Abscisic acid determines arbuscule development and functionality in the tomato arbuscular mycorrhiza

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Summary

- The role of abscisic acid (ABA) during the establishment of the arbuscular mycorrhiza (AM) was studied using ABA *sitiens* tomato (*Lycopersicon esculentum*) mutants with reduced ABA concentrations.
- *Sitiens* plants and wild-type (WT) plants were colonized by *Glomus intraradices*. Trypan blue and alkaline phosphatase histochemical staining procedures were used to determine both root colonization and fungal efficiency. Exogenous ABA and silver thiosulfate (STS) were applied to establish the role of ABA and putative antagonistic cross-talk between ABA and ethylene during AM formation, respectively.
- *Sitiens* plants were less susceptible to the AM fungus than WT plants. Microscopic observations and arbuscule quantification showed differences in arbuscule morphology between WT and *sitiens* plants. Both ABA and STS increased susceptibility to the AM fungus in WT and *sitiens* plants. Fungal alkaline phosphate activity in *sitiens* mutants was completely restored by ABA application.
- The results demonstrate that ABA contributes to the susceptibility of tomato to infection by AM fungi, and that it seems to play an important role in the development of the complete arbuscule and its functionality. Ethylene perception is crucial to AM regulation, and the impairment of mycorrhiza development in ABA-deficient plants is at least partly attributable to ethylene.

Key words: abscisic acid, arbuscular mycorrhiza, ethylene, *sitiens*, tomato (*Lycopersicon esculentum*).

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Introduction

The endosymbiosis formed between plant roots and arbuscular mycorrhizal (AM) fungi is the most widespread symbiotic association in the plant kingdom. The establishment of this mutualistic association is a successful strategy to improve the nutritional status of both partners. Fungal penetration and establishment in the host roots involve a complex sequence of events and intracellular modifications (Bonfante-Fasolo & Perotto, 1992). 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.

The key to understanding the phenomenon of compatibility is the elucidation of the recognition mechanisms and molecules involved in the AM interaction. In this context, biochemical and morphogenetic events mediated by phytohormones during AM formation have been suggested, and certain roles for phytohormones in ectomycorrhizas and arbuscular mycorrhizas have been proposed (Beyrle, 1995; Ludwig-Müller, 2000; Herrera-Medina *et al.*, 2003).

In view of the role of abscisic acid (ABA) in the regulation of the signalling pathways involved in plant growth and development, both of which processes are altered in AM symbiosis, it is of interest to study the putative role of ABA in the process

of AM formation. Studies of ABA and AM development have been conducted, mainly through experiments involving measurements of hormone content in AM plants. Contradictory results have been obtained, and in the case of *Zea mays* infected with *Glomus*, the ABA contents in leaves and roots of mycorrhizal plants were always higher than in control plants (Danneberg *et al.*, 1992). Similar data were obtained for ABA contents in roots of soybean (*Glycine max*) plants. In mycorrhizal roots, the ABA content was always clearly increased compared with ABA contents in roots of nonmycorrhizal control plants (Murakami-Mizukami *et al.*, 1991; Meixner *et al.*, 2005). However, infection of *Bouteloua gracilis* with AM fungi resulted in decreased ABA contents in leaves, and unchanged ABA contents in roots (Allen *et al.*, 1982). ABA has also been detected in fungal hyphae at contents higher than in roots (Esch *et al.*, 1994), and it was speculated that ABA in AM fungi could control the flux of water and mineral salts from the soil to the hyphae or from other fungal structures such as arbuscules to the root cells.

A genetic interaction between ABA and other signalling pathways such as those for ethylene, sugar and salicylic acid, which are important in AM symbiosis, has also been reported (Gazzarrini & McCourt, 2001; Audenaert *et al.*, 2002; León & Sheen, 2003). The ABA signalling pathway interacts antagonistically with the ethylene signalling pathway, and vice versa, to modulate plant development (Beaudoin *et al.*, 2000; Ghassemian *et al.*, 2000) and plant disease resistance (Anderson *et al.*, 2004). Furthermore, ABA regulates plant responses to several abiotic stresses, such as drought, salt and cold, and some of these stresses are alleviated in mycorrhizal plants, suggesting that the putative mechanism of alleviation in mycorrhizal plants could be mediated by ABA. ABA appears to negatively modulate the salicylic acid-dependent defence pathway in tomato (*Lycopersicon esculentum*), determining susceptibility to *Botrytis cinerea* (Audenaert *et al.*, 2002), and it is tempting to speculate that the detected increases in ABA production in mycorrhizal roots could contribute to the suppression of the salicylic acid-dependent defence mechanisms activated in AM roots (Blilou *et al.*, 1999; Herrera-Medina *et al.*, 2003).

Nevertheless, no specific role has been demonstrated for ABA during AM symbiosis, and our understanding of how the ABA pathway interacts with the sugar and ethylene pathways during AM formation is minimal. In the present study, using ABA *sitiens* tomato mutants with reduced ABA concentrations, we first demonstrate that ABA increases the susceptibility of tomatoes to AM infection. The results further suggest that ABA is necessary for the process of arbuscule formation.

Materials and Methods

Plant growth and AM inoculation

Seeds of *Lycopersicon esculentum* Mill. cvs Rheinlands Ruhm (accession LA0535) and ABA-deficient *sitiens* mutants (Taylor *et al.*, 1988) (accession LA0575) were obtained from the Tomato Genetics Resource Center (TGRC) at the University of California, Davis, CA, USA. Seeds were surface-sterilized by soaking in 0.75% sodium hypochlorite for 5 min, after which they were rinsed with tap water and soaked again in 7% H_2O_2 for 10 min, and then rinsed three times with sterile deionized water and germinated on a sterilized moistened filter paper for 3–4 d at 25°C. After germination, plants were grown in a steam-sterilized (40 min at 120°C) mixture of silicate sand, peat, soil and vermiculite (1 : 1 : 1 : 1, by volume). Plant growth and treatments took place in a growth chamber (day:night cycle, 16 h, 25°C : 8 h, 19°C; relative humidity 50%). Every day, *sitiens* plants were sprayed with water to prevent them from wilting.

