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A dipeptide transporter from the arbuscular mycorrhizal fungus *Rhizophagus irregularis* is upregulated in the intraradical phase

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Planta

The Medicago truncatula MtRbohE gene is activated in arbusculated cells and is involved in root cortex colonization --Manuscript Draft--

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Abstract:	<p>Plant NADPH oxidases, known as respiratory burst oxidase homologs (RBOH), belong to a multigenic family that plays an important role in the regulation of plant development and responses to biotic and abiotic stresses. In this study we monitored the expression profiles of five Rboh genes (MtRbohA, MtRbohB, MtRbohE, MtRbohG, MtRbohF) in the roots of the model species Medicago truncatula upon colonization by arbuscular mycorrhizal fungi. A complementary cellular and molecular approach was used to monitor changes in mRNA abundance and localize transcripts in different cell types from mycorrhizal roots. Rboh transcript levels did not drastically change in total RNA extractions from whole mycorrhizal and non mycorrhizal roots. Nevertheless, the analysis of laser microdissected cells and Agrobacterium rhizogenes-transformed roots expressing a GUS transcriptional fusion construct highlighted the MtRbohE expression in arbuscule-containing cells. Furthermore, the down regulation of MtRbohE by an RNAi approach generated an altered colonization pattern in the root cortex, when compared to control roots, with fewer arbuscules and multiple penetration attempts. Altogether our data indicate a transient up-regulation of MtRbohE expression in cortical cells colonized by arbuscules and suggest a role for MtRbohE in arbuscule accommodation within cortical cells.</p>	
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8 5 **Title:** The *Medicago truncatula* *MtRbohE* gene is activated in arbusculated cells and is involved in
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45 23 **Main Conclusion**
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47 24 Our study demonstrated that the NADPH oxidase gene *MtRbohE* is expressed in arbusculated cells and
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49 25 plays a role in arbuscule development.
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Abstract

Plant NADPH oxidases, known as respiratory burst oxidase homologs (RBOH), belong to a multigenic family that plays an important role in the regulation of plant development and responses to biotic and abiotic stresses. In this study we monitored the expression profiles of five *Rboh* genes (*MtRbohA*, *MtRbohB*, *MtRbohE*, *MtRbohG*, *MtRbohF*) in the roots of the model species *Medicago truncatula* upon colonization by arbuscular mycorrhizal fungi. A complementary cellular and molecular approach was used to monitor changes in mRNA abundance and localize transcripts in different cell types from mycorrhizal roots. *Rboh* transcript levels did not drastically change in total RNA extractions from whole mycorrhizal and non mycorrhizal roots. Nevertheless, the analysis of laser microdissected cells and *Agrobacterium rhizogenes*-transformed roots expressing a GUS transcriptional fusion construct highlighted the *MtRbohE* expression in arbuscule-containing cells. Furthermore, the down regulation of *MtRbohE* by an RNAi approach generated an altered colonization pattern in the root cortex, when compared to control roots, with fewer arbuscules and multiple penetration attempts. Altogether our data indicate a transient up-regulation of *MtRbohE* expression in cortical cells colonized by arbuscules and suggest a role for *MtRbohE* in arbuscule accommodation within cortical cells.

Keywords: Arbuscular mycorrhizal symbiosis, NADPH oxidase, *Medicago truncatula*, gene expression, reactive oxygen species, respiratory burst oxidase homolog

Introduction

Plants generate reactive oxygen species (ROS) as signaling molecules to control various cellular mechanisms (Neill et al. 2002; Apel and Hirt 2004). Pharmacological, molecular, and genetic studies strongly support that the primary source of ROS is a superoxide-generating membrane-bound NADPH oxidase (Torres and Dangel 2005) that catalyzes the production of superoxide by transferring electrons from NADPH to molecular oxygen, with secondary generation of H₂O₂. Seven mammalian NADPH oxidase enzymes have been identified and characterized: the best studied member of this family is the mammalian gp91phox (NOX2), which is responsible for high-level production of superoxide in phagocytic cells in response to microbial invasion (Aguirre and Lambeth 2010).

The NADPH oxidase homologs in plants, designated RBOH (respiratory burst oxidase homolog), are a family of enzymes, structurally more similar to mammalian calcium-regulated NADPH oxidase NOX5, which has an N-terminal calcium binding EF-hand motif (Oda et al. 2010; Suzuki et al. 2011; Marino et al. 2012). *Arabidopsis thaliana* possesses 10 RBOH homologues which differ in their expression profile and involvement in diverse processes of plant growth and metabolism (Sagi and Fluhr 2006; Suzuki et al. 2011). Only a few of these genes have been characterized: *AtRbohD* and *AtRbohF* are involved in ROS-dependent abscissic acid signalling in guard cells (Kwak et al. 2003), while *AtRbohC* plays a key role in root hair development (Foreman et al. 2003) and *AtRbohB* in seed germination (Müller et al. 2009). More recently, ROS production by *AtRbohH* and *AtRbohJ* has been clearly shown to be essential for proper pollen tube tip growth (Kaya et al. 2014).

RBOH-dependent superoxide generation by plants in response to microbial pathogen colonization is a well-known plant defense mechanism. ROS generation is associated to the oxidative burst linked to the perception of microbe/pathogen-associated molecular patterns and to the hypersensitive response coupled to the recognition of specific pathogens avirulence factors (Torres et al. 2006; Torres 2010). The activation of particular RBOH isoforms is responsible for ROS accumulation in several plant-microbe interactions (Simon-Plas et al. 2002; Torres et al. 2002; Torres and Dangel 2005; Yoshioka et al. 2003; for review see Torres 2010).

The involvement of ROS and RBOH enzymes in the legume-rhizobium symbiotic interaction has also been proved (for review Puppo et al. 2013). ROS accumulation has been detected in the wall of infected cells and infection threads, in both early steps of the interaction (Santos et al. 2001; Ramu et al. 2002; Rubio et al. 2004; Lohar et al. 2006; Peleg-Grossman et al. 2007, 2012) and in mature nodules

(Santos et al. 2001; Rubio et al. 2004). Delayed nodulation has also been observed in *Medicago truncatula* inoculated with a catalase-overexpressing *S. meliloti* strain, which acts as a H₂O₂ scavenger (Jamet et al. 2007). Recently, *Rboh* genes were shown to regulate the early steps of rhizobial infection in *Phaseolus vulgaris* (common bean; Montiel et al. 2012) and affect nodule function in *M. truncatula* (Marino et al. 2011). ROS therefore appear to be produced in response to rhizobial infection, in association with nodule development, and are essential for optimal symbiosis establishment (Puppo et al. 2013).

