

Mycorrhization of the *notabilis* and *sitiens* tomato mutants in relation to abscisic acid and ethylene contents

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ABSTRACT

We examined whether the reduced mycorrhization of abscisic acid (ABA)-deficient tomato mutants correlates with their incapacity in ABA biosynthesis and whether this effect is dependent on ethylene production. The mycorrhization of *notabilis* and *sitiens* mutants, which have different ABA deficiencies and an excess of ethylene production, was analyzed. Comparative analysis of the ABA-deficient tomato mutants showed both quantitative and qualitative differences in the pattern of arbuscular mycorrhiza (AM) colonization between the two tomato mutant phenotypes. The *sitiens* mutant showed a great limitation in fungal colonization (mycorrhizal intensity and arbuscule formation) well correlated with their incapacity in ABA biosynthesis. The *notabilis* plants, which maintained normal ABA levels in roots under our experimental conditions, appeared to be less affected in their capacity for AM formation, and only a decrease in mycorrhizal intensity was noted at the end of the mycorrhization process. Blockage of ABA formation after tungstate application resulted in a reduction in mycorrhization of wild-type tomato plants. The transcript accumulation of the mycorrhiza-responsive *LePT4* gene (tomato phosphate transporter) was clearly associated with the ABA content and mycorrhiza development in roots, as the tungstate treatment in wild-type plants and the inherent ABA deficiency in *sitiens* mutants led to a complete abolishment of their expression. Our results suggest that the decrease in arbuscular abundance in mycorrhizal *sitiens* roots is directly associated with their ABA biosynthesis deficiency, and the accumulation of ethylene, as a consequence of ABA deficiency in the mutants, primarily affects mycorrhizal intensity.

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Introduction

The establishment of the arbuscular mycorrhiza (AM) mutualistic association is a successful strategy to improve the nutritional status of both partners (the plant and fungus). Fungal penetration and establishment in the host roots depend on a complex sequence of events and intracellular modifications (Bonfante-Fasolo and Perotto, 1992) leading to a nutrient interchange involving soil mineral nutrient uptake by the fungus in return for plant carbohydrates. Moreover, AM formation often improves growth, fitness and stress tolerance of the plant (Linderman, 2000).

Compatibility between plant roots and AM fungi implies a clear and selective recognition by the plant host that distinguishes the beneficial features of AM fungi from pathogens or other soil microbes. The key to understanding the phenomenon of

compatibility is the elucidation of the recognition mechanisms and molecules involved in the AM interaction. Recently, discoveries regarding the molecular communication between plants and AM fungi have shown that a group of compounds (strigolactones) exuded from the plant root induce hyphal branching of AM fungi (Akiyama et al., 2005; Gómez-Roldán et al., 2008). Moreover, a symbiotic plant signal-transduction cascade is activated in plant roots after both Rhizobia and AM fungi recognition by the host (Parniske, 2004). However, once the fungus has entered the root, the biochemical and morphogenetic events that take place in plant roots are unknown, and alterations in the homeostasis of plant hormones have been implicated in this process (Beyrle, 1995; Ludwig-Müller, 2000; Herrera-Medina et al., 2003; Hause et al., 2007).

The levels of plant hormones such as cytokinin, jasmonate (JA), auxin, auxin-related compounds and abscisic acid (ABA) change upon the establishment of AM (Hause et al., 2007), suggesting a change in the hormonal balance in an established AM. The majority of studies on this issue do not provide clear functional proof relating the importance of the changes in hormone levels

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observed with the establishment of the AM symbiosis. However, in this respect, experiments using transgenic and mutant approaches have shown that the plant hormones jasmonic acid and ABA play a role in the establishment of a functional AM symbiosis (Isayenkov et al., 2005; Herrera-Medina et al., 2007), and the plant ABA phenotype mediated the effects of the AM symbiosis on plant responses and resistance to water deficit (Aroca et al., 2008).

The analysis of the AM colonization in the tomato mutant *sitiens*, in which ABA levels are reduced to 8% of those in wild-type tomato plants (Herde et al., 1999), has shown that ABA is necessary for complete and fully functional arbuscule development and for sustained colonization of the plant root (Herrera-Medina et al., 2007). The arbuscule is the symbiotic structure involved in nutrient transfer in AM, and several reports have shown that their development and functionality are related with other phytohormone pathways other than ABA, such as jasmonic acid (Isayenkov et al., 2005; Herrera-Medina et al., 2008). The reduced AM formation in the *sitiens* mutant can be restored by the application of ABA, correlating with the increased mycorrhization observed following treatment of wild-type roots with ABA (Herrera-Medina et al., 2007).