Inoculation with *Glomus intraradices* (DAOM 197198) 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 (ICN Biochemicals, Aurora, OH, USA) containing 50 spores of *G. intraradices* and infected carrot (*Daucus carota*) roots. The monoxenic culture (*G. intraradices* and carrot roots) was produced according to Chabot *et al.* (1992). In the noninoculated treatment, the plants were inoculated with a piece of Gel-Gro medium containing only uninfected carrot roots.

One week after planting and weekly thereafter, the pots were given 20 ml of a modified Long Ashton nutrient solution containing 25% of the standard phosphorus (P) concentration (Hewitt, 1966) to prevent mycorrhizal inhibition as a result of an excess of 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 nature of the experiments. In each experiment, five independent plants were analysed per treatment.

Estimation of root colonization

Two histochemical staining procedures were used to determine total colonization and alkaline phosphatase activity, respectively. The nonvital trypan blue stain was made up according to the method of Phillips & Hayman (1970), and the vital stain for alkaline phosphatase allowing detection of active AM fungi was made up as described by Tisserant *et al.* (1993). Alkaline phosphatase localized in the vacuole of the intraradical mycelium (Gianinazzi *et al.*, 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 *et al.*, 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 *et al.* (1986) using MYCOCALC software (http://www.dijon.inra.fr/mychintec/Mycocal-prg/download.html). The parameters measured according to this method were the frequency (F) and intensity (M) of root cortex colonization, and the

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arbuscular abundance in the mycorrhized root cortex (*a*) (each as a percentage). In some cases, mycorrhiza development was evaluated by the grid-line intersect method described by Giovannetti & Mosse (1980). To sort the arbuscules into the three morphologically different stages of arbuscule formation, at least 100 arbuscular units were observed in root sections and each arbuscular unit was placed in one of the three classes.

RNA extractions and gene expression

A semiquantitative reverse transcriptase–polymerase chain reaction (RT-PCR) was carried out to measure the transcript levels of *G. intraradices* 18S ribosomal RNA (rRNA) and the *le4* and polyubiquitin plant genes in tomato roots. Total RNA was isolated from roots frozen at –80°C using the RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA) 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 avian myeloblastosis virus (AMV) reverse transcriptase (Roche, Mannheim, Germany), 400 ng of random hexamer primers, 1 mM of each dNTP, 50 U of RNase inhibitor, and 1 × reverse transcription buffer. After reverse transcription, 80 µl of MilliQ water was added to obtain a final volume of 100 µl of each cDNA solution. Five µl 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 $MgCl_2$ and 50 mM KCl, pH 8.3). Primers LE4F (5′-ACTCAAGGCATGGGTACTGG-3′) and LE4R (5′-CCTTCTTTCTCCTCCCACCT-3′) were designed to amplify a 112-bp region of the *le4* ABA-responsive gene from tomato (Cohen *et al*., 1991), and primers RMF (5′-TGTTAATAAAAATCGGTGCGTTGC-3′) and RMR (5′-AAAACGCAAATGAT CAACCGGAC-3′) described by González-Guerrero *et al.* (2005) were used to amplify a DNA fragment from the *G. intraradices* 18S rRNA. As an internal control for plant RNA quantity and quality, the same single-stranded cDNA was PCR-amplified with specific primers for the polyubiquitin gene (Ubi-1 : 5′-ATGCAGAT(C/T)TTT-GTGAAGAC-3′ and Ubi-2: 5′-ACGCAGACCGAGGT-GGAG-3′) that specifically bind to the 228-bp monomer fragment of the tomato polyubiquitin gene (Rollfinke & Pfitzner, 1994). The PCR programme 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 (100 mM Tris, 90 mM boric acid, 1 mM EDTA, pH8.3) buffer.

Chemical treatments

Tomato plants were treated with ABA (Sigma-Aldrich, Steinheim, Germany) and silver thiosulfate (STS) by application to the soil. The solutions were prepared by dilution from a stock solution. 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 1 wk after AM fungus inoculation. Stock solutions contained 1 mM ABA in 1% ethanol and 40 mM STS in water. Control treatments used a 0.1% ethanol solution. The final ABA and STS concentrations used were within the range of concentrations used previously in other studies (Fujino *et al*., 1989; 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. Measurements were carried out in a gas chromatograph (Hewlett Packard 5890; Hewlett Packard, Wilmington, DE, USA) fitted with a flame ionization detector, using commercial ethylene as a standard for identification and quantification. The ethylene concentration was expressed per g of root fresh weight.

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

Mycorrhiza development in *sitiens* tomato mutants impaired in ABA biosynthesis

Rheinlands Ruhm wild-type (WT) tomato and *sitiens* mutant plants were inoculated with *G. intraradices* and the susceptibility of the plants to fungal colonization and the efficiency of the fungus in colonized plant roots were determined using histochemical trypan blue and alkaline phosphatase staining, respectively. Assessment of the frequency and intensity of root colonization and measurement of fungal tissue with phosphatase activity in the intraradical mycelium were carried out. Both quantitative and qualitative changes in the pattern of colonization were observed between WT plants and ABA-deficient mutants. Table 1 shows the comparative values of AM colonization for WT and *sitiens* mutants after 40 d of fungal inoculation. *Sitiens* plants appeared to be less susceptible to *G. intraradices* infection, as a considerable decrease in the percentage of root colonization was observed. When the frequency and intensity of colonization were measured, both were clearly higher in WT than in mutant plants. Furthermore, the percentages of vesicles and arbuscules in the colonized roots were markedly reduced in *sitiens* plants (Table 1).

