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Defense Related Phytohormones Regulation in Arbuscular Mycorrhizal Symbioses Depends on the Partner Genotypes

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Abstract

Arbuscular mycorrhizal (AM) symbioses are mutualistic associations between soil fungi and most vascular plants. Modulation of the hormonal and transcriptional profiles, including changes related to defense signalling, has been reported in many host plants during AM symbioses. These changes have been often related to the improved stress tolerance common in mycorrhizal plants. However, results on the alterations in phytohormones content and their role on the symbiosis are controversial. Here, an integrative analysis of the response of phylogenetically diverse plants (i.e., tomato, soybean, and maize) to two mycorrhizal fungi -Funneliformis mosseae and Rhizophagus irregularis- was performed. The analysis of the defense-related hormones salicylic acid, abscisic acid, and jasmonates, and the expression of marker genes of the pathways they regulate, revealed significant changes in the roots of mycorrhizal plants. These changes depended on both the plant and the AM fungus (AMF) involved. However, general trends can be identified: roots associated with the most effective colonizer R. irregularis showed fewer changes in these defense-related traits, while the colonization by F. mosseae led to significant modifications in all plants tested. The up-regulation of the jasmonate pathway by F. mosseae was found to be highly conserved among the different plant species, suggesting an important role of jasmonates during this AM interaction. Our study evidences a strong influence of the AMF genotype on the modulation of host defense signalling, and offers hints on the role of these changes in the symbiosis.

Keywords: Abscisic acid Arbuscular mycorrhiza Defense Jasmonates Oxylipins Salicylic acid

Introduction

Plants continuously interact with a broad range of organisms present in their environment, giving rise to a large variety of associations. They are frequently challenged by microbial pathogens that threaten their fitness, but they also interact with neutral or beneficial microorganisms that positively affect multiple vital parameters, such as plant nutrition, growth, and stress tolerance (Barea et al. 2005; Raaijmakers et al. 2009). In fact, a microbe-free plant may be considered an “exotic exception” (Partida-Martínez and Heil 2012). Thus, plants constantly have to fine-tune their defense mechanisms, combating deleterious organisms while allowing associations with beneficials (Zamioudis and Pieterse 2012). The effective regulation of defense mechanisms upon recognition of the microbe relies on the phytohormones salicylic acid (SA), jasmonic acid (JA) and derivatives (JAs), ethylene (ET), and abscisic acid (ABA), which play central roles in coordinating plant responses (Pieterse et al. 2012).

Among plant associations with beneficial microorganisms, arbuscular mycorrhizas (AM) are one of the most widespread, with a major impact on plant health and

ecosystem dynamics (Smith and Read 2008). About 80 % of all land plants are able to form this mutualistic association with soil-borne fungi from the phylum Glomeromycota, known as arbuscular mycorrhizal fungi (AMF) (Smith and Read 2008). They are obligate biotrophs that obtain carbohydrates from their host, while they improve mineral nutrition and water acquisition by the host plant (Parniske 2008). Additionally, the symbiosis impacts the plant's ability to overcome biotic and abiotic stresses, commonly improving host resistance to pathogens and tolerance to unfavourable environmental conditions (Jung et al. 2012; Pozo and Azcón-Aguilar 2007; Ruiz-Lozano et al. 2012).

Although AMF are considered non specific with respect to host range, there is evidence for certain "functional diversity". That is, both the plant and fungal genotypes determine the benefits of the interaction, some combinations being more efficient than others in terms of nutrition and/or stress resistance improvement (Cavagnaro et al. 2001; Feddermann et al. 2010; Pozo et al. 2002; Smith et al. 2004). The interaction requires a high degree of coordination between both partners, and bidirectional (plant and fungal) control assure a fair trade of resources between the symbionts (Kiers et al. 2011). Indeed, the plant is able to prevent excessive colonization by the AMF, avoiding costs that out-weigh the benefits of the interaction (Breuillin et al. 2010).

Several studies have evidenced alterations in the transcriptional profile of the plant associated with AM development and functioning (Güimil et al. 2005; Hause et al. 2007; Liu et al. 2003; Requena et al. 2007), and many of the changes are related to defense mechanisms (Fiorilli et al. 2009; Gallou et al. 2012; Liu et al. 2007; López-Ráez et al. 2010a) likely contributing to the plant maintaining control over the symbiotic partner. The impact of the symbiosis on plant defense mechanisms may also have consequences on their interactions with other organisms, commonly priming defenses against potential attacks (Campos-Soriano et al. 2012; Pozo and Azcón-Aguilar 2007). Alterations in phytohormones homeostasis may also affect plant tolerance against abiotic stresses, such as salinity, drought, and heavy metals (Aroca et al. 2013; Pineda et al. 2013; Ruiz-Lozano et al. 2012). Thus, alterations in host phytohormone levels in well established mycorrhizas may contribute to the improved plant stress tolerance associated with the symbiosis.

Plant-AMF communication starts prior to the physical contact between both symbionts and continues during the entire symbiotic phase (Bonfante and Genre 2010; Gutjahr and Parniske 2013; López-Ráez et al. 2011). Initially, upon mutual recognition, the fungus has to deal with the plant immune system to proceed with a successful colonization (Zamioudis and Pieterse 2012). Then, a symbiotic program is triggered and the plant actively accommodates the fungus. During root colonization, AMF proliferate within the cortex and form specialized structures called arbuscules where the exchange of nutrients between the partners takes place. The growth of the fungal symbiont inside the root implies the alteration of multiple host cellular processes (Bonfante and Genre 2010). Accordingly, gene expression and hormonal profiles should be precisely regulated even at the cellular level (Balestrini et al. 2007). The symbiotic status is maintained throughout the life of the plant.

Among plant stress-related hormones, ABA, SA, and JAs are believed to play a key role not only in the establishment, but also in the functioning of the AM symbiosis (Hause et al. 2007; Herrera-Medina et al. 2007; López-Ráez et al. 2010a; Ludwig-Müller 2010). Salicylic acid is known to have a major role in plant defense against microorganisms with a biotrophic lifestyle (Pieterse et al. 2009). Therefore, AMF as obligate biotrophs are expected to be negatively affected by SA (Gutjahr and

Paszkowski 2009; Pozo and Azcón-Aguilar 2007). Indeed, previous studies have shown a negative, although transient, effect of SA on fungal root colonization (de Roman et al. 2011; Herrera-Medina et al. 2007). However, contrasting results are reported concerning SA content in well established associations, with higher (Khaosaad et al. 2007; López-Ráez et al. 2010a), unaltered (Campos-Soriano and Segundo 2011), or lower (Herrera-Medina et al. 2003) SA levels in mycorrhizal than in non mycorrhizal roots. As for SA, ET has been shown to have a negative regulatory role on the AM symbioses. Ethylene inhibits root colonization (Fracetto et al. 2013; Geil and Guinel 2002), and reduced ET levels have been reported in mycorrhizal roots (López-Ráez et al. 2010a). In contrast to the negative regulatory role of SA and ET, ABA is necessary for AM development and functionality (Herrera-Medina et al. 2007; Martín-Rodriguez et al. 2010). Again, controversial results have been obtained in relation to the ABA content in AM roots, ranging from an increase (Meixner et al. 2005), no change (López-Ráez et al. 2010a; Martínez-Medina et al. 2011), or decrease (Aroca et al. 2008).

