Late activation of the 9-oxylipin pathway during arbuscular mycorrhiza formation in tomato and its regulation by jasmonate signalling

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Abstract

The establishment of an arbuscular mycorrhizal (AM) symbiotic interaction is a successful strategy for the promotion of substantial plant growth, development, and fitness. Numerous studies have supported the hypothesis that plant hormones play an important role in the establishment of functional AM symbiosis. Particular attention has been devoted to jasmonic acid (JA) and its derivates, which are believed to play a major role in AM symbiosis. Jasmonates belong to a diverse class of lipid metabolites known as oxylipins that include other biologically active molecules. Recent transcriptional analyses revealed up-regulation of the oxylipin pathway during AM symbiosis in mycorrhizal tomato roots and indicate a key regulatory role for oxylipins during AM symbiosis in tomato, particularly those derived from the action of 9-lipoxygenases (9-LOXs). Continuing with the tomato as a model, the spatial and temporal expression pattern of genes involved in the 9-LOX pathway during the different stages of AM formation in tomato was analysed. The effects of JA signalling pathway changes on AM fungal colonization were assessed and correlated with the modifications in the transcriptional profiles of 9-LOX genes. The up-regulation of the 9-LOX oxylipin pathway in mycorrhizal wild-type roots seems to depend on a particular degree of AM fungal colonization and is restricted to the colonized part of the roots, suggesting that these genes could play a role in controlling fungal spread in roots. In addition, the results suggest that this strategy of the plant to control AM fungi development within the roots is at least partly dependent on JA pathway activation.

Key words: Arbuscular mycorrhiza, jasmonate mutants, 9-LOX pathway, oxylipins.

Introduction

Arbuscular mycorrhizal (AM) fungi are soil micro-organisms that establish mutual symbiosis with the majority of higher plants. This symbiotic interaction is highly beneficial and substantially promotes plant growth, development, and fitness (Smith and Read, 2008). It enables plants to grow and reproduce in relatively harsh mineral stress environments. In exchange, the fungi obtain their carbon source from the host plant in the form of plant photosynthates. This whole process of bidirectional nutrient exchange between plant and fungus is tightly linked and highly dependent upon environmental and biological variables (Koltai et al., 2010).

The establishment of AM symbiosis requires the host root cells to undergo significant structural and functional modifications that eventually lead to reciprocal beneficial effects. A combination of genetic, molecular, and cellular approaches actually reveals that functional symbiosis appears to occur at the end of a series of plant-controlled checkpoints in which different classes of plant hormones play an essential role.

Plant hormones are critical for plant development and also constitute signals for the interaction of plants with microbes, including both pathogens and mutualistic...
symbionts. They are known to regulate processes of plant adaptation and change, and participate in the processes of plant interaction with their environment. In general terms, they are multifunctional molecules and excellent plant growth regulators. It is therefore not surprising that this type of molecule is involved in the process of AM development, which involves mutual recognition as well as morphological and physiological adjustments in the root in order to support the fungus.

In recent years, tremendous efforts have been made to unravel the role of plant hormones during the interaction of plants with AM fungi. However, there are still considerable gaps in our knowledge of how both partners in the symbiosis contribute to the regulation of hormone production and how plant hormones regulate the different stages and processes of symbiosis. Some of the changes in the host are linked to modifications in the relative abundance of plant hormones, most of which are thought to play a role in the symbiosis (Ludwig-Müller, 2010). It is accepted that ethylene (ET) and salicylic acid (SA) negatively affect root colonization (Bilou et al., 1999; Herrera-Medina et al., 2003; Riedel et al., 2008; Gutjarh and Paszkowski, 2009), and there is evidence to show that abscisic acid (ABA) has a positive impact on the establishment and functionality of mycorrhiza (Herrera-Medina et al., 2007; Martín Rodríguez et al., 2010, 2011).

Particular attention has been devoted to jasmonic acid (JA) and its derivates, which are believed to play a major role in AM symbiosis (Hause and Schaaranschmidt, 2009). Experimental data on the pattern of JA accumulation in mycorrhizal roots as compared with non-mycorrhizal controls are highly variable and have been reported to increase in certain studies (Hause et al., 2002; Meixner et al., 2005) and to remain unchanged in others (Riedel et al., 2008). Studies using plant mutants affected in JA biosynthesis or signalling have highlighted the positive and negative regulatory roles played by the JA pathway in AM symbiosis (Isayenkov et al., 2005; Herrera-Medina et al., 2008; Tejeda-Sartorius et al., 2008). These contradictory findings could be mainly explained by the different plant and experimental systems used in these studies. However, the possibility that these results are partly due to the overlapping, yet distinct, signalling activities of precursors and/or jasmonate derivates can be ruled out. Jasmonates belong to a diverse class of lipid metabolites known as oxylipins that include other biologically active molecules (Wasternack, 2007; Mosblech et al., 2009).

