strains (Fig 7): in particular, a stimulation of root branching was observed. Roots colonized by the transgenic strains formed lateral roots up to the 6^{th} order while the roots colonized by the wt strain only developed up to 4^{th} order lateral roots (Fig. 7 C).

DISCUSSION

Among plant-associated microbes, AM fungi form the most ecologically and agriculturally important mutualistic association with plant roots in terrestrial ecosystems. Yet, the genetic determinants that control the fungal development *in planta,* which are necessary to support a long-lasting interaction between the partners, are still largely unknown. Transcriptomic (Tisserant et al. 2012) and genomic (Tisserant et al. 2013; Lin et al. 2014) data recently obtained for *R. irregularis* have offered new opportunities to decipher the contribution of the fungal partner to the establishment of the symbiotic association. A large number of genes expressed in the intraradical and the extraradical phases have been described as orphan and lineage-specific. This may be an indicator of the uniqueness of Glomeromycota. In this work, we have focused on the characterization of an orphan gene that was called *RiPEIP1* based on its expression profiles.

Based on bioinformatics analyses, RiPEIP1 is a protein with no similarity to known sequences; consistently with *in silico* predictions, RiPEIP1 seems to be a transmembrane protein localized in particular in the endoplasmic reticulum and nuclear envelope when expressed as a GFP fusion in yeast cells. It is worth to note that a similar sequence was found in the recently published transcriptome (Salvioli et al. 2016) of another AM fungus, *G. margarita*. Even if the sequence is only partial, lacking the C-terminus portion, and the percentage of identity is relatively low (22%),

248 the two proteins share the same 4 transmembrane domains topology. Moreover, RNA-seq data
249 showed an up-regulation of about 4 folds in mycorrhizal roots, as compared to germinating spores
250 (Salvioli et al. 2016). Only the search within genomic and transcriptomic data from other AM fungi,
251 once available, will clarify whether *RiPEIP1*-related sequences are a specific feature of
252 Glomeromycota and whether they may have a general role in the *in planta* phase.
253 *RiPEIP1* expression is strongly induced in the intraradical phase, and the time course experiment
254 showed that the highest expression levels were reached in mature mycorrhizal roots; *RiPEIP1*
255 mRNA abundance perfectly matches the expression profile of *MtPT4* (at 28 and 60 dpi), a
256 phosphate transporter essential for the acquisition of Pi delivered by the AM fungus (Javot et al.
257 2007) and thus considered a marker of a functional symbiosis. We therefore suggest that *RiPEIP1*
258 expression is, to some extent, related to arbuscules formation. This hypothesis is supported by the
259 fact that *RiPEIP1* transcripts were detected in laser microdissected arbuscule-containing cells, while
260 they were absent in adjacent cortical cells that likely contained only intercellular hyphae. To better
261 understand the relationship with arbuscule development, we analysed *RiPEIP1* expression in the *M.*
262 *truncatula mtpt4-2* mutant line that is defective of MtPT4. MtPT4 function was shown to be critical
263 for the AM symbiosis since its inactivation led to an altered arbuscules morphology, with premature
264 senescence (Javot et al. 2007). As expected, we observed a reduced level of fungal colonization in
265 *mtpt4-2* mutants, as monitored by *RiTEFα* transcripts abundance, in comparison with colonized
266 roots of wt plants. Interestingly, *mtpt4-2* mycorrhizal roots showed lower levels of *RiPEIP1*
267 mRNAs. Transcripts for *MtBCP1*, coding for a protein localized in the periarbuscular membrane
268 and considered a molecular marker of arbuscule development and of colonization of the root system
269 (Pumplin and Harrison 2009), were also found expressed at lower levels in the *mtpt4-2* genotype.
270 Overall the data support a relationship between *RiPEIP1* and arbuscule differentiation.
271 The involvement of *RiPEIP1* in the intraradical phase of the colonization process was also
272 supported by the heterologous expression of *RiPEIP1* in the ericoid mycorrhizal fungus *O. maiu*s.
273 In the absence of genetic transformation protocols for AM fungi, AM fungal genes have been

characterized by heterologous expression in filamentous fungi in few studies. To our knowledge, this approach was limited to pathogenic systems such as *Magnaporthe oryzae* (Kloppholz et al. 2011) or *Colletotrichum lindemuthianum* (Tollot et al. 2009). We suggest that *O. maius* could represent an additional, possibly more suitable biological system for the characterization of AM fungal genes, as it shares with AM fungi the capability to form an endomycorrhizal association and to colonize root cells at an intracellular level.

Roots colonized by *O. maius RiPEIP1*-expressing strains showed a higher mycorrhization degree compared to roots colonized by the wt strain. Although no *RiPEIP1* homolog has been found in the complete *O. maius* genome sequence (Kohler et al. 2015) and *V. myrtillus* is not a host for AM fungi, the stimulation of mycorrhization observed in this heterologous mycorrhizal system suggests a general role for *RiPEIP1* in endosymbiosis establishment or functioning. Remarkably, *O. maius* transgenic strains induced changes on *V. myrtillus* root morphology with the stimulation of lateral roots formation up to the 6^{th} order. The molecular basis of this phenomenon are unknown and deserve further investigations.