Even if the arbuscular mycorrhizal (AM) symbiosis is known to share several cellular and molecular features with legume nitrogen-fixing symbiosis (Guthjar and Parniske 2013; Venkateshwaran et al. 2013), little is known about ROS and RBOH-related processes in AM associations. Salzer et al. (1999) provided the first evidence for the accumulation of H₂O₂ in *M. truncatula*-*Rhizophagus irregularis* (formerly *Glomus intraradices*) mycorrhizal interaction, in particular in arbuscule-containing cortical cells, and hypothesized the involvement of a plant plasma membrane NADPH oxidase. H₂O₂ production was also observed in roots of *M. truncatula* and *Lotus japonicus* colonized by another AM fungus, *Gigaspora margarita*. In this case H₂O₂ accumulation was mainly associated with the fungal structures and this was mirrored by the up-regulation of a gene encoding a superoxide dismutase (SOD) in intraradical fungal structures (Lanfranco et al. 2005). SODs are the primary line of defence against oxidative stress also preventing hydroxyl radical formation through the Fenton reaction (Winterbourn 1995). In this frame, Fester and Hause (2005), using three independent staining techniques, suggested that both AM-colonized root cortical cells and fungal structures were involved in the H₂O₂ production.

Direct evidence of a specific role for an *Rboh* gene in the AM symbiosis has recently been provided by Arthikala et al. (2013), who demonstrated, through an RNA interference (RNAi) approach, that *PvRbohB* negatively regulates AM colonization in *Phaseolus vulgaris*. In line with this, the silencing of *MtROP9*, a small GTPase considered to be a positive regulator of RBOH enzymes, was shown to stimulate early mycorrhizal colonization in *M. truncatula* (Kiirika et al. 2012). Nevertheless, many questions remain open as to the involvement of other RBOH-encoding genes in the AM symbiosis.

We here investigated the expression profiles of five *Rboh* genes (*MtRbohA*, *MtRbohB*, *MtRbohE*, *MtRbohG*, *MtRbohF*) in mycorrhizal roots and root organ cultures (ROCs) of the model species *M. truncatula*: we used a complementary cellular and molecular approach to monitor changes in mRNA abundance and localize transcripts within different root cell types. Our results show that *MtRbohE* is

transiently expressed in arbusculated cells and has a role in root cortex colonization and arbuscule accommodation.

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115 **Materials and Methods**

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117 Biological materials, growth conditions and inoculation methods

118 *Rhizophagus irregularis* (Syn. *Glomus intraradices*, DAOM 197198) inoculum for seedlings and ROC
119 (root organ cultures) mycorrhization, was produced through *in vitro* monoxenic cultures. These were
120 established in bi-compartmental Petri dishes with a watertight plastic wall separating the root
121 compartment (RC) from the hyphal compartment (HC) (Fortin et al. 2002). The RC was filled with 25
122 ml of M minimal medium and an explant of transgenic chicory (*Cichorium intybus*) roots colonized
123 with the AM fungus was added. The HC was filled with 25 ml of solid M Minimal medium lacking
124 sugar. Once the mycelium of *R. irregularis* had grown over the plastic wall and completely filled the
125 HC compartment, the medium was dissolved with sterile citrate buffer 10 mM, pH 6.0 (mix 0.018 ml
126 of citric acid 0.1 M and 0.082 ml of sodium citrate 0.1 M and reach the final volume of 50 ml with
127 sterile distilled water). Spores were then collected and used for plant colonization.

128 Spores of *Gigaspora margarita* (BEG34) were collected from *Trifolium repens* L. pot cultures.
129 Aliquots of 100 spores were surface sterilized twice for 10 minutes with 3% chloramine-T and 0.03%
130 streptomycine sulfate, then rinsed several times with sterile distilled water.

131 To obtain seedlings colonized by *R. irregularis* or *G. margarita* the Millipore sandwich method
132 (Giovannetti et al. 1993) was used. Seeds of *Medicago truncatula* Gaertn cv Jemalong were first
133 scarified using sandpaper P180-200, sterilized with 5% commercial bleach for 3 minutes and rinsed
134 three times for 10 minutes with sterile distilled water. Germination was induced under sterile
135 conditions in 0.6% agar/water, incubated for 5 days in the dark (25°C) and then exposed at the light for
136 4 days. Plants were watered with Long-Ashton solution containing a low phosphorus concentration (3.2
137 μM $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$) (Hewitt 1966) and they were grown in a growth chamber under 14 h light
138 (24°C)/10 h dark (20°C) regime. Plants were harvested 60 days post-inoculation (dpi). For mycorrhizal
139 plants, only portions of the root system showing extraradical fungal structures were collected under a
140 stereomicroscope. The colonization level was assessed according to Trouvelot et al. (1986). For the
141 molecular analyses, roots were immediately frozen in liquid nitrogen and stored at -80°C.

Agrobacterium rhizogenes-transformed lines of *M. truncatula* expressing GUS-GFP reporter genes under the promoter of 5 different *Rboh* genes (*MtRbohA*, *MtRbohB*, *MtRbohE*, *MtRbohF* and *MtRbohG*; Marino et al. 2011) were inoculated with AM fungi. Spores of *R. irregularis* were collected and placed in Petri dishes containing the transgenic roots and cultured for 2 months at 26°C in the dark. For *G. margarita* the targeted AM inoculation technique developed by Chabaud et al. (2002) was chosen since it allows the direct observation of colonization events and easy collection of samples. Spores were placed in M medium and incubated at 30°C to induce germination. About ten germinated spores were then transferred to Petri dishes containing three growing *M. truncatula* hairy root explants and positioned below the growing lateral roots, to facilitate reciprocal contacts. Root cultures were incubated at 26°C in the dark and vertically oriented. Root and hyphal growth was followed daily under a stereomicroscope. After inoculation, *G. margarita* germ tubes grew upwards and branched, contacting root epidermis. Hyphopodia were generally observed after 5 days and first arbuscules were observed after 10 days. Root fragments surrounded by extraradical mycelium and displaying hyphopodia were selected under a stereomicroscope, excised and stored at -80°C.

Nucleic acid extraction and RT-qPCR assays

Total genomic DNA was extracted from *R. irregularis* extraradical structures, *G. margarita* spores and *M. truncatula* leaves using the DNeasy Plant Mini Kit (Qiagen), according to the manufacturer's instructions. Conventional PCR assay were set up to exclude cross-hybridization of *MtRboh* specific primers described by Marino et al. (2011) on fungal DNA.

Total RNA was isolated from about 20 mg root fragments for hairy roots and about 100 mg for seedling roots using the RNeasy Plant Mini Kit (Qiagen). Samples were treated with TURBO™ DNase (Ambion) according to the manufacturer's instructions. The RNA samples were routinely checked for DNA contamination by RT-PCR analysis, using primers MtTef-f 5'AAGCTAGGAGGTATTGACAAG 3' and MtTef-r 5' ACTGTGCAGTAGTACTTGGTG 3' for *MtTef* (Vieweg et al. 2005) and the One-Step RT-PCR kit (Qiagen) by omitting the incubation step at 50°C. The *MtPT4* phosphate transporter gene was amplified using MtPT4F (5'TCGCGCGCCATGTTTGTGT3') and MtPT4R (5'CGCAAGAAGAATGTTAGCCC3') primers (Fiorilli et al. 2013). For single-strand cDNA synthesis about 500 ng of total RNA were 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 µ M random primers, 0.5 mM dNTPs, 4 µl 5X buffer, 2 µl 0.1 M DTT, and 1 µl Super-ScriptII (Invitrogen).

qRT-PCR experiments were carried out in a final volume of 20 µl containing 10 µl of Power Sybr Green PCR master mix (Applied Biosystems), 1 µl of 3 µM *MtRboh* specific primers, and 1 µl of cDNA. Samples were run in the StepOne Real-Time PCR system (Applied Biosystems) using the following program: 10 min pre-incubation at 95°C, followed by 40 cycles of 15 s at 95°C, and 1 min at 60°C. Each amplification was followed by melting curve analysis (60°C to 94°C) with a heating rate of 0.3°C every 15 s. All reactions were performed with three technical replicates and only Ct values with a standard deviation that did not exceed 0.3 were considered. The comparative threshold cycle method (Rasmussen 2001) was used to calculate relative expression level using the *MtTef* as reference gene. The analyses were performed on at least three independent biological replicates. Statistical tests were carried out through one-way analysis of variance (one-way ANOVA) and Tukey's post hoc test, using a probability level of $p<0.05$.

