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The Jasmonic Acid Signalling Pathway Restricts the Development of the Arbuscular Mycorrhizal Association in Tomato

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Abstract The role of the jasmonate signalling pathway in modulating the establishment of the arbuscular mycorrhiza (AM) symbiosis between tomato plants and *Glomus intraradices* fungus was studied. The consequences of AM formation due to the blockage of the jasmonate signalling pathway were studied in experiments with plant mutants impaired in JA perception. The tomato *jai-1* mutant (jasmonic acid insensitive 1) failed to regulate colonization and was more susceptible to fungal infection, showing accelerated colonization. The frequency and the intensity of fungal colonization were greatly increased in the *jai-1* insensitive mutant plants. In parallel, the systemic effects on mycorrhization due to the activation of the jasmonate signalling pathway by foliar application of MeJA were evaluated and histochemical and molecular parameters of mycorrhizal intensity and efficiency were measured. Histochemical determination of fungal infectivity and fungal alkaline phosphatase activity reveal that the systemic application of MeJA was effective in reducing mycorrhization and mainly affected fungal phosphate metabolism and arbuscule formation, analyzed by the expression of *GiALP* and the AM-specific gene *LePT4*, respectively. The results of the present study clearly show that JA participates in the susceptibility of tomato to infection by arbuscular mycorrhizal fungi, and it seems that arbuscular colonization in tomato is tightly controlled by the jasmonate signalling pathway.

Keywords Tomato · Arbuscular mycorrhiza · Jasmonic acid · MeJA-insensitive mutants

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Introduction

Arbuscular mycorrhiza (AM) is a mutualistic endosymbiosis formed between soil-borne fungi and the vast majority of terrestrial plants, mostly angiosperms. A successful establishment of the fungus in the root improves the nutritional status of both partners. The fungi receive fixed carbon compounds from the host plant, while the plant benefits from the association by an increased nutrient uptake (mainly phosphorus), enhanced tolerance to abiotic stress, and resistance to pathogens (Smith and Read 1997).

The formation of the AM symbiosis is a complex developmental event requiring the coordination of gene expression in two partner organisms. Major progress has been made recently in the study of the pathway giving rise to arbuscular mycorrhiza formation, especially with respect to the plant partner. The use of genetic tools such as mutant plants affected in different stages of AM formation (Peterson and Guinel 2000; Marsh and Schultze 2001) or more recently the use of DNA array methodologies (Brechenmacher and others 2004; Güimil and others 2005; Hohnjec and others 2005) has led to an advance in the knowledge of the pathway leading to mycorrhization. Compared to the genetics and molecular biology of AM, not much is known about the biochemical and morphogenetic events mediated by phytohormones during AM formation. Certain roles for phyto-

hormones in ecto- and arbuscular mycorrhiza have been suggested (Beyrle 1995; Ludwig-Müller 2000; Hause and others 2007; Herrera-Medina and others 2007), and the idea that a fine balance between hormones and nutrient availability (phosphorus, carbon, or nitrogen) is probably important for the regulation of mycorrhizal formation and functioning was debated. Most of these putative functions are speculations based on few data, mainly obtained by experiments involving hormone application or measurements of the hormone content in AM plants. There is evidence that phytohormones are involved in signalling events between AM fungi (AMF) and host plants, including processes of symbiosis stimulation such as lateral root formation (Fang and Hirsch 1998; Hirsch and others 1997) and arbuscule development (Herrera-Medina and others 2007).

Events similar to those found in plant-pathogen interactions have also been found in the plant interaction with AMF, including signal perception, signal transduction, and defense gene activation (García Garrido and Ocampo 2002; Garcia-Garrido and Vierheilig 2008). As hormones such as jasmonic acid (JA), salicylic acid (SA), ethylene, and abscisic acid (ABA) play a key role as signalling compounds in plant-pathogen interactions, their participation in the development of AM has been postulated (Garcia-Garrido and Vierheilig, 2008; Hause and others 2007).

In the case of JA, recent experiments involving endogenous measurements of this compound in arbuscular mycorrhizal roots provided evidence of a rise in jasmonates correlated with mycorrhization in *Hordeum vulgare* (Hause and others 2002), *Cucumis sativus* (Vierheilig and Piché 2002), *Glycine max* (Meixner and others 2005), and *Medicago truncatula* (Stumpe and others 2005). Studies of *H. vulgare* and *M. truncatula* have shown a temporal correlation between increases in the JA level and the expression of genes coding for JA-biosynthetic enzymes and jasmonate-responsive genes in mycorrhizal roots (Hause and others 2002; Isayenkov and others 2005). Because JA levels increase after the initial stage of mycorrhizal fungal infection and due to the cell-specific expression of JA-biosynthetic enzymes and JA-induced proteins in arbuscule-containing cells (Hause and others 2002), it has been assumed that enhanced JA levels in mycorrhizal roots could be linked to the arbuscule development in roots. Other authors have suggested that JA levels could be linked with the number of collapsed arbuscules in mycorrhizal roots (Vierheilig 2004). On the other hand, in a reverse genetic approach, partial suppression of the JA-biosynthetic gene allene oxide cyclase (AOC1) in *M. truncatula* roots showed that both the mycorrhization rate and the arbuscule formation were negatively affected by the reduction of JA levels (Isayenkov and others 2005), indicating an essential role of JA in the colonization of roots by AM fungi.