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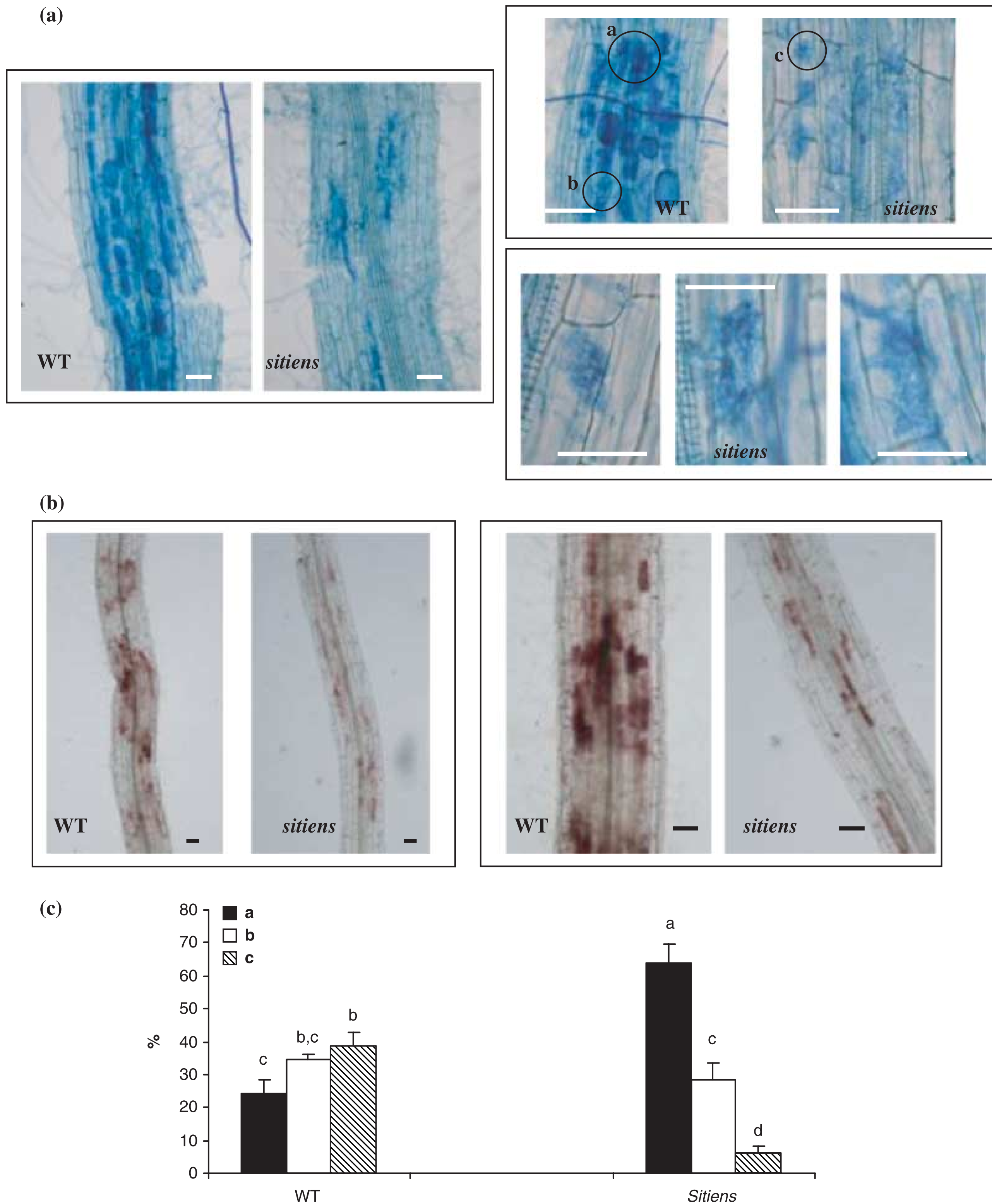

Fig. 1 (a) Trypan blue histochemical staining and (b) fungal alkaline phosphatase activity in roots of Rheinlands Ruhm (wild-type) and *sitiens* mutant tomato (*Lycopersicon esculentum*) plants colonized by *Glomus intraradices.* The root pieces were photographed at different optical magnifications, to show a typical colonized root, a root section with several arbuscules, *sitiens* arbuscules in detail, and representative arbuscules of the three different morphological classes (circles) defined in the text. Bar, 50 µm. (c) Percentage of arbuscules at three different morphological stages of arbuscule formation. Values are the means ± standard error of five biological replications. Bars with similar letters are not significantly different ($P = 0.05$) according to Duncan's multiple range test.

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Table 1 Mycorrhiza development parameters in roots of tomato (*Lycopersicon esculentum*) Rheinlands Ruhm wild-type (WT) and *sitiens* mutant plants 40 d after inoculation with *Glomus intraradices*

	Root colonization		Alkaline phosphatase activity	
	WT	*Sitiens*	WT	*Sitiens*
Frequency (*F*; %)	75 a	25 b	45.5 a	2.4 b
Intensity (*M*; %)	25.2 a	4.3 b	7.6 a	0.5 b
Arbuscules (*a*; %)	60 a	15 b		
Vesicles (%)	43 a	9 b		

Within root colonization and within alkaline phosphatase activity rows, values followed by the same letter are not significantly different ($P = 0.05$) according to Duncan's multiple range test.

In terms of quantitative measurements, when stained fungal structures were compared between WT plants and mutants, clear differences were observed (Fig. 1a). Mycorrhiza development was less abundant in mutant roots and trypan blue spots corresponding to arbuscules in roots showed lower intensity. The arbuscules formed in the roots of the mutant showed anomalies. Essentially they seemed incompletely formed and were less branched (Fig. 1a).

In these comparative assays, *sitiens* roots appeared to be much less susceptible to *G. intraradices* infection than WT roots, and both quantitative and qualitative morphological changes were observed. Experiments were performed subsequently to determine how the observed morphological alterations affected physiological parameters of the symbiosis. We measured fungal alkaline phosphatase activity (AP) in mycorrhizal roots of WT and mutant plants to analyse the efficiency of the AM symbiosis. The data shown in Table 1 clearly illustrate that both the frequency and the intensity of fungal tissue with AP activity were greatly reduced in *sitiens* mutants. In both WT and mutant plants, the histochemical AP activity was mostly associated with arbuscules (Fig. 1b). In *sitiens* roots, less AP activity was observed compared with the WT. The colour intensity of spots with histochemical AP activity was clearly lower in mutant than in WT roots (Fig. 1b).