Semi-quantitative RT-PCR on laser microdissected cells

Roots colonized by *R. irregularis* and uninoculated roots obtained with the millipore sandwich system, as described above, were dissected into 5-10 mm pieces and treated with ethanol and glacial acetic acid (3:1) under vacuum for 30 min, then placed at 4°C overnight. Roots were subsequently dehydrated in a graded series of ethanol (50%-70%-90% in sterilized water and 100% twice) followed by Neoclear (twice) with each step on ice for 30 min. Neoclear was gradually replaced with paraffin (Paraplast Plus; Sigma-Aldrich, St. Louis) according to PPlus; Pérez-Tienda et al. (2011). A Leica AS LMD system (Leica Microsystem, Inc.) was used to collect cortical cells from paraffin root sections as described by Balestrini et al. (2007).

RNA was extracted using the PicoPure kit protocol (Arcturus Engineering) and treated with RNase-free DNase (Promega Corp., Madison, WI, USA) following the manufacturer's instructions. RNAs were precipitated using Na-acetate-ethanol, and resuspended in 21 µl of sterile water.

All RT-PCR assays were carried out using the One Step RT-PCR kit (Qiagen). DNA contaminations

199 were assessed using the *MtTef* primers described above. RNAs extracted from the three different cell
200 populations were then calibrated using *MtTef* as housekeeping gene. In detail, reactions were carried
201 out in a final volume of 25 µl containing 5 µl of 5X buffer, 1.2 µl of 10 mM dNTPs, 0.6 µl of each
202 primer 10 mM, 0.5 µl of One Step RT-PCR enzyme mix and 1 µl of RNA. Samples were incubated for
203 30 min at 50°C, followed by 15 min incubation at 95°C. Amplification reactions were run for 40 cycles
204 of 94°C for 30 sec, 60°C for 30 sec, and 72°C for 40 sec. An aliquot of the PCR reaction was taken
205 after the 36, 38 and 40 cycles.

206 Reactions with *MtRboh* specific primers (Marino et al. 2011) were carried out in a final volume of 10
207 µl containing 2 µl of 5X buffer, 0.4 µl of 10 mM dNTPs, 1 µl of each primer 10 mM, 0.2 µl of One
208 Step RT-PCR enzyme mix, and 1 µl of a total RNA diluted 1:1. The samples were incubated for 30 min
209 at 50°C, followed by 15 min incubation at 95°C. Amplification reactions were run for 40 cycles of
210 94°C for 30 sec, 60°C for 30 sec, and 72°C for 40 sec. RT-PCR experiments were conducted on two
211 different biological replicates of 1500-2000 microdissected cells each.

212 GUS histochemical assay

214 Root fragments from *Agrobacterium rhizogenes*-transformed lines showing extraradical fungal
215 structures and hyphopodia were selected under a stereomicroscope, excised and placed in a single wells
216 of a Multiwell plate and covered with freshly prepared GUS-buffer (0.1 M sodium phosphate buffer pH
217 7.0, 5 mM K₄Fe(CN)₆, 5 mM K₃Fe(CN)₆, 0.3% Triton X, 0.3% x-Gluc). The samples were incubated at
218 37°C for 16 hours in the dark, destained with distilled water and observed under an optical microscope
219 (Nikon eclipse E300). Two independent lines were analysed for each construct; GUS assays were
220 conducted on about 20 root fragments for each condition collected from 5 distinct Petri dishes in two
221 independent colonization experiments.

222 RNAi lines

224 A 344-bp cDNA fragment of 3'-UTR of *MtRbohE* (Mt4.0, Medtr8g095520.1) was amplified by PCR
225 using the following primers: forward 5'-TGAGGATAACAGTGGAAGG-3' and reverse primer 5'-
226 TCTCCTGGGACGACTATAA-3' and cloned into the pDNOR207 vector using the BP Gateway
227 technology (Invitrogen). The resulting vector was recombined with the pK7GWIWG2D(II) vector

(Karimi et al. 2002) using the LR Gateway technology (Invitrogen), according to the manufacturer's recommendations. The construct was checked by DNA sequencing introduced by electroporation into *Agrobacterium rhizogenes* strain ARqua1, and used for *M. truncatula* root transformation as previously described (Medicago handbook). The pK7GWIWG2D(II) empty vector has been used as control. After 2 weeks, transgenic roots were selected under fluorescent microscope (Leica), and transferred on SHb10 medium (Medicago handbook) supplemented with 200 mg L⁻¹ augmentin (amoxicillin:clavulanic acid [5:1]) and 20 mg L⁻¹ kanamycin (Sigma-Aldrich). Root cultures were kept at 20°C in the dark and subcultured each 3 weeks on new medium. After 3 subculturing, augmentin was removed and the level of *MtRbohE* was evaluated by qRT-PCR (Fig. S2).

Confocal microscopy

Mycorrhizal roots were counterstained with 0.01% (w/v) acid fuchsin in lactoglycerol (lactic acid-glycerol-water, 14:1:1; Kormanik and McGraw 1982) and screened under an optical microscope (Nikon eclipse E300). 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.

Alternatively, colonized root segments were excised under a stereomicroscope, rapidly embedded in 8% agarose Type III (Sigma-Aldrich) and cut into 100 µm-thick longitudinal sections using a Vibratome 1000 microtome. Sections were stained with FITC-labelled wheat germ agglutinin to mark the fungal cell wall and imaged in confocal microscopy. FITC fluorescence was excited at 488 nm and recorded at 500-550 nm. Transmitted light images were acquired concomitantly and overlaid to the fluorescence images through the Leica Confocal Software.

Results

MtRbohE is upregulated in arbuscule-containing cells

To analyse *Rboh* gene expression profiles in *Medicago truncatula* arbuscular mycorrhizas we focused our attention on five genes, *MtRbohA*, *MtRbohB*, *MtRbohE*, *MtRbohF* and *MtRbohG*, since they were shown to be expressed in the *M. truncatula* root system by Marino et al. (2011). To this purpose qRT-PCR assays were first set up on fresh biological material obtained from whole roots of seedlings

inoculated in the semi-sterile sandwich system with *Rhizophagus irregularis* (DAOM 197198) over a time course of 7, 14, 28 and 60 days post-inoculation (dpi). Morphological analyses of roots revealed almost no fungal structures at 7 or 14 dpi, while mycorrhization frequency increased from 28 to 60 dpi. Arbuscules were detected starting from 28 dpi while decreased at 60 dpi (Table 1). These data were confirmed by molecular analyses showing the parallel accumulation of *MtPT4* mRNA, the *M. truncatula* phosphate transporter which is considered a molecular marker for functional arbuscules (Harrison et al. 2002; Fig. 1a).