In contrast to the reverse genetic data, exogenous application of JA to leaves of *Carica papaya*, *Tropeaolum majus*, and *C. sativus* resulted in a clear reduction of AM root colonization (Ludwig-Müller and others 2002). However, an exogenous treatment of *Allium sativum* with low concentrations of JA stimulated mycorrhization (Regvar and others 1996), suggesting a concentration-dependent effect of JA on mycorrhizal development (Hause and others 2007).

In the present work we tested the hypothesis of regulation of mycorrhization by the plant hormone JA with two complementary approaches. To test the importance of JA perception and signalling, different mycorrhizal root colonization parameters were followed over a time course in a jasmonate-insensitive tomato mutant. In parallel, we studied the effect on mycorrhization of the systemic activation of the JA pathway by foliar application of MeJA. Different parameters of mycorrhization such as mycorrhizal intensity and mycorrhizal efficiency together with the analysis of MA and JA marker gene expression were used to assess mycorrhizal status.

Materials and Methods

Plant Growth and AM Inoculation

Tomato (*Solanum lycopersicum*, syn. *Lycopersicon esculentum*) cvs Moneymaker (MM) wild type and *jai-1* mutants (Li and others 2001) were used. Seeds were surface-sterilized by soaking in 0.75% sodium hypochlorite for 5 min, rinsed with tap water, and soaked again in 7% H_2O_2 for 10 min. Thereafter seeds were rinsed three times with sterile deionized water and germinated on a sterilized moistened filter paper for 3–4 days at 25°C. After germination, plants were grown in a steam-sterilized (40 min, 120°C) mixture of silicate sand, peat, soil, and vermiculite (1:1:1:1/v:v:v:v). The plant growth and the treatments were performed in a growth chamber (day/night cycle: 16 h, 25°C/8 h, 19°C; relative humidity: 50%).

The recessive *jai-1* mutant is female sterile and, therefore, cannot be maintained as a homozygous line. Homozygous *jai-1/jai-1* seedlings had to be selected in the F_2 populations. The segregating homozygous *Jai-1/Jai-1* and heterozygous *Jai-1/jai-1* plants are jasmonate-sensitive and were used as control plants in all experiments. The selection of homozygous mutants (Li and others 2004) is based on two criteria: root growth and anthocyanin accumulation (violet color). After seed germination in the moistened filter paper, the excess water was drained and the paper was resaturated with a solution of 1 mM of MeJA. After growing the seedlings for 1-1.5 days, the MeJA treatment caused root growth inhibition and

anthocyanin accumulation in the hypocotyls of sensitive seedlings, in contrast to *jai-1/jai-1* insensitive lines that do not accumulate anthocyanin and do show root growth in presence of MeJA.

The inoculation with *Glomus intraradices* (DAOM 197198) was carried out in 200-ml pots. Each seedling was growing in one pot and was inoculated with a piece of Gel-Gro medium from a monoxenic culture containing 50 spores of *G. intraradices* and colonized carrot roots. The monoxenic culture (*G. intraradices* and carrot roots) was made according to Chabot and others (1992).

One week after planting in pots and weekly thereafter, 20 ml of the modified Long Ashton nutrient solution was added to each pot. The modified Long Ashton nutrient solution contained 25% of the original P concentration (Hewitt 1966) to prevent mycorrhizal inhibition due to 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. In each experiment five independent plants were analyzed per treatment.

Estimation of Root Colonization

Two staining procedures were used to determine total colonization and alkaline phosphatase activity. The non-vital trypan blue stain was made according to the method of Phillips and Hayman (1970), and the vital stain for alkaline phosphatase allowing detection of active AM fungi was made as described by Tisserant and others (1993). Alkaline phosphatase localized in the vacuole of the intraradical mycelium (Gianinazzi and others 1979) has been reported as a potential marker for the efficiency of the mycorrhizal symbiosis and it was suggested that it might be involved in P transport (Tisserant and others 1993). Stained roots were observed with a light microscope and the intensity of root cortex colonization by AM fungus was determined as described by Trouvelot and others (1986) using MYCOCALC software (http://www.dijon.inra.fr/mychintec/Mycocal-prg/download.html). The parameters measured according to this method were mycorrhizal frequency (*F* [%]) and intensity (*M* [%]) in the root, and arbuscular abundance in the colonized root (*a* [%]). Mycorrhization was also evaluated by the gridline intersect method described by Giovannetti and Mosse (1980).

Chemical Treatments

Leaves of tomato plants were sprayed two times per week with 5- and 50-µM solutions of methyl jasmonate (MeJA) (Aldrich). The pH of each solution was adjusted to 7. The aqueous stock solution of MeJA contains 1 mM MeJA and 0.01% Tween 20.

Ethylene Quantification

The ethylene content in roots was measured by incubating the excised root system for 1 h at room temperature in a 16-ml tube closed with a rubber stopper. The accumulation of ethylene in each tube was determined from three different samples by taking 1 ml from the internal space of the tube with a syringe. The samples were placed in a gas chromatograph (Hewlett Packard 5890) fitted with a flame ionization detector, using commercial ethylene as a standard for identification and quantification.