The tomato mutants *notabilis*, *flacca* and *sitiens* are deficient in ABA, and the poor growth and strong leaf epinasty in these mutants occurs even under non-wilting conditions, and has been attributed, at least partially, to an excess of ethylene (Sharp et al., 2000). It has been proposed that restriction of ethylene production may be a widespread function of ABA. In fact, it is well known that ethylene and ABA mutually antagonize to modulate development (Beaudoin et al., 2000), shoot growth (Sharp et al., 2000) and disease resistance in plants (Anderson et al., 2004). In the same way, Herrera-Medina et al. (2007) showed that the impairment of mycorrhiza development in the tomato ABA-deficient *sitiens* mutant is partially attributable to an excess of ethylene.

Using genetic tools such as plant mutants defective in hormone production in combination with histochemical and molecular biology techniques for the analysis of AM formation, we studied mycorrhization in the ABA-deficient tomato mutants *notabilis* and *sitiens*, which have different ABA deficiency rates and an excess of ethylene production. Our results identified differences in the patterns of AM colonization between mutants according to their root ABA content. The use of the ABA-biosynthesis inhibitor sodium tungstate allowed us to determine its effect on mycorrhiza development in the tomato wild-type and whether it simulates the limitation in mycorrhiza development found in *sitiens* plants.

Material and methods

Plant growth and AM inoculation

Seeds of *Solanum lycopersicum* wild-type Rheinlands Ruhm (Rhe) (Accession LA0535) and Ailsa Craig (Accession LA2838A) and ABA-deficient mutants (Taylor et al., 1988) *sitiens* (*sit*) (Accession LA0575) and *notabilis* (*not*) (Accession LA3614) were obtained from the tomato genetics resource center (TGRC) at the University of California. Tomato seed sterilization, AM fungi inoculation and plant growth were carried out as previously described by Herrera-Medina et al., (2007).

The inoculation with *Glomus intraradices* (DAOM 197198), reclassified as *G. irregulare* according to Stockinger et al. (2009), was carried out in 200 mL pots. Each seedling was grown in a separate pot and was inoculated with a piece of monoxenic culture in Gel-Gro medium containing 50 spores of *G. irregulare*

and infected carrot roots. The monoxenic culture (*G. irregulare* and carrot roots) was made according to Chabot et al. (1992). In the non-inoculated treatment, the plants were inoculated with a piece of Gel-Gro medium containing only uninfected carrot roots.

One week after planting in pots and weekly thereafter, the pots were given 20 mL of a modified Long Ashton nutrient solution containing 25% of the P concentration (Hewitt, 1966) to prevent mycorrhizal inhibition due to excess of P. Plants were harvested at different times after inoculation (35, 55 and 65 d), 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 nature of the experiments. In each experiment, five independent plants were analyzed per treatment.

Estimation of root colonization

The non-vital trypan blue histochemical staining procedure was performed 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 according to this method were: mycorrhizal intensity (M%) and arbuscular abundance in mycorrhizal root fragments (a%). In some cases mycorrhization was evaluated by the grid-line intersect method described by Giovanetti and Mosse (1980).

RNA extraction and gene expression

Semiquantitative RT-PCR was carried out to measure the transcript levels of the 18S rRNA gene from *G. irregulare* (*Gi18S*), and elongation factor 1 α (*EF1 α*) and phosphate transporter 4 (*LePT4*) tomato genes in roots. Total RNA was isolated from the roots stored at -80°C using the RNeasy Plant Mini Kit (Qiagen, Maryland USA) following the manufacturer's instructions. To perform PCR experiments, total RNA was isolated from 1 g of pooled material that contained representative portions of roots from at least five different plants. cDNAs were obtained from 1 μg of total DNase-treated RNA in a 20 μL reaction volume. cDNA synthesis and PCR reaction conditions were the same as described previously (Herrera-Medina et al., 2008). Primers RMF and RMR, described by González-Guerrero and others (2005), were used to amplify a DNA fragment from the *G. irregulare* 18S rRNA. The gene-specific primers used for *LePT4* amplification were previously described by Nagy et al. (2005). As internal control of plant RNA amounts and quality the same single-strand cDNA was PCR amplified with specific primers for tomato *LeEF-1* mRNA (X14449). 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 the optimal annealing temperature and 90 s at 72°C . The synthesized DNA was separated in 1.25% agarose gels in TBE buffer. Each RT-PCR experiment was performed twice with different RNA extractions.

Quantitative RT-PCR was carried out with an iCycler apparatus (Bio-Rad, Hercules, CA, USA). Each 20 μL PCR reaction contained 1 μL of diluted cDNA (1:10), 10 μL 2x SYBR Green Supermix (Bio-Rad, Hercules, CA, USA), and 200 nM 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 $58\text{--}63^{\circ}\text{C}$ and 30 s at 72°C . The specificity of the PCR amplification procedure was checked with a melting curve after the final cycle of the PCR (70 steps of 30 s from 60 to 95°C with a heating rate of 0.5°C). Real-time PCR experiments were carried out from three biological replicates and the threshold cycle (C_T) was determined in

triplicate. The relative levels of transcription were calculated by using the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001). C_T values of all genes were normalized to the C_T value of the *LeEF-1* (X14449) housekeeping gene.