In summary, although the mechanism of action still remain obscure, we showed that *RiPEIP1*, an orphan gene from the AM fungus *R. irregularis*, is preferentially expressed *in planta* and may play a role in the root accommodation of fungal structures. Our data also underlies the potential of the endomycorrhizal fungus *O. maius* to characterize genes from AM fungi.

MATERIALS AND METHODS

Plant and fungal material

Seeds of *Medicago truncatula* Gaertn cv Jemalong and the *mtpt4-2* TILLING mutant (Javot et al., 2011) were treated as described in Fiorilli et al. (2013). *Rhizophagus irregularis* (Syn. *Glomus intraradices*, DAOM 197198) inoculum was obtained from *in vitro* monoxenic cultures of *Agrobacterium rhizogenes*-transformed chicory roots (Bécard and Fortin 1988) in two-compartment Petri plates, as described in Belmondo et al. (2014). Plates were incubated in the dark at 24°C until

300 the fungal compartment, containing a solid M medium without sucrose (M-C medium), was
301 profusely colonized by the fungus (approximately 6 weeks).
302 *M. truncatula* wilde type (WT) and *mtpt4-2* mycorrhizal plants were obtained in the sandwich
303 system (Giovannetti et al. 1993), inoculating seedlings with *R. irregularis* extraradical mycelium
304 (ERM) between two sterile nitrocellulose membranes. Plants were fertilized with Long-Ashton
305 nutrient solution containing 32 μM KH_2PO_4 and grown in climate-controlled rooms at 22°C with a
306 photoperiod of 14-h light and 10-h dark.
307 *Oidiodendron maius* (CLM1381.98 strain; Martino et al. 2000) was grown in Czapek-Dox medium
308 (supplemented with 1% agar for the solid medium). Petri dishes were kept in the dark at 25°C in a
309 dark room at 25°C for 2 months. Flasks were kept under the same conditions on an orbital shaker.
310 *In vitro* endomycorrhizas were synthesized as described by Abbà et al. (2009) with some
311 modifications. Axenic *V. myrtillus* seedlings (Les Semences du Puy, Le Puy-En-Velay, France)
312 were inoculated on modified Ingestad's medium (Ingestad, 1971), where four mycelium plug of wt
313 or transformants strains were previously grown for two months. Plates were placed in a growth
314 chamber (16 h photoperiod, light at 170 μmol m^{-2} s^{-1}, temperatures at 23°C day and 21°C night),
315 and roots were observed after 2 months of incubation. Four Petri dishes, each containing three
316 seedlings (for a total of twelve replicates), were used for each fungal genotype.
317
318 **Quantification of mycorrhizal colonization**
319 *M. truncatula* WT and *mtpt4-2* mycorrhizal roots were stained after two months of inoculation with
320 0.1% cotton blue in lactic acid and the estimation of mycorrhizal parameters was performed as
321 described by Trouvelot et al. (1986) using MYCOCALC (http://
322 www2.dijon.inra.fr/mychintec/Mycocalc-prg/download.html).
323 The degree of *V. myrtillus* mycorrhization was recorded after two months of inoculation by *O.*
324 *maius* Zn WT or *RiPEIP1*-expressing strains. The magnified intersections method (Villarreal-Ruiz
325 et al. 2004) was adapted to quantify the percentage of infection of *V. myrtillus* roots after staining

with acid fuchsin. The root system was examined under the microscope using the rectangle around the cross-hair as intersection area at 200X magnification. A total of 100 intersections per seedling root system were scored. Counts were recorded as percentage of root colonized (RC) by the fungus using the formula: RC% = 100 × Σ of coils counted for all the intersections, where Σ is the number of epidermal cells for all the intersections.

Biomass analysis

Conidia were harvested from two month-agar cultures of both WT and transformants by gently scraping cultures in 9 cm Petri dishes flooded with 1 ml of sterilized water. Conidia were counted in a Bürker counting chamber (Marienfeld, Germany) and, for each fungus, an aliquot containing a comparable number of conidia was transferred into liquid medium. After one month, fungal mycelia were harvested and the biomass weight evaluated. Analyses were carried out on three technical replicates for each biological condition.