RNA Extractions and Gene Expression

Semiquantitative RT-PCR was carried out to measure the transcript levels of 18S rRNA and alkaline phosphatase genes from *G. intraradices* (*Gi18S*, *GiALP*) and polyubiquitin, apoplastic invertase 6, protein inhibitor-II, and the phosphate transporter 4 transcripts (*PolyUbi*, *LIN6*, *PI-PII*, *LePT4*) in tomato roots. Total RNA was isolated from the roots stored at −80°C using the RNeasy Plant Mini Kit (Qiagen) following the manufacturer's instructions. cDNAs were obtained from 1 µg of total DNAse-treated RNA in a 20-µl reaction volume containing 20 U of AMV reverse transcriptase (Roche), 400 ng of random hexamer primers, 1 mM each dNTP, 50 U of RNase inhibitor, and 1 × reverse transcription buffer. After reverse transcription, 80 µl of MilliQ water was added to get a final volume of 100 µl of each cDNA solution. Five microliters of the synthesized cDNA was PCR-amplified using a specific primer set. Each 25 µl of PCR reaction contained 2.5 mM of $MgCl_2$, 120 µM of each dNTP, 1 µM of each primer, 2.5 µl of 1% Triton X-100, and 1 U of *Taq* DNA polymerase (Roche) in 1 × PCR buffer (10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl, pH 8.3). Primers RMF and RMR, described by González-Guerrero and others (2005), were used to amplify a DNA fragment from the *G. intraradices* 18S rRNA, and primers ALP1 and ALP2 were designated to amplify the *G. intraradices* alkaline phosphatase gene (*GiALP*) previously identified by Aono and others (2004). The gene-specific primers used for *LePT4*, *LIN6*, and *PI-II* amplification were previously described by Nagy and others (2005), Goetz and others (2000), and Kandoth and others (2007), respectively. As an internal control of plant RNA amounts and quality, the same single-strand cDNA was PCR-amplified with specific primers for the polyubiquitin gene (Ubi-1 and Ubi-2) that specifically bind to the 228-bp monomer fragment of the tomato polyubiquitin gene (Rollfinke and Pfitzner 1994). All primer names and the corresponding sequences are

Table 1 Primers Used in the Present Study

Primer name	Organism	Target gene	Primer sequence (5′-3′)
RMF	*Glomus intraradices*	18S rDNA	(5′-TGTTAATAAAAATCGGTGCGTTGC-3′)
RMR	*Glomus intraradices*	18S rDNA	(5′-AAAACGCAAATGATCAACCGGAC-3′)
ALP1	*Glomus intraradices*	GiALP	(5′-ATCGTAGCCGCAGTAATAGT-3′)
ALP2	*Glomus intraradices*	GiALP	(5′-CTAAGTGTTTGGCAGATTCC-3′)
PT4F	*Solanum lycopersicum*	LePT4	(5′-GAAGGGGAGCCATTTAATGTGG-3′)
PT4R	*Solanum lycopersicum*	LePT4	(5′-CCATCTTGTGTGTATTGTTGTATC-3′)
Ubi-1	*Solanum lycopersicum*	PolyUbi	(5′-ATGCAGAT(C/T)TTTGTGAAGAC-3′)
Ubi-2	*Solanum lycopersicum*	PolyUbi	(5′-ACGCAGACCGAGGTGGAG-3′)
TIN9	*Solanum lycopersicum*	LIN6	(5′-ACCCAAAAGGAGCAACATGGGGC-3′)
TIN10	*Solanum lycopersicum*	LIN6	(5′-CCATCAATAGAAGTGTTATCCGG-3′)
PI-IIF	*Solanum lycopersicum*	PI-II	(5′-CCCACGTTCAGAAGGAAGTC-3′)
PI-IIR	*Solanum lycopersicum*	PI-II	(5′-TTTTGGGCAATCCAGAAGAT-3′)

listed in Table 1. The PCR program consisted of a 5-min incubation at 95°C followed by 30 cycles of 30 s at 95°C, 60 s at 55°C, and 90 s at 72°C. The synthesized DNA was separated in 1.25% agarose gels in TBE buffer.

To perform RT-PCR experiments, total RNA was isolated from 1 g of pooled material that contained representative portions of roots from at least five different plants. Each RT-PCR experiment was repeated twice with different RNA extractions and the data presented show the results from a representative experiment.

Statistical Analysis

The data were subjected to one-way analysis of variance (ANOVA). The mean values of five replicate samples were compared using Duncan's multiple-range test ($P = 0.05$).

Results

Suppression of Mycorrhization Through the Impairment of Jasmonic Acid Signalling

To study the consequences of impaired JA signalling, MeJA-sensitive (*Jai-1/-*) or insensitive (*jai-1/jai-1*) seedlings from a segregating F_2 population were inoculated with *G. intraradices* and AM colonization was determined. Figure 1 shows the AM colonization parameters in the mutants at two different time-points. Forty-five days after inoculation, only a slight increase in the frequency (*F* [%]) was observed, whereas the intensity (*M* [%]) of root colonization was highly increased in the MeJA-insensitive *jai-s1/jai-1* mutant compared to the MeJA-sensitive *Jai-1/-* plant. Forty-five days after inoculation, in the MeJA-insensitive mutant the mycorrhizal intensity was three times higher and after 60 days two times higher than in the MeJA-sensitive plants. Parallel to the increase in mycorrhizal intensity, MeJA-insensitive mutants showed a higher level of arbuscule intensity (*a* [%]) than the MeJA-sensitive line.