In order to perform a statistical analysis of the differences in arbuscule morphology between WT and *sitiens* plants, the percentage of arbuscules at three different morphological stages of arbuscule formation was calculated. The three arbuscule classes were defined as follows: class a, arbuscules in formation (or degradation) with no fine branches partially occupying the plant cell; class b, arbuscules with at least some fine branches, with intermediate intensity of trypan blue stain occupying almost all of the plant cell; class c, arbuscules with many branches and a high intensity of trypan blue stain occupying the whole of the plant cell. The data for the percentages of each arbuscule class in the two types of plant were completely different and confirmed the histochemical observations. The three classes of arbuscules were present in similar proportions in WT plant roots (Fig. 1c). In these plants, the percentage ranged from 25% for class a to 35% and 40% for classes b and c, respectively. Conversely, in *sitiens* plants the majority of the arbuscules were of class a (60%) and only 10% were of class c (Fig. 1c).

ABA increases susceptibility of tomato to AM fungi

Experiments using exogenous application of ABA were performed to determine its effect on mycorrhiza development of WT tomato and whether it restores the extent of mycorrhiza development found in *sitiens* plants. Figure 2 shows the pattern of *G. intraradices* colonization in WT Rheinlands Ruhm and *sitiens* plants in an experiment consisting of ABA application and sequential determination of root colonization. ABA application increased AM colonization in WT and mutant plants (Fig. 2a). When the frequency and intensity of root colonization were measured (Fig. 2b), the data demonstrated that in WT plants the frequency of mycorrhiza development was only weakly affected by exogenous ABA application. Nevertheless, in WT plants a clear increase in the intensity of AM colonization was observed in the presence of exogenous ABA. This increase in intensity was parallel to an increase in arbuscule abundance in the colonized zones of the root. An increase in the frequency and intensity of AM colonization in *sitiens* roots as a result of ABA application was also observed, but the values were smaller than those for WT plants. No detectable increase in arbuscule number was observed in *sitiens* roots after ABA application.

A semiquantitative RT-PCR experiment was carried out to verify at the molecular level the microscopic measurements of mycorrhiza development in ABA-treated and nontreated WT and *sitiens* plants. In this experiment, we quantified fungal colonization as the accumulation of *G. intraradices* 18S rRNA in tomato roots relative to amounts of plant mRNA, measured as polyubiquitin mRNA accumulation. The quantity of fungal rRNA 18S in colonized roots (Fig. 2c) clearly confirmed the histochemical data. The quantities of fungal rRNA 18S were significantly lower in *sitiens* than in WT plants, and ABA treatment increased these quantities in both types of plant (Fig. 2c). The values of fungal 18S rRNA accumulation in *sitiens* plants were smaller than those in WT plants in all cases.

The deficiency of growth inherent to *sitiens* mutant plants was effectively eliminated by the dose of applied ABA (Fig. 3a). At all times assayed, the shoot and root fresh weights of *sitiens* plants treated with ABA were similar to the shoot and root fresh weights of WT Rheinlands Ruhm plants. Nevertheless, nontreated *sitiens* plants showed a clear deficiency in their growth capacity (Fig. 3a).

To confirm the negative effect on ethylene accumulation in roots produced by ABA application, we performed ethylene

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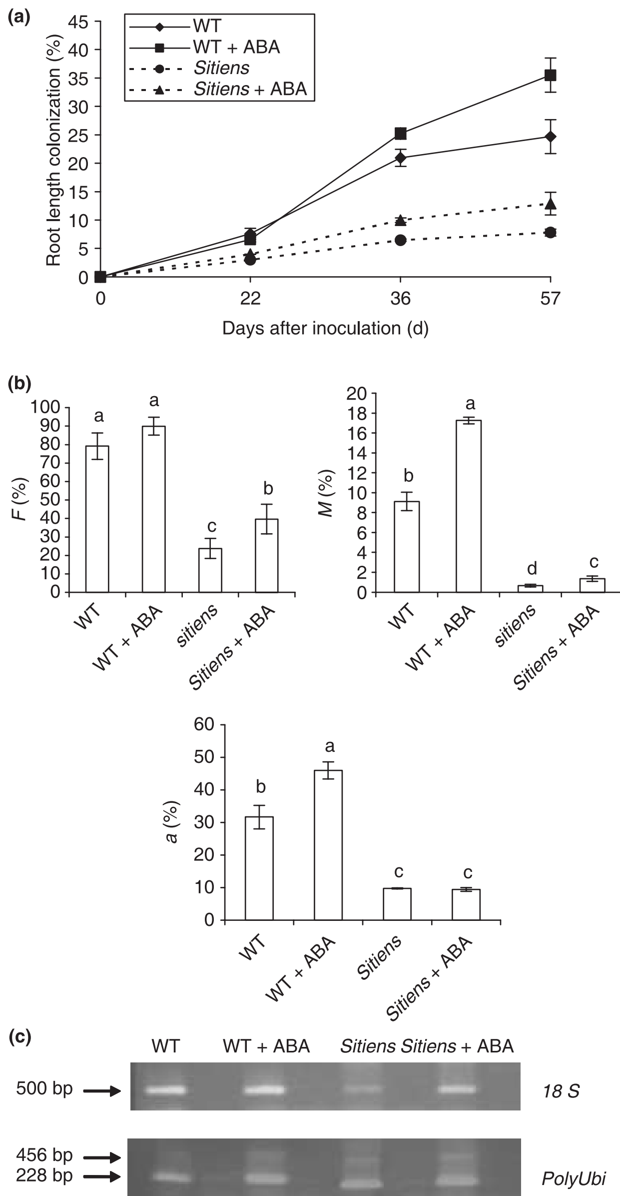

Fig. 2 Effect of abscisic acid (ABA) on the colonization of *sitiens* mutant and Rheinlands Ruhm wild-type (WT) tomato (*Lycopersicon esculentum*) plant roots by *Glomus intraradices*. An ABA solution (50 μM) was applied to the soil twice per week, and untreated control plants were washed with 0.1% ethanol solution. (a) Time–course experiment consisting of ABA application and measurement of the percentage of root colonization using the grid-line intersect method to determine mycorrhiza development. (b) The following mycorrhiza development parameters were determined 57 d after inoculation using MYCOCALC software: the frequency (*F*) and intensity (*M*) of root cortex colonization, and the arbuscular abundance of the mycorrhized root cortex (*a*) (each as a percentage). (c) Analysis by semiquantitative reverse transcriptase–polymerase chain reaction (RT-PCR) of the expression of fungal 18S and plant polyubiquitin genes in WT and *sitiens* tomato roots 57 d after inoculation with *G. intraradices* which were exposed or not exposed to 50 μM ABA. Values are the means ± standard error of five biological replications. Bars with similar letters are not significantly different ($P = 0.05$) according to Duncan's multiple range test.