Oxylipins are a group of biologically active compounds that perform a variety of functions in plants (Howe and Schilmiller, 2002). The structural and functional diversity of oxylipins is governed by the coordinated expression of specific members of the lipoxygenase (LOX) and cytochrome P450 (CYP74) enzyme families. The relative specificity of these enzymes for either 9- or 13-hydroperoxides supports the concept that oxylipin metabolism is organized into discrete 9-LOX and 13-LOX pathways. In the 13-LOX branch of oxylipin metabolism, allene oxide synthase (AOS) transforms 13-hydroperoxide linolenic acid (13-HPOT) to an epoxide intermediate (EOT) that converts spontaneously into 9- and γ-ketols that have unknown function in plants. In the presence of allene oxide cyclase (AOC), however, EOT is converted to 12-oxophytodienoic acid (OPDA) and then to JA.

Two recent microarray analyses of tomato mycorrhizal roots revealed a strong up-regulation of genes coding for the key LOXA and AOS3 enzymes, directly involved in the metabolism of 9-LOX oxylipins (García-Garrido et al., 2010; López-Ráez et al., 2010). It is plausible that the activation of the 9-LOX pathway is part of the plant’s strategy to control AM fungus development within the roots given that a defensive role for 9-LOX oxylipins in restricting pathogen spread in roots has previously been proposed (Blécé, 2002; Vellosillo et al., 2007; Mosblech et al., 2009).

In this study, a spatial and temporal expression pattern analysis of genes involved in the 9-LOX pathway during the different stages of AM formation in tomato was carried out. The effects of JA signalling pathway changes on AM fungal colonization were assessed and correlated with the modifications in the transcriptional profiles of 9-LOX genes. The results provide an original contribution to understanding the impact of the 9-LOX oxylipin pathway on AM symbiosis and the regulatory network controlling this interaction.

Materials and methods

Plant growth and AM inoculation

Seeds of Solanum lycopersicum wild-type Ailsa Craig (LA2838A), Money Maker (LA2706), and Castlemart (LA2400) were obtained from the Tomato Genetics Resource Centre (TGRC) at the University of California. Mutant tomato seed lines jai-1 (Li et al., 2001), spr-2 (Li et al., 2003), and def-1 (Howe et al., 1996) were kindly provided by Dr G.A. Howe at the Plant Research Laboratory, Michigan State University, USA.

Tomato seed sterilization, AM fungi inoculation, and plant growth were carried out according to techniques described by Martin-Rodriguez et al. (2010). Plant growth and treatments were carried out in a growth chamber (day/night cycle: 16 h, 25 °C/8 h, 19 °C; relative humidity 50%). Inoculation with Glomus intraradices (DAOM 197198), reclassified as G. irregularare according to Stockinger et al. (2009), was carried out in 200 ml pots. Each seedling was grown in a separate pot and inoculated with a piece of monoxenic culture in a Gel-Gro medium containing 50 G. intraradices spores and infected carrot roots. One week after planting in pots and weekly thereafter, 20 ml of a modified Long Ashton nutrient solution containing 25% of the P concentration (Hewitt, 1966) was added to prevent mycorrhizal inhibition caused by excess P. Plants were harvested at different times after inoculation, and the root system was washed and rinsed several times with sterilized distilled water. The root system was weighed and used for the different measurements according to the type of experiment carried out. In each experiment, five independent plants were analysed per treatment.

The jasmonate-insensitive recessive jai-1 mutant is female sterile and, therefore, cannot be maintained as a homozygous line. Homozygous jai-1/jai-1 seedlings were selected from F2 populations after G. intraradices inoculation. The segregating homozygous JAI-1/JAI-1 and heterozygous jai-1/jai-1 plants are jasmonate sensitive and were used as control plants in the experiments. The selection of homozygous seedlings (jai-1/jai-1) is based on a PCR technique using DNA from a leaf of each seedling (Li et al., 2004).
In experiments on exogenous methyl jasmonate (MeJA; Sigma-Aldrich) applications, tomato Spr-2 plant mutants were inoculated and grown in 200 ml pots. A 25 ml aliquot of 0.1 μM and 5 μM solutions of MeJA was applied once a week to each pot. The pH of each solution was adjusted to 7. The aqueous stock solution of MeJA contained 1 mM MeJA and 0.01% Tween-20.

Phytohormone treatments
After germination, tomato plantlets (Money Maker) were grown in 200 ml pots filled with a mixture of silicate sand, peat, soil, and vermiculite (1:1:1:1, v:v:v) for 40 d, and the phytohormone solutions were then applied to soil in a volume of 25 ml per pot. Plants were harvested 6, 12, and 24 h later, and the root system was washed and immediately frozen in liquid nitrogen. The solutions applied contained H2O, MeJA 50 μM, ABA (Sigma) 75 μM, ethephon (Sigma) 70 μM, gibberellic acid (GA3; Sigma) 5 μM, and SA (Sigma-Aldrich) 0.5 mM. All were prepared from stock solutions, and the pH of each solution was adjusted to 7 prior to use.

Estimation of mycorrhizal root colonization
The non-vital trypan blue histochemical staining procedure was used according to the method of Phillips and Hayman (1970). Stained roots were observed with a light microscope, and the intensity of root cortex colonization by AM fungus was determined as described by Trouvelot et al. (1986) using MYCOCALC software (http://www.dijon.inra.fr/mychintec/Mycocalc-prg/download.html). The parameters measured were colonization intensity (M%) and arbuscular abundance (a%) in mycorrhizal root fragments. Three microscope slides were analysed per biological replicate, and each slide contains thirty 1 cm root pieces.