To measure the efficiency of the AM symbiosis we determined the percentage of roots with AM fungal alkaline phosphatase activity (AP). The data shown in Figure 1 clearly illustrate that 45 days after inoculation the intensity (M-AP [%]) of fungal tissue with AP activity was greatly increased in the MeJA-insensitive mutant. Forty-five days after inoculation, the intensity of the AP activity was four times higher in MeJA-insensitive *jai-1/ja-1* plants compared to sensitive *Jai-1/-* plants, and after 60 days the AP intensity in MeJA-insensitive plants was double compared to MeJA-sensitive plants.

The mycorrhization status was also examined by the expression of marker genes in semiquantitative RT-PCR experiments (Figure 2). Fungal colonization was quantified as the accumulation of *G. intraradices* 18S rRNA in tomato roots relative to the amount of plant mRNA, measured as polyubiquitin mRNA accumulation. The metabolic activity of the AM fungus and the arbuscular development in mycorrhizal roots were quantified at the molecular level by the detection of transcripts for *GiALP* (alkaline phosphatase gene) and the arbuscular-specific tomato phosphate transporter *LePT4*. No difference in transcript accumulation was found between sensitive and insensitive plants 60 days after inoculation with *G. intraradices* for all tested marker genes. At this time the intensity of colonization and the intensity of fungal tissue with AP activity, histochemically quantified (Figure 1), was 1.5 times higher in sensitive plants than in insensitive *jai-1* mutants. Nevertheless, 45 days after inoculation, when the intensity of AM colonization and the intensity of fungal tissue with AP activity (Figure 1) was 4 times higher in MeJA-insensitive plants than in MeJA-sensitive plants, the transcript

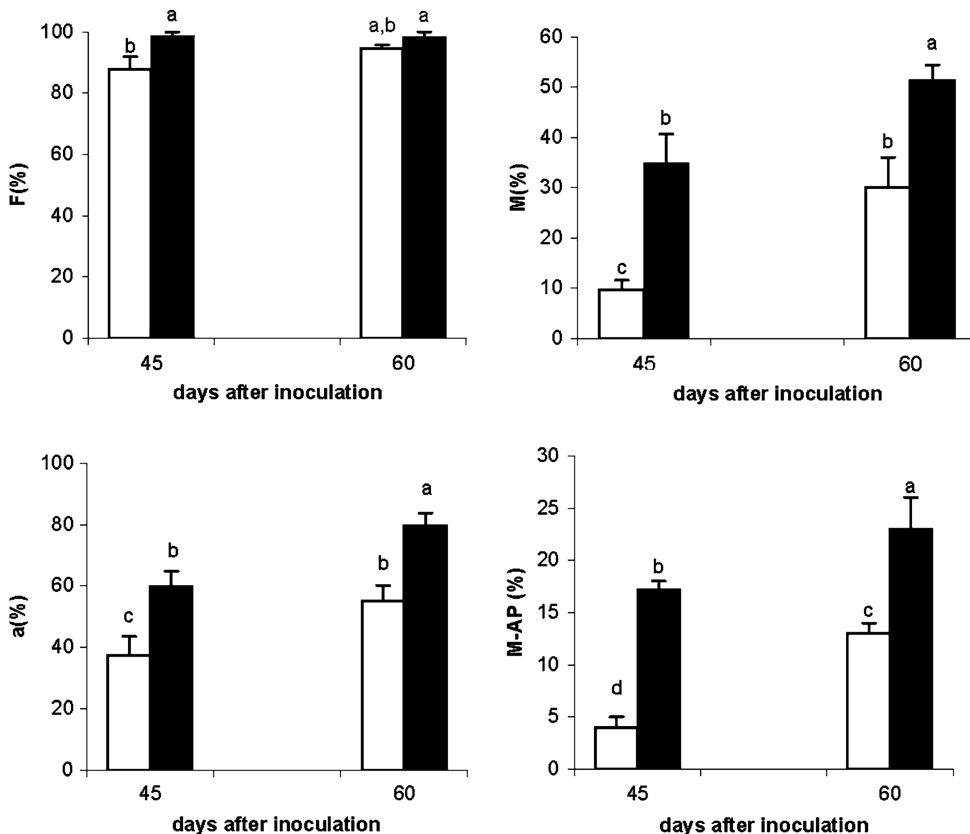

Fig. 1 Mycorrhization of *Jai-1/-* MeJA-sensitive (white bars) and *jai-1/jai-1* MeJA-insensitive tomato mutants (black bars) by *G. intraradices*. The frequency (*F* [%]), intensity (*M* [%]), arbuscule abundance (*a* [%]), and intensity of fungal alkaline phosphatase activity (M-AP [%]) were determined 45 and 60 days after inoculation with *G. intraradices* using MYCOCALC software. Values are the means ± SE of five biological replications. Bars with similar letters are not significantly different ($P = 0.05$) according to Duncan's multiple-range test

accumulation of *Gi18S*, and mainly *GiALP* and *LePT4*, was clearly higher in the *jai-1/jai-1* insensitive mutant (Figure 2), corroborating the histochemical measurements.

