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Main Conclusion

Our study demonstrated that the NAPDH oxidase gene *MtRbohE* is expressed in arbusculated cells and plays a role in arbuscule development.

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 transgenic 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 Dangl 2005) that catalyzes the production of superoxide by transferring electrons from NADPH to molecular oxygen, with secondary generation of H_2O_2. 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 Dangl 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 H_2O_2 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 H_2O_2 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. H_2O_2 production was also observed in roots of *M. truncatula* and *Lotus japonicus* colonized by another AM fungus, *Gigaspora margarita*. In this case H_2O_2 accumulation was mainly associated with the fungal structures and this was mirrored by the up-regulation of a gene encoding a superoxide dismutase in intraradical fungal structures (Lanfranco et al. 2005). 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 H_2O_2 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 Pv*RbohB* 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 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.

Materials and Methods

Biological materials, growth conditions and inoculation methods

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

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

To obtain seedlings colonized by *R. irregularis* or *G. margarita* the Millipore sandwich method (Giovannetti et al. 1993) was used. Seeds of *Medicago truncatula* Gaertn cv Jemalong were first scarified using sandpaper P180-200, sterilized with 5% commercial bleach for 3 minutes and rinsed three times for 10 minutes with sterile distilled water. Germination was induced under sterile conditions in 0.6% agar/water, incubated for 5 days in the dark (25°C) and then exposed at the light for 4 days. Plants were watered with Long-Ashton solution containing a low phosphorus concentration (3.2 µM $Na_2HPO_4 \cdot 12H_2O$) (Hewitt 1966) and they were grown in a growth chamber under 14 h light (24°C)/10 h dark (20°C) regime. Plants were harvested 60 days post-inoculation (dpi). For mycorrhizal plants, only portions of the root system showing extraradical fungal structures were collected under a stereomicroscope. The colonization level was assessed according to Trouvelot et al. (1986). For the 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). The *MtPT4* phosphate transporter gene was amplified using MtPT4F (5′TCGCGCGCCATGTTTGTTGT3′) and MtPT4R

(5′CGCAAGAAGAATGTTAGCCC3′) primers. 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 were assessed using the *MtTef* primers described above. RNAs extracted from the three different cell populations were then calibrated using *MtTef* as housekeeping gene. In detail, reactions were carried 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 primer 10 mM, 0.5 µl of One Step RT-PCR enzyme mix and 1 µl of RNA. Samples were incubated for 30 min at 50°C, followed by 15 min incubation at 95°C. Amplification reactions were run for 40 cycles 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 after the 36, 38 and 40 cycles.

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

GUS histochemical assay

Root fragments from *Agrobacterium rhizogenes*-transformed lines showing extraradical fungal structures and hyphopodia were selected under a stereomicroscope, excised and placed in a single wells of a Multiwell plate and covered with freshly prepared GUS-buffer (0.1 M sodium phosphate buffer pH 7.0, 5 mM $K_4Fe(CN)_6$, 5 mM $K_3Fe(CN)_6$, 0.3% Triton X, 0.3% x-Gluc). The samples were incubated at 37°C for 16 hours in the dark, destained with distilled water and observed under an optical microscope (Nikon eclipse E300). Two independent lines were analysed for each construct; GUS assays were

conducted on about 20 root fragments for each condition collected from 5 distinct Petri dishes in two independent colonization experiments.

RNAi lines

A 344-bp cDNA fragment of 3'-UTR of *MtRbohE* (Mt4.0, Medtr8g095520.1) was amplified by PCR using the following primers: forward 5'-TGAGGATAACAGTGGAAGG-3' and reverse primer 5'-TCTCCTGGGACGACTATAA-3' and cloned into the pDNOR207 vector using the BP Gateway 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 keept 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.