All primer names (for semiquantitative RT-PCR and qRT-PCR) and the corresponding sequences are listed in Table 1.

Chemical treatments

Tomato plants were treated in soil with (\pm) ABA (Sigma) and/or sodium tungstate (Na_2WO_4). The solutions were prepared by dilution from a stock. Twenty mL of the corresponding diluted solution was applied twice a week to each 200 mL pot containing one tomato plant. The first application started one week after AM fungus inoculation. Stock solutions contained 1 mM ABA in 1% ethanol, and 10 mM of sodium tungstate in water. Control treatments used 0.1% ethanol solution. The final ABA and tungstate concentrations used were in the range of concentrations used previously in other studies (Hansen and Grossmann, 2000; Audenaert et al., 2002).

Ethylene quantification

The ethylene content in roots was measured by placing excised root systems in a 16 mL tube sealed with a rubber stopper, and incubated for 1 h at room temperature. The accumulation of ethylene in each tube was determined from three different samples of 1 mL taken with a syringe from the tube. Measurement was carried out in a gas chromatograph (Hewlett Packard 5890) fitted with a flame ionization detector, using commercial ethylene as standard for identification and quantification. The ethylene concentration was expressed per g of root fresh weight.

ABA quantification

Plants were harvested, the roots were carefully rinsed with water and the root fresh weight was determined. The apical and upper parts of the root system of each plant were removed and root aliquots of 1 g of pooled material that contained representative portions of roots from at least three different plants were separated for the determination of root colonization and ABA analysis. ABA quantification was repeated three times with different pooled root material.

For ABA quantification, freeze-dried roots (the equivalent of minimum 100 mg fresh weight of root material) were extracted with a mixture of iso-propanol and acetic acid (95:5, v/v). 100 ng (d_6)-ABA (Plant Biotechnology Institute, National Research Council of Canada, Saskatoon, Canada) was added to each sample. Sample preparation was performed according to Meixner et al. (2005). Briefly, the samples were incubated under continuous shaking for 2 h at 4 °C, centrifuged for 10 min at 10,000g, then the

supernatant was removed and evaporated to dryness under a stream of N_2 . The residue was resuspended in methanol, centrifuged again for 10 min at 10,000g, and the supernatant was removed and placed in a glass vial. The methanol was evaporated under a stream of N_2 and the sample was resuspended in 100 μL ethyl acetate. Methylation was carried out according to Cohen (1984) with freshly prepared diazomethane.

GC-MS analysis was carried out on a Varian Saturn 2100 ion-trap mass spectrometer using electron impact ionization at 70 eV, connected to a Varian CP-3900 gas chromatograph equipped with a CP-8400 autosampler (Varian, Walnut Creek, CA, USA). For the analysis, 2 μL of the methylated sample dissolved in 30 μL ethyl acetate was injected in the splitless mode (splitter opening 1:100 after 1 min) onto a Phenomenex (Aschaffenburg, Germany) ZB-5 column (30 m \times 0.25 mm \times 0.25 μm) using He carrier gas at 1 ml min^{-1} . The injector temperature was 250 °C and the temperature program was 60 °C for 1 min, followed by an increase of 25 °C min^{-1} to 180 °C, 5 °C min^{-1} to 250 °C, 25 °C min^{-1} to 280 °C, then 5 min isothermally at 280 °C. The methyl ester of ABA eluted under these conditions at 13.5 min. For higher sensitivity, the μSIS mode (Varian Manual; Wells and Huston, 1995) was used.

The endogenous ABA concentration was calculated by the principles of isotope dilution using the ions of the methylated substance at m/z 190/194 (ions deriving from endogenous and d_6 -ABA; Walker-Simmons et al., 2000).

Statistical analysis

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

Results

Analysis of mycorrhization in different ABA-deficient tomato mutants

In order to investigate the possible correlation between AM fungal colonization and ABA content in roots, wild-type and tomato mutant plants were inoculated with *G. irregulare* and the degree of fungal colonization was determined using histochemical trypan blue staining. We assessed the intensity of root colonization and the percentage of arbuscules in the colonized roots. The comparative analysis of different ABA-deficient tomato mutants showed both quantitative and qualitative differences in the pattern of colonization between tomato mutant phenotypes. The *sitiens* mutant showed great limitations in fungal colonization (mycorrhizal intensity) and arbuscule formation (Fig. 1A). These limitations correlated with their incapacity in ABA biosynthesis; the roots of these mutants contained only about 16% and 21% of

Table 1
Primers used in the present study.