Specific primers designed for *MtRboh* genes (Marino et al. 2011) were first tested on *M. truncatula* DNA as a positive control and also on *R. irregularis* genomic DNAs to exclude any cross-hybridization. All the primers amplified a DNA fragment of the expected size from *M. truncatula* genomic DNA while no signal was detected from fungal DNA (data not shown). The *M. truncatula* *MtTef* was used as a housekeeping gene for the normalization of the *Rboh* expression levels. Among the 5 genes *MtRbohG* showed the highest expression levels in all the samples and slight fluctuations between control and mycorrhizal roots along the 4 time points. Almost no significant variation in transcripts abundance for the other genes (*MtRbohA*, *MtRbohB*, *MtRbohE*, and *MtRbohF*) was observed in most of the time points (Fig. 1b).

Since the AM colonization is an asynchronous process, whole roots analyses may hinder expression patterns limited to specific tissues or cell sub-populations (Balestrini et al. 2007). We therefore chose to monitor gene expression in different cell types, by using laser microdissection (LMD) coupled to semi-quantitative RT-PCR. Three different cell populations were collected from *M. truncatula* roots: cortical cells from control non-inoculated roots (C), non-colonized cells (NcMyc) and arbusculated cells (Arb) from roots colonized by *R. irregularis*. The quality of LMD samples was verified by monitoring *MtPT4* expression. The corresponding PCR product was only detected in the arbuscule-containing cells while no signal was obtained from the other two samples (Fig. 2b). In order to calibrate the amount of RNAs in the three different samples, RT-PCR assays using *MtTef* primers were performed (Fig. 2a).

MtRbohB and *MtRbohF* mRNAs were not detected in any cell type, while a weak *MtRbohA* signal was detected in cortical cells from control roots (Fig. 2b). By contrast, *MtRbohE* and *MtRbohG* transcripts were detected in all three cell types. *MtRbohE* displayed a slightly stronger signal in arbusculated cells compared to the other two cell types (Fig. 2b). These results were confirmed in a second independent

experiment (data not shown).

To better localize gene expression in the root, histochemical staining was performed using a promoter transcriptional fusion approach based on *Agrobacterium rhizogenes*-transformed root of *M. truncatula* expressing the GUS reporter gene under *Rboh* promoters (Marino et al. 2011). Hairy roots expressing *pRboh*:GUS constructs were colonized by the AM fungus *G. margarita* using the targeted inoculation technique and then subjected to the GUS histochemical reaction. GUS staining was observed in root tips and central cylinders for all transgenic lines, in both control and mycorrhizal roots (Fig. 3). These results are largely in line with the observations by Marino et al. (2011) in composite plant roots. Altogether, these experiments highlight the role of *Rboh* genes during root growth, where they could have a role in cell wall extension (Monshausen et al. 2007; Macpherson et al. 2008).

When mycorrhizal roots were analyzed, the pattern of GUS activity largely overlapped to that observed in non mycorrhizal roots. Nevertheless, an additional GUS-positive district was found in the *MtRbohE* transgenic line, corresponding to some of the inner cortical cells (Fig. 3q,r). The same pattern was confirmed in a second set of experiments, where all five transgenic lines were inoculated with *R. irregularis*; a detail for the *MtRbohE* line is shown in Fig. 4. Since inner cortical cells are the site of arbuscule development, we verified the presence of fungal structures in GUS-positive cortical cells. To this aim, roots were counterstained with acid fuchsin. Since the blue GUS staining dominated the acid fuchsin pink color, a more detailed visualization of arbuscules was obtained with confocal microscope observations, taking advantage of acid fuchsin fluorescence. The results clearly showed that the cortical cells where GUS activity was detected also contained arbuscules (Fig. 5).

In short, different approaches consistently suggest that *MtRbohE* is upregulated in arbuscule-containing cells.

***MtRbohE* silencing affects arbuscule development**

To better understand the role of *MtRbohE* in the AM symbiosis, hairy roots expressing an RNA interference (RNAi) construct for the *MtRbohE* gene or the empty vector (EV) were obtained by *A. rhizogenes* transformation. Different independent transgenic lines displayed an average of 50% *MtRbohE* silencing (Fig. S1). To study their mycorrhizal phenotype, these roots were inoculated with *G. margarita* by the targeted inoculation method (Chabaud et al. 2002). EV roots showed the typical AM colonization pattern with abundant, fully developed arbuscules in the root cortex (Fig. 6). By

contrast, *MtRbohE* RNAi lines displayed abundant intercellular hyphae, but rare arbuscules. In more detail, short - and occasionally multiple - hyphal protrusions were observed along the cell wall of cortical cells (Fig. 6; Fig. S2). We interpret these structures as cell penetration attempts from the intercellular hyphae that did not result in arbuscule formation. These observations strongly suggest that *MtRbohE* plays a role in arbuscule accommodation in the root cortex. Interestingly, inoculation of *MtRbohE* RNAi roots of composite plants with *S. meliloti* showed no significant effect on nodule number (Fig. S3). Altogether, these results suggest that *MtRbohE* is involved in mycorrhizal but not in the rhizobial symbiosis.

Discussion

Many processes in the AM symbiosis have been hypothesized to be mediated by ROS: from the control of host compatibility and fungal morphogenesis (Lanfranco et al. 2005; Fester and Hause 2005) to the regulation of plant responses to biotic (Pozo and Azcon-Aguilar, 2007) and abiotic stress conditions (Dumas-Gaudot et al. 2000; Linderman 2000), but the precise underlying mechanisms are far from being completely elucidated.

Since the literature reports evidence of H₂O₂ accumulation in arbuscular mycorrhizas (Salzer et al. 1999; Fester and Hause 2005; Lanfranco et al. 2005) in this work we monitored the expression profiles of five *M. truncatula* *Rboh* genes during the AM interaction.

Investigations on RNA from whole roots revealed that *Rboh* transcript levels did not drastically change in *M. truncatula* roots upon colonization by *R. irregularis*. Similar results were obtained in mycorrhizal roots of *Phaseolus vulgaris* (Arthikala et al. 2013) although a weak up-regulation of putative orthologs of *MtRbohB*, *MtRbohA*, *MtRbohE* and *MtRbohF* (*PvRbohB*, *PvRbohD*, *PvRbohA* and *PvRbohH* respectively; Montiel et al. 2012) was reported.