Among all defense related phytohormones analyzed in mycorrhizal plants, particular interest has been devoted to oxylipins, particularly JAs, as they seem to have a dual positive and negative role on the symbioses (Hause and Schaarschmidt 2009; León-Morcillo et al. 2012; López-Ráez et al. 2010a). The oxilipins include biologically active derivatives and intermediates of JA metabolism, such as JA-Ile and OPDA (oxo-phytodienoic acid), respectively. These compounds are widely distributed in plants and affect multiple processes involved in development and defense (Pozo et al. 2004; Santino et al. 2013; Wasternack 2014). Increased levels of JA have been reported in mycorrhizal roots of several monocot and dicot species (Hause et al. 2002; López-Ráez et al. 2010a; Meixner et al. 2005; Stumpe et al. 2005; Vierheilig and Piche 2002), although there also are reports illustrating unaltered JA levels (Riedel et al. 2008). Still, the regulatory role of JAs and other oxylipins on the symbiosis is far from clear. Studies involving foliar application of JA and wounding have resulted in promotion of mycorrhizal root colonization (Landgraf et al. 2012; Regvar et al. 1996), while the use of JA-impaired mutants in different plant species has resulted in both promotion (Herrera-Medina et al. 2008; Ludwig-Müller et al. 2002) and reduction (Isayenkov et al. 2005) of mycorrhizal colonization, supporting a multifunctional role on the symbiosis.

In summary, current data on defense-related phytohormone homeostasis in AM roots are often fragmented and highly contradictory, opening the hypothesis that the changes in mycorrhizal roots are dependent on the plant and the AMF genotype. To verify such a hypothesis and to identify common features conserved across plant species, we combined targeted metabolomics and transcriptional approaches to analyze changes in SA, ABA, and JA signalling pathways associated with well established mycorrhizas in different host plants. We compared plants belonging to distant families, including maize, soybean, and tomato, and their interaction with two different AMF, F. mosseae and R. irregularis. The results provide insight into the conservation of defense signalling regulation in AM symbioses among phylogenetically distant plant species, and provide hints on the mechanisms underlying functional diversity in AM interactions.

Methods and Materials

Plant Growth and AM Inoculation

Tomato (*Solanum lycopersicum* L. cv. MoneyMaker), soybean (*Glycine max* L. Merr. cv.Williams 82), and maize (*Zea mays* L.) seeds were surface sterilized in 4 % sodium hypochlorite, rinsed thoroughly with sterile water, and germinated for 3 days in a container with sterile vermiculite at 25 °C in darkness. Subsequently, individual seedlings were transferred to 0.25 L pots for tomato and soybean and 0.4 L for maize containing a sterile sand:soil (4:1, v:v) mixture. At transplanting, three treatments were set: non mycorrhizal uninoculated controls (Nm) or plants inoculated by adding 10 % (v:v) inoculum of either *F. mosseae* (Fm) or *R. irregularis* (Ri). For each plant species, at least 6 plants per treatment were set up. The AMF *Rhizophagous irregularis* DAOM 197198 -formerly known as *Glomus intraradices* DAOM 197198- and *Funneliformis mosseae* BEG12 -formerly known as *G. mosseae* BEG12- were maintained as a soil–sand-based inoculum. The inoculum consisted of thoroughly mixed rhizosphere samples containing spores, hyphae, and mycorrhizal root fragments. The same amount of soil:sand mix but free from AMF was added to control plants. All plants received an aliquot of a filtrate (20 ml) of both AMF inocula to homogenize the microbial populations. Plants were randomly distributed and grown in a greenhouse at 24/16 °C with a 16/8 h photoperiod and 70 % humidity. All plants were watered three times a week with water for the first 3 weeks, and from the 4th week on with Long Ashton solution (Hewitt 1966), which contained 25 % of the standard phosphorus concentration. Plants were harvested 8 weeks post inoculation, corresponding to a well established mycorrhizal symbiosis, and the fresh weight of shoots and roots was determined. An aliquot of each individual root system was reserved for mycorrhizal quantification. Root of all plants were frozen in liquid nitrogen and stored at −80 °C until use for metabolite and gene expression analyses.

Mycorrhizal Colonization Determination

Roots were stained with 10 ml of trypan blue solution (Phillips and Hayman 1970) and examined using a Nikon Eclipse 50i microscope and brightfield conditions. The percentage of total root colonization was determined by the gridline intersection method (Giovannetti and Mosse 1980).

Hormone Quantification

OPDA, JA, JA–Ile, ABA, and SA were analyzed by ultraperformance liquid chromatography coupled to mass spectrometry (UPLC-MS/MS) as described by Flors et al. (2008). The hormone content shown for tomato roots corresponds to the experiment described in López-Ráez et al. 2010a. Briefly, a 100 mg aliquot of dry tissue was used per sample. A mixture of internal standards containing 100 ng of [2H6]ABA, 100 ng of dihydrojasmonic acid, 100 ng of prostaglandin B1, and 100 ng of [2H5]SA was added to each sample prior to extraction. Individual calibration curves for each tested compound and internal standard were performed before the analysis. Root tissues were homogenized immediately in 2.5 ml of ultra pure water and centrifuged at 5,000 g for 40 min. Then, the supernatant was acidified and partitioned against diethyl-ether, dried, and resuspended in 1 ml of water/methanol (90:10, v/v). A 20 µl aliquot of this solution was injected into a Waters Acquity UPLC system (Waters). The UPLC was interfaced into a triple quadrupole tandem mass spectrometer (TQD, Waters). LC separation was performed using an Acquity UPLC

BEH C18 analytical column (Waters) at a flow rate of 300 µl min−1. Quantifications were carried out with MassLynx 4.1 software (Waters) using the internal standards as a reference for extraction recovery and the standard curves as quantifiers.

RNA Isolation

Total RNA was extracted using Tri-Reagent (Sigma-Aldrich) according to the manufacturer's instructions. The RNA was treated with RQ1 DNase (Promega), purified through a silica column using the NucleoSpin RNA Clean-up kit (Macherey-Nagel), and stored at –80ºC until use.