Primer name	Organism	Target gene	Primer sequence (5'–3')
RMF	<i>Glomus irregulare</i>	18S rDNA	(5'-TGTTAATAAAAATCGGTGCGTTGC-3')
RMR	<i>Glomus irregulare</i>	8S rDNA	(5'-AAAACGCCAATGATCAACCGGAC-3')
PT4F	<i>Solanum lycopersicum</i>	<i>LePT4</i>	(5'-GAAGGGGAGCCATTTAATGTGG-3')
PT4R	<i>Solanum lycopersicum</i>	<i>LePT4</i>	(5'-CCATCITGTGTATTGTTGTATC-3')
EF-1 α F	<i>Solanum lycopersicum</i>	<i>LeEF-1α</i>	(5'-CCGTTGAGACTGGTGTGAT-3')
EF-1 α R	<i>Solanum lycopersicum</i>	<i>LeEF-1α</i>	(5'-GATGATGACCTGGGAGTG-3')
qEF α F	<i>Solanum lycopersicum</i>	<i>LeEF-1α</i>	(5'-GAT TGG TGG TAT TGG AAC TGT C-3')
qEF α R	<i>Solanum lycopersicum</i>	<i>LeEF-1α</i>	(5'-AGC TTC GTG GTG CAT CTC-3')
qPT4F	<i>Solanum lycopersicum</i>	<i>LePT4</i>	(5'-GAA GGG GAG CCA TTT AAT GTG G-3')
qPT4R	<i>Solanum lycopersicum</i>	<i>LePT4</i>	(5'-ATC GCG GCT TGT TTA GCA TTT CC-3')

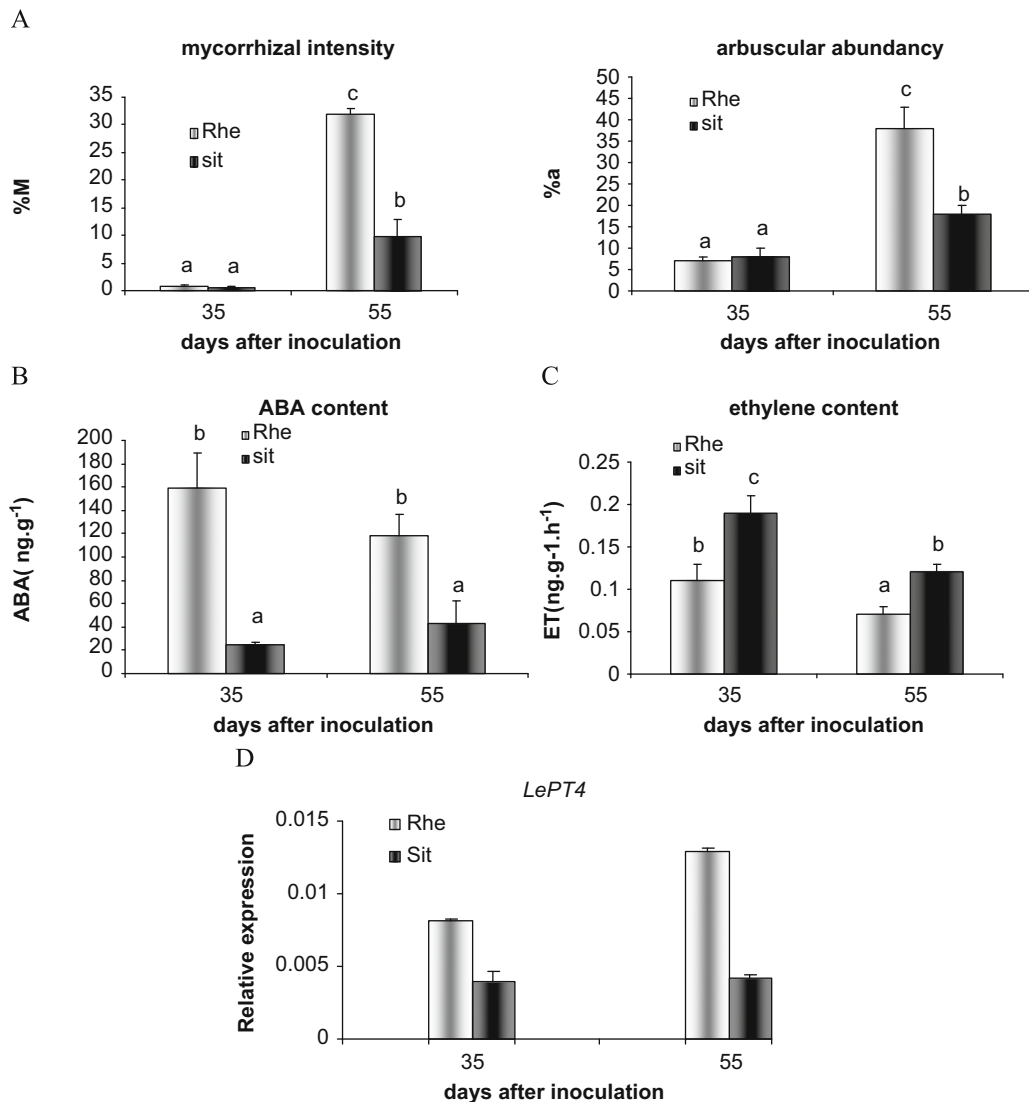


Fig. 1. Time course of mycorrhization parameters (A), ABA (B) and ethylene (C) contents in roots, and qRT-PCR analysis of *LePT4* gene expression (D) in roots of wild-type Rheinlands Ruhm and *sitiens* ABA-deficient mutant tomato plants colonized by *G. irregulare*. Mycorrhization parameters of mycorrhizal intensity (M%) and arbuscular abundance in mycorrhizal zones of the roots (a%) were determined using MYCOCALC software. Values are the mean \pm SE of five biological replications. Bars with similar letters are not significantly different ($P=0.05$) according to Duncan's multiple range test.