RNA isolation and gene expression analysis by real-time quantitative PCR
For the quantitative reverse transcription-PCR (qRT-PCR) experiments, total RNA was isolated from a 0.5 g sample taken from the root, which was a representative part of the root system for each plant and was treated as a biological replicate.

Total RNA was isolated from the roots stored at –80°C using the RNeasy Plant Mini Kit (Qiagen, MD, USA) following the manufacturer’s instructions. cDNAs were obtained from 1 μg of total DNase-treated RNA in a 20 μl reaction volume using the iScript™ cDNA synthesis kit following the supplier’s protocol (Bio-Rad, Hercules, CA, USA). qRT-PCR was carried out with an iCycler apparatus (Bio-Rad). Each 20 μl PCR contained 1 μl of diluted cDNA (1:10), 10 μl of 2× SYBR Green Supermix (Bio-Rad), and 200 nM of each primer using a 96-well plate. The PCR program consisted of a 3 min incubation at 95°C, followed by 35 cycles of 30 s at 95°C, 30 s at 58–63°C, and 30 s at 72°C. The specificity of the PCR amplification procedure was checked using a melting curve after the final PCR cycle (70 steps of 30 s from 60°C to 95°C at a heating rate of 0.5°C). Experiments were carried out on three biological replicates, and the threshold cycle (Ct) was determined in triplicate. The relative transcription levels were calculated by using the 2^{Ct ΔΔCt} method (Livak and Schmittgen, 2001). The Ct values of all genes were normalized to the Ct value of the LeEF-1 (X14449) housekeeping gene.

Real-time PCR was carried out to measure the transcript levels of the G. intraradices elongation factor 1α (GinEF) and glutamine synthase (GinGS) genes in mycorrhizal roots as well as mycorrhiza-responsive phosphate transporter 4 (LePT4), lipoxygenase A (LOXA), allene oxide synthase 3 (AOS3), divinyl ether synthase (LeDES), and proteinase inhibitor II (PIN II) genes from tomato. All primer names and corresponding sequences are listed in Table 1.

Statistical analysis
The data were subjected to one- or two-way analysis of variance (ANOVA). The mean values of five biological replication samples for mycorrhization parameters and three replications for qRT-PCR experiments were compared using Duncan’s multiple range test (P = 0.05).

Results
Expression pattern of genes involved in 9-LOX metabolism during AM development
Previous array analysis of mycorrhizal tomato revealed that a set of up-regulated genes corresponded to genes involved in 9-oxylipin metabolism (Fig. 1). In order to investigate the possible correlation between the degree of AM fungal colonization and 9-LOX-related genes, wild-type Ailsa Craig tomatoes were inoculated with G. intraradices, and the degree of fungal colonization and gene expression were determined in a time course experiment.

Table 1. Primers used in this study

| Primer name | Organism                  | Target gene | Primer sequence (5′–3′)          | Reference               |
|-------------|---------------------------|-------------|----------------------------------|-------------------------|
| PT4F        | Solanum lycopersicum      | LePT4       | 5′-GAAGGGGGGAGCATATAATGGTG3′      | Nagy et al. (2005)      |
| PT4R        |                           |             | 5′-CCATCTTGTTGTATTTGTGA3′        |                         |
| EF-1xF      | Solanum lycopersicum      | LeEF-1x     | 5′-GGCGTTGAGACTGTTGTGAT3′        | Martín Rodríguez et al. (2010) |
| EF-1xR      |                           |             | 5′-GATGATGACCTGGTCAGT3′          |                         |
| Aos3-F      | Solanum lycopersicum      | AOS3        | 5′-TCGAGATAGATTGTGGGGG3′         | García-Garrido et al. (2010) |
| Aos3-R      |                           |             | 5′-CTACTACACCTTTCCCAAA-3′        |                         |
| LoxA-F      | Solanum lycopersicum      | LOXA        | 5′-GAAAACCCCGAAGGACAT3′          | García-Garrido et al. (2010) |
| LoxA-R      |                           |             | 5′-AGGAGACTTCCTGTTGTGCA-3′       |                         |
| LeDes-F     | Solanum lycopersicum      | LeDES       | 5′-CCGAGATGATTGACATGTA-3′        | López-Raéz et al. (2010) |
| LeDes-R     |                           |             | 5′-ATCTTTTGCCTGAGCATTGCT-3′      |                         |
| PIN II-F    | Solanum lycopersicum      | PIN II      | 5′-GAAATGGATTAATTATCCAC-3′       | López-Raéz et al. (2010) |
| PIN II-R    |                           |             | 5′-ACATACAACCTTTCCATTTT-3′       |                         |
| GinGS-F     | Glomus intraradices       | GinGS       | 5′-CCTCAAGTGTCACCTTATGTTTG-3′    | Gomez et al. (2009)     |
| GinGS-R     |                           |             | 5′-AGCTTTTGAGCATCAAGCACC-3′      |                         |
| GinEF-F     | Glomus intraradices       | GinEF       | 5′-GACATATCGTATCACCAGGC-3′       | Benabdellah et al. (2009) |
The intensity of root colonization and the percentage of arbuscules in the colonized roots were assessed (Fig. 2A). A typical mycorrhization dynamic was found in the experiments, where values for the percentage of colonized roots with AM fungal structures ranged from 10% at 36 dai (days after inoculation) to 55% at 65 dai, with the percentage of arbuscule abundance (%a) ranging from 9% at 36 dai to 37% at 65 dai. Similar values for %a were found at 50 and 65 dai. Fungal colonization was also analysed using the qRT-PCR technique, since arbuscular functionality in mycorrhizal roots was quantified at the molecular level by detecting transcripts for the AM-specific tomato phosphate transporter LePT4 (Fig. 2B). Transcript accumulation of LePT4 was clearly associated with mycorrhizal development and functionality in roots, since the M data for LePT4 correlated with the data for arbuscule abundance in roots (%a). The data in Fig. 2B show the M values for LePT4 gene induction (log2 fold change ratio) in treated plants with respect to gene expression for non-treated plants. A similar and significant change in LePT4 expression was observed at 50 and 65 dai, where M values were found to be between 5 and 6 (30- and 60-fold up-regulated, respectively).