Gene Expression Analysis by Real-Time Quantitative RT-PCR (qPCR)

Real-time quantitative RT-PCR (qPCR) was performed using the iCycler iQ5 system (Bio-Rad) and gene-specific primers (Table S1). Routinely, the first-strand cDNA was synthesized from 1 µg of purified total RNA using the iScript cDNA Synthesis kit (Bio-Rad) according to the manufacturer's instructions. For the analysis of genes with low expression level, the first-strand cDNA was synthesized with 3 µg of purified total RNA using the BioScript cDNA Synthesis kit (Bioline), according to the manufacturer's instructions. At least three independent biological replicates were analyzed per treatment, each of them corresponding to root systems of individually grown plants, and qPCR reactions were performed in duplicates. Relative quantification of specific mRNA levels was performed using the comparative method of Livak and Schmittgen (2001). Expression values were normalized using the housekeeping genes SlEF, encoding for the tomato elongation factor-1α, GmEF1, which encodes for the soybean elongation factor-1β; and ZmEF1, which encodes for the maize elongation factor-1α. Amplification reactions were run for 35 cycles at 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 40 s. The specificity of each PCR amplification procedure was verified by the melt curve analysis of the PCR product with a heat dissociation protocol (from 58 to 95 °C).

Laser Capture Microdissection

Roots from 8-wk-old tomato plants inoculated or not with AMF (as described above) were dissected into 5–10 mm pieces and fixed in 100 % acetone at 4 °C overnight for paraffin embedding. Root pieces were placed in acetone under vacuum for 15 min, and then kept at 4 °C overnight. The next day they were gradually dehydrated in a graded series of acetone: Neoclear (Merck, Darmstadt, Germany) (3:1, 1:1, and 1:3) followed by Neoclear 100 % (twice) with each step being carried out on ice for 1 h. The Neoclear was gradually replaced with paraffin (Paraplast Plus; Sigma-Aldrich, St Louis, MO, USA). The embedding step was as described in Balestrini et al. (2007). Sections of 14 µm were cut using a rotary microtome (Microm Hm325) and placed on Leica RNase-free PEN foil slides (Leica Microsystem, Inc., Bensheim, Germany) with diethyl pyrocarbonate-distilled water. The sections were dried at 40 °C in a warming plate, stored at 4 °C, and used within 2 days. Laser microdissection was performed using a Leica AS laser capture microdissection system (Leica Microsystem, Inc.). Samples were deparaffinized in xylene for 10 min, dipped in 100 % ethanol for 2 min, and then air-dried. After collection of around 1,000 cells per cell type, RNA extraction buffer from a Pico Pure kit (Arcturus Engineering, Montain View, CA, USA) was added. Samples were incubated at 42ºC for 30 min, centrifugated at 800 g for 2 min, and stored at 80 °C. RNA was extracted with the Pico Pure kit (Arcturus Engineering), as described by Balestrini et al. (2007), and quantified using a NanoDrop 1,000 (Thermo Scientific, Wilmington, DE, USA) spectrophotometer.

Absence of DNA contamination was confirmed by PCR assays in samples without retrotranscription. Retrotranscription and PCR amplification were carried out using the One Step RT-PCR kit (Qiagen). Samples were incubated for 30 min at 50 °C, followed by 15 min of incubation at 95 °C. Amplification reactions were run in a termocycler Flexcyler (AnalytikJena) for 40 cycles at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 40 s.

Statistical Analysis

Data for mycorrhizal root colonization and gene expression levels were subjected to one-way analysis of variance (ANOVA) using the software SPSS Statistics v. 19 for Windows. When appropriate, DMS's test was applied. Data for hormone content and gene expression were subjected to two-way analysis of variance (two-factor ANOVA, fungus and plant). Significance levels were set at 5 or 0.1 % as indicated.

Results

Root Colonization by F. mosseae and R. irregularis in Different Plant Families

To compare changes in defense related signalling associated to the AM symbiosis in different plant species, tomato, soybean, and maize were inoculated with the AMF *F. mosseae* and *R. irregularis*. Only maize showed a positive growth response to the inoculation with both AMF (Fig. S1). Both fungi were able to colonize the three host plant species and established AM symbiosis, with abundant intraradical colonization and arbuscules in all samples at harvesting. Mycorrhizal colonization levels for tomato and maize were similar (over 20 and 40 % for *F. mosseae* and *R. irregularis*, respectively), while in soybean their colonization levels were 45 and 80 % (Fig. 1). Thus, *R. irregularis* was a more effective root colonizer than *F. mosseae* in all plants tested.

Mycorrhiza Associated Changes Related to SA Signalling

UPLC-MS/MS analysis revealed that SA concentration was slightly higher in roots of tomato plants colonized by *F. mosseae* compared with non-mycorrhizal control plants (Fig. 2a). Interestingly, SA content also was higher in maize roots colonized by *R. irregularis* compared to non-mycorrhizal control plants, while they remained unaltered in *F. mosseae* mycorrhizal plants (Fig. 2c). However, in soybeans, no significant differences were found (Fig. 2b).

We analyzed by qPCR the expression of PR-1a genes, markers of SA regulated defenses that encode for an acidic form of the pathogenesis related protein PR1. In agreement with the changes in the SA content of the plants, a significant induction of tomato PR-1a was observed in *F. mosseae*-colonized roots, with no detectable changes upon colonization with *R. irregularis* (Fig. 2d). The same expression pattern was observed in soybean, with about 2-fold higher levels in plants colonized by *F. mosseae* (Fig. 2e). In contrast, only *R. irregularis* induced PR-1a expression in maize roots (Fig. 2f).

Taken on the whole, the data reveal that the AM symbiosis can result in elevated levels of SA in the three host species, but the changes depend on the plant-AMF combination.

Mycorrhiza Associated Changes Related to ABA Signalling

Free ABA was quantified by UPLC-MS/MS in tomato, soybean, and maize roots colonized by *F. mosseae* or *R. irregularis*. Abscisic acid content was not significantly

altered in either mycorrhizal tomato or soybean roots, compared with non-mycorrhizal control plants, regardless of the colonizing fungi (Fig. 3a and b). Conversely, in maize, lower ABA levels were observed in plants colonized by both AMF (Fig. 3c). To monitor changes in gene expression related to ABA signalling, we compared the transcript levels of ABA response marker genes: Le4, Lea, and ABP9 for tomato, soybean, and maize, respectively. These genes encode for dehydrins, proteins known to be associated with drought stress responses (Hanin et al. 2011). As in the case of ABA content, no changes in Le4 expression levels were observed in tomato roots (Fig. 3d). Similarly, no significant changes were detected for the ABP9 gene in maize (Fig. 3f). However, the expression of Lea in soybean was lower in *F. mosseae*-colonized plants, while *R. irregularis* colonization did not change its expression (Fig. 3e).