the ABA levels measured in roots of Rheinlands Ruhm wild-type tomatoes at 35 and 55 days after inoculation, respectively (Fig. 1B). The *notabilis* plants, which maintained normal ABA levels in roots under our experimental conditions (Fig. 2B), appeared to be less affected in their capacity for AM formation, and only a decrease in mycorrhizal intensity was noted at 65 days after inoculation (Fig. 2A). The level of percentage of arbuscules in colonized roots did not differ between Ailsa Craig wild-type and the *notabilis* mutant (Fig. 2A).

Determinations of ethylene accumulation in colonized WT and ABA mutant plant roots showed that root ethylene concentrations were higher in ABA mutant phenotypes than in WT, with the increases being similar in *sitiens* (Fig. 1C) and *notabilis* (Fig. 2 C).

To examine arbuscule functionality in *sitiens* roots, qRT-PCR experiments of *LePT4* gene expression were carried out with cDNA from wild-type and *sitiens* plants 35 and 55 days after inoculation (Fig. 1D). At two harvest points, the level of *LePT4* gene expression was higher in wild-type roots inoculated with *G. irregulare* than in *sitiens* inoculated roots. Whereas wild-type plants showed an elevation of *LePT4* expression from 35 to 55 days after inoculation,

sitiens roots at 35 days and 55 after inoculation showed similar *LePT4* gene expression levels.

Analysis of mycorrhization in plants treated with an ABA-biosynthesis inhibitor

Experiments using exogenous application of the ABA-biosynthesis inhibitor sodium tungstate, which blocks the formation of ABA from ABA-aldehyde by impairing ABA-aldehyde oxidase (Hansen and Grossmann, 2000), were performed to determine its effect on mycorrhiza development in the tomato wild-type and to assess whether it was related to the limitation in mycorrhiza development found in *sitiens* plants. Also, to test whether the effects of tungstate could be overridden by exogenously supplied ABA, a group of these plants were pre-treated with tungstate plus ABA. Treatment with the ABA-biosynthesis inhibitor tungstate substantially diminished the accumulation of ABA in roots; the amount of ABA was reduced to 50% of those in non-treated plants (Fig. 3B). The deficiency of ABA in *sitiens*, which had around 30% of