No differences between sensitive and insensitive plants were observed at the level of transcript accumulation of the MeJA-inducible *LIN6* invertase gene at the two different time-points assayed (Figure 2). Nevertheless, the analysis of the MeJA-inducible proteinase inhibitor *PI-II* gene expression showed that this gene was downregulated in MeJA-insensitive *jai-1/jai-1* plants compared to sensitive *Jai-1/-* plants.

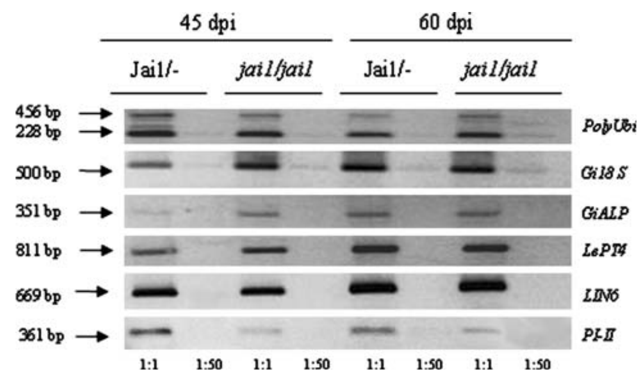

Fig. 2 Analysis of gene expression by semiquantitative RT-PCR of fungal *GiALP* and *Gi18S* rRNA and plant polyubiquitin, *LePt4*, *LIN6*, and *PI-II* genes. ARN was extracted from roots of *Jai-1/-* MeJA-sensitive and *jai-1/jai-1* MeJA-insensitive plants harvested 45 and 60 days after inoculation with *G. intraradices*. Two cDNA dilutions (1 and 1:50) were used to perform the PCR reactions

Systemic Suppression of AM Formation in Roots by Jasmonic Acid Applied to Leaves

Exogenous MeJA application experiments were performed to compare the effects on AM formation as a consequence of the systemic activation of the JA signalling pathway with the effects on AM formation due to the blockage of the jasmonate signalling pathway. Leaves of tomato were sprayed twice a week with MeJA solutions with different concentrations, and the percentage of root colonization by *G. intraradices* was evaluated in a time-course experiment. Sixty days after inoculation, in all MeJA treatments AM root colonization was reduced (Figure 3a), however, after 25 and 45 days MeJA-treated plants exhibited similar colonization levels as control plants. The foliar application of MeJA reduced the colonization of the root by the arbuscular mycorrhizal fungus after the initial stages, thus affecting the final rate of colonization.

At all times assayed the root fresh weight of plants treated with MeJA was similar to the root fresh weight of nontreated control plants (Figure 3b). To check a possible effect on the ethylene accumulation in the roots due to MeJA application, we performed ethylene determinations

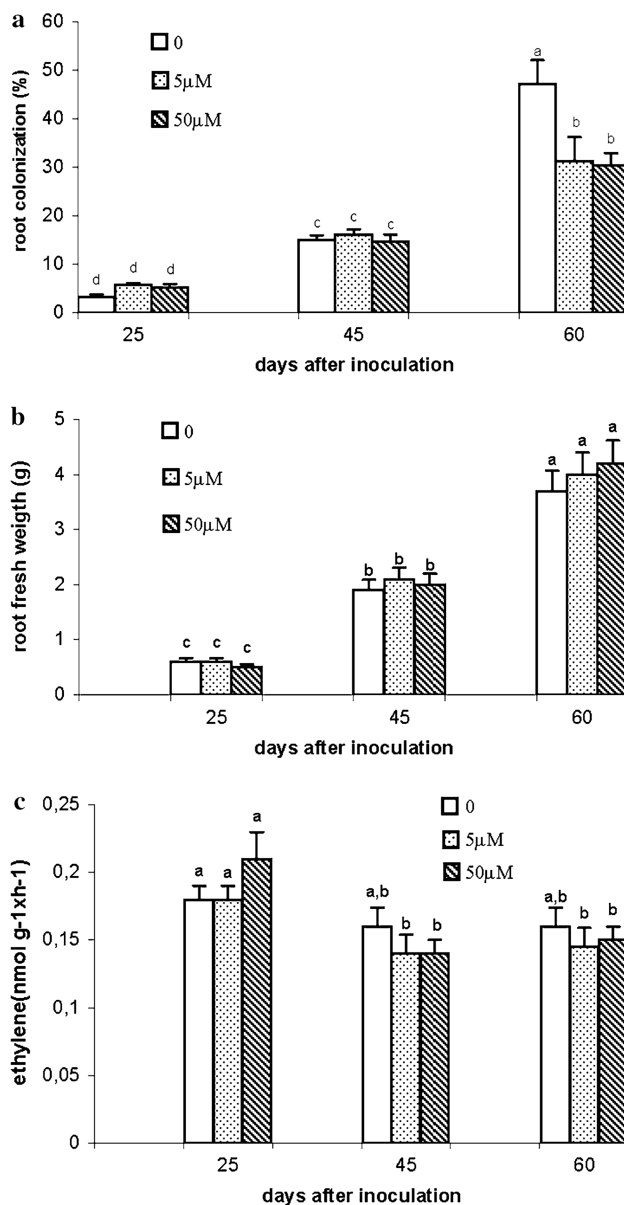

Fig. 3 Effect of foliar MeJA application on the colonization of tomato roots by *G. intraradices*. MeJA solutions were sprayed on leaves twice a week. Untreated control plants were sprayed with 0.1% ethanol solution. Time-course experiment of MeJA application and measurement of the percentage of root colonization using the gridline intersect method to determine mycorrhization (**a**). Effect of exogenous MeJA application on root fresh weight (**b**) and ethylene accumulation (**c**). Values are the means ± SE of five biological replications. Bars with similar letters are not significantly different ($P = 0.05$) according to Duncan's multiple-range test