Since a mycorrhizal root is a heterogeneous environment where different cell types, either colonized or not by the AM fungus, coexist, we took advantage of the laser microdissection technique to investigate whether a fine tuning of gene expression was associated to specific cells. In recent years this method has been successfully applied to identify transcripts specifically or preferentially associated to the key structures of the AM symbiosis, that is arbusculated cells (Fiorilli et al. 2009; Gomez et al. 2009; Guether et al. 2009; Hogeekamp et al. 2011; Gaude et al. 2012). Cortical parenchyma, the exclusive niche of arbuscule differentiation, was therefore the target of our analysis. Semi-quantitative RT-PCR

showed that *MtRbohE* and *MtRbohG* were expressed in cortical cells from both mycorrhizal and control roots, but a slightly stronger intensity was detected for *MtRbohE* in arbusculated cells compared to adjacent, non-colonized, cortical cells or cortical cells from non mycorrhizal roots. This result is in agreement with data obtained from transcriptional fusion ROC lines. ROCs are a well-established experimental system for the study of host responses to AM fungi (Chabaud et al. 2002); while their application should be carefully evaluated for hormonal, metabolic or physiologic investigations, ROCs display a normal process of root colonization and were demonstrated to be powerful tools for cellular and molecular studies (Genre et al. 2013; Kuhn et al. 2009). Upon colonization by AM fungi the *MtRbohE* line showed GUS activity in certain cells of the inner cortical parenchyma where arbuscules were present. This promoter activity appears a common response to colonization by AM fungi since it was observed with both *G. margarita* or *R. irregularis* inoculation.

In their whole the data suggest that *MtRbohE* gene is activated in cortical cells possibly in relation to specific events of formation, differentiation and/or senescence of arbuscules. We propose a transient activation of *MtRbohE* since the up-regulation was not evident in the qRT-PCR from whole roots. The molecular mechanisms underlying the formation and the turnover of arbuscules are largely unknown and remain a very enigmatic aspect of the AM symbiosis (Guthjar and Parniske 2013). It can be hypothesized that the spatio-temporal localized production of ROS mediated by *MtRbohE* may contribute to control the formation or the life span of these ephemeral intracellular structures. This hypothesis is also supported by the mycorrhizal phenotype of *MtRbohE* silenced lines; although the down regulation was only partial (about 50%) *MtRbohE* RNAi roots displayed abundant intercellular hyphae with many cell penetration attempts, but rare arbuscules.

As far as concerns the five genes under analysis, the situation is clearly different from that described in the nitrogen fixing symbiosis (Marino et al. 2011; 2012) and adds some points of divergence between the two root symbioses. Based on our expression data *MtRbohA* does not seem to have a role in the AM symbiosis. This is not surprising since *MtRbohA* was shown to control nodule nitrogen fixation activity also through the modulation of genes encoding the microsymbiont nitrogenase, a process which is indeed specific to the rhizobial symbiosis. However, we can not exclude regulations occurring at post-translational level (Marino et al. 2012). On the other hand, it has been recently demonstrated that the *PvRbohB* gene from *Phaseolus vulgaris* (homolog of *MtRbohG*), is required for root infection by rhizobia but acts as a negative regulator of the AM symbiosis (Arthikala et al. 2013). Also our data,

showing that *MtRbohE* silencing did not impact the nodulation process, further hint at a divergent role for Rboh/ROS-related processes in the two root symbioses. Interestingly, our results point to a positive role of *MtRbohE* on the AM colonization while *PvRbohB* was shown to negatively affect the symbiosis. It is worth to note that from a phylogenetic analysis *MtRbohE* is not the homolog of *PvRbohB*. Although only two *Rboh* genes from two different plants have been characterized so far, these findings suggest that different gene members of the RBOH family might play distinct functions in the AM symbiosis.

Two recent works on the model legume *M. truncatula* have also provided indirect evidence for a role of *Rboh* genes in the AM symbiosis. The silencing of *MtROP9*, a small GTPase considered a positive regulator of RBOH enzymes, led to a stimulation of early mycorrhizal colonization (Kiirika et al. 2012). However, the specific RBOH isoform involved remain to be identified.

In a second publication, *M. truncatula* transcriptomic responses to a combined phosphate and nitrogen limited condition were supposed to be mediated by the action of a *Rboh* gene which would lead to alteration of plant defence and, in the end, to a better AM root colonization. Interestingly, the same authors, based on *in silico* analyses of expression profiles, identified *MtRbohE* as a candidate gene that deserves further investigation (Bonneau et al. 2013): our current results are in line.

Based on genomic analyses, *M. truncatula* possesses 10 *Rboh* genes some of which were proposed to have arisen by a recent whole genome duplication event (Shoemaker et al. 2006). It will be interesting to extend this investigation to other members of the *MtRboh* family to highlight potential specific roles of ROS in controlling root symbioses. Furthermore, given the emerging role of fungal NADPH oxidases in controlling many aspects of fungal development and interactions with plants (Tudzynski et al. 2012), NADPH oxidases in AM fungi will also deserve careful investigation.

Author Contribution Statement

LL, NP and AP conceived and designed research. SB and CC conducted experiments and contributed to data elaboration. AG contributed to the confocal microscopy analyses. LL wrote the manuscript. All authors read and approved the manuscript.

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Figure legends

Fig. 1 Relative expression values of *MtPT4* (a) and *MtRbohA*, *B*, *E*, *F* and *G* genes (b) in roots of *M. truncatula* colonized with *R. irregularis* at 7, 14, 28 and 60 days post-inoculation (dpi). Expression values (bars = standard deviations) are normalized to the *MtTef* housekeeping gene. In panel b, Y axis has a log10 scale. Letters or asterisks indicate statistically significant difference ($p < 0.05$, ANOVA).

Fig. 2 Gel electrophoresis of RT-PCR products obtained from laser microdissected samples from roots colonized by *R. irregularis* (Arb: arbusculated cortical cells; NcMyc: non colonized cortical cells from mycorrhizal roots; C: cortical cells from non mycorrhizal roots) using primers specific for the housekeeping gene *MtTef* (a) and for the different *MtRboh* genes or for *MtPT4*, used as marker of arbuscule-containing cells (b).

Fig. 3 GUS histochemical staining of *M. truncatula* hairy roots expressing *pRboh:GUS* transcriptional fusion colonized (Myc) or not (Non myc) with the AM fungus *G. margarita*. In non mycorrhizal roots (figures from a to l) the GUS staining is localized in the root apex (arrows) and in the vascular system (asterisks). In roots colonized by the AM fungus *G. margarita* (figures from m to v) the GUS staining is localized in all the transgenic lines (as in the non mycorrhizal roots) in the root tips (arrows) and in the vascular system (asterisks). Only in *MtRbohE* transgenic roots the GUS staining is also localized in some cells of the inner cortex (arrowheads) where usually arbuscules are formed.

Fig. 4 GUS histochemical staining of *M. truncatula* *MtRbohE* transgenic hairy roots expressing *pRboh:GUS* transcriptional fusion colonized (Myc) or not (Non myc) with the AM fungus *R. irregularis*. In non mycorrhizal roots (figures a and b) the GUS staining is localized in the root apex (arrowhead) and in the vascular system (asterisks). In roots colonized by the AM fungus *R. irregularis* (figures c and d) the GUS staining is localized in the vascular system (asterisks) and in some cells of the inner cortex (arrows) where usually arbuscules are formed.

Fig. 5 GUS histochemical staining of *M. truncatula* *MtRbohE* transgenic hairy roots expressing *pRboh:GUS* transcriptional fusion colonized with the AM fungus *G. margarita* and counterstained with

acid fuchsin to localize the intraradical hyphae. Under a confocal microscope (figures **a**, **b** and **c**) the acid fuchsin autofluorescence allows to localize the arbuscules (A) in some cortical cells. The observation of the same cortical cells under a light microscope (figures **d**, **e** and **f**) allow to appreciate the overlap of the GUS staining (blue) and the acid fuchsin staining (pink), clearly indicating the co-localization of the GUS staining and the arbuscules. Bars: 20 μ m.