246

247 **Results**

248

249 ***MtRbohE is upregulated in arbuscule-containing cells***

250 To analyse *Rboh* gene expression profiles in *Medicago truncatula* arbuscular mycorrhizas we focused
251 our attention on five genes, *MtRbohA, MtRbohB, MtRbohE, MtRbohF* and *MtRbohG*, since they were
252 shown to be expressed in the *M. truncatula* root system by Marino et al. (2011). To this purpose qRT-
253 PCR assays were first set up on fresh biological material obtained from whole roots of seedlings
254 inoculated in the semi-sterile sandwich system with *Rhizophagus irregularis* (DAOM 197198) over a
255 time course of 7, 14, 28 and 60 days post-inoculation (dpi). Morphological analyses of roots revealed
256 almost no fungal structures at 7 or 14 dpi, while mycorrhization frequency increased from 28 to 60 dpi.
257 Arbuscules were detected starting from 28 dpi while decreased at 60 dpi (Table 1). These data were
258 confirmed by molecular analyses showing the parallel accumulation of *MtPT4* mRNA, the *M.*
259 *truncatula* phosphate transporter which is considered a molecular marker for functional arbuscules
260 (Harrison et al. 2002; Fig. 1a).
261 Specific primers designed for *MtRboh* genes (Marino et al. 2011) were first tested on *M. truncatula*
262 DNA as a positive control and also on *R. irregularis* genomic DNAs to exclude any cross-
263 hybridization. All the primers amplified a DNA fragment of the expected size from *M. truncatula*
264 genomic DNA while no signal was detected from fungal DNA (data not shown). The *M. truncatula*
265 *MtTef* was used as a housekeeping gene for the normalization of the *Rboh* expression levels. Among
266 the 5 genes *MtRbohG* showed the highest expression levels in all the samples and slight fluctuations
267 between control and mycorrhizal roots along the 4 time points. Almost no significant variation in
268 transcripts abundance for the other genes (*MtRbohA, MtRbohB, MtRbohE,* and *MtRbohF) was observed*
269 in most of the time points (Fig. 1b).
270 Since the AM colonization is an asynchronous process, whole roots analyses may hinder expression
271 patterns limited to specific tissues or cell sub-populations (Balestrini et al. 2007). We therefore chose
272 to monitor gene expression in different cell types, by using laser microdissection (LMD) coupled to
273 semi-quantitative RT-PCR. Three different cell populations were collected from *M. truncatula* roots:
274 cortical cells from control non-inoculated roots (C), non-colonized cells (NcMyc) and arbusculated
275 cells (Arb) from roots colonized by *R. irregularis*. The quality of LMD samples was verified by

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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 p*Rboh*: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.

306

307 ***MtRbohE silencing affects arbuscule development***

308 To better understand the role of *MtRbohE* in the AM symbiosis, hairy roots expressing an RNA
309 interference (RNAi) construct for the *MtRbohE* gene or the empty vector (EV) were obtained by *A.*
310 *rhizogenes* transformation. Different independent transgenic lines displayed an average of 50%
311 *MtRbohE* silencing (Fig. S1). To study their mycorrhizal phenotype, these roots were inoculated with
312 *G. margarita* by the targeted inoculation method (Chabaud et al. 2002). EV roots showed the typical
313 AM colonization pattern with abundant, fully developed arbuscules in the root cortex (Fig. 6). By
314 contrast, *MtRbohE* RNAi lines displayed abundant intercellular hyphae, but rare arbuscules. In more
315 detail, short - and occasionally multiple - hyphal protrusions were observed along the cell wall of
316 cortical cells (Fig. 6; Fig. S2). We interpret these structures as cell penetration attempts from the
317 intercellular hyphae that did not result in arbuscule formation. These observations strongly suggest that
318 *MtRBohE* plays a role in arbuscule accommodation in the root cortex.
319 Intestingly, inoculation of *MtRbohE* RNAi roots of composite plants with *S. meliloti* showed no
320 significant effect on nodule number (Fig. S3). Altogether, these results suggest that *MtRbohE* is
321 involved in mycorrhizal but not in the rhizobial symbiosis.
322