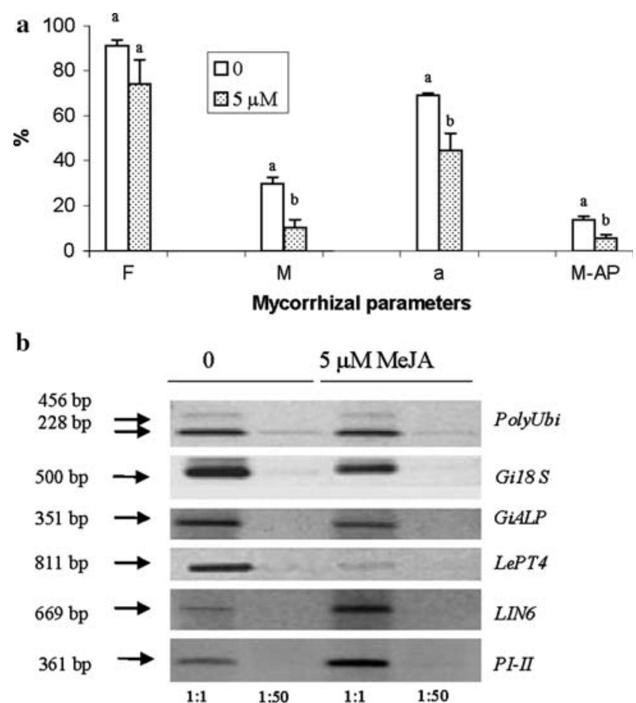

Fig. 4 Effect of foliar MeJA application on different colonization parameters determined 60 days after inoculation. Tomato plants were inoculated with *G. intraradices* and a 5-µM MeJA solution was sprayed on leaves twice a week. Untreated control plants (0) were sprayed with 0.1% ethanol solution. Mycorrhization parameters of frequency (*F* [%]), intensity (*M* [%]), arbuscule intensity (*a* [%]), and intensity of fungal alkaline phosphatase activity in roots (M-AP [%]) (**a**). Analysis of gene expression by semiquantitative RT-PCR of fungal *GiALP* and *Gi18S* rRNA and plant polyubiquitin, *LePt4*, *LIN6*, *and PI-II* genes after inoculation with *G. intraradices* in the control and the 5-µM MeJA treatment (**b**). Two cDNA dilutions (1 and 1:50) were used to perform the PCR reactions. Values are the means ± SE of five biological replications. Bars with similar letters are not significantly different ($P = 0.05$) according to Duncan's multiple-range test

in the control and the MeJA-treated plants. No differences in the ethylene content were observed (Figure 3c).

To better characterize the effects on mycorrhizal suppression due to the systemic JA application, we performed experiments of MeJA application and quantification of the mycorrhizal frequency and intensity and arbuscule abundance in roots. To check arbuscular mycorrhiza efficiency, the intensity (M-AP [%]) of fungal tissue with AP activity was also tested. Based on the previous results, we applied 5 µM of MeJA to leaves twice per week, and 60 days after inoculation with *G. intraradices* the mycorrhization parameters were evaluated (Figure 4a). The frequency (*F* [%]) of mycorrhiza development was not significantly affected by the MeJA treatment (Figure 4a). Nevertheless, as a consequence of foliar treatment with MeJA a clear suppression of the intensity (*M* [%]) of fungal colonization was observed and this decrease was paralleled by a decrease of the arbuscule abundance (*a* [%]) (Figure 4a). Fungal alkaline phosphatase activity was analyzed and compared between mycorrhizal roots of foliar MeJA-treated versus nontreated plants. The intensity of fungal AP decreased nearly 60% with the foliar application of MeJA (Figure 4a). The reduction in AP intensity due to MeJA application was parallelled by arbuscule abundance suppression (about the 50%) in the treated mycorrhizal roots (Figure 4a).

To verify the microscopic measurements at the molecular level, a semiquantitative RT-PCR experiment was performed. The quantity of fungal rRNA 18S (*Gi18S*) in roots was slightly higher in nontreated than in MeJA-treated plants. Nevertheless, the reduction in mycorrhizal parameters observed by histochemical measurements (Figure 4a) was more evident than the reduction reflected by the level of rRNA 18S accumulation in roots (Figure 4b). *GiALP* transcripts showed higher levels in nontreated than in MeJA-treated plants (Figure 4b). The clearest difference between nontreated and MeJA-treated plants could be observed at the *LePT4* transcript level. Plants treated with 5 μM exhibited low levels of *LePT4* transcripts in roots which hardly could be detected, whereas in nontreated plants the *LePT4* transcript accumulation was clearly detectable (Figure 4b).