Mycorrhiza Associated Changes Related to JA Signalling

The levels of different jasmonates were determined in tomato, soybean, and maize colonized by either *F. mosseae* or *R. irregularis*. The levels of OPDA were significantly ($P<0.05$) higher in *F. mosseae* colonized roots in the three host plants (Fig. 4a, b and c). However, a different behavior was observed in plants colonized by *R. irregularis*. In tomato, *R. irregularis* induced an increase in OPDA content similar to that induced by *F. mosseae* (Fig. 4a). In contrast, OPDA levels were lower with R. irregularis colonized soybean, and not altered in maize plants (Fig. 4b and c). The content of free JA correlated with those of OPDA in maize (Fig. 4f), but no alteration was observed in tomato between mycorrhizal and non-mycorrhizal plants (Fig. 4d). In additions, free JA level was non-altered by *F. mosseae* or was decreased by *R. irregularis* in soybean (Fig. 4e). When JA-Ile was analyzed, higher levels were detected in tomato and soybean roots colonized by *F. mosseae*, but no changes in roots colonized by *R. irregularis* were observed (Fig. 4g and h). In maize, no significant differences in JA-Ile were observed between mycorrhizal and non-mycorrhizal plants (Fig. 4i).

Lipoxygenases are key enzymes in the biosynthesis of JA and other oxylipins, and they are positively regulated by JA. Here, LOX genes encoding for the lipoxygenases LOXA, LOX, and LOX10, confirmed as induced by JA in tomato, soybean, and maize, respectively, were used as markers (Christensen et al. 2013; López-Ráez et al. 2010a; Moy et al. 2004). In all three species, a higher expression of these genes was found in roots colonized by *F. mosseae* compared to the non mycorrhizal controls (Fig. 4j, k, and l). In contrast, colonization by *R. irregularis* did not significantly alter its expression in any of the host plants (Fig. 4j, k, and l).

The results illustrate a common pattern of regulation of the JA signalling pathway in mycorrhizal roots that is dependent on the AM fungi involved, and which is in general conserved across the three plant species tested.

General Analysis of Mycorrhiza Associated Changes in Defense-Related Signalling

On the whole, the results described above illustrate differences in the impact of the symbiosis on the host hormonal profiles depending on both the host plant and the AMF. Figure 5 summarizes the results regarding the impact of root colonization on all three plants by *F. mosseae* or *R. irregularis* on the defense-related pathways regulated by ABA, SA, and JAs. For each signalling pathway, the information obtained from the parameters analyzed (hormone content and changes in the expression of marker genes) is integrated using a color code (green = downregulation, red = upregulation of the pathway, intense colors if both metabolite and the corresponding marker gene expression are altered, and light green or orange when only one of the parameters -

metabolite or marker gene expression- is altered). The two-way ANOVA analysis of the data confirmed that ABA, OPDA, and JA levels were dependent on the plant and fungal genotypes and their interaction, while for SA the values were mostly affected by the plant (Table 1). Globally, the analysis reveals a general positive regulation of the SA- and JA-related pathways in mycorrhizal plants, while the modulation of the ABA-related pathway was neutral or negative (Fig. 5). This conserved enhancement of SA and JA levels in a well established mycorrhiza could mediate the plant control over AMF proliferation within the roots.

The overall analysis revealed a higher impact on defense-related signalling of *F. mosseae* compared to *R. irregularis* colonization in all three plants tested, with JA signalling being consistently induced (Fig. 5). Thus, the data suggest a more exhaustive control of F. mosseae by the host plant, which may underlie the reduced colonization achieved by this fungus when compared with *R. irregularis*.

Cell Specific Analysis of Gene Expression

Mycorrhization is a highly dynamic and asynchronous process with different colonization stages occurring simultaneously within the same root. Laser capture microdissection (LCM) offers an effective way to monitor gene expression in individual cells (Balestrini et al. 2009; Guether et al. 2009; Hogekamp et al. 2011). Aiming to better understand the regulatory role of JA signalling in the symbiosis, we investigated the spatial distribution of the changes to determine if the increase was restricted to arbuscule containing cells. Therefore, LCM was used to analyze changes in gene expression of different JA-responsive genes at the cellular level in the root cortex of tomato plants upon colonization by *F. mosseae*, the AMF leading to stronger and more consistent changes (Fig. 5). Arbuscule containing cells (Fm+) and cortical cells from *F. mosseae* colonized roots without arbuscules (Fm-) were obtained, and compared with cortical cells from non mycorrhizal plants (Nm) (Fig. 6a). Absence of DNA contamination was confirmed by PCR in RNA samples (data not shown). Equal loading was assessed by analysis of transcript levels of the tomato housekeeping gene SlUbiquitin (Fig. 6b). Expression of the AM fungal specific gene Fm 18S rRNA was detected only in cells from mycorrhizal roots, and especially in arbusculated cells (Fm+) (Fig. 6b). The light band detected in non-arbusculated cells from mycorrhizal roots (Fm-) indicated a limited contamination with fungal DNA, probably due to the presence of intercellular hyphae in the thickness of the section. SlPT4 encodes a phosphate transporter specifically associated to cells with arbuscules (Gómez-Ariza et al. 2009), and it is used as a marker of a functional symbiosis. Indeed, SlPT4 expression was detected only in Fm + cells (Fig. 6b), confirming a well established mycorrhization and confirming the discrimination between cells with and without arbuscules. The analysis of different JA regulated genes showed an upregulation in mycorrhizal roots. The JA responsive LoxA and AOS3 genes, involved in the biosynthesis of oxilipins of the 9 LOX branch, were induced in mycorrhizal roots, both in Fm + and Fm- cells, compared to non-mycorrhizal cells (Fig. 6b). The same pattern was observed for the JA biosynthesis gene AOS1 (Fig. 6b). In contrast, the JA-regulated defense gene MC, encoding for a multicystatin, was over the detection limit only in arbuscule containing cells (Fm+) (Fig. 6b). Lin6, encoding for an invertase with a regulatory role in mycorrhiza, and reported to be JA inducible was induced in mycorrhizal roots in both Fm + and Fm- cells (Fig. 6b), although to a higher extent in arbusculated cells. These data confirm the activation of JA metabolism and responses in the roots colonized by *F. mosseae*, not only in the arbusculated cells but also in the neighboring cells.