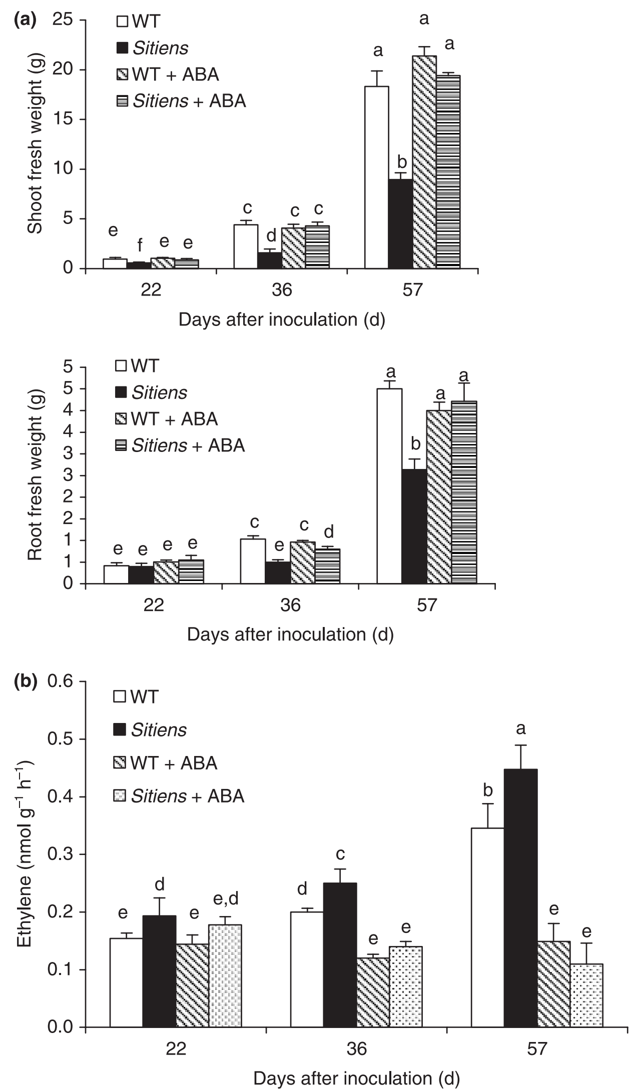

Fig. 3 Influence of exogenous abscisic acid (ABA) on shoot and root fresh weights (a) and ethylene accumulation in roots (b) of *sitiens* mutant and Rheinlands Ruhm wild-type (WT) tomato (*Lycopersicon esculentum*) plants inoculated with *Glomus intraradices.* An ABA solution (50 μM) was applied to the soil twice per week, and untreated control plants were washed with a 0.1% solution of ethanol. Values are the means ± standard error of five biological replications. Bars with similar letters are not significantly different ($P = 0.05$) according to Duncan's multiple range test.

determinations in colonized WT and *sitiens* mutant plants treated or not treated with ABA. As expected, nontreated *sitiens* plants with constitutively reduced ABA contents showed an increase in root ethylene concentration compared with nontreated WT plants (Fig. 3b). As a consequence of ABA application, both WT and *sitiens* plants showed a reduction in root ethylene content (Fig. 3b).

To determine the concentration of exogenous ABA necessary to completely restore the susceptibility to *G. intraradices* in *sitiens* tomato, two different concentrations of ABA were

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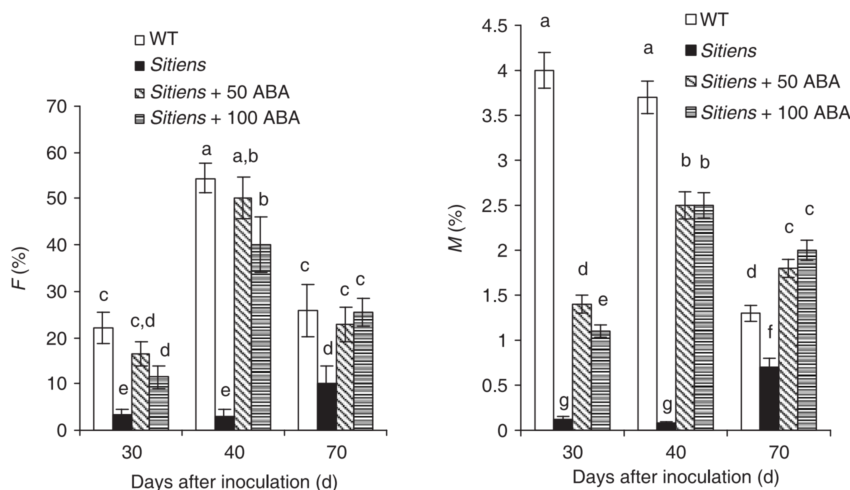

Fig. 4 Time–course experiment consisting of abscisic acid (ABA) application and measurements of alkaline phosphatase in fungal structures. The frequency (*F*; %) and intensity (*M*; %) of colonization by *Glomus intraradices* in untreated Rheinlands Ruhm wild-type (WT) plants and *sitiens* mutant tomato (*Lycopersicon esculentum*) plants treated with two doses of ABA were determined. ABA solutions (50 and 100 μM) were applied to the soil twice per week, and untreated control plants were washed with a 0.1% ethanol solution. Values are the means ± standard error of five biological replications. Bars with similar letters are not significantly different ($P = 0.05$) according to Duncan's multiple range test.