The expression pattern of 9-LOX metabolism-related genes (LOXA, AOS3, and LeDES) was also quantified using qRT-PCR. The expression patterns of selected genes were characterized in the roots of mycorrhizal and non-mycorrhizal plants, respectively, at 36, 50, and 65 dai. The M value was calculated for mycorrhizal plants with respect to gene expression in non-mycorrhizal plants for each gene at each harvest time (Fig. 2C). The expression of all three genes was lower in mycorrhizal than in non-mycorrhizal plant roots at 36 dai. The expression of LOXA was higher in mycorrhizal roots than in non-mycorrhizal roots at 50 and 65 dai. AOS3 gene expression was found to have increased in mycorrhizal plants only at 65 dai. No increase in gene expression in mycorrhizal plants with respect to non-mycorrhizal plants was found at any time for the LeDES gene. Even at 36 dai, LeDES expression was twice as low in non-mycorrhizal plants as in mycorrhizal plants (M value reaching –1) (Fig. 2C).

**Analysis of the mycorrhizal systemic effect on the expression profiles for 9-LOX genes**

Split-root systems are a useful tool for studying systemic effects of mycorrhizal roots, including autoregulatory
mechanisms (Catford et al., 2003), and the systemic bioprotectional effect against fungal pathogen (Khaosaad et al., 2007). In this study, whether the establishment of symbiosis in one compartment of the split-root system regulates expression of 9-LOX genes in the other compartment was determined. The first half of the split-root system was inoculated with the mycorrhizal fungus *G. intraradices* and not inoculated for the control plants, and the development of AM symbiosis was followed in a time course experiment. Gene expression was determined on both sides by qRT-PCR. Fungal colonization was quantified by qRT-PCR at the molecular level by measuring the accumulation of mRNA for the *GinEF* gene in tomato roots in order to determine the rate of fungal colonization. The presence of arbuscules in the whole mycorrhizal root was quantified at the molecular level through transcript detection in the *GinGS* gene, which has been described elsewhere as being expressed in the arbuscule (Gomez et al., 2009).

A diagram of the split-root system is shown in Fig. 3A. The data in Fig. 3B show the M values for fungal genes on the inoculated and non-inoculated side of mycorrhizal plants (sides A and B) with respect to gene expression on the inoculated side of mycorrhizal plants at 40 dai (side A of 40 dai plants). As expected, no gene expression for fungal genes was found on the non-inoculated side of mycorrhizal plants (side B), confirming that the fungus was retained on side A of the split-root system. The evolution of fungal *GinEF* and *GinGS* expression on side A was similar. The expression of both genes was found to have increased ~6-fold (M value of 2.5) at 54 dai with respect to that at 40 dai. At 65 dai, the expression of *GinEF* and *GinGS* was 2–3 times higher than at 40 dai (M value of 1–1.5).
Data in Fig. 3C show the M values for 9-LOX gene induction in the inoculated (side A) and non-inoculated zone (side B) of mycorrhizal plants, and the proximal (side C) and distal zone (side D) of non-mycorrhizal plants. All M values were calculated with respect to gene expression on the proximal side (side C) of the split-root system’s non-mycorrhizal plants at 40 d harvest time. The evolution in gene expression for LOXA and AOS3 was quite similar. The expression of these genes did not change in relation to side or mycorrhizal status at 40 dai, and clearly increased only on the inoculated side of mycorrhizal plants (side A) at 54 dai. For the LOXA gene, the non-inoculated zone (side B) of mycorrhizal plants and the proximal (side C) and distal zone (side D) of non-mycorrhizal plants showed an expression level similar to that at 40 dai. In all the non-mycorrhizal parts of the split-root system, the expression of the AOS3 gene at 54 dai diminished with respect to the expression on the proximal side (side C) of the split-root system’s non-mycorrhizal plants at 40 d harvest time. The non-inoculated zone (side B) of mycorrhizal plants showed
similar levels of AOS3 gene expression at 40 and 54 dai. At 75 dai, the expression of LOXA and AOS3 genes diminished in all parts of plants regardless of the side or mycorrhizal status. The increase in LOXA and AOS3 gene expression at 54 dai coincided with maximum fungal gene expression in roots. No major differences in the evolution of LeDES gene expression were associated with the side or mycorrhizal status of the plant in the split-root experiment, as the M value ranged from 1 to –1 (a <2-fold increase/decrease) in all cases.