323 **Discussion**

324 Many processes in the AM symbiosis have been hypothesized to be mediated by ROS: from the control
325 of host compatibility and fungal morphogenesis (Lanfranco et al. 2005; Fester and Hause 2005) to the
326 regulation of plant responses to biotic (Pozo and Azcon-Aguilar, 2007) and abiotic stress conditions
327 (Dumas-Gaudot et al. 2000; Linderman 2000), but the precise underlying mechanisms are far from
328 being completely elucidated.
329 Since the literature reports evidence of H_2O_2 accumulation in arbuscular mycorrhizas (Salzer et al.
330 1999; Fester and Hause 2005; Lanfranco et al. 2005) in this work we monitored the expression profiles
331 of five *M. truncatula Rboh* genes during the AM interaction.
332 Investigations on RNA from whole roots revealed that *Rboh* transcript levels did not drastically change
333 in *M. truncatula* roots upon colonization by *R. irregularis*. Similar results were obtained in mycorrhizal
334 roots of *Phaseolus vulgaris* (Arthikala et al. 2013) although a weak up-regulation of putative orthologs
335 of *MtRbohB, MtRbohA, MtRbohE* and *MtRbohF* (*PvRbohB, PvRbohD, PvRbohA* and *PvRbohH*

336 respectively; Montiel et al. 2012) was reported.

337 Since a mycorrhizal root is a heterogeneous environment where different cell types, either colonized or
338 not by the AM fungus, coexist, we took advantage of the laser microdissection technique to investigate
339 whether a fine tuning of gene expression was associated to specific cells. In recent years this method
340 has been successfully applied to identify transcripts specifically or preferentially associated to the key
341 structures of the AM symbiosis, that is arbusculated cells (Fiorilli et al. 2009; Gomez et al. 2009;
342 Guether et al. 2009; Hogekamp et al. 2011; Gaude et al. 2012). Cortical parenchyma, the exclusive
343 niche of arbuscule differentiation, was therefore the target of our analysis. Semi-quantitative RT-PCR
344 showed that *MtRbohE* and *MtRbohG* were expressed in cortical cells from both mycorrhizal and
345 control roots, but a slightly stronger intensity was detected for *MtRbohE* in arbusculated cells compared
346 to adjacent, non-colonized, cortical cells or cortical cells from non mycorrhizal roots. This result is in
347 agreement with data obtained from transcriptional fusion lines: upon colonization by AM fungi the
348 *MtRbohE* line showed GUS activity in certain cells of the inner cortical parenchyma where arbuscules
349 were present. This promoter activity appears a common response to colonization by AM fungi since it
350 was observed with both *G. margarita* or *R. irregularis* inoculation.

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

361 As far as concerns the five genes under analysis, the situation is clearly different from that described in
362 the nitrogen fixing symbiosis (Marino et al. 2011; 2012) and adds some points of divergence between
363 the two root symbioses. Based on our expression data *MtRbohA* does not seem to have a role in the AM
364 symbiosis. This is not surprising since *MtRbohA* was shown to control nodule nitrogen fixation activity
365 also through the modulation of genes encoding the microsymbiont nitrogenase, a process which is