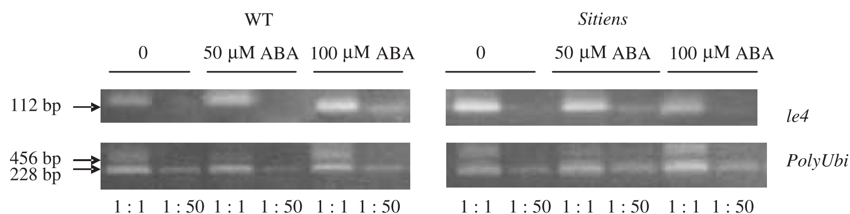

Fig. 5 Analysis by semiquantitative reverse transcriptase–polymerase chain reaction (RT-PCR) of the expression of the *le4* and polyubiquitin genes in wild-type (WT) and *sitiens* tomato (*Lycopersicon esculentum*) roots, exposed to different abscisic acid (ABA) concentrations, after 40 d of growth. Two cDNA dilutions (1 : 1 and 1 : 50) were used to perform the PCR reactions. ABA solutions (50 and 100 μM) were applied to the soil twice per week, and untreated plants were washed with a 0.1% ethanol solution.

applied to *sitiens* plants and the effects on fungal alkaline phosphatase activity in roots were analysed and compared with those in non-ABA-treated WT plants. Figure 4 shows results for mycorrhiza development in a time–course experiment in which ABA concentrations of 50 and 100 μM were applied to the soil in which *sitiens* plants were growing. Analysis of AP activity in the AM fungal tissue of these roots produced interesting results. Both the frequency and the intensity of fungal AP in *sitiens* plants increased considerably when 50 and 100 μM ABA were applied, and in some cases values reached those observed in WT plants. The addition of ABA restored the frequency of AP activity in *sitiens* plants at all times assayed (Fig. 4). The recovery in the intensity of AP activity produced by ABA application reached values of 25, 70 and 150% at 30, 44 and 72 d after inoculation, respectively (Fig. 4). Microscopic observation of alkaline phosphatase in roots of *sitiens* plants treated with 50 μM ABA showed that arbuscules recovered their normal stain intensity (data not shown).

In order to investigate whether WT and *sitiens* plants have different responses to the doses of exogenous ABA applied in our experimental conditions, we performed an experiment in which ABA was applied and expression of the *le4* ABA-inducible gene in roots was analysed. We chose the *le4* ABA-responsive gene because its mRNA abundance increases in the roots of ABA-treated plants, as described by Cohen *et al.* (1991). The analysis of *le4* gene expression by semiquantitative RT-PCR (Fig. 5) showed that mRNA abundance increased in WT plants in response to ABA. In WT plant roots, the amount of *le4* mRNA accumulation was correlated with the increases in ABA concentration. However, in *sitiens* plants the pattern of mRNA accumulation was different and a large amount of accumulation was detected in roots of nontreated control plants. Even treatment with 100 μM ABA produced a decrease in mRNA accumulation (Fig. 5). In nontreated control plants, the basal level of *le4* mRNA was higher in *sitiens* than in WT roots.

ABA and ethylene signalling pathways during AM formation

Genetic analyses have demonstrated that the ABA signalling pathway interacts antagonistically with the ethylene signalling pathway and vice versa (Gazzarrini & McCourt, 2001). In our experimental system *sitiens* plants showed more ethylene in the roots than WT plants, and ABA application caused a reduction in root ethylene content in both WT and *sitiens* plants (Fig. 3b). To determine whether the reduced susceptibility to AM fungal colonization shown by *sitiens* mutants

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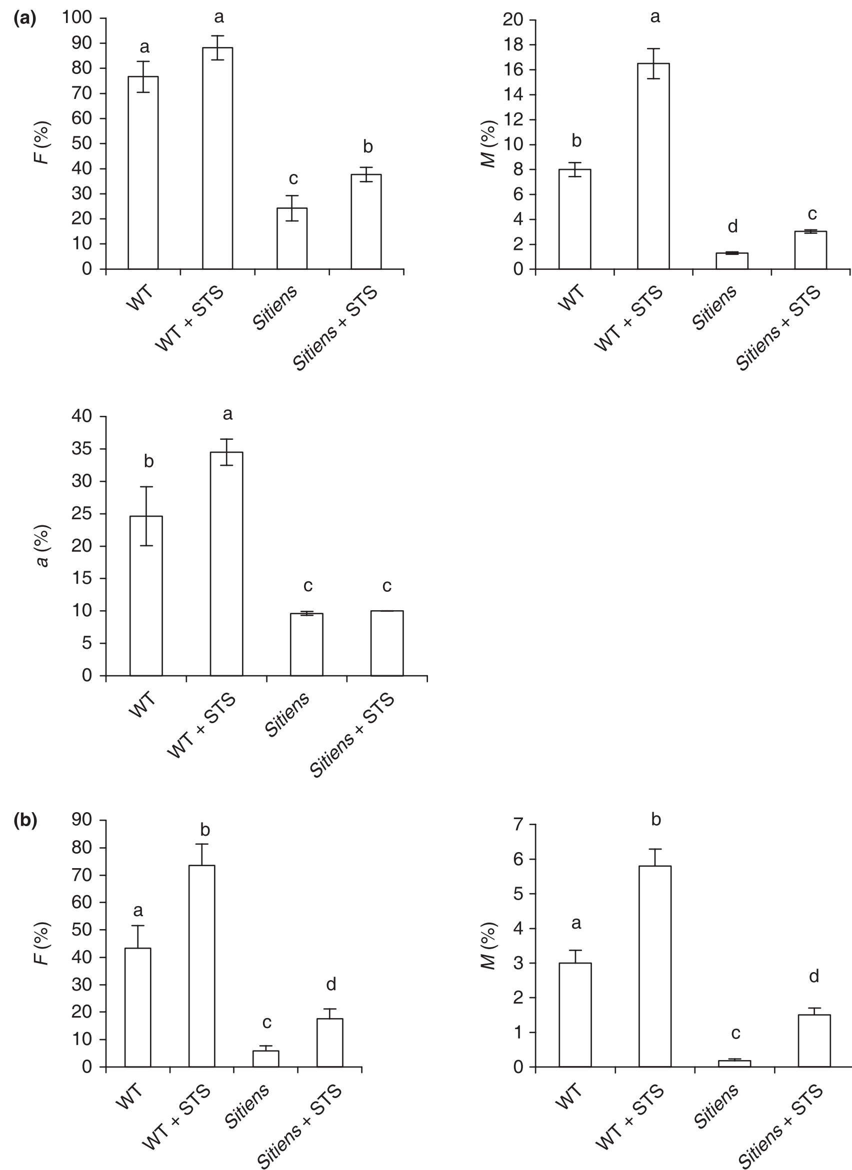

