Microb Ecol (2008) 56:704–719
DOI 10.1007/s00248-008-9390-y

ORIGINAL ARTICLE

Plant Responses to Drought Stress and Exogenous ABA Application are Modulated Differently by Mycorrhization in Tomato and an ABA-deficient Mutant (*Sitiens*)

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Received: 11 October 2007 / Accepted: 12 March 2008 / Published online: 29 April 2008

Abstract The aims of the present study are to find out whether the effects of arbuscular mycorrhizal (AM) symbiosis on plant resistance to water deficit are mediated by the endogenous abscisic acid (ABA) content of the host plant and whether the exogenous ABA application modifies such effects. The ABA-deficient tomato mutant *sitiens* and its near-isogenic wild-type parental line were used. Plant development, physiology, and expression of plant genes expected to be modulated by AM symbiosis, drought, and ABA were studied. Results showed that only wild-type tomato plants responded positively to mycorrhizal inoculation, while AM symbiosis was not observed to have any effect on plant development in *sitiens* plants grown under well-watered conditions. The application of ABA to *sitiens* plants enhanced plant growth both under well-watered and drought stress conditions. In respect to *sitiens* plants subjected to drought stress, the addition of ABA had a cumulative effect in relation to that of inoculation with *G. intraradices*. Most of the genes analyzed in this study showed different regulation patterns in wild-type and *sitiens* plants, suggesting that their gene expression is modulated by the plant ABA phenotype. In the same way, the colonization of roots with the AM fungus *G. intraradices* differently regulated the expression of these genes in wild-type and in *sitiens* plants, which could explain the distinctive effect of the symbiosis on each plant ABA phenotype. This also suggests that the effects of the AM symbiosis on plant responses and resistance to water deficit are mediated by the plant ABA phenotype.

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Introduction

Plants are constantly faced with environmental constraints of both biotic and abiotic origin. Abiotic stresses such as drought, salinity, and extreme temperatures are the most common environmental stress factors experienced by terrestrial plants [61]. All these stresses share a common osmotic component since they cause dehydration in plant tissues. There is a broad consensus that climate change will continue to occur and that stresses caused by climatic extremes will continue and probably increase and thus cause major difficulties for plant and crop growth in many parts of the world. These difficulties will be particularly pronounced in currently semiarid agricultural zones [2, 18].

Plant adaptation to environmental stress is regulated through multiple physiological mechanisms at the cellular, tissue, and whole-plant levels, which are controlled by changes in gene expression [29, 72]. Traditional explanations of drought-induced regulation of plant responses have emphasized the importance of the decline in shoot water status, which commonly accompanies severe soil dehydration. It is now accepted, however, that many of the

plant's responses to soil dehydration can occur in the absence of changes in shoot water status, via chemical signals such as abscisic acid (ABA) [70]. In this respect, it is well known that the endogenous levels of ABA in vegetative plant tissues rise in response to stresses causing a plant water deficit [12, 73]. Moreover, a clear relationship between plant ABA content and plant tolerance to water deficit has also been demonstrated [36]. The protective effect of ABA is explained by the fact that ABA primarily promotes stomatal closure to minimize transpirational water loss and then mitigates stress damage through the activation of many stress-responsive genes that collectively increase plant stress tolerance [12, 73]. *Cis*- and *trans*-acting factors have been identified that are involved in the transcriptional regulation of genes by ABA through interactions with the so-called ABA response element [53]. Two other deoxyribonucleic acid elements, MYC-like and MYB-like elements, are also involved in the regulation of gene expression by ABA in response to water deficit [12]. ABA is also involved in the regulation of gene expression in post-transcriptional events [16].