Discussion

Phytohormones are essential regulators of plant development and immunity (Pieterse et al. 2012; Vanstraelen and Benkov 2012), and many of the deep physiological changes described in mycorrhizal roots may be related to altered hormone levels in the host plant (Hause et al. 2007; Pozo and Azcón-Aguilar 2007; Ruiz-Lozano et al. 2012). However, their role in mycorrhiza establishment and functioning is poorly understood (Ludwig-Müller 2010). Notwithstanding, targeted metabolomic and transcriptomic analyses combined with pharmacological and genetic approaches have yielded abundant information in recent years (Hause et al. 2007; Ludwig-Müller 2010), although data are often fragmented and contradictory. The different experimental set ups, symbiotic stages analysed, and methodologies used for quantification make direct comparisons and conclusions difficult. In this work, we focused on well-established mycorrhizas, and tested the hypothesis that hormone content and hormone-related gene expression levels are dependent on the plant and fungal genotypes. Focussing on stress-related hormones associated with functional mycorrhizas in different AMF- host combinations and using high resolution techniques (UPLC-MS and qRT-PCR), we demonstrated that altered phytohormone-related patterns in mycorrhizal roots are a common feature across plant species. In addition, the two-way ANOVA analysis revealed that some hormonal related changes, mainly those related to ABA and JA, depend on both partner genotypes and their interactions.

Despite the high promiscuity of AMF species capable of forming a successful symbiosis with most host plants, a high functional diversity among different combinations has been reported in terms of morphology, efficiency, and gene expression patterns of the symbiosis (Feddermann et al. 2010). The two AMF species used in this study, *Funneliformis mosseae* and *Rhizophagus irregularis*, −formerly *Glomus mosseae* and *Glomus intraradices*, both within the Glomeraceae family (Krüger et al. 2012)- are the most common AMF used in functional and biodiversity studies, and they are present across drastically different environments (Smith and Read 2008). A different level of mycorrhizal colonization was observed when comparing both fungi, *R. irregularis* being the most effective colonizer in the three host plants tomato, soybean, and maize. Differences in the colonization by both AMF have been reported previously in several other plant species (Feddermann et al. 2008), evidencing differences in their colonization strategies. The quantification of the defense-related hormones ABA, SA, and JA, and the analysis of marker genes of the pathways they regulate, revealed significant changes in their basal levels in roots of mycorrhizal plants compared to non mycorrhizal controls, depending on the AMF and/or the plant involved.

The two AMF had different influence on the levels of SA and its related marker genes (Fig. 5). Only *F. mosseae* induced the SA-related pathway in tomato, while R. irregularis had no effect. A similar trend was observed in soybean. In contrast in maize, the SA pathway was induced exclusively by *R. irregularis* root colonization. Salicylic acid is a key phytohormone in the regulation of defense responses against biotrophs (Gutjahr and Paszkowski 2009), and its negative effect on AM colonization has been shown previously (García-Garrido and Ocampo 2002; Herrera-Medina et al. 2003). Thus, elevated SA levels in the roots could mediate plant control over AMF proliferation, and therefore, spatial and temporal regulation of SA within the roots is probably required following the dynamics of the association. Although SA increases have been related to initial stages of the AM interaction, we report here higher SA

levels associated to well established symbioses in tomato and maize. Elevated levels also have been reported in *F. mosseae* colonized clover (Zhang et al. 2013) and barley (Khaosaad et al. 2007). It has been proposed that SA signalling has a biphasic induction during AM symbiosis, with a first increase in presymbiotic stages that level off as the colonization initiates, and a second induction at later stages of root colonization likely to control colonization extension (Blilou et al. 1999; Gallou et al. 2012).

Regardless of the AMF involved, the ABA-pathway appeared unaltered (in tomato) or slightly repressed (soybean and maize) upon mycorrhization. Similarly, previous studies have reported no changes or repression of the ABA-related pathway in roots (Aroca et al. 2008, 2013; López-Ráez et al. 2010a) and leaves (Asensio et al. 2012; Fiorilli et al. 2009) from mycorrhizal plants. Abscisic acid is known to be a key signal in plant responses to stresses (Hirayama and Shinozaki 2007). Therefore, a reduction of ABA in plants may reflect the consequence of mycorrhiza on improved plant fitness (Barker and Tagu 2000; Ruiz-Lozano 2003). However, ABA also has been shown to play a role in the development of the arbuscules and their functionality (Herrera-Medina et al. 2007), and may be important in pre-symbiosis signalling through the regulation of strigolactone biosynthesis (López-Ráez et al. 2010b). Moreover, ABA is able to modulate other defense-related pathways through positive and negative interactions (Pieterse et al. 2012). Accordingly, the role of ABA in regulating mycorrhizas may depend on both its positive effect on arbuscules and its crosstalk with the SA/JA/ET pathways (Herrera-Medina et al. 2007; Martín-Rodríguez et al. 2011). Interestingly, Meixner et al. (2005) using a split-root system showed a local increase in ABA content in AMF colonized Medicago roots, but a decrease in the systemic, non colonized parts of the root system. Overall, it seems that mycorrhizal plants have to finely regulate ABA levels to promote arbuscule development, and at the same time, to efficiently regulate stress responses.

Finally, the effect of AM symbiosis on the JA-signalling pathway showed a conserved pattern among the plant species analyzed, but it was clearly dependent on the colonizing AMF (Fig. 5). Root colonization by *F. mosseae* led to an increase of JA-related compounds and the up-regulation of JA responsive genes in all plants tested. Oxo-phytodienoic acid levels rose in the three plants, together with increases in JA-Ile in tomato and soybean, and free JA in maize. Since the diverse JA-related compounds may have different functions, this differential accumulation pattern may indicate diverse roles in orchestrating the plant responses to mycorrhizal fungi, according to the host plant species. Interestingly, no major alterations in JA-signalling were observed in R. irregularis mycorrhizal plants (with higher colonization levels) compared to non-mycorrhizal plants, regardless of the plant species involved. Similarly, no changes in JA content have been described in R. irregularis colonized Nicotiana roots (Riedel et al. 2008). These observations demonstrate a strong dependence of the modulation of JA-signalling on the AMF genotype, with a negative correlation between JA increases and mycorrhizal extension, as shown for F. mosseae. The negative role of JA in the extension of AM colonization has been confirmed in the tomato JA insensitive mutant, jai1, that shows higher colonization levels than the wild-type (Fernandez et al., unpublished data; Herrera-Medina et al. 2008). Recently, JA signalling has been shown to be induced in late stages of ectomycorrhiza formation to limit, together with ET, intraradical fungal growth. Moreover, exogenous JA or ET application did not affect the early interaction events (mantle formation), but they affected the later stages, inhibiting the development of the Hartig net (Plett et al. 2014a).