Fig. 6 Confocal microscopy images representative of hyphal and arbuscule development in WT (**a-c**) and *MtRbohE* RNAi-silenced lines (**d-f**). Both hyphae (h) and arbuscules (ar) are abundant in WT roots (**a**). Higher magnifications (**b** and **c**) show details of arbuscule morphology, with the fine branches occupying most of the cell volume and the large trunk (t) branching from the intercellular hypha (arrowhead). By contrast, arbuscules are much more sparse in *MtRbohE* RNAi-silenced lines (**d**), where extensive areas are mostly colonized by intercellular hyphae (h). When present, arbuscule have a WT-like morphology (**e**, **f**), while intercellular hyphae often show short protrusions that we interpret as recursive, aborted cell penetration attempts (arrowheads). Bars = 80 μ m in **a**, **d**, **e**; 40 μ m in **b**, **f**; 20 μ m in **c**.

Table 1 Mycorrhization level in *M. truncatula* roots at different days post inoculation (dpi). Different letters indicate statistically significant differences ($p>0.05$, ANOVA).

Fig. S1 Relative expression of *MtRbohE* in transgenic root cultures. *MtRbohE* expression was evaluated by qRT-PCR on established transgenic root cultures expressing either an empty (EV) or a *MtRbohE* RNAi construct (RNAi). Values were normalized against the 40S2 ribosomal protein (Andrio et al. 2013). Data are reported as mean \pm standard error. Asterisk indicates a statistically significant difference between control (EV) and RNAi lines using t-test ($p<0.05$).

Fig. S2 Details of intraradical fungal structures in the cortex of EV (**a**) or *MtRbohE* RNAi-silenced lines (**b**). A young arbuscule is displayed in **a**, filling up most of the host cell lumen with its trunk (t) and branches (b). A root segment only containing intercellular hyphae is shown in **b**: several short branches are evident, protruding from an intercellular hypha, corresponding to aborted cell penetration events. Bars = 20 μ m in **a**; 80 μ m in **b**.

647 **Fig. S3** Nodulation of composite plants expressing either an empty (EV) or a *MtRbohE* RNAi
648 construct. Nodule number was evaluated 4 and 10 days after inoculation with *Sinorhizobium meliloti*.
649 Data are reported as mean \pm standard error. Values are representative of two independent biological
650 replicates (n>30). Differences are not statistically significant (t-test).