Agrobacterium tumefaciens*-mediated transformation of *Oidiodendron maius

The pCAMBIA0380 (CAMBIA) was used as a backbone to construct the p*RiPEIP1* expression vector. The hygromycin resistance cassette (containing the *A. nidulans gpdA* promoter, the *hph* gene encoding resistance to hygromycin, and the *trpC* terminator from *A. nidulans*) was excised from pAN7-1 (Punt et al. 1987) with *Hind*III and BglII and inserted into the pCAMBIA030 at the HindIII/BglII sites to create the pCAMBIA0380_HYG. The insertion of the Hygromycin resistance cassette introduced at the end of the trpC terminator a XbaI restriction site that was not originally present in the pCAMBIA0380 vector. The vector was then *XbaI-XmaI* digested to insert another copy of the *A. nidulans gpdA* constitutive promoter, which was modified at the 3' end to carry also a *KpnI* restriction site before the *XmaI* site. *RiPEIP1* full length cDNA was then amplified using a forward primer (*RiPEIP1-K)* containing a *Kpn*I site and a reverse primer (*RiPEIP1-X)* containing a *Xma*I site (Table S1). The PCR product was *Kpn*I and *Xma*I digested and inserted into the *Kpn*I-

352 *Xma*I-digested pCAMBIA0380_HYG plasmid under the A. *nidulans gpdA* constitutive promoter.
353 The resulting recombinant plasmid was introduced into *A. tumefaciens* LBA1100 strain, which was
354 used to transform *O. maius* ungerminated conidia according to the protocol described by Abbà et al.
355 (2009). Transformants were isolated and transferred into 24-well plates with Czapek-Dox agar
356 medium supplemented with 100 µg/ml hygromycin B. Transgenic strains were confirmed by PCR
357 assays and Southern blot hybridization.
358 Genomic DNA of transformants strains was extracted using the CTAB protocol from mycelium
359 grown for 30 days in liquid Czapek-2% DOX medium. For Southern blot, 10 micrograms of
360 genomic DNA was digested with *Bgl*II restriction enzyme, size-fractionated on 1% (w/v) agarose
361 gel and blotted onto nylon membranes following standard procedures (Sambrook and Russel 2001).
362 Hybridization with a chemiluminescent detection system (ELC Direct DNA labelling and Detection
363 System, Amersham) was performed according to the manufacturer's recommendation using a probe
364 corresponding to the full length *RiPEIP1* cDNA sequence using *RiPEIP1* full length (RiPEIP1fl)
365 forward/reverse primer (Table 1). Probe labelling and high stringency hybridization were carried
366 out using ECL protocol (GE Healthcare, Chalfont St. Giles, U.K.).
367
368 **5′- and 3′ RACE**
369 Both 5′- and 3′ RACE were performed on total RNA extracted from the mycorrhizal roots with the
370 SMART RACE cDNA amplification kit (Clontech). The PCR product was obtained using the
371 primers *RiPEIP1*-race-forward/reverse (Table 1). PCR was performed according to the Clontech
372 protocol using the Advantage 2 PCR enzyme system and 35 cycles of 95°C for 30 sec, 60°C for 30
373 sec and 72°C for 2 min, with a final extension at 72°C for 10 min. The RACE products were cloned
374 into pCRII vector (TOPO cloning kit; Invitrogen) and sequenced.
375
376 **Nucleic acid extraction, cDNA synthesis, RT-PCR assay**

Total genomic DNA was extracted from *R. irregularis* ERM and *M. truncatula* shoot using the DNeasy Plant Mini Kit (Qiagen), according to the manufacturer's instructions. Each primer pair was first tested on plant or fungal genomic DNA as a positive control and to exclude cross hybridization.

Total RNA was extracted from *R. irregularis* ERM and mycorrhizal *M. truncatula* roots using the Plant RNeasy Kit (Qiagen), according to the manufacturer's instructions.

Total RNA was extracted from *O. maius* transformats and WT mycelia using a Tris-HCl extraction buffer (Tris-HCl 100 mM pH 8, NaCl 100 mM, Na-EDTA 20 mM, PVP 0.1 %, Na-laurylsarcosine 1 % in DEPC-treated H_2O), followed by phenol (Roti-Phenol, Roth) extraction, phenol:chloroform:isoamyl alcool (25:24:1) extraction, chloroform extraction and isopropanol precipitation (30 min at -80°C). The pellet was then resuspended in DEPC-treated water and precipitated in 6M LiCl (12 hours at 4°C). Finally, RNA was pelleted by centrifugation, rinsed with 70 % ethanol and resuspended in DEPC-treated H_2O.

Samples were treated with TURBO™ DNase (Ambion) according to the manifacturer's instructions. RNA samples were routinely checked for DNA contamination by means of RT-PCR (One-RT-PCR, Qiagen) analysis, using for *R. irregularis RiEF1α*, *M. trucatula MtTef*, and *O. maius OmTubulin* primers (Table 1).

For conventional RT-PCR analyses, RNAs were amplified with *RiPEIP1-qpcr* forward/reverse primers and for the *O. maius* tubulin housekeeping gene (Table 1) using the One Step RT-PCR kit (Qiagen). RNA samples were incubated for 30 min at 50°C, followed by 15 min of incubation at 95°C. Amplification reactions were run for 40 cycles at 94°C for 30 s, 60°C for 30 s, and 72°C for 40 s.

cDNA synthesis was carried out on about 700 ng of total RNA which was denatured at 65°C for 5 min and then reverse-transcribed at 25°C for 10 min, 42°C for 50 min and 70° for 15 min in a final volume of 20 µl containing 10 µM random primers, 0.5 mM dNTPs, 4 µl 5X buffer, 2 µl 0.1 M DTT, and 1 µl Super-ScriptII (Invitrogen).