Fig. 6 Effect of silver thiosulfate (STS) application on the colonization of *sitiens* mutant and Rheinlands Ruhm wild-type (WT) tomato (*Lycopersicon esculentum*) plant roots by *Glomus intraradices*. The STS solution (0.3 mM STS) was applied to the soil twice per week, and the mycorrhiza development parameters (a) frequency (*F*; %), intensity (*M*; %) and arbuscular abundance (*a*; %) of root colonization and (b) frequency (*F*; %) and intensity (*M*; %) of fungal alkaline phosphatase were determined 40 d after inoculation. Untreated control plants were washed with water. Values are the means ± standard error of five biological replications. Bars with similar letters are not significantly different ($P = 0.05$) according to Duncan's multiple range test.

could be attributed to ABA suppression or to ethylene enhancement, and to further elucidate the putative antagonistic cross-talk between ABA and ethylene during AM formation, we performed experiments in which ethylene perception was blocked by STS application. STS was applied to soil in which *sitiens* and WT plants were growing and data for root colonization and AP fungal activity were collected. Results presented in Fig. 6(a) clearly illustrate that STS increased the susceptibility of WT plants to *G. intraradices* infection, measured as the frequency and intensity of mycorrhiza development and arbuscule abundance. Similar to the ABA application experiment described in Fig. 2, the data presented here demonstrated that in WT plants a clear increase in the intensity of mycorrhiza development was produced by STS application. The value of *M* was doubled in WT plants treated with STS compared with nontreated WT plants, and the increase in fungal intensity was parallel to an increase (40%) in arbuscule abundance in mycorrhizal roots. No significant increase in the frequency of mycorrhiza development was observed in WT plants after STS treatment (Fig. 6a). The *sitiens* mutants treated with STS also showed an increased frequency and intensity of colonization compared with nontreated *sitiens* control plants, but no detectable increase in arbuscule abundance was observed (Fig. 6a). The data for AP activity determined in nontreated vs STS-treated roots are presented in Fig. 6(b). In mutant and WT mycorrhizal plant roots, the ability of STS to restore the frequency and intensity of AP activity was similar (40–50%), but mutant plants always showed less activity than WT. In both WT and *sitiens* plants, the increase in frequency of AP activity after STS treatment was as relevant as the increase in AP intensity (Fig. 6b).

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Discussion

In this study, we investigated the effect of ABA on the establishment of AM symbiosis. We chose the model plant *L. esculentum*, for which mutants in the biosynthesis of plant ABA have been described and successfully used in comparative studies of the role of endogenous plant ABA in the maintenance of plant shoot growth (Sharp *et al.*, 2000) and susceptibility to plant pathogens (Audenaert *et al.*, 2002). Here, we used ABA biosynthesis *sitiens* tomato mutants to elucidate the role of ABA in interactions of AM fungi with the plant; to our knowledge, ABA-negative mutants have not previously been used in studies of AM symbiosis. These mutants have a residual ABA concentration (no more than 8% of that of the WT plants) and are unable to increase their ABA concentration upon elicitation by wounding, heat or electrical current (Herde *et al.*, 1999).

In the present study, we have shown that *sitiens* plants are less susceptible to *G. intraradices* infection than WT plants (Table 1, Fig. 2). Exogenous ABA application increased the susceptibility of WT tomato to the AM fungus. Therefore, it is clear that endogenous plant ABA is necessary for proper AM establishment, and ABA exogenously applied can modulate the colonization ratio in tomato. In *sitiens* roots, the parameters of frequency and intensity of root colonization and arbuscule abundance were reduced by approximately 70–80% compared with WT plants, and these results could mean that ABA is necessary for both fungal penetration and fungal spread through the root cortex. When ABA was applied to roots of WT plants, the increase in frequency of colonization was only 10% in treated compared with nontreated plants, but increases of around 90% in the intensity of colonization and 40% in arbuscule abundance were obtained. These data support the idea that ABA acts positively mainly in fungal spread in the root. Thus it seems that ABA enhances the growth of AM fungi, although the possibility that low concentrations of ABA in mutant plants lead to a degradation of symbiotic structures cannot be discounted. Furthermore, results of microscopic observation of stained fungal structures together with the measurement of AP activity of arbuscular fungus in the root demonstrate that endogenous plant ABA is necessary for complete arbuscule development and full functionality in roots of tomato (Fig. 1). In *sitiens* plants, the majority of the arbuscules were arbuscules in formation (or degradation) with no fine branches partially occupying the plant cell, suggesting that incomplete arbuscule development and/or enhanced degradation of symbiotic structures could occur in these plants.

Some studies have been conducted on the alteration of ABA concentrations in AM plants. Enhanced concentrations of ABA have been shown in mycorrhizal plants (Danneberg *et al.*, 1992; Bothe *et al.*, 1994; Meixner *et al.*, 2005), and higher amounts of ABA in fungal hyphae than in roots have been detected (Esch *et al.*, 1994). These results indicate that the increase in ABA detected in mycorrhizal roots may be, at least in part, a result of the synthesis of this hormone by the AM fungus. Nevertheless, our results in tomato plants show that, apart from the possible contribution of ABA from AM fungi, an active mechanism of plant biosynthesis of ABA is necessary to assure proper mycorrhiza development.