Figure 1

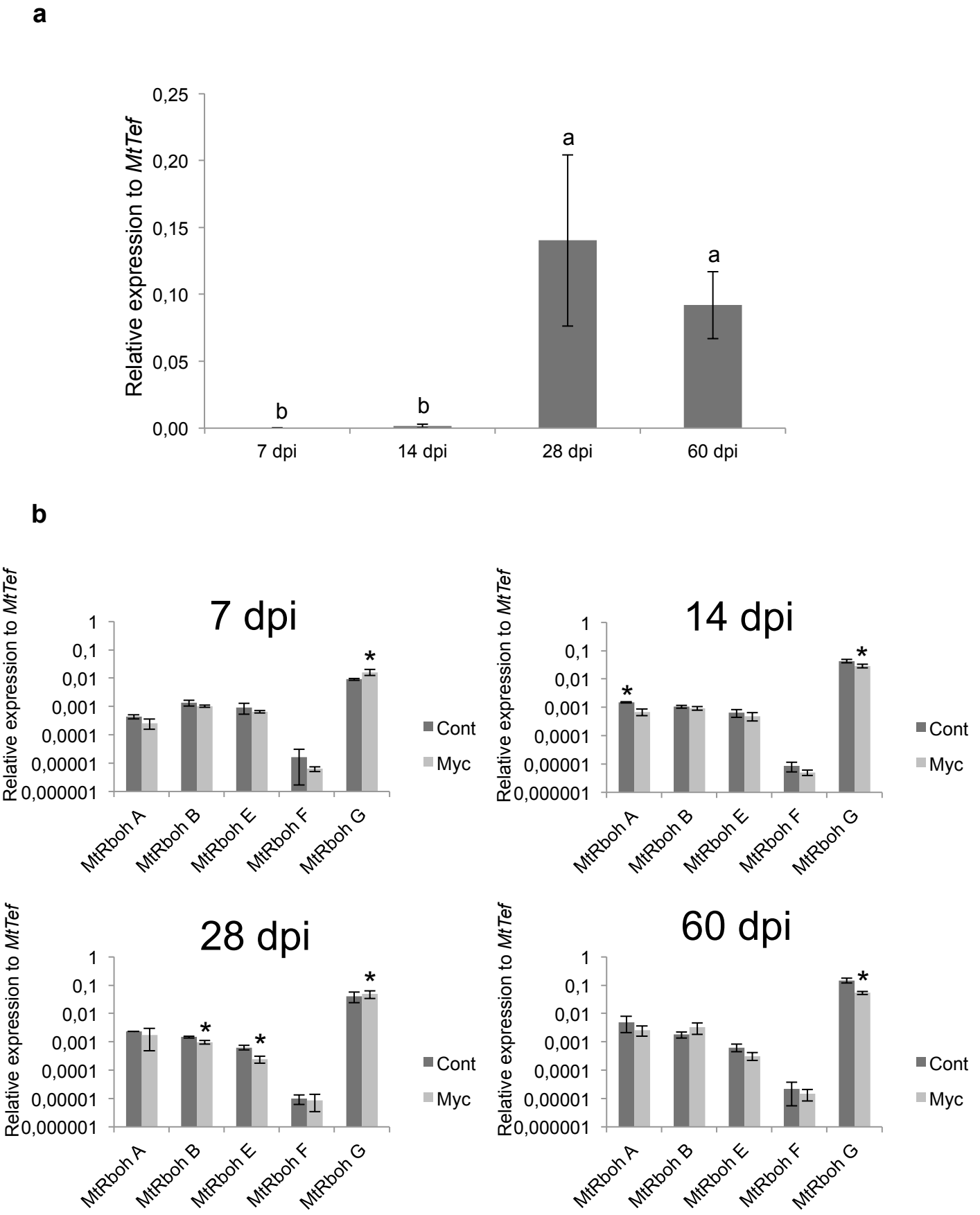


Fig. 1

Figure 2

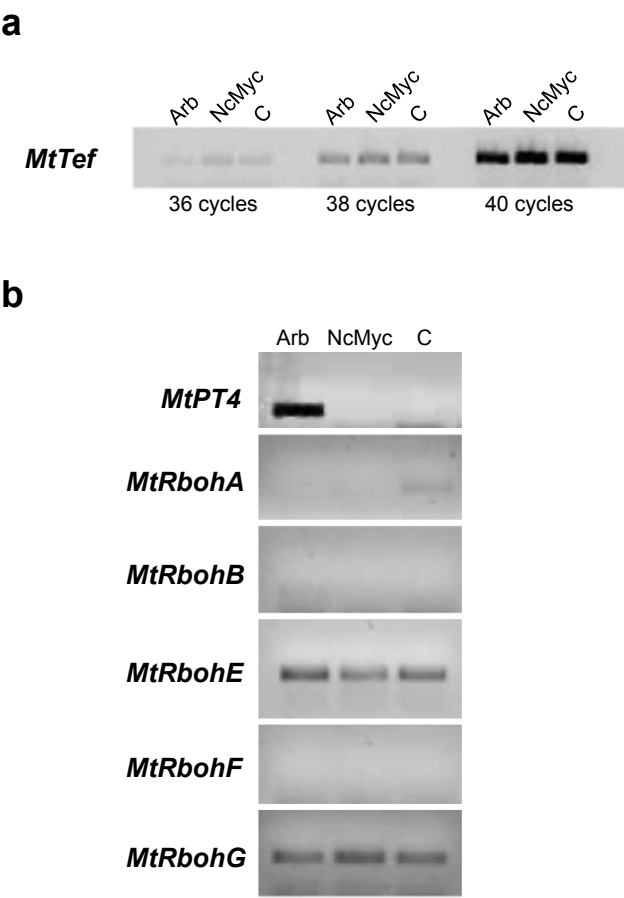


Fig. 2

Figure 3

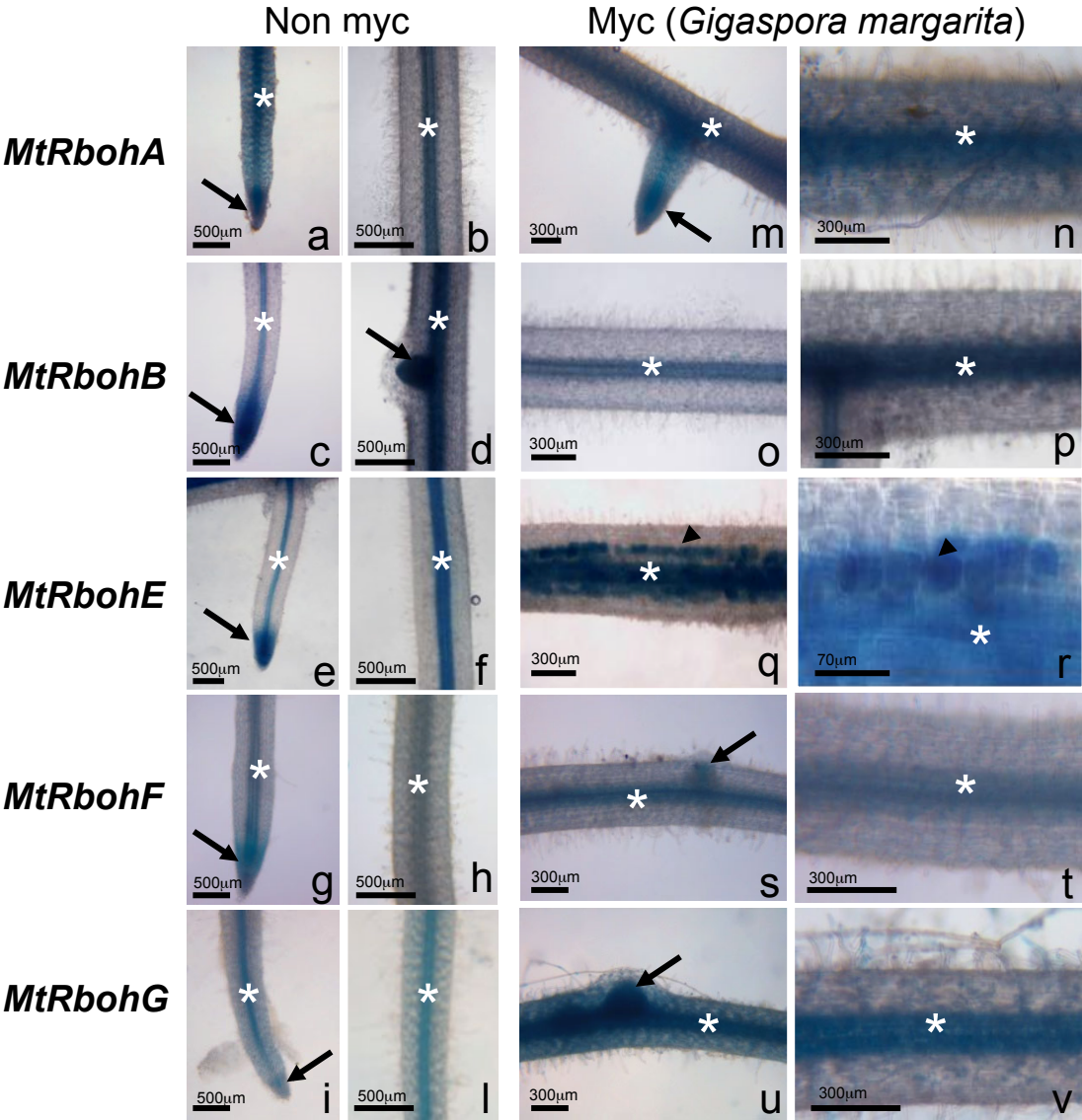


Fig. 3

Figure 4

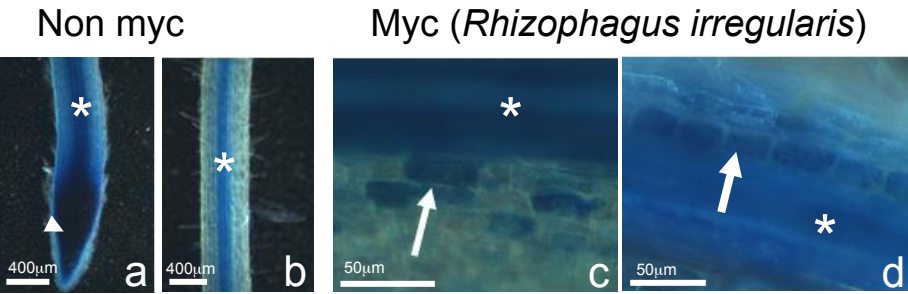


Fig. 4

Figure 5

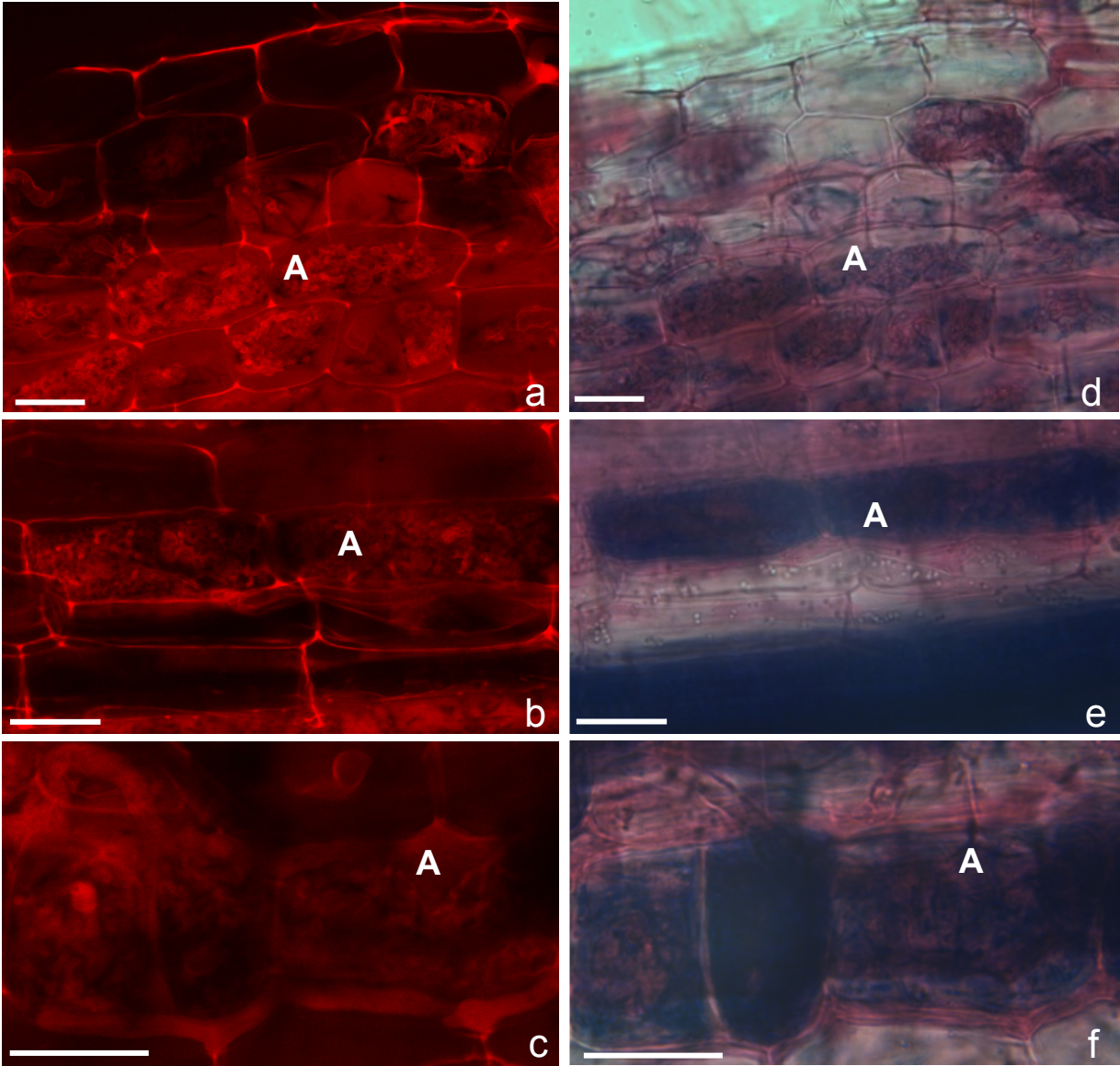


Fig. 5

Figure 6

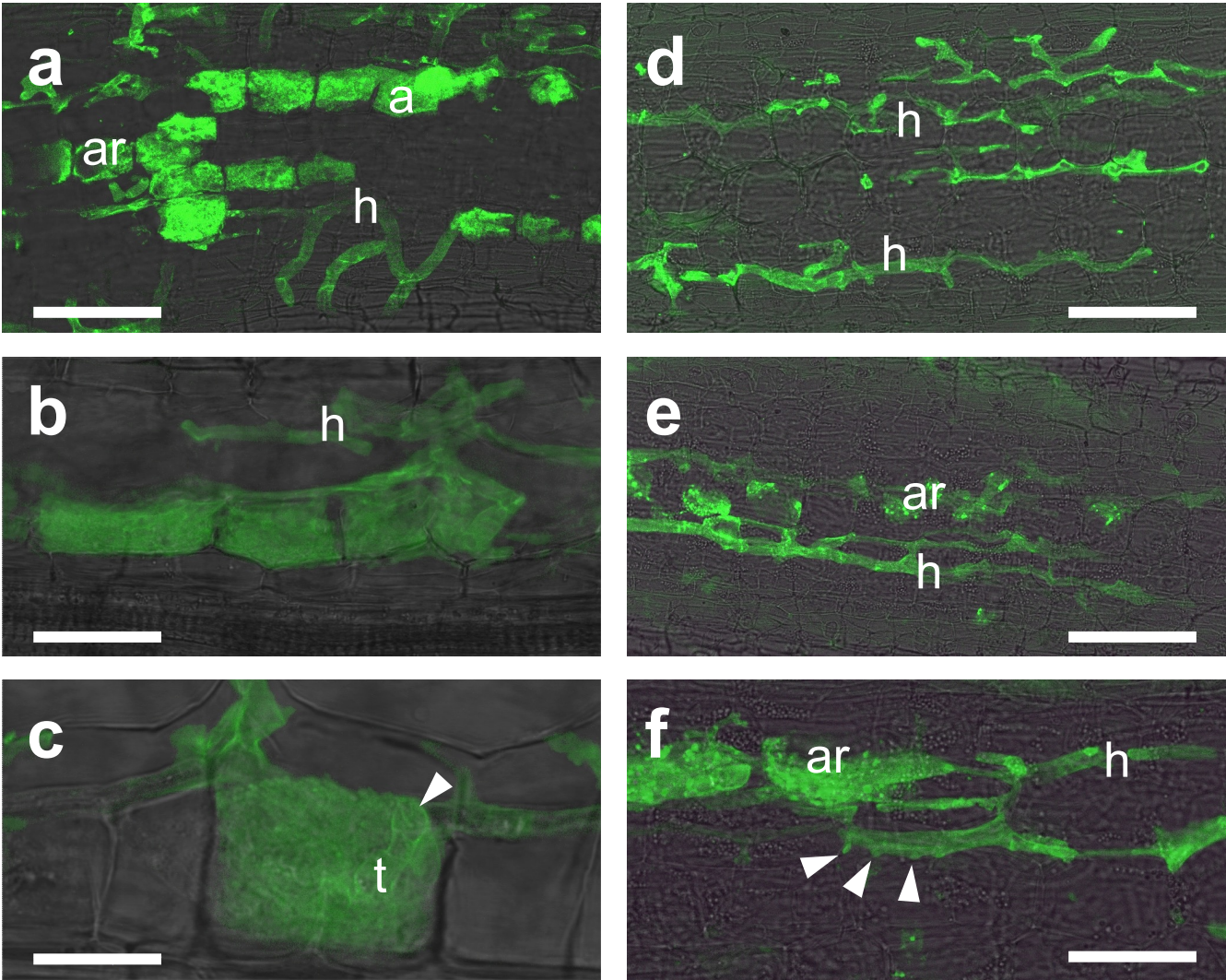


Fig. 6

Table 1.
Mycorrhization level in *M. truncatula* roots at different days post inoculation (dpi).
Different letters indicate statistically significant differences ($p>0.05$, ANOVA).

dpi	F%	a%
7	0	0
14	0	0
28	49.4±2.5 ^a	87.8±7.7 ^a
60	62.8±2.6 ^b	48.8±18.7 ^b

F: Indicates the percentage of root segments showing internal colonization.
a: Indicates the average presence of arbuscules within the infected areas.

Supplementary Material Rebuttal letter

[Click here to download Supplementary Material: Rebuttal letter Belmondo et al.pdf](#)