Besides the natural responses of plants to water deficit to increase their tolerance, most terrestrial plants can establish a symbiotic association with the so-called arbuscular mycorrhizal (AM) fungi. Several eco-physiological studies investigating the role of AM symbiosis in the protection against drought stress have demonstrated that the symbiosis often results in altered rates of water movement into, through, and out of the host plants, which affect tissue hydration and improve plant physiology (for reviews, see [6, 55]). It is currently accepted that AM symbiosis protects host plants against the detrimental effects of water deficit and that the contribution of AM symbiosis to plant drought tolerance is due to a combination of physical, nutritional, and cellular effects [55]. The cellular effects include changes in some of the genes involved in the response of plants to osmotic stresses such as genes encoding aquaporins or the gene encoding *9-cis*-epoxycarotenoid dioxygenase (NCED), the enzyme catalyzing the rate-limiting step in the biosynthesis of ABA [31, 48, 57].

So far, no studies have been conducted aimed specifically at explaining the reciprocal influence of AM symbiosis and ABA on the enhanced tolerance of AM plants in relation to water deficit. However, studies carried out on AM symbiosis or on plant water relations have shown that mycorrhization can alter ABA levels in the host plant, although contradictory results were also obtained. Studies of plants growing under normal conditions showed increased ABA levels in AM as compared to non-AM maize [17] and soybeans [43, 44]. On the other hand, Allen et al. [1] reported decreased ABA levels in leaves from AM *Bouteloua gracilis* plants and an unchanged ABA content in their roots. ABA has also been detected in fungal hyphae at higher levels than in roots [20], and it was suggested that ABA in AM fungi may control the flow of water and mineral salts from the soil to the hyphae or from the fungus to the root cells. On the other hand, it has been shown that when plants are subjected to drought stress, the levels of ABA are lower in AM than in non-AM plants [21, 24, 39]. Similarly, ectomycorrhizal larch plants subjected to osmotic stress due to the addition of polyethylene glycol had lower ABA levels than their nonmycorrhizal counterparts [52]. Thus, the aims of the present work are to show whether the effects of the AM symbiosis on plant tolerance in relation to water deficit are mediated by the endogenous ABA content of the host plant and whether the exogenous application of ABA modifies such effects. The initial hypotheses are: (1) endogenous plant ABA levels will affect the response of AM symbiosis to water deficit, (2) the application of exogenous ABA will alter the responses of the plant to water deficit, and (3) this alteration will depend on the plant genotype.

In our study, tomato was chosen as the host plant. The tomato has a number of well-known ABA pathway mutants and represents an appropriate model for studying the role of ABA in plant responses to several stressful conditions [13]. The ABA-deficient tomato mutant *sitiens* and its near isogenic wild-type parental line were used in the study. Previous research has shown that the mutants have residual ABA levels (no more than 8% of the wild-type plants) and are unable to increase their ABA levels upon elicitation by several stresses [26].

Materials and Methods

Experimental Design and Statistical Analysis

The experiment consisted of a factorial design with two tomato plant cultivars: (1) the ABA-defective mutant *sitiens* and (2) the near-isogenic parental wild-type line *Rheinlands Ruhm*. Plants were either inoculated with the AM fungus *Glomus intraradices* (Schenck and Smith) (Gi) or remained uninoculated. Half of the plants were supplied weekly with exogenous ABA, while the other half remained ABA-free. Ten replicates represent each treatment, totaling 80 pots (one plant per pot), so that half of the plants were cultivated under well-watered conditions throughout the entire experiment and the other half were subjected to drought stress for 12 days before harvest.

For each plant cultivar, data were subjected to analysis of variance with microbial treatment, ABA treatment, and water regime as sources of variation, and followed by Duncan's multiple-range test [19]. Percentage values were arc-sin transformed before statistical analysis.

Substratum and Biological Materials

Loamy soil was collected from the Zaidin Experimental Station (Granada, Spain), sieved (2 mm), mixed with quartz sand (<1 mm; 1:1, soil/sand, *v*/*v*) to get the experimental substratum, and sterilized by steaming (100°C for 1 h on 3 consecutive days). This substratum had a pH of 8.1 (water); 1.81% organic matter, nutrient concentrations (mg kg^{-1}): N, 2.5; P, 6.2 ($NaHCO_3$-extractable P); and K, 132.0, and a texture of 35.8% sand, 43.6% silt, and 20.5% clay.