Analysis of genes involved in 9-oxylipin metabolism in response to phytohormone treatment

The results suggest that the expression of LOXA and AOS3, although not that of LeDES, seems to depend on a certain degree of AM fungal colonization. On the other hand, phytohormones have been reported to be involved in AM fungal colonization (Hause et al., 2007), and LOXA and AOS3 genes have previously been reported to be JA inducible (Garcı´a-Garrido et al., 2010). In this study, the capacity of the selected 9-LOX genes to respond to the exogenous application of certain phytohormones involved in the formation of fungal structures and the establishment of functional symbiosis was determined and compared.

Tomato plants were treated with phytohormone solutions, and RNA was extracted from roots after 6, 12, and 24 h of treatment. The experiments were designed to show the capacity of selected genes to respond early on (6 h) and at a late stage (12–24 h) to the exogenous application of phytohormone solutions. LOXA and AOS3 genes showed similar patterns of response to phytohormone treatment, while the LeDES gene had a different response pattern (Fig. 4). MeJA treatment caused high up-regulation of the gene expression for LOXA and AOS3. Gene expression peaked after 6 h of treatment and later decreased after 24 h. Apart from MeJA, two slight increases in AOS3 gene expression were observed in plant roots after 12 h of SA and ET treatment. No other phytohormone treatments caused significant differences in LOXA and AOS3 gene expression compared with control roots treated with water (Fig. 4).

Treatment of plants with MeJA caused early, moderate, and transient expression of the LeDES gene. This gene was also ABA, ET, and SA responsive. ABA application caused a slight but significant 3-fold increase in LeDES expression. ET treatment resulted in an early, large, and transient increase in gene expression, whereas SA caused a moderate but more permanent increase in LeDES expression (Fig. 4).

AM formation and expression profiles of 9-LOX genes in plant mutants with altered JA signalling pathways

To examine the extent and significance of the interaction between the JA pathway and 9-LOX gene expression in relation to the process of AM formation, the pattern of mycorrhization and 9-LOX gene expression was studied in tomato mutant plants with altered JA biosynthesis and/or perception pathways. Two different kinds of mutant lines with altered JA pathways were used. The spr-2 and def-1 lines correspond to plants whose JA response has been affected by JA synthesis impairment (Howe et al., 1996; Li et al., 2003). The tomato jai-1 line has deficient JA perception due to the blockage of the jasmonate signalling pathway (Li et al., 2001).

The intensity of root colonization and the percentage of arbuscules in the colonized roots were assessed in an initial experiment using wild-type Castlemart tomatoes and spr-2 and def-1 mutant plants (Fig. 5A). The wild-type plants showed a typical mycorrhization dynamic, with values for the intensity of AM fungal colonization ranging from 15% at 38 dai to 55% at 52 dai, and an arbuscule abundance (%a) ranging from 30% at 38 dai to 45% at 52 dai. Spr-2 plants were unaffected in terms of mycorrhizal intensity at 38 dai, although a sharper reduction in this mycorrhization parameter was observed at 52 dai. A slight increase in the intensity of mycorrhization was detected in the roots of mycorrhizal def-1 plants at 38 dai as compared with wild-type plants, and, as with spr-2, a marked reduction in this parameter was also observed at 52 dai. No differences in the values for arbuscular abundance (%a) in mycorrhizal roots were found at 38 and 52 dai for all three plant phenotypes (Fig. 5A).

The expression pattern of 9-LOX metabolism-related genes was quantified using the qRT-PCR technique. The patterns of selected genes were characterized in the roots of mycorrhizal and non-mycorrhizal plants, respectively, at 38 and 52 dai. The M value was calculated for mycorrhizal plants with respect to gene expression in non-mycorrhizal plants for each plant line, gene, and harvest time (Fig. 5B). In these experiments, the expression pattern of all three genes was found to be quite similar. For all three plant lines, no significant changes in gene expression in mycorrhizal plants with respect to non-mycorrhizal plants were found at 38 dai for the genes analysed, except for a slight increase in LeDES expression in spr-2 mycorrhizal plants. However, the expression of LOXA, AOS3, and LeDES was higher in the mycorrhizal roots than in the non-mycorrhizal roots of wild-type plants at 52 dai. For the spr-2 and def-1 mutants at this harvest time, it was observed that LOXA and AOS3 gene expression remained unaltered (<2-fold up- or down-regulated, with an M value in the +1/–1 range), and LeDES expression was significantly down-regulated in the mycorrhizal def-1 mutant (Fig. 5B).

To examine the capacity of spr-2 and def-1 plant mutants to activate a JA response against AM fungus, the pattern of PIN II gene expression was also studied in the roots of mycorrhizal and non-mycorrhizal plants at 38 and 52 dai, respectively. The M value for gene expression in mycorrhizal plants was calculated with respect to gene expression in non-mycorrhizal plants for wild-type Castlemart and each mutant line at the two harvest times (Fig. 5B). The expression pattern of the PIN II gene was found to be quite similar to the expression patterns of LOXA and AOS3 (Fig. 5B). No significant changes in gene expression for mycorrhizal plants with respect to non-mycorrhizal plants were found at 38 dai for the PIN II gene except for a slight increase in def-1 mycorrhizal plants. The expression of PIN II was higher in the mycorrhizal roots than in the non-mycorrhizal roots of
wild-type plants at 52 dai (~300 times greater, with an M value of 8), and def-1 mutant plants (eight times higher, with a M value of 3). In relation to the spr-2 mutant, at both 52 and 38 dai, it was observed that PIN II gene expression in mycorrhizal plants remained unaltered with respect to non-mycorrhizal plants (Fig. 5B).