403 **Quantitative RT-PCR**

404 Quantitative RT-PCR (qRT-PCR) assays were performed using an iCycler apparatus (Bio-Rad) as
405 described in Belmondo et al. (2014) with primers pairs listed in Table S1. All reactions were
406 performed on three technical replicates and on at least three biological replicates. Baseline range
407 and Ct values were automatically calculated using iCycler software. Transcript levels were
408 normalized to Ct values registered for the *RiEF1α* (Gonzàlez-Guerrero et al. 2010) fungal gene and
409 the *MtTef* (Hohnjec et al. 2005) plant gene. Only Ct values leading to a Ct mean with a standard
410 deviation below 0.5 were considered.

411

412 **Laser microdissection (LMD)**

413 *M. truncatula* mycorrhizal roots, obtained using the sandwich method, were dissected, fixed and
414 embedded in paraffin according to the method described in Perez-Tienda et al. (2011). A Leica AS
415 LMD system (Leica Microsystem, Inc.) was used to collect arbuscule-colonized cortical cells
416 (ARB) and non-colonized cortical cells (MNM) from paraffin root sections, as described by
417 Balestrini et al. (2007). Two thousand ARB and MNM cells (for each biological replicate) from *M.*
418 *truncatula* roots were collected. RNA was extracted following the Pico Pure kit (Arcturus
419 Engineering) protocol. A DNAse treatment was performed using an RNA-free DNase Set (Qiagen)
420 in a Pico Pure column, according to the manufacturer's instructions. RNA was then quantified using
421 a NanoDrop 1000 spectrophotometer. DNA contamination in RNA samples was evaluated using
422 *RiEF1α* (Table 1) by means of RT-PCR assays carried out using One Step RT-PCR kit (Qiagen).

423

424 **Construction of GFP fusion proteins for expression in yeast**

425 The full length cDNA of *RiPEIP1* was amplified from *R. irregularis* cDNA by PCR using the
426 Phusion DNA-Polymerase (Finnzymes, Espoo, Finland). cDNAs were amplified using a forward
427 primer containing the *Kpn*I site (*RiPEIP1-K*) and a reverse primer containing the *Not*I site
428 (*RiPEIP1-N)* (Table S1). The PCR products were *Kpn*I and *Not*I -digested and inserted into the

*Kpn*I-*Not*I-digested pYES2-GFP plasmid (Blaudez et al. 2003) under the control of the GAL1 promoter and allowing a 3' fusion with the enhanced GFP reporter gene. *Saccharomyces cerevisiae* (BY4742 strain) transformation was performed using the lithium acetate based method described by Gietz et al. (1992). As a control of subcellular localization, two yeast strains constitutively expressing the red fluorescent protein fused to Sec13 (marker of both the endoplasmic reticulum and Golgi stacks) or Cop1 (marker of early Golgi) were analysed in parallel (http://yeastgfp.yeastgenome.org/info.php).

Microscopy

Fluorescence emission from yeast cells expressing RiPEIP1::GFP, Sec13::RFP or Cop1::RFP was examined with a Leica TCS-SP2 confocal laser-scanning microscope equipped with a 63x water immersion objective. GFP was excited at 488 nm (Ar laser) and fluorescence was detected at 515-530 nm. RFP was excited at 546 nm and fluorescence was detected at 570-650 nm.

For acid fuchsin staining mycorrhizal roots were stained with 0.01% (w/v) acid fuchsin in lactoglycerol (lactic acid-glycerol-water, 14:1:1; Kormanik and McGraw 1982). Confocal microscopy observations were done using a Leica TCS-SP2 microscope equipped with a 40x long-distance objective. Acid fuchsin fluorescence was excited at 488nm and detected using a 560-680 nm emission window.

For calcofluor white (CFW; Fluorescent Brightener 28, F3543; Sigma-Aldrich) staining *O. maius* mycelia were let grown on a coverslip. The CFW solution was dropped onto the coverslips immediately before observation. CFW fluorescence was visualized with a Leica TCS-SP2 confocal laser-scanning microscope using a 405nm diode and an emission window at 410-460nm.

Statistical analyses

453 Statistical analyses were performed through one-way ANOVA and Tukey's post hoc test, using a
454 probability level of $p<0.05$. All statistical elaborations were performed using PAST statistical
455 package (version 2.16; Hammer et al. 2001).
456
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FIGURE LEGENDS

Figure 1 *RiPEIP1* is up-regulated in the intraradical mycelium. Expression of *RiPEIP1* (relative to *RiTef*) assessed by qRT-PCR in intraradical mycelium (IRM) and extraradical mycelium (ERM) from mycorrhizal roots of *M. truncatula* grown in the sandwich system (n= 5). Single data for each condition are shown as dots and the median as black bars. Asterisks indicate a statistically significant difference ($p < 0.01$, ANOVA)