The genotypes used in this study were *Solanum lycopersicum* Mill. cv. Rheinlands Ruhm (accession LA0535) and its near-isogenic ABA-deficient mutant, *sitiens* (accession LA0575). The mutation of *sitiens* affects the final step of ABA biosynthesis [66]. Seeds were obtained from the Tomato Genetic Resources Centre, University of California, Davis. Seeds were germinated on wet sand, and seedlings were transplanted to pots containing 900 g of the same soil/sand mixture as described above.

Mycorrhizal inoculum was bulked in an open-pot culture of *Zea mays* L. and consisted of soil, spores, mycelia, and colonized root fragments. The AM species was *G. intraradices* (Schenck and Smith) isolate BEG 121, obtained from the collection of the Estación Experimental del Zaidín. Ten grams of inoculum with about 80 infective propagules per gram (according to the most probable number test) were added to appropriate pots at sowing time, just below seedlings.

Uninoculated control plants received the same amount of autoclaved mycorrhizal inoculum together with a 2-ml aliquot of a filtrate (<20 μm) of the AM inoculum to provide a general microbial population free of AM propagules.

Growth Conditions

The experiment was carried out under greenhouse conditions with temperatures ranging from 19°C to 25°C, 16/8-h light/dark period, and a relative humidity of 70–80%. A photosynthetic photon flux density of 800 μE m^{-2} s^{-1} was measured with a light meter (LICOR, Lincoln, NE, USA, model LI-188B). Water was supplied daily to maintain the soil at field capacity during the entire period of plant growth.

One week after planting and weekly thereafter, half of the plants from both cultivars received 10 ml per pot of an aqueous solution 100 μM of ABA, applied directly on the pot substratum.

Soil moisture was measured with a ML2 ThetaProbe (AT Delta-T Devices, Cambridge, UK), which measures volumetric soil moisture content by responding to changes in the apparent dielectric constant of moist soil [50]. Water was supplied daily to maintain constant soil water content close to field capacity (20% volumetric soil moisture, as determined experimentally using a pressure plate apparatus and applying a pressure of one third atmosphere for 48 h and then determining the volumetric soil moisture) during the first 6 weeks of plant growth. At this time, half of the plants were allowed to dry until soil water content reached 70% field capacity (2 days needed), which corresponded to 12% volumetric soil moisture (also determined experimentally in a previous assay). The other half of the plants was maintained at field capacity. Plants were maintained under such conditions for an additional 10 days. To control the level of drought stress, the soil water content was daily measured with the ThetaProbe ML2 (at the end of the afternoon), and the amount of water lost was added to each pot to return soil water content at the desired 12% of volumetric soil moisture (70% of field capacity). However, during the 24-h period comprised between each rewatering, the soil water content was progressively decreasing until a minimum value of 60% of field capacity was obtained.

Variables Measured

Biomass Production

At harvest (52 days after planting), the shoot and root systems were separated, and the shoot dry weight (DW) was measured after drying in a forced hot-air oven at 70°C for 2 days.

Symbiotic Development

The percentage of mycorrhizal root infection in tomato plants was estimated by visual observation of fungal colonization after clearing washed roots in 10% KOH and staining with 0.05% trypan blue in lactic acid (*v*/*v*), according to Phillips and Hayman [47]. The extent of mycorrhizal colonization was calculated according to the gridline intersect method [23].

Relative Water Content and Leaf Water Potential

The relative water content (RWC) in plant leaves was determined at the harvest time as previously described by Ruiz-Lozano and Azcón [56].

The leaf water potential (Ψ) was determined with a C-52 thermocouple psychrometer chamber and a HR-33T dew point microvoltmeter (Wescor, Logan, UT, USA). Leaf disks (0.7 cm diameter) were cut, placed inside the psychrometer chamber, and allowed to reach temperature and water vapor equilibrium for 15 min before measurements were made by the dew point method.

Leaf Transpiration Rate

The leaf transpiration rate was determined by a gravimetric method [4]. Surfaces of the pots were covered with aluminum foil. The pot–plant system was weighed and referred as W_0. The pot–plant system was weighed again after 2 h and referred as W_f. Leaf transpiration rate was calculated as: $(W_0