Fig. 4. Analysis of the response to phytohormone treatments of genes involved in 9-LOX metabolism. qRT-PCR analysis was performed on 40-day-old tomato plants after 6, 12, and 24 h of water and phytohormone treatment. M values (log2 ratio) for qRT-PCR data are represented. The value of M is 0 if there is no change and +1 or −1 if there is a 2-fold induction or reduction, respectively, with respect to the expression of each of the genes at time 0 of control plants. Values correspond to the means ± SE of three biological replications. Bars with the same letters are not significantly different (\( P = 0.05 \)) according to Duncan’s multiple range test.
These results reveal that there is a significant interaction between plant genotype and date of sampling factors for the four genes analysed, LOXA (two-way ANOVA, $F_{2,6}=2744, P=0.0001$), AOS3 (two-way ANOVA, $F_{2,6}=129.64, P=0.0001$), LeDES (two-way ANOVA, $F_{2,12}=913.94, P=0.0001$), and PIN II (two-way ANOVA, $F_{2,12}=1362.91, P=0.0001$), demonstrating that these factors interact, and the effect on gene expression at each date of sampling was dependent on plant genotype.

The consequences for AM formation and 9-LOX gene expression due to blockage of jasmonate signalling pathway were studied in experiments using the MeJA-sensitive line (JAI/–), and the MeJA-insensitive tomato jai-1 line deficient in JA perception. As the single recessive mutation in the jai-1 line causes female sterility, a PCR test was performed to select jai1/jai1 homozygote seedlings from an F2 segregated population of seeds (see the Materials and methods). After this selection, the sensitive (JAI/–) and insensitive (jai1/jai1) seedlings were inoculated with G. intraradices, and AM colonization was determined. Figure 6A shows the AM colonization parameters of mycorrhizal colonization intensity and arbuscule abundance in the plants at two different harvest points. At 38 dai, the intensity of root colonization was five times greater in the MeJA-insensitive jai-1/jai-1 mutant than in MeJA-sensitive JAI/– plants. At 52 dai, mycorrhizal intensity was three times higher in the MeJA-insensitive mutant plants than in MeJA-sensitive plants. The jai1-jai1 MeJA-insensitive mutants showed an arbuscule intensity (%a) twice that for the MeJA-sensitive plants (Fig. 6A), which was in line with the increase in mycorrhizal intensity.

The expression patterns of 9-LOX metabolism-related genes LOXA, AOS3, and LeDES were quantified in the roots of mycorrhizal and non-mycorrhizal plants, respectively, at 38 and 52 dai. The M value was calculated for mycorrhizal plants with respect to gene expression in non-mycorrhizal plants for MeJA-sensitive and MeJA-insensitive mutants for each gene at each harvest time (Fig. 6B). A similar expression pattern was found for LOXA and AOS3 genes in the roots of Me-JA-sensitive plants. In these plants, the expression of both genes was lower at 38 dai but was higher at 52 dai in mycorrhizal plants with respect to non-mycorrhizal plants. No changes in gene expression in MeJA-sensitive mycorrhizal plants with respect to non-mycorrhizal plants were found at any time for the LeDES gene.

Fig. 5. Mycorrhizal colonization and expression analysis of genes involved in 9-LOX metabolism and the JA response in tomato mutant plants spr-2 and def-1. (A) Intensity of colonization (%M) and arbuscule abundance (%a) in wild-type Castlemart tomatoes (cast) and spr-2 and def-1 mutant plants inoculated with G. intraradices. (B) Gene expression analysis by real-time qRT-PCR for genes involved in 9-LOX metabolism and the JA response. M values (log2 ratio) for qRT-PCR data are represented. The value of M is 0 if there is no change and +1 or –1 if there is a 2-fold induction or reduction, respectively, with respect to the expression of each gene in non-mycorrhizal plants for each plant line and harvest time. Values correspond to the means $\pm$SE of five biological replications in A, and three replications in B. Bars with the same letters are not significantly different ($P=0.05$) according to Duncan’s multiple range test.
The blockage of the jasmonate signalling pathway in MeJA-insensitive jai-1/jai-1 plants slightly affected the expression pattern of 9-LOX metabolism-related genes. In these plants, no significant differences in the expression pattern of LOXA and AOS3 genes were found in mycorrhizal plants with respect to non-mycorrhizal plants at both 38 and 52 dai, except for a slight 2-fold up-regulation of LOXA at 38 dai. Mycorrhizal MeJA-insensitive jai-1/jai-1 plants showed lower LeDES gene expression than non-mycorrhizal plants at 38 dai (2-fold down-regulation) and 52 dai (4-fold down-regulation) (Fig 6B).