Figure 2 Confocal imaging of RiPEIP1::GFP localization in yeast cells. The observed fluorescence pattern (**a**) is compatible with GFP localization in the endoplasmic reticulum and nuclear (n) envelope. A brightfield image of the same cells is shown in panel **b**. RiPEIP1::GFP localization is confirmed by a comparison with the fluorescence pattern from constitutively expressed Sec13::RFP (**c**), which labels the endoplasmic reticulum/nuclear envelope (n) and Golgi stacks, and Cop1::GFP (**d**), which targets the early Golgi stacks (arrowheads). Bars: 5 µm

Figure 3 *RiPEIP1* gene expression along the AM colonization process. Relative expression of *MtPT4* (**a**) and *RiPEIP1* (**b**) assessed by qRT-PCR in a time course experiment of root colonization at 7, 14, 28 and 60 days post-inoculation (dpi). Single data for each condition are shown as dots and the median as black bars. Different letters indicate statistically significant difference ($p < 0.05$, ANOVA). (**c**) Gel electrophoresis of RT-PCR products obtained from two independent samples of RNA from laser-microdissected arbuscule-containing cells (ARB) and one sample of not colonized cortical cells from mycorrhizal roots (MNM) using primers specific for *MtPT4*, *RiTef* or *RiPEIP1*. No RNA sample (-)

Figure 4 *RiPEIP1* gene expression is affected in the *mtpt4-2* mutant line. The AM colonization of wt and *mtpt4-2* genotypes was evaluated through the assessment of fungal *RiTef* mRNA abundance (**a**); *MtBCP* expression was used as a marker of AM intraradical phase (**b**). The relative expression of *RiPEIP1* in wt and *mtpt4-2* roots is shown in panel **c**. The positive correlation on a linear regression model between the expression values of *RiPEIP* and *MtBCP* and the high R-squared

value demonstrated that the two genes have similar expression profiles in wt (grey rhombi) or *mtpt4-2* (black rhombi) genotypes (**d**). Asterisks indicate a statistically significant difference ($p < 0.01$, ANOVA)

Figure 5 Expression of RiPEIP1 in *Oidiodendron maius* does not affect the growth of free-living mycelia. Gel electrophoresis of RT-PCR products obtained from wt and three *RiPEIP1*-expressing (BA2, BA4, BC6) free-living strains using *RiPEIP1* specific primers (**a**). Dry weight of free-living mycelia of wt and transgenic strains grown in liquid cultures (**b**)

Figure 6 *RiPEIP1*-expressing strains led to a higher *Vaccinum myrtillus* root colonization level compared to the WT strain. (**a**) *In vitro* mycorrhization system between *V. myrtillus* seedlings and *O. maius.* Non inoculated *V. myrtillus* seedlings were unable to correctly develop (right panel) (**b**) The percentage of root colonization of *V. myrtillus* seedlings colonized with the *O. maius* wt or the three *RiPEIP1*-expressing strains was quantified after staining with acid fuchsin. Different letters indicate statistically significant difference ($p < 0.05$, ANOVA)

Figure 7 Representative details of the *V. myrtillus* root system colonized by wt (**a**) or BC6 RiPEIP1-expressing (**b**) strains after two months of inoculation. Note the stimulation of root branching in roots colonized by the RiPEIP1-expressing strain. (**C**) Average values and standard deviation of the number of lateral roots of the different orders in plant colonized by the wt or the transgenic strains (BA2, BA4, BC6). Bar = 1 cm

Supplementary material

Figure S1 Nucleotide and deduced amino acid sequences of *RiPEIP1*. **a**) *RiPEIP1* genomic DNA sequence showing the presence of five introns. **b**) RiPEIP1 protein sequence showing the 4 transmembrane domains (underlined), the typical ER-retention/retrieval motifs (bold) and the predicted phosphorylation sites (red). See text for details

Figure S2 Colonization level of *M. truncatula* roots at 28 and 60 days post inoculation (dpi) assessed accordingly to Trouvelot et al (1986). F%: frequency of mycorrhization in the root system (**a**), a%: arbuscules abundance in mycorrhizal pars of root fragments (**b**). Different letters indicate statistically significant difference ($p < 0.05$ ANOVA)

Figure S3 Mycorrhizal phenotype of *M. truncatula* wt (**a, b**) and *mtpt4-2* (**c, d**) roots colonized by *R. irregularis*. Roots were harvested 60 dpi, stained with acid fuchsin and observed with a confocal microscope. Arbuscules in *mtpt4-2* are degenerated as described in Javot et al. (2011). Bars = 25 µm

Figure S4 Molecular analyses of *O. maius* transgenic strains expressing *RiPEIP1*. (**a**) Gel electrophoresis of PCR products obtained from genomic DNA of wt and transgenic strains using *RiPEIP1* specific primers (**b**) Southern blot of genomic DNA from wt and transgenic strains restricted with *Bgl*II enzyme and hybridized with the *RiPEIP1* probe. Lanes corresponding to BC6, BA2, BA4 samples exhibited a single genomic insertion

Figure S5 Phenotype of *O. maius* free living mycelia from wt and *RiPEIP1*-expressing strain BA2 as revealed by calcoflour white staining. Laser-scanning microscope observation of one-month-old *O. maius* wt and transgenic strains stained with calcofluor white. Transmitted light images are shown on the right (**b, d**) and the corresponding fluorescence images on the left (**a, c**). Bars: 10 µm

Figure S6 Mycorrhizal phenotype of *O. maius* wt and *RiPEIP1*-expressing strain BC6. Mycorrhized *V. myrtillus* roots were stained with acid fuchsin and observed 2 months after fungal inoculation. *O. maius* transgenic strains showed an increased number of coils (asterisk) in epidermal cells compared to the wt.