The activation of some plant defenses during AMF colonization seems to occur at the cellular level, and probably different regulation patterns may coexist in time in different parts of the root system. Indeed, transcript dilution in whole root system analyses may mask some transcriptional changes occurring locally. Here, the induction of JA related gene expression in roots colonized by *F. mosseae* was consistent at the whole root scale and the colonized cortical cells. Jasmonic acid biosynthetic genes have been reported previously to be induced in arbuscule containing cells (Hause et al. 2002). Here, the analysis of the allene oxydase synthase encoding genes AOS1 and AOS3 at the cellular level in *F. mosseae* colonized tomato roots revealed an induction in arbuscule containing and non-containing cortical cells of mycorrhizal roots compared to those from non mycorrhizal roots. However, the induction of the JA-responsive marker genes MC and Lin6 was stronger in arbuscule-containing cells, thus supporting a spatial expression pattern of JA responsive genes. MC codes for a multicystatin involved in plant defense responses, and Lin6 encodes for an extracellular invertase induced in mycorrhizal roots with an important role in the symbiosis (Schaarschmidt et al. 2006, 2007). Both functions, defense and carbon metabolism, have been pointed out as the major roles of JAs in the symbiosis (Hause et al. 2007). In this regard, JA seems to contribute to the regulation of colonization in different ways, positively, by increasing the sink strength of mycorrhizal roots for carbohydrates that support fungal growth (Schaarschmidt et al. 2006) and negatively by restricting excessive colonization (Herrera-Medina et al. 2008). Besides that, JAs have been proposed to contribute to the reorganization of the cytoskeleton needed for fungal accommodation (Genre and Bonfante 2002; Genre et al. 2005). More recently, it has been proposed that JA may act by fine tuning gibberellins signalling, contributing to the regulation of the AM symbiosis according to phosphate availability (Gutjahr and Parniske 2013). The multifunctionality of these hormones, the existence of different active forms, and the spatial and temporal regulation of genes related to JA and other oxylipins (Gallou et al. 2012; Hause et al. 2002; León-Morcillo et al. 2012) suggest a precise control of the JAs homeostasis for fine-tuning the symbiosis.

In summary, we found that *R. irregularis* showed higher root colonization rates in all plants tested, while the changes in the host roots related to defense signalling, and mainly in the JA pathway, were lower than in *F. mosseae* colonized roots. This finding suggests a lessor control by the plant over *R. irregularis* colonization that may be related to the fungal ability to suppress or evade the plant defense response, thus achieving a higher root invasion (Campos-Soriano et al. 2010). Indeed, it has been reported that *R. irregularis* can deliver the effector protein SP7, which attenuates the plant immune response that enables the accommodation of the fungus within plant roots (Kloppholz et al. 2011), as it occurs in ectomycorrhizas (Plett et al. 2014b). How conserved and efficient this mechanism is in other AMF remains to be determined. It is possible that the different impact of each AMF on host defense signalling partially explains the functional diversity observed in terms of plant induced stress resistance. For example, it is tempting to speculate that the stronger activation by *F. mosseae* of JA signalling may underlie the higher ability of this AMF to induce mycorrhiza induced resistance (Jung et al. 2012; Pozo et al. 2002). Our results provide an overview of the changes related to stress related hormones in a well established mycorrhizal symbiosis, confirming our hypothesis that the changes depend on the partners genotypes and more specifically on the colonizing fungus.

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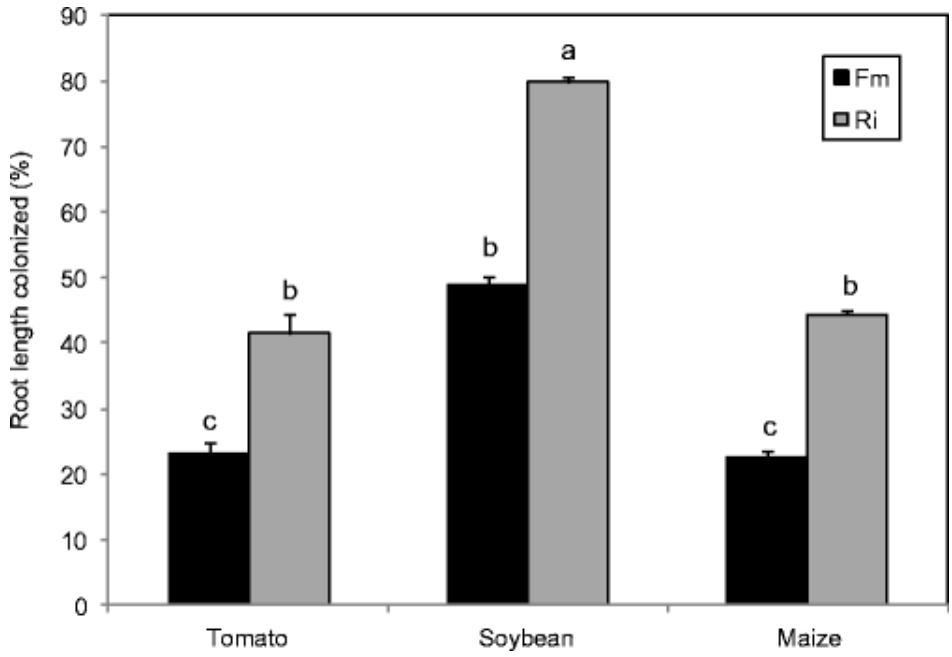

Figure 1
Quantification of mycorrhizal colonization of tomato, soybean and maize roots inoculated with Funneliformis mosseae (Fm) or Rhizophagus irregularis (Ri). Data represent the means of 4 independent replicates ± SE. Data not sharing a letter in common differ significantly ($P \leq 0.05$) according to DMS's test