The results reflects that for LOXA (two-way ANOVA, $F_{1,4}=1842.67$, $P=0.0001$), AOS3 (two-way ANOVA, $F_{1,4}=295.29$, $P=0.0001$), and LeDES (two-way ANOVA, $F_{1,8}=33.14$, $P=0.0004$), there was a significant interaction between genotype and date of sampling, demonstrating that for each date of sampling the gene expression in mycorrhizal plants was dependent on the plant genotype studied.

**Response of spr-2 mutant plants to MeJA treatment**

The results suggest that the expression of LOXA, AOS3, and PIN II, though different from that of LeDES, seems to depend on a certain degree of AM fungal colonization. On the other hand, although an increase in mycorrhization could be expected to occur in tomato mutants defective in JA synthesis such as in jai-1 plants, these plants showed less intense AM colonization than wild-type plants. This apparent contradiction could be due to the different roles played by JA in the colonization of roots by AM fungi.

In order to compare the role of JA in AM formation and JA response activation in spr-2 plants, two different concentrations of exogenous MeJA applications were applied once a week to soil. The expression patterns of 9-LOX metabolism-related genes LOXA and AOS3, the MeJA-inducible proteinase inhibitor PIN II gene, and the G. intraradices glutamine synthase gene (GinGS) were quantified using qRT-PCR. Tomato plants were treated with MeJA solutions, and RNA was extracted from roots after a 40 d period of inoculation. The M value was calculated for gene expression in treated mycorrhizal plants with respect to gene expression in non-treated mycorrhizal plants for each gene.

LOXA, AOS3, and PIN II genes showed patterns of response to MeJA treatment, while the GinGS gene had a contrary response pattern (Fig. 7). MeJA treatment at
0.1 μM caused only up-regulation of GinGS gene expression and had no effect on LOXA, AOS3, and PIN II expression. Although the application of 5 μM MeJA provoked up-regulation of LOXA, AOS3, and PIN II gene expression, it did not alter GinGS expression.

Discussion

In plants, there are two main branches of the oxylipin pathway, which are determined by two different types of LOXs, 9-LOX and 13-LOX (Fig. 1). The 13-LOX pathway leads to the biosynthesis of JA and its derivatives, and some genes in this pathway were up-regulated during AM interaction (López-Raúez et al., 2010). Free JA increased up to several fold in the roots of barley, cucumber, Medicago truncatula, and soybean upon mycorrhization (Hause et al., 2002; Vierheilig and Piche, 2002; Meixner et al., 2005; Stumpe et al., 2005), and an increase in OPDA (precursor of JA) was detected in mycorrhizal tomato plants (López-Raúez et al., 2010). In the case of M. truncatula, the 9- and 13-LOX products of linoleic and α-linolenic acid were present in root samples, but did not show significant differences between mycorrhizal and non-mycorrhizal roots, except for JA (a 13-LOX oxylipin) which reached elevated levels in mycorrhizal roots (Stumpe et al., 2005). Although there are no published data on 9-LOX products in mycorrhizal tomato roots, two recent microarray analyses reveal a strong up-regulation in the roots of AM plant genes coding for key enzymes directly involved in the metabolism of 9-LOX oxylipins (García-Garrido et al., 2010; López-Raúez et al., 2010). Apart from this, little information is available on the importance of lipid peroxide metabolism in AM symbiosis, and the above-mentioned results suggest that there may be some differences between Solanaceae plants and other plant families in terms of the relevance and importance of 9-LOX and 13-LOX metabolic branches during mycorrhizal formation.

In this study, the expression pattern of three genes associated with the biosynthesis of 9-LOX products in tomato mycorrhizal plants was analysed. The results reveal an induction in the late stages of mycorrhization of genes coding for LOXA and AOS3, key enzymes in the 9-LOX branch of the oxylipin pathway, giving rise to the formation of ketols and 10-OPDA, and also indicate that their expression in mycorrhizal roots seems to depend on a certain degree of AM fungal colonization. Interestingly, the up-regulation of these genes in mycorrhizal tomato roots was located in the inoculated part of the mycorrhizal split-root plants, affecting only that part of the root where AM fungi are growing and developing. Thus, if the activation of the 9-LOX pathway is part of the plant’s strategy to control AMF development within the roots, as suggested elsewhere (García-Garrido et al., 2010; López-Raúez et al., 2010), its effects should be local and not contribute to systemic mechanisms of fungal regulation such as autoregulation of AM or systemic resistance to fungal pathogens.

The present findings reveal that the expression pattern of the gene coding for LeDES, a 9-LOX desaturase that catalyses the biosynthesis of colnelenic and colnleic acids, differed from the pattern for the LOXA and AOS3 genes. In relation to LeDES, no major differences in gene expression in mycorrhizal plants with respect to non-mycorrhizal plants were found in the experiments, except in the case of mycorrhizal Castlemart plants at 52 dai which showed similar and higher up-regulation of LeDES, LOXA, and AOS3 genes. LeDES has been described as a mycorrhizal up-regulated tomato gene in a transcriptional analysis of tomato roots by López-Raúez et al. (2010), but two other sets of array data published by Fiorilli et al. (2009) and García-Garrido et al. (2010) did not refer to this up-regulation. It is plausible that the transcriptional regulation of LeDES during AM may be different from the regulation of LOXA and AOS3 and not so dependent on the degree of colonization, as seems to be the case for LOXA and AOS3. LOXA and AOS3 actually showed similar patterns of response to phytohormone treatment, which differed from the pattern found for LeDES. Although MeJA treatment caused an early, moderate, and transient increase in LeDES expression, it caused a sharper and more permanent increase in LOXA and AOS3 expression. In addition, ABA, ET, and SA positively regulated LeDES expression even more than MeJA and had no effect on LOXA and a minimal impact on AOS3 expression. This demonstrates that LeDES is more versatile in its response to defence-related phytohormones, and its expression could be subject to fine adjustment.