735 **Table S1.** List of primers used in this study. Sites for GATEWAY recombination or restriction

736 enzymes are underlined.

Primer ID	Primer sequences [5'-3']
RiEFαf	GCTATTTTGATCATTGCCGCC
RiEFαr	TCATTAAAACGTTCTTCCGACC
MtTEFf	AAGCTAGGAGGTATTGACAAG
MtTEFr	ACTGTGCAGTAGTACTTGGTG
MtPT4 f	TCGCGCGCCATGTTTGTTGT
MtPT4r	CGCAAGAAGAATGTTAGCCC
RiPEIP1-attB-forward	GGGGACAAGTTTGTACAAAAAAGCAGGCTATGTCAGCTAAATTTATCAAGC
RiPEIP1-attB-reverse	GGGGACCACTTTGTACAAGAAAGCTGGGTCTTTAAACATTTTCATTAACACTC
RiPEIP1flF:	ATGTCAGCTAAATTTATCAAGC
RiPEIP1flR:	CTTTAAACATTTTCATTAACACTCATCTCG
RiPEIP1-N	GTCAGCGGCCGCCTTTAAACATTTTCATTAAC
RiPEIP1-K	GATCGGTACCATGTCAGCTAAATTTATC
RiPEIP1-X	GCATCCCGGGCTTTAAACATTTTCATTAAC
RiPEIP1-race-forward	AGTAGAAGCACTAAAGGTGCCAAGAAAAGT
RiPEIP1-race-reverse	TAACACTCATCTCGGGACTGACTTCATTCT
RiPEIP1-qpcrF	AAGAAAGTAAACGTGTGGCT
RiPEIP1-qpcrR	TAACACTCATCTCGGGACTG
OmTubulin-forward	GTTTCCATGAAGGAGGTTGAGG
OmTubulin-reverse	CAGAGAGCAGTCTGGACGTTGT