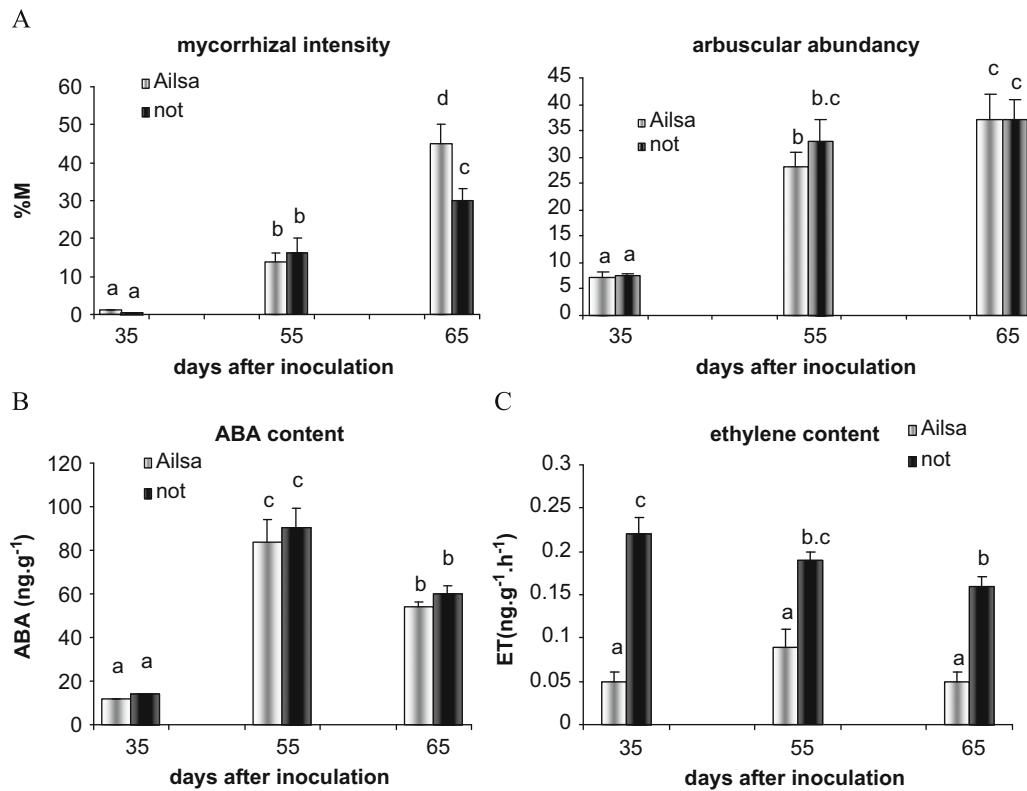


Fig. 2. Time course of mycorrhization parameters (A), ABA (B) and ethylene (C) contents in roots of wild-type Ailsa Craig (WT) and *notabilis* ABA-deficient mutant tomato plants colonized by *G. irregulare*. Mycorrhization parameters of mycorrhizal intensity (M%) and arbuscular abundance in mycorrhizal zones of the roots (a%) were determined using MYCOCALC software. Values are the mean \pm SE of five biological replications. Bars with similar letters are not significantly different ($P=0.05$) according to Duncan's multiple range test.

the wild-type content, and tungstate-treated WT plants was eliminated when ABA was applied alone (in *sitiens* plants) or together with tungstate (in WT plants) (Fig. 3B).

The extent of mycorrhization was reduced following the application of sodium tungstate in wild-type tomato plants (Fig. 3A). The degree of reduction, in terms of percentage, was similar for ABA content and root length colonization in wild-type tungstate-treated plants (Fig. 3A). Application of ABA restored mycorrhization in Rheinlands Ruhm wild-type plants treated with tungstate and increased the capacity of mycorrhization in *sitiens* plants.

To assess the effect on ethylene accumulation in roots produced by tungstate application, we performed ethylene determinations in roots of WT plants treated or not treated with tungstate alone and in combination with ABA, and in roots of *sitiens* plants treated with ABA. As expected, non-treated *sitiens* plants with constitutively reduced ABA contents, and WT tungstate-treated plants that diminished the accumulation of ABA showed an increase in root ethylene concentration. The increase in ethylene was blocked when ABA was applied to *sitiens* plants and WT tungstate-treated plants (Fig. 3C).

Fungal colonization was also quantified by the semiquantitative RT-PCR technique. These experiments were carried out to verify, at the molecular level, the microscopic measurements of mycorrhization in tungstate and ABA-treated and non-treated wild-type and *sitiens* plants. We quantified fungal colonization as the accumulation of *G. irregulare* 18S rRNA in tomato roots relative to the level of plant mRNA amounts measured as EF 1 α mRNA accumulation. The quantity of fungal rRNA 18S in colonized roots (Fig. 4) clearly confirms the histochemical data. The levels of fungal rRNA 18S were significantly lower in tungstate-treated wild-type and in non-treated *sitiens* than in non-treated wild-type plants, and ABA treatment increased these

levels in both types of plants (Fig. 4). The values for fungal 18S rRNA accumulation in *sitiens* plants were less than those of wild-type. The arbuscular functionality in mycorrhizal roots was quantified at the molecular level by the detection of transcripts for the arbuscular-specific tomato phosphate transporter *LePT4*. The transcript accumulation of *LePT4* was clearly associated with the ABA content and mycorrhiza development in roots, as the tungstate treatment in wild-type plants and the inherent ABA deficiency in *sitiens* mutants led to a complete abolishment of their accumulation (Fig. 4).

Discussion

Previous work has shown that ABA is necessary for sustainable colonization of the plant root during the establishment of the AM (Herrera-Medina et al., 2007). Here we investigated the behavior of distinct ABA-deficient mutants in terms of their mycorrhizal capacities. As in previous studies on plant growth and development, ABA-deficient mutants were used here as vehicles to investigate the role of ABA during AM formation. *Notabilis* (*not*), *flacca* (*flc*) and *sitiens* (*sit*) ABA-deficient tomato mutants have been intensively studied. Both *flacca* and *sitiens* were shown to be impaired in their ability to convert xanthoxin (Xan) to ABA, specifically at the stage of ABA-aldehyde oxidation (Parry et al., 1988; Sindhu and Walton, 1988; Taylor et al., 1988). In contrast, *notabilis* converted both Xan and ABA-aldehyde to ABA in detached leaves and cell-free systems as efficiently as the wild-type (Parry et al., 1988; Sindhu and Walton, 1988; Taylor et al., 1988). *Notabilis* has been characterized by its "leakiness", and unlike *flacca* and *sitiens* it can respond to stress by synthesizing some ABA (Parry et al., 1988). In our experiments, *notabilis* plants had normal ABA levels in roots compared with wild-type plants