The effect of increasing susceptibility to AM fungi by ABA application in tomato was similar to the reactions described for various plant species to fungal pathogens and Oomycetes, such as *Phytophthora infestans* in potato (*Solanum tuberosum*) (Henfling *et al.*, 1980), *Phytophthora megasperma* in soybean (Ward *et al.*, 1989), *Peronospora tabacina* in tobacco (*Nicotiana tabacum*) (Salt *et al.*, 1986) and *Botrytis cinerea* in tomato (Kettner & Dörffling, 1995; Audenaert *et al.*, 2002). This indicates that the ABA signalling pathway could mediate both pathogenic and mutualistic interactions between plants and fungi. Furthermore, it has been reported that ABA exogenously injected into leaves of bean (*Phaseolus vulgaris*) plants increased the incidence of haustoria produced by rust fungus (Li & Heath, 1990). Note here that both arbuscules of AM fungi and rust fungi haustoria are primary sites of nutrient transfer between the plant and biotrophic fungi, and the two fungal structures show similar morphological and functional features (Harrison, 1999; Hahn & Mengen, 2001).

ABA has been considered a negative regulator of disease resistance, and this effect appears to be a result of the interference of ABA with biotic stress signalling regulated by salicylic acid, jasmonic acid and ethylene (Mauch-Mani & Mauch, 2005). These signal molecules are plant regulators of AM development (see review by Hause *et al.*, 2006), and it is possible that the role of ABA in regulating mycorrhiza development may depend on its interaction with the salicylic acid/jasmonate/ethylene pathways.

It is well documented that ABA and ethylene mutually antagonize to modulate development (Beaudoin *et al.*, 2000; Ghassemian *et al.*, 2000), shoot growth (Sharp *et al.*, 2000) and disease resistance in plants (Anderson *et al.*, 2004). Results similar to those presented here were reported in a previous study in which ethylene production was found to be enhanced in ABA-deficient tomato mutants (Hussain *et al.*, 1999). In our experiments, the application of STS demonstrated that ethylene blockage enhanced mycorrhiza development, demonstrating that ethylene perception is essential for AM formation. The decrease in ethylene sensitivity caused by blocking ethylene perception, without altering ethylene concentration, was sufficient to increase AM colonization. This result supports the hypothesis that ethylene regulates AM formation (Guinel & Geil, 2002) but it also provides new, strong evidence for putative AM ethylene regulation at the level of ethylene receptors.

Because ABA and ethylene have antagonistic actions in regulating AM colonization, and because the ABA signalling pathway interacts antagonistically with the ethylene signalling pathway, it is logical to speculate that the role of ABA in regulating mycorrhiza development depends on its action in

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regulating ethylene concentration. In the present work, we have shown that, in WT tomato plants, the effect on mycorrhiza development of ABA application was similar to that of STS application, supporting a dependent and antagonistic relationship between ABA and ethylene during arbuscular mycorrhizal development. In WT tomato plants the independent application of both ABA and STS increased the intensity and arbuscular abundance in mycorrhized roots, suggesting a role for ethylene in the regulation of fungal spreading in roots. The increase in AM colonization and AP activity in STS-treated *sitiens* plants compared with nontreated *sitiens* plants also supports the hypothesis that, at least in part, the impairment of mycorrhiza development in ABA deficiency is attributable to ethylene.

Nevertheless, the fact that ABA application restored AP activity in *sitiens* roots but not fungal spread, as measured by trypan blue staining, together with the higher recovery of AP activity in the *sitiens* mutant produced by ABA application compared with STS application, suggests that ABA could have a complementary role in the functionality of the arbuscules apart from its function as an inhibitor of ethylene production. It is clear that, in the zones of the *sitiens* mycorrhizal roots colonized by the fungus, the exogenously applied ABA restored the capacity of arbuscule formation and functioning, as measured by AP activity. In this context, further cytological and histological studies using microscopic observation of stained fungal structures in *sitiens* mutant plants after STS or ABA application, together with the measurement of transcript abundances of fungal marker genes, are necessary to elucidate which fungal colonization steps are dependent on both the ethylene and ABA pathways.

In our experiments, the application of ABA to *sitiens* plants normalized growth, and allowed an almost complete recovery in the frequency and intensity of AP activity. Nevertheless, the exogenous ABA was incapable of restoring the frequency and intensity of mycorrhiza development. Ethylene production was decreased in the WT and the *sitiens* mutants by exogenous ABA application (Fig. 3b), although the dose of ABA applied was not able to fully restore the mycorrhizal susceptibility of *sitiens* plants (Fig. 2b), suggesting a plant phenotype-dependent response to this ABA application. The results showing the effect of ABA on *le4* ABA-responsive gene expression (Fig. 5) support this hypothesis, because a clear plant phenotype-dependent response to ABA was evident. It is also possible that the sensitivity to ethylene was also increased by the inherent ABA deficiency in the mutant, and the observed differential response to exogenous ABA in WT and *sitiens* plants could be attributable to their different sensitivities to ethylene. It is also possible that the uptake of exogenous ABA by the mutant was not sufficient to restore all the physiological deficiencies caused by the mutation. Furthermore, *sitiens* mutants are known to be impaired in the oxidation of ABA aldehyde to ABA, and accumulate ABA alcohol instead of ABA in response to water stress (Linforth *et al.*, 1987). It is possible that the accumulation of these compounds negatively affects mycorrhiza development.

In conclusion, the present study has clearly shown a hormonal regulation of AM formation. To our knowledge, this is the first attempt to elucidate the role of ABA during AM formation using genetic tools, such as plant mutants defective in the production of hormonal signals. We have shown that ABA participates in the susceptibility of tomato to infection by AM fungi, and that it seems to play an important role in the development of the complete arbuscule and its functionality. One of the mechanisms by which ABA determines susceptibility to fungal infection is by negative modulation of the ethylene pathway, although an additional regulating role of ABA cannot be excluded and requires further research.

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