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Figure 4

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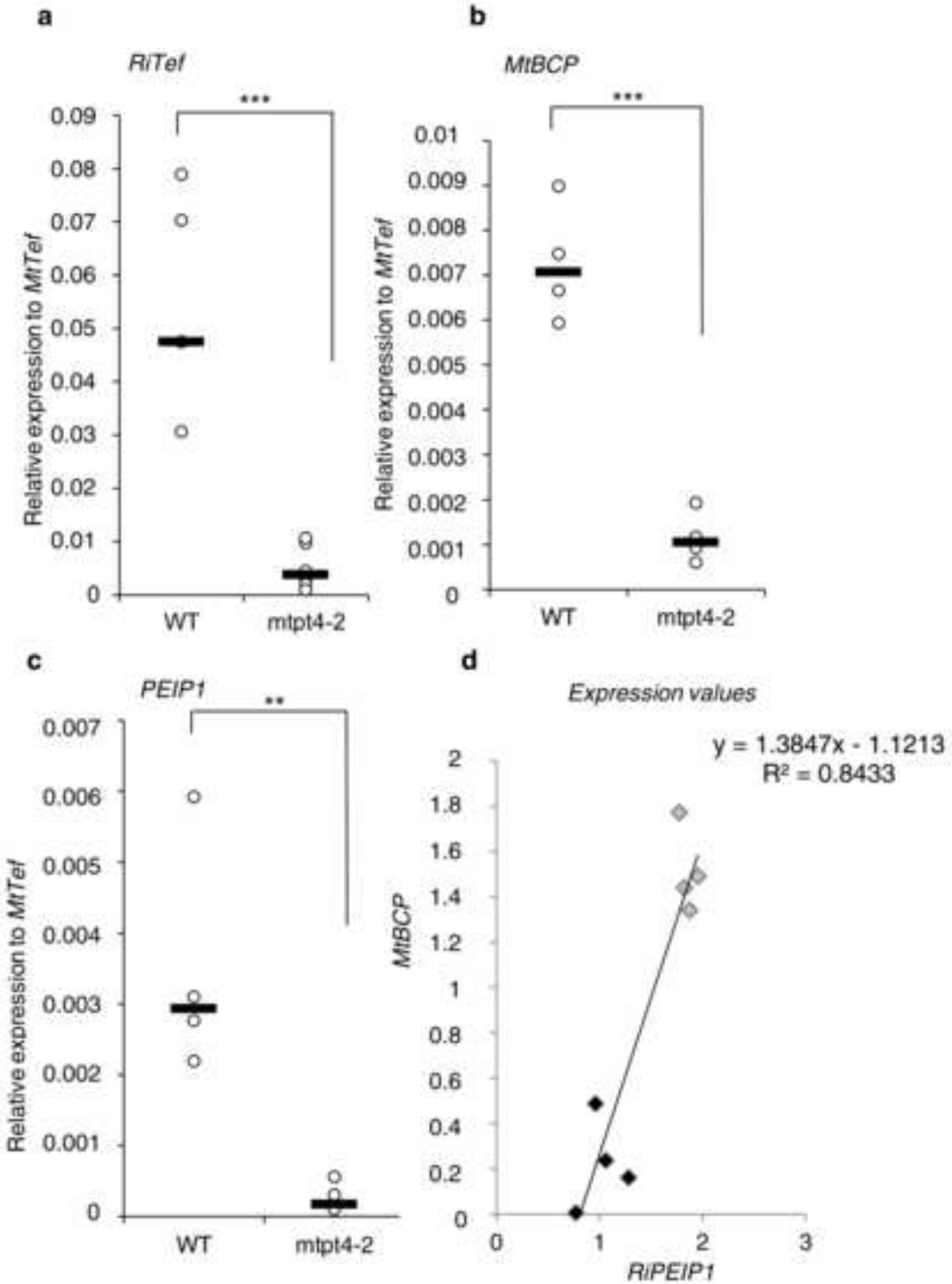
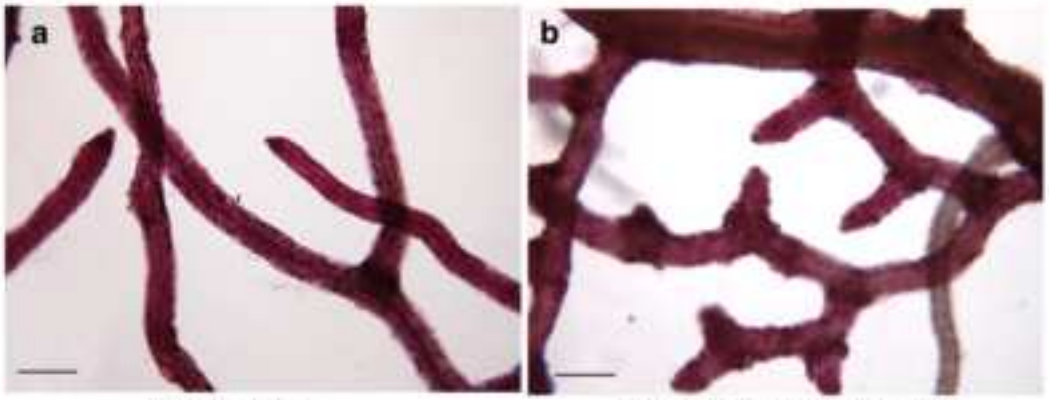


Figure 4

Figure 7



V. myrtillus + O. maius wt

V. myrtillus + O. maius BC6 - RiPEPI1-expressing strain

c

Lateral roots average values	Root orders	samples			
		BA2	BA4	BC6	WT
	1°	14 ± 7	14.6 ± 7.2	16 ± 10.3	9.6 ± 2
	2°	33.3 ± 25	39 ± 14.9	51.6 ± 38.4	17.3 ± 4
	3°	23 ± 24	31 ± 18-1	52 ± 28.7	10.3 ± 1.1
	4°	8.6 ± 9.8	15 ± 14	27 ± 18.5	2.3 ± 4
	5°	0.7 ± 1.1	6.3 ± 7	13.3 ± 8.7	0
	6°	0.3 ± 0.5	1.7 ± 1.5	2.3 ± 1.5	0