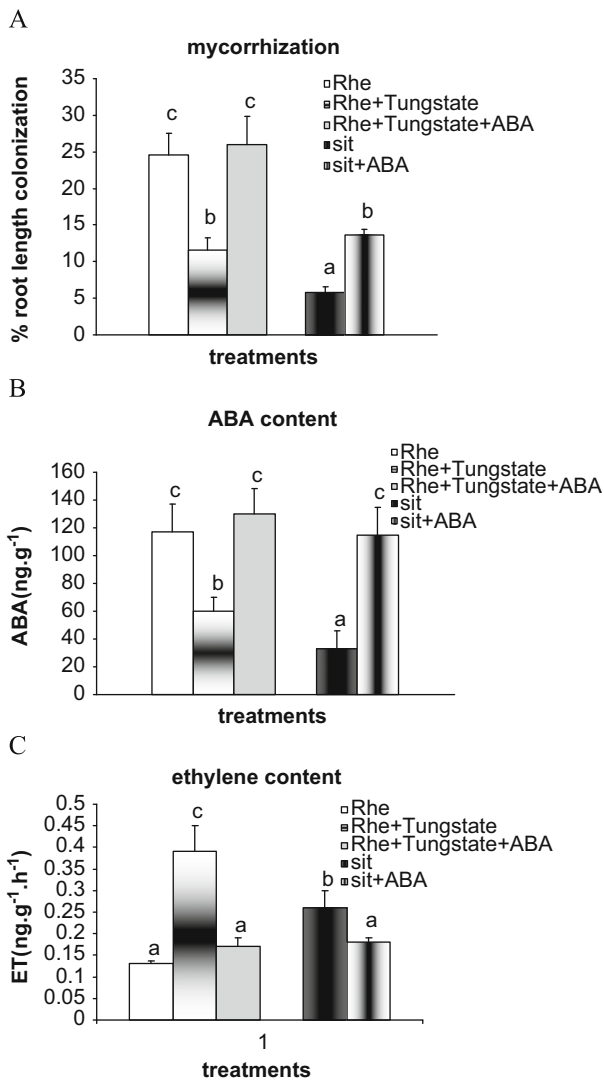


Fig. 3. Effect of ABA and sodium tungstate application on mycorrhization (A), ABA (B) and ethylene (C) contents in roots of wild-type Rheinlands Ruhm and ABA-deficient *sitiens* tomato plants colonized by *G. irregulare*. After one week of inoculation, tomato plants were root treated with compounds. Mycorrhization and ABA content were measured 50 days after inoculation. ABA (75 μ M) and sodium tungstate (1.5 mM) solutions were applied to the soil twice per week. Values are the mean \pm SE of five biological replications. Bars with similar letters are not significantly different ($P=0.05$) according to Duncan's multiple range test.

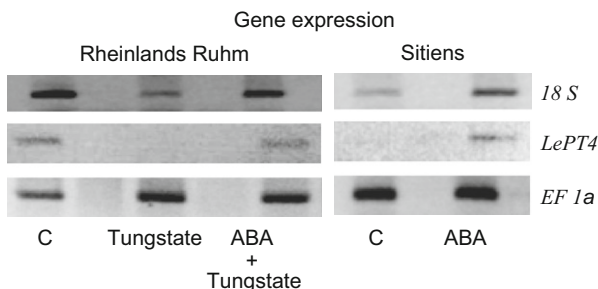


Fig. 4. Effect of ABA and sodium tungstate application on gene expression in roots of wild-type Rheinlands Ruhm and ABA-deficient *sitiens* tomato plants colonized by *G. irregulare*. After one week of inoculation tomato plants were root treated with compounds. Gene expression by semiquantitative RT-PCR of tomato *LePT4* and *EF 1 α* and fungal *18 S* was measured 50 days after inoculation. ABA (75 μ M) and sodium tungstate (1.5 mM) solutions were applied to the soil twice per week.

under well-irrigated and non-stressed experimental conditions, though they maintained an excess of ethylene, as has been described previously (Sharp et al., 2000).

The comparative analysis of *notabilis* and *sitiens* ABA-deficient tomato mutants showed both quantitative and qualitative differences in the pattern of AM colonization between the two tomato mutant phenotypes. The *sitiens* mutants showed a great limitation in fungal colonization (mycorrhizal intensity and arbuscule formation) well correlated with their incapacity in ABA biosynthesis. *Notabilis* plants, which maintained normal ABA levels in roots under our experimental conditions, appeared to be less affected in their capacity of AM formation, and only a decrease in mycorrhizal intensity was noted at the end of the mycorrhization process. The correlation between ABA root content and limitation in AM fungal colonization, together with the fact that *notabilis* and *sitiens* mutants showed similar increases in root ethylene content compared with their respective wild-type plants, suggest that ABA has a role in the functionality of the symbiosis that is independent of ethylene.