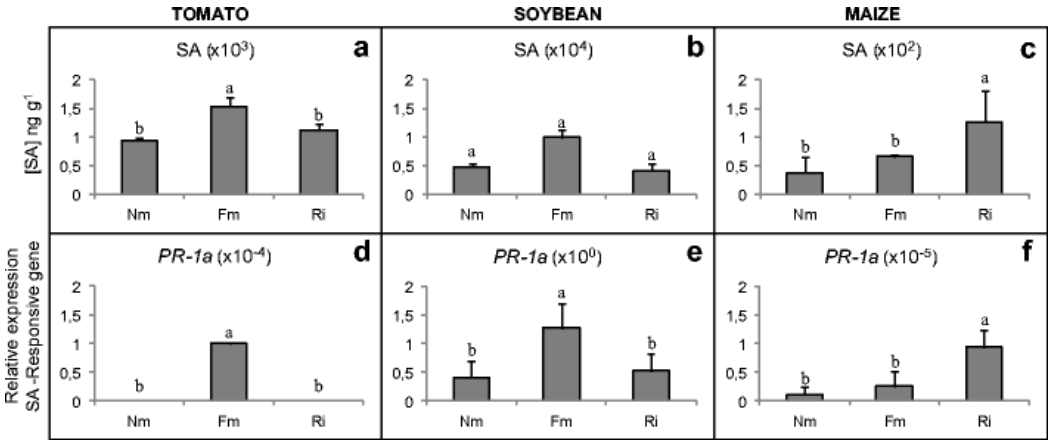

Figure 2
Effect of mycorrhizal colonization on the salicylic acid (SA) signalling pathway in tomato, soybean, and maize roots colonized by either *Funneliformis mosseae* (Fm) or *Rhizophagus irregularis* (Ri). a, b, c, SA levels in roots of non mycorrhizal plants (Nm) or plants colonized by *F. mosseae* (Fm) or *R. irregularis* (Ri). d, e, f, relative expression of the SA-responsive PR-1a genes. Gene expression was normalized to the expression of constitutively expressed genes selected as reference for each plant (encoding for elongation factors SlEF1, GmEF1, and ZmEF1). Data points represent the means of five (UPLC-MS/MS) or three (qPCR) independent biological replicates ± SE. Data not sharing a letter in common differ significantly ($P \leq 0.05$) according to DMS's test

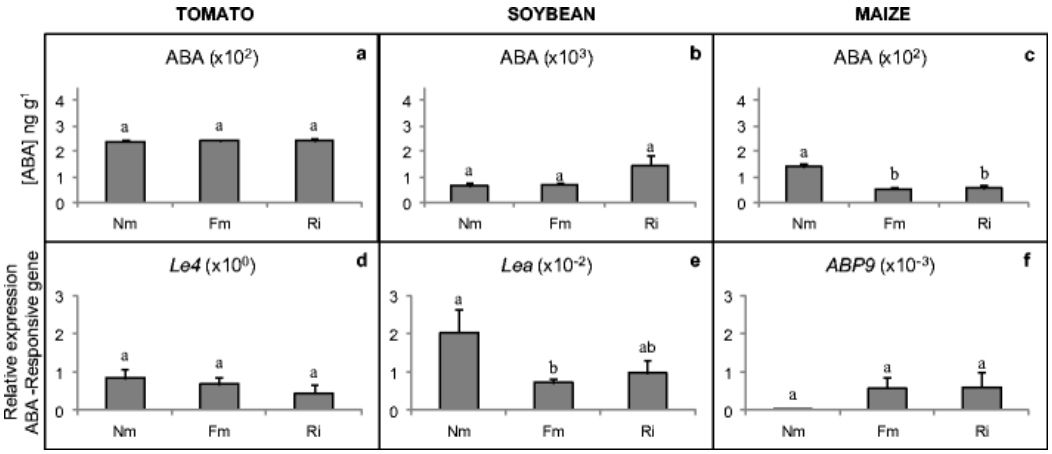

Figure 3
Effect of mycorrhizal colonization on the abscisic acid (ABA) signalling pathway in tomato, soybean, and maize roots colonized by either Funneliformis mosseae (Fm) or Rhizophagus irregularis (Ri). a, b, c, ABA levels in roots of non mycorrhizal plants (Nm) or plants colonized by F. mosseae (Fm) or R. irregularis (Ri). d, e, f, relative expression of the ABA-responsive genes Le4, Lea and ABP8 in tomato, soybean and maize roots, respectively. Replication, normalization and statistical analysis as described in Fig. 2.

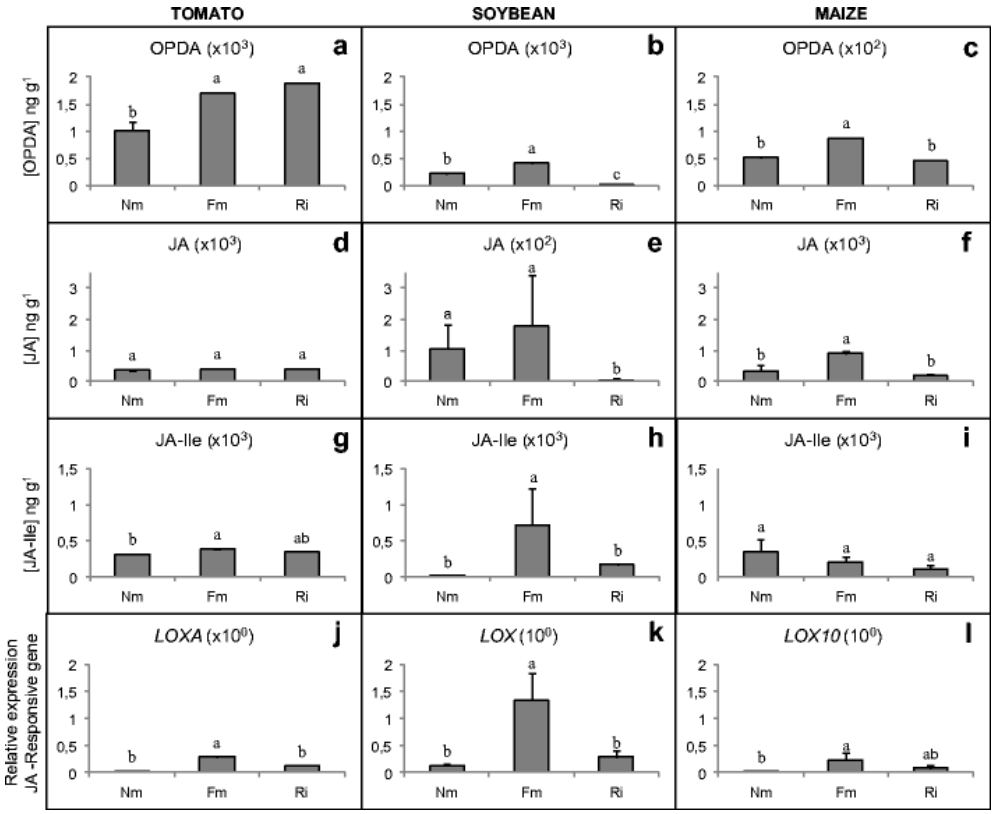

Figure 4

Effect of mycorrhizal colonization on the oxylipin/jasmonate signalling pathway in tomato, soybean and maize roots colonized by either *Funneliformis mosseae* (Fm) or *Rhizophagus irregularis* (Ri). Levels of oxo-phytodienoic acid (OPDA) (a, b, c), free JA (d, e, f) and JA-Ile (g, h, i) in roots of non mycorrhizal plants (Nm) or plants colonized by F. mosseae (Fm) or R. irregularis (Ri). j, k, l, relative gene expression for the JA-responsive genes LOXA, LOX, and LOX10 in tomato, soybean and maize roots respectively. Replication, normalization and statistical analysis as described in Fig. 2.