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366 indeed specific to the rhizobial symbiosis. However, we can not exclude regulations occurring at post-
367 translational level (Marino et al. 2012). On the other hand, it has been recently demonstrated that the
368 *PvRbohB* gene from *Phaseolus vulgaris* (homolog of *MtRbohG*), is required for root infection by
369 rhizobia but acts as a negative regulator of the AM symbiosis (Arthikala et al. 2013). Also our data,
370 showing that *MtRbohE* silencing did not impact the nodulation process, further hint at a divergent role
371 for Rboh/ROS-related processes in the two root symbioses.
372 Two recent works on the model legume *M. truncatula* have provided indirect evidence for a role of
373 *Rboh* genes in the AM symbiosis. The silencing of *MtROP9*, a small GTPase considered a positive
374 regulator of Rboh enzymes, led to a stimulation of early mycorrhizal colonization (Kiirika et al. 2012).
375 However, the specific Rboh isoform involved remain to be identified.
376 In a second publication, *M. truncatula* transcriptomic responses to a combined phosphate and nitrogen
377 limited condition were supposed to be mediated by the action of a *Rboh* gene which would lead to
378 alteration of plant defence and, in the end, to a better AM root colonization. Interestingly, the same
379 authors, based on *in silico* analyses of expression profiles, identified *MtRbohE* as a candidate gene that
380 deserves further investigation (Bonneau et al. 2013): our current results are in line.
381 Based on genomic analyses, *M. truncatula* possesses 10 *Rboh* genes some of which were proposed to
382 have arisen by a recent whole genome duplication event (Shoemaker et al. 2006). It will be interesting
383 to extend this investigation to other members of the *MtRboh* family to highlight potential specific roles
384 of ROS in controlling root symbioses. Furthermore, given the emerging role of fungal NADPH
385 oxidases in controlling many aspects of fungal development and interactions with plants (Tudzynski et
386 al. 2012), NADPH oxidases in AM fungi will also deserve careful investigation.
387
388 **Author Contribution Statement**
389 LL, NP and AP conceived and designed research. SB and CC conducted experiments and contributed
390 to data elaboration. AG contributed to the confocal microscopy analyses. LL wrote the manuscript. All
391 authors read and approved the manuscript.
392
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553
554
555
556 **Figure legends**
557
558 **Fig. 1** Relative expression values of *MtPT4* (**a**) and *MtRbohA, B, E, F* and *G* genes (**b**) in roots of *M.*
559 *truncatula* colonized with *R. irregularis* at 7, 14, 28 and 60 days post-inoculation (dpi). Expression
560 values (bars = standard deviations) are normalized to the *MtTef* housekeeping gene. In panel b, Y axis
561 has a log10 scale. Letters or asterisks indicate statistically significant difference (p<0.05, ANOVA).
562
563 **Fig. 2** Gel electrophoresis of RT-PCR products obtained from laser microdissected samples from roots
564 colonized by *R. irregularis* (Arb: arbusculated cortical cells; NcMyc: non colonized cortical cells from
565 mycorrhizal roots; C: cortical cells from non mycorrhizal roots) using primers specific for the
566 housekeeping gene *MtTef* (**a**) and for the different *MtRboh* genes or for *MtPT4,* used as marker of
567 arbuscule-containing cells (**b**).
568
569 **Fig. 3** GUS histochemical staining of *M. truncatula* hairy roots expressing *pRboh:GUS* transcriptional
570 fusion colonized (Myc) or not (Non myc) with the AM fungus *G. margarita*. In non mycorrhizal roots
571 (figures from a to l) the GUS staining is localized in the root apex (arrows) and in the vascular system
572 (asterisks). In roots colonized by the AM fungus *G. margarita* (figures from m to v) the GUS staining
573 is localized in all the transgenic lines (as in the non mycorrhizal roots) in the root tips (arrows) and in
574 the vascular system (asterisks). Only in *MtRbohE* transgenic roots the GUS staining is also localized in
575 some cells of the inner cortex (arrowheads) where usually arbuscules are formed.

576

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

583

584 **Fig. 5** GUS histochemical staining of *M. truncatula MtRbohE* transgenic hairy roots expressing
585 *pRboh:GUS* transcriptional fusion colonized with the AM fungus *G. margarita* and counterstained with
586 acid fuchsin to localize the intraradical hyphae. Under a confocal microscope (figures **a**, **b** and **c**) the
587 acid fuchsin autofluorescence allows to localize the arbuscules (A) in some cortical cells. The
588 observation of the same cortical cells under a light microscope (figures **d**, **e** and **f**) allow to appreciate
589 the overlap of the GUS staining (blue) and the acid fuchsin staining (pink), clearly indicating the co-
590 localization of the GUS staining and the arbuscules. Bars: 20 µm.

591

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

601

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

604

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 ± 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 = 20µm in **a**; 80µm in **b**.

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