At all harvest times, *notabilis* plants showed constitutively higher ethylene levels compared to WT plants, though only 65 days after inoculation colonization rates of *notabilis* roots were lower than in WT roots. Furthermore, at 65 days after inoculation, the ethylene concentration in roots of *notabilis* plants was significantly lower compared to 35 days after inoculation. This apparent contradiction could be explained by the fact that ethylene mainly affects the maintenance of the fungus in the root and not the establishment, and it might be that the regulation mediated by ethylene could be linked with certain degree of AM root colonization. In this case, a certain infection threshold should be necessary to lead to an ethylene response. There are some indications in favor of this hypothesis. There is experimental evidence that other phenomena control fungal spread in the root. For example, mycorrhizal autoregulation and mycorrhiza bioprotection against root-fungal pathogens are linked with a certain degree of AM root colonization (Vierheilig et al., 2008). In our experiments there was no growth depression in *notabilis* plants at late stages of development (data not shown) due to their higher ethylene content. Therefore, the decrease in fungal colonization was not due to a decrease in growth in older plants. Nevertheless, the possibility other factors participate in the regulation of the mycorrhization in combination with ethylene cannot be ruled out.

The blockage in the formation of ABA after tungstate application resulted in a reduction in mycorrhization of wild-type tomato plants. In addition, the application of ABA restored mycorrhization in these wild-type plants treated with tungstate and increased the capacity of mycorrhization in *sitiens* plants. Interestingly, the transcript accumulation of *LePT4* was clearly associated with the ABA content and mycorrhiza development in roots, as the tungstate treatment in wild-type plants and the inherent ABA deficiency in *sitiens* mutants led to a complete abolishment of their accumulation.

The spatial expression pattern of the mycorrhiza-specific *LePT4* tomato phosphate transporter has been investigated previously, and it is confined exclusively to the colonized cells containing arbuscules in tomato mycorrhizal roots (Nagy et al., 2005; Balestrini et al., 2007), being a marker gene for arbuscular functionality in arbuscular mycorrhizal roots. Our qRT-PCR experiments of *LePT4* gene expression showed that *sitiens* plants levels of arbuscules similar to those of WT plants have almost no *LePT4* expression, demonstrating that arbuscules in *sitiens* are less functioning than in WT. The expression of *LePT4* was associated with the ABA content in roots and, consequently, the application of ABA also restored *LePT4* expression in *sitiens*. Previous data showed that the application of ABA also restored fungal alkaline

phosphatase activity (Herrera-Medina et al., 2007). Both *LePT4* and alkaline phosphatase are molecular markers for arbuscular functionality. A recent transcriptome analysis focused on the identification of *M. truncatula* and *G. intraradices* genes expressed in colonized cortical cells and in arbuscules (Gomez et al., 2009), and it might be interesting to investigate whether these genes respond in a similar way to *LePT4* in *sitiens* plants.

In the present investigation, the application of ABA to mycorrhizal *sitiens* roots resulted in recovery of the arbuscular frequency. Recent results suggest that a balance between biosynthesis and catabolism of ABA is crucial for the differentiation of arbuscules (Fiorilli et al., 2009). Therefore, a direct effect of ABA on the arbuscule formation can be postulated. The opposite effect can be observed by gibberellic acid (GA) application, since a dose-dependent phenomenon of suppression of arbuscule formation has been described in pea plants treated with GA3 (El Ghachtouli et al., 1996). The data point to a possible antagonistic role for ABA and GA during AM formation.

The use of ABA-deficient mutants with different wilting phenotypes allowed us to confirm that ABA plays a role in AM development. In previous work, the limitation of fungal colonization in *sitiens* mutants has been attributed, at least partially, to an excess of ethylene (Herrera-Medina et al., 2007). Though we do not further elucidate the discrete roles of ethylene and ABA in AM, our results suggest that the decrease in arbuscular abundance in mycorrhizal *sitiens* roots is directly associated with their ABA biosynthesis deficiency, and indicate that the accumulation of ethylene, as a consequence of ABA deficiency in the mutants, mainly affects mycorrhizal intensity. There is clear evidence for an inhibitory role of exogenous ethylene in the process of mycorrhization (Guinel and Geil, 2002). Putative roles can be hypothesized for this hormone during AM formation: (i) regulation of the calcium spiking response in plant cells; (ii) Control of cell wall deposition that could affect localized resistance (Abeles et al., 1992) and (iii) activation of defense responses to control fungal infection (Adie et al., 2007). In this respect, it has been suggested that ethylene defines the nature (strength and frequency) of the calcium spiking response in plants cells (Oldroyd et al., 2001), which has been described as an essential stage in both Nod-factor perception and AM fungal-mediated signaling (Levy et al., 2004).

In future work, to further elucidate the discrete roles of ethylene and ABA in AM, we will carry out cytological and histological studies attempting to discriminate the ethylene-dependent and independent role of ABA during AM formation, analyzing the pattern of mycorrhization in transgenic tomato plants modified in the ethylene pathway and comparing the consequences for mycorrhization of ABA-deficient plants treated either with ethylene biosynthesis inhibitor or ABA.

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