Figure 7

Fig S2

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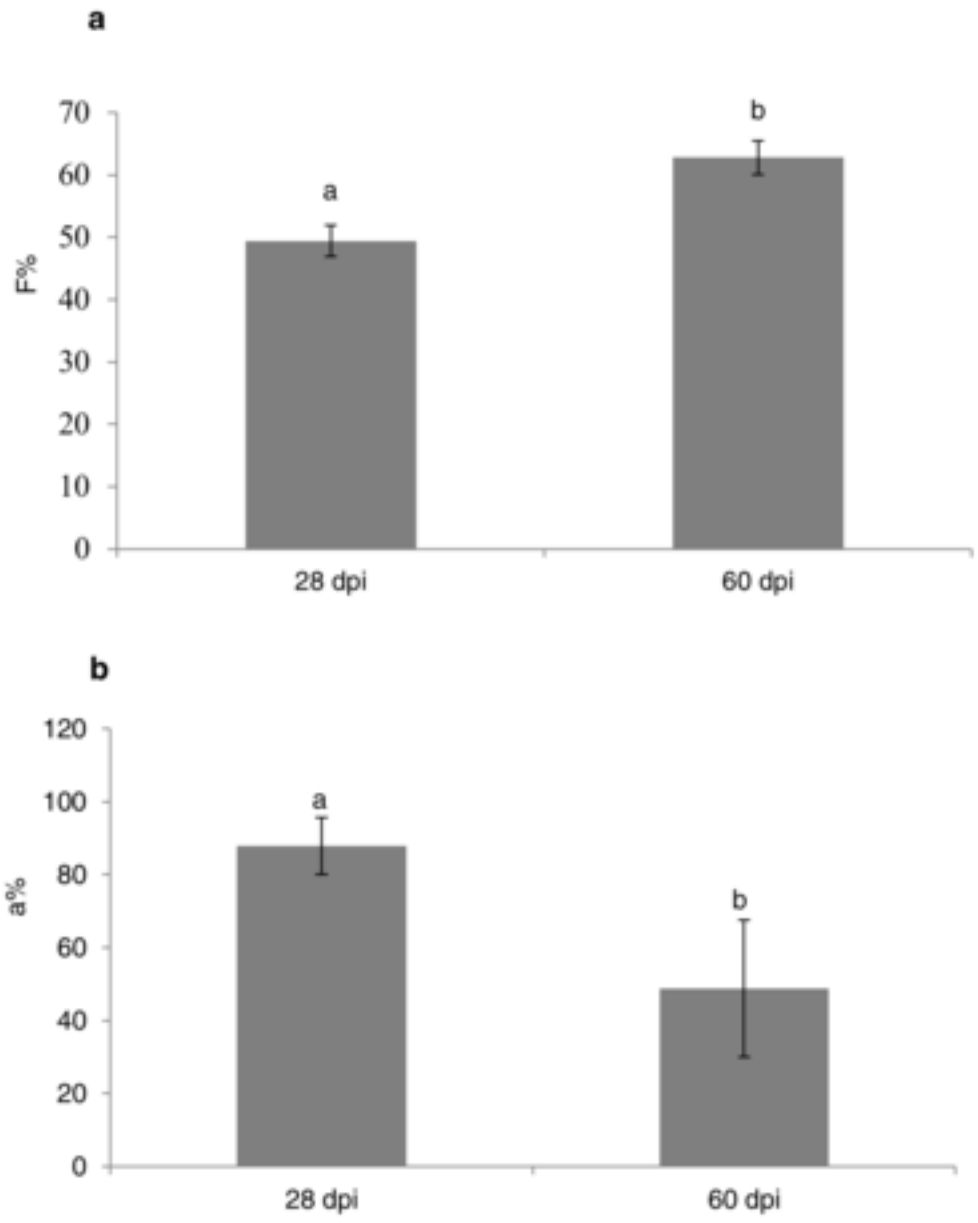

Figure S2

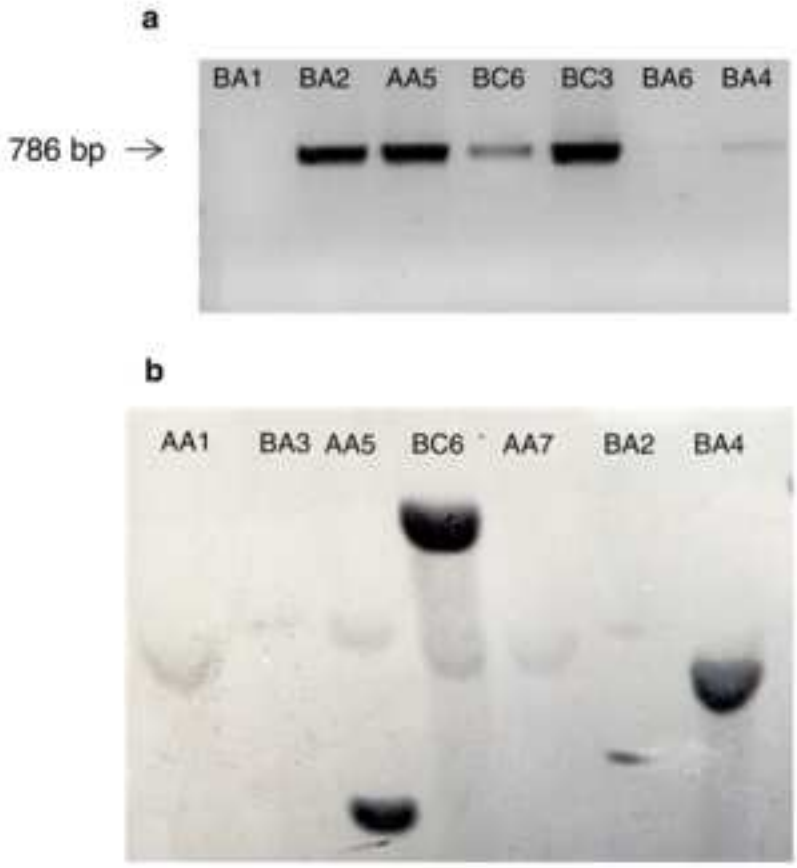

Figure S4