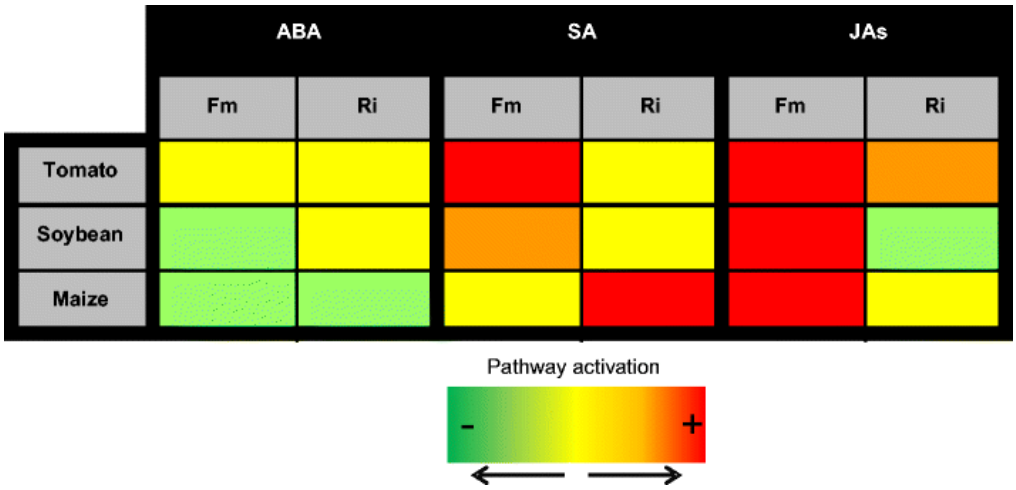

Figure 5
Summary of the changes in the different hormone-related pathways induced in the host plants by the arbuscular mycorrhizal fungi (AMF) *Funneliformis mosseae* and *Rhizophagus irregularis* [abscisic acid (ABA), salicylic acid (SA), and jasmonic acids (JAs)]. Red indicates up-regulation and green indicates down-regulation of the different hormonal pathways. Changes only in one of the parameters analyzed (hormone content or expression level of marker genes) are indicated in orange when up-regulated and light green when down-regulated. Yellow indicates no changes compared to non-mycorrhizal control plants.

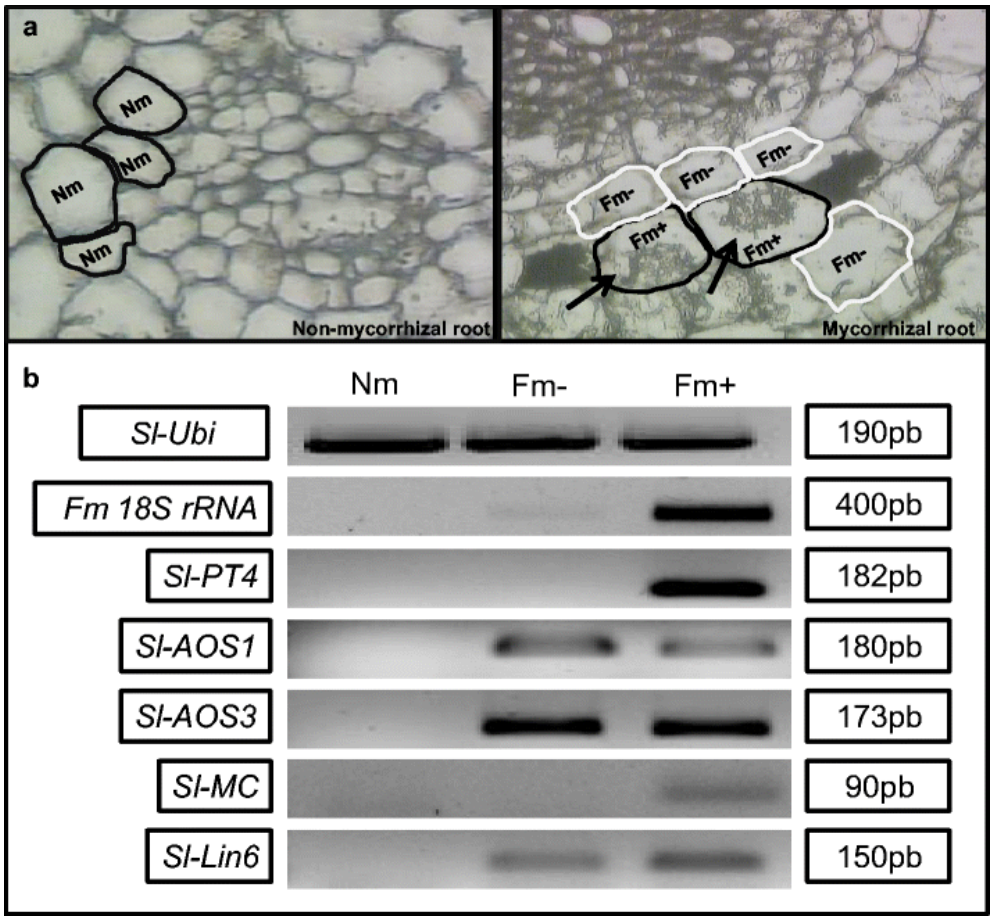

Figure 6
Detection of marker gene transcripts in laser microdissected cell types in non mycorrhizal or *F. mosseae* colonized tomato roots. The analysis was performed by One step RT-PCR in three different cell types: cortical cells from non mycorrhizal controls (Nm), non colonized cortical cells of mycorrhizal roots (Fm-), and arbuscule containing cortical cells (Fm+) (arrows point to the arbuscules) (a). The figure shows gel electrophoresis of the amplification products, and their corresponding size is indicated on the right (b). SlUbi, Ubiquitin, Fm18SrRNA,AMF 18S ribosomic RNA, SlPT4, Phosphate transporter4, SlLOXA, lipoxygenase A, SlAOS1, allene oxide synthase 1, SlAOS3, allene oxydase synthase 3, SlMC, multicistatin, SlLin6 extracellular invertase 6

Table 1

Significance of the sources of variation after *two-way anova* analysis for the hormone content and expression levels of related responsive (r-gene) genes

P-value							
	ABA		SA		JAs		
	[ABA]	R-Gene	[SA]	R-Gene	[OPDA]	[JA]	[JA-Ile]
Plant	<0,001**	<0,001**	<0,001**	<0,001**	<0,001**	<0,001**	0,655
Fungus	0,002*	0,116	0,158	0,054	<0,001**	<0,001**	0,189
Plant x Fungus	<0,001**	0,091	0,204	0,029*	<0,001**	<0,001**	0,142

P-values were considered significant, * $P < 0.05$, ** $P < 0.001$