As a positive control for the systemic activity of MeJA after foliar application, the expression of *LIN6* and *PI-II* MeJA-inducible genes was analyzed in roots. Clear differences between nontreated and MeJA-treated plants could be observed at the level of transcript accumulation. In roots of MeJA-treated plants *LIN6* and *PI-II* mRNAs were clearly upregulated (Figure 4b).

Discussion

Although much research has focused on understanding the response of plants to pathogenic microorganisms, relatively little is known about how plants regulate and control defense responses in symbiotic mutualistic associations. In the case of the arbuscular mycorrhizal association, it has been suggested that similar to plant-pathogen interactions, mechanisms associated with plant defense play a key role in the compatibility of the two partners, the fungus and its host plant. A number of regulatory mechanisms of the plant defense system have been described during the establishment of the AM symbiosis (reviewed by García Garrido and Ocampo 2002 and García-Garrido and Vierheilig 2008), and the coordination of multiple hormone signalling pathways seems to be essential for the regulation of mycorrhizal establishment and functioning (Beyrle 1995; Ludwig-Müller 2000). Roles for ethylene, ABA, JA, and SA have been suggested in AM formation in various plant systems (Esch and others 1994; Blilou and others 2000; Geil and Guinel 2002; Herrera-Medina and others 2003, 2007; Hause and others 2007); however, most of these hypotheses about the putative functions of these plant hormones in AM formation are derived only from experiments involving hormone application or the measurements of the hormone content in AM plants.

Plant mutants impaired in the perception or biosynthesis of plant hormones have been essential tools in studies of plant-microbe interactions and plant hormone biology. Several mutants lacking antiherbivore defenses or defense against pathogens have been shown to be deficient in either JA synthesis or JA perception (Li and others 2001, 2002; Zhao and others 2003). The *jai-1* mutation disrupts the function of the tomato homolog (LeCOI1) of COI1 (coronatine insensitive 1) in *Arabidopsis* (Feys and others 1994). *Jai-1* tomato plants exhibit several defense-related phenotypes, including the inability to express JA-responsive genes. *Jai-1* plant mutants are deficient in wound-inducible systemic expression of proteinase inhibitor (*PI*) genes and also lack *PI* expression in response to MeJA application, but they are capable of synthesizing JA (Li and others 2001, 2004; Zhao and others 2003).

In our experiments the use of *jai-1* mutant tomato plants showed that the disruption of JA signalling enhances mycorrhization, demonstrating that JA signalling affects AM formation. Our results constitute the first direct and clear evidence that supports the hypothesis that JA contributes to an effective suppression of AM colonization, but it is also new and strong evidence of putative AM regulation at the level of the JA signalling pathway. MeJA-insensitive plants showed accelerated infection by *G. intraradices*. AM fungal colonization intensity, arbuscule intensity, and consequently fungal alkaline phosphatase activity were increased in *jai-1* MeJA-insensitive plants. The levels of *GiALP* and *LePT4* transcripts markedly increased in *jai-1* MeJA-insensitive plants compared to MeJA-sensitive plants, coinciding with high increases in arbuscule abundance and enzymatic alkaline phosphatase fungal activity, suggesting that MeJA insensitivity mainly affects arbuscule formation.

The expression of the JA-responsive gene *LIN6* was similar in wild-type sensitive and *jai-1* insensitive plants, suggesting that LeCOI1 mutation does not affect *LIN6* expression, at least in non-JA-treated conditions. In contrast, *PI-II* gene expression was dependent on the plant genotype. The expression of *PI-II* in roots of MeJA-sensitive plants was higher than the expression in the roots of *jai-1* mutants, suggesting that the mutant plants are truly impaired in JA signalling under the conditions of the study. In this sense, a significant level of expression of some early JA-inducible genes was maintained in *jai-1* plants, although the gene defined by *jai-1* mutation plays a major role in promoting expression of the JA- and wound-responsive genes (Li and others 2004).

Parallel to the studies with *jai-1* mutants, the effects on mycorrhization from the systemic activation of the jasmonate signalling pathway by foliar application of MeJA were evaluated. Some studies on the alteration of AM colonization by exogenous application of jasmonates have been conducted, and a concentration-dependent effect of exogenous JA on mycorrhizal plants has been proposed