The JA-insensitive tomato mutant jai-1 was more susceptible to fungal infection (Herrera-Medina et al., 2008; this
study) and recorded increases in fungal colonization intensity and arbuscule frequency. In parallel with the increase in AM colonization, the insensitive response to JA in jai-1 plants eliminated the increases in LOXA and AOS3 gene expression typically found in wild-type JA-sensitive plants. These patterns of LOXA and AOS3 gene expression in JA-sensitive plants were similar to the previously reported pattern of the MeJA-inducible proteinase inhibitor PIN II gene which was down-regulated in MeJA-insensitive jai-1/jai-1 plants compared with JA-sensitive plants (Herrera-Medina et al., 2008). These results suggest that the activation of LOXA and AOS3 gene expression, though not that of LeDES, during mycorrhization is dependent on JA pathway activation and bear out the findings of previous studies in which AOS3 expression is undetectable in jai-1 plants (Itoh et al., 2002). In addition, this suggests that AOS3 and LOXA are not necessary for successful root mycorrhization, since the low levels of LOXA and AOS3 expression in jai-1 do not negatively affect AM formation.

As in the case of JA-insensitive plants, tomato mutant plants defective in JA synthesis eliminated the increases in LOXA gene expression observed in wild-type plants in the late stage of mycorrhization and showed only a weak induction (<2-fold) for AOS3. Although mycorrhization for tomato mutants defective in JA synthesis such as in jai-1 plants would be expected to increase, these mutants showed less intense AM colonization than wild-type plants. This apparent contradiction could be due to the different roles played by JA in the colonization of roots by AM fungi. As reported elsewhere (Tejeda-Sartorius et al., 2008), spr-2 mutants defective in JA synthesis recorded reduced mycorrhizal intensity. It is shown that the other defective JA mutant, def-1, also had reduced AM colonization, although, in both cases, this deficiency did not affect the capacity of the AM fungus *G. intraradices* to form arbuscules. It has been reported that JA levels increase after the initial stage of mycorrhizal fungal infection, and a reverse genetic approach, involving partial suppression of the JA biosynthetic gene allene oxide cyclase (*AOC1*) in *M. truncatula* roots, showed that both the mycorrhization rate and arbuscule formation were negatively affected by the reduction in JA levels (Isayenkov et al., 2005). This indicates that JA plays an essential role in the colonization of roots by AM fungi. In the present experiments, although spr-2 and def-1 plants showed a reduction in mycorrhizal intensity, they maintained the capacity to form arbuscules, suggesting that the level of JA in these plants was sufficient to form arbuscules but insufficient to generate maximum mycorrhizal intensity in roots.

It is shown that JA has a concentration-dependent effect on mycorrhizal development and JA response activation in spr-2 plant mutants. LOXA, AOS3, and PIN II genes showed similar patterns of response to MeJA treatment, while the GinGS gene marker for fungal development had a converse response pattern. It is therefore plausible that the basal level of JA in def-1 and spr-2 was responsible for the reduction in AM formation, as the reduction in JA levels reduces the presence of a jasmonate-responsive plant signal compound that positively enhances fungal infectivity. However, the failure of these plants to accumulate JA and to express a JA-responsive defence, as in the case of jai-1 plant mutants, could explain their inability to induce LOXA and AOS3 expression dependent on JA pathway activation. In this respect, the ability of def-1 and spr-2 plants to accumulate JA in response to elicitation is undermined (Howe et al., 1996; Li et al., 2003). In this study, it is shown that spr-2 failed and def-1 was reduced in the induction of the expression of the tomato PIN II gene, a typical gene marker for the JA pathway, which is activated during the late stages of mycorrhization in wild-type plants.

As reported with respect to *Phytophthora parasitica* (Fammartino et al., 2007) and other fungal pathogens (Blée, 2002; Vellosillo et al., 2007), it is conceivable that the 9-LOX pathway plays a defensive role and restricts fungal spread in roots. The up-regulation of the expression of these genes in mycorrhizal wild-type roots seems to depend on a particular degree of AM fungal colonization. These genes run parallel to the expression of the JA-responsive PIN II gene and are restricted to the colonized part of the roots. This suggests that these genes may play a role in controlling fungal spread in roots and that a certain infection threshold would be necessary in tomato mycorrhizal root plants in order to provoke a gene expression response. This strategy of the plant to control AM fungal development within the roots at least partly depends on JA pathway activation. However, it remains to be determined whether the activation of the 9-LOX pathway in mycorrhizal tomato is important in solanaceous plants and whether it correlates with the accumulation of specific 9-LOX oxylipin components.

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