(Hause and others 2007). The results we obtained from foliar application of MeJA seem to be in agreement with the results of Ludwig-Müller and others (2002) who, after applying a 50-, 500-, or 5×10^3-μM JA solution to the leaves of *T. majus*, *C. papaya*, and cucumber plants every second day, found suppression of AM root colonization. We found a similar suppressive effect on AM root colonization with 5 and 50 μM of MeJA. The foliar application of MeJA reduced the colonization of the root by the arbuscular mycorrhizal fungus after the initial stages, affecting mainly the final rate of colonization. A similar effect was observed with 5 and 50 μM, suggesting that the low concentration is sufficient to induce the suppressive mechanism. Application of low-concentration MeJA to leaves suppresses mycorrhization. That suppression affects mainly fungal colonization intensity and arbuscule abundance in roots and has serious consequences in the development of fungal efficiency in colonized roots, measured as alkaline phosphatase activity. The reduction in mycorrhizal parameters observed by histochemical methods was not exactly reflected at the level of gene rRNA 18S accumulation in roots, because the reduction in the amount of fungal rRNA in treated plants with respect to control plants was not considerable. Nevertheless, the amounts of *GiALP* and mainly *LePT4* transcripts were markedly reduced in treated plants, confirming that the exogenously applied MeJA negatively affected phosphate fungal metabolism and arbuscule formation rather than fungal root spread and penetration capacities. *LePT4* is a mycorrhiza-specific phosphate transporter from tomato (Nagy and others 2005), orthologous to the mycorrhiza-specific *MtPT4* Pi transporter from *M. truncatula* (Harrison and others 2002), which has been used as a marker of arbuscule formation (Isayenkov and others 2004) and is indispensable for arbuscular mycorrhizal symbiosis development because the loss of *MtPT4* function leads to premature death of the arbuscule (Javot and others 2007). On the other hand, although it is not confirmed that the *GiALP* gene encodes the ALP observed with the enzymatic-histochemical technique, previous results on *GiALP* gene expression suggest that it may have a role in nutrient exchange of the AM fungus with the host plant rather than in nutrient uptake from the rhizosphere (Aono and others 2004). These previous results showed that *GiALP* expression does not depend on infection rate. In our experiments, the plants with less than 10% of mycorrhizal intensity (*M* [%]) showed a significant reduction in the level of *GiALP* gene expression.

This systemic suppression of AM colonization in jasmonate-treated plants could be due to the role of jasmonates in the induction of the expression of genes coding for defense-related proteins that might regulate AM fungal spread in the host root. In our experiments, JA-inducible genes such as *LIN6* and *PI-II* were upregulated in roots as a result of MeJA application to leaves, confirming a systemic induction of the JA pathway in roots. The *LIN6* gene encodes an extracelluar apoplastic invertase induced by external stress-related stimuli, including wounding and elicitor treatment (Godt and Roitsch 1997), and by internal plant regulatory substances such as brassinosteroids (Goetz and others 2000), cytokinin (Godt and Roisch 1997), and jasmonic acid (Thoma and others 2003). Interestingly, arbuscular mycorrhiza induces *LIN6* gene expression in tomato roots, albeit to a moderate degree compared with stress-stimulated induction (Schaarschmidt and others 2006).

As the role of jasmonates in signal transduction in relation to defense gene induction has been shown to frequently include a synergistic effect with ethylene (Rojo and others 2003; Lorenzo and Solano 2005), we investigated whether the applied MeJA systematically induced ethylene production in roots. The results obtained from ethylene measurements in roots showed that, at least in our experiments, the application of MeJA to tomato leaves does not induce an increase in ethylene accumulation in roots, although we cannot exclude a transitory increase of ethylene not detected by our measurements.

Hause and others (2007) pointed toward the importance of jasmonate levels in root on mycorrhiza and suggested several mechanisms of action for jasmonates, including induction of flavonoid biosynthesis, reorganization and alterations in the cytoskeleton, the sink status of roots, and an increase in the plant fitness, which may participate in the development of AM. In this respect, Isayenkov and others (2005) observed that the reduction of JA levels as a result of partial suppression of gene expression of the JA biosynthetic enzyme allene oxide cyclase (AOC1) in *M. truncatula* roots leads to a reduction in the mycorrhization rate and arbuscule formation. Our results seem to disagree with the work by Isayenkov and others (2005). However, variation in methodology and the multiplicity of functions and concentration-dependent effect of JA application on mycorrhization might explain the differences between the results. Although Isayenkov and others (2005) observed the most obvious effect early on, at 21 days after inoculation, as a result of the reduction in JA levels we detected a restriction in colonization by the arbuscular mycorrhizal fungus after the initial phase, in later stages, and as a consequence of systemic activation of the jasmonate signalling pathway. It is possible that the reduction in JA levels reduces the presence of a jasmonate-responsive plant-signal compound that positively enhances fungal infectivity and consequently the fungal colonization decreases. Moreover, in the present study the most obvious effect of suppression of arbuscule formation was obtained by the systemic application of MeJA, because the expression of *LePT4* transcript was clearly downregulated

when we applied 5 μM of MeJA to leaves. The effect of suppression of arbuscule formation was attributable to the jasmonate signalling pathway rather than the JA concentration in roots.

In conclusion, the present study shows regulation of AM formation by JA. We provide strong evidence that JA participates in the susceptibility of tomato plants to infection by arbuscular mycorrhizal fungi, and that it plays a regulatory role in the development of AM. The systemic effect of foliar application of MeJA on mycorrhization and the fact that in the *jai-1* mutant the colonization characteristics by an AM fungus are altered indicate that arbuscular colonization in tomato is tightly controlled by the jasmonate signalling pathway. We demonstrated that *jai-1* MeJA-insensitive plants are more susceptible to AM fungal infection, indicating that the JA signalling pathway limits not only pathogenic interactions but also mutualistic interactions, although many of the responses and target genes regulated by the pathway may be specific to the particular plant-microorganism interaction. In this sense the specific role of JA-responsive genes with an altered expression in *jai-1* mutants (Li and others 2004) during the mycorrhization process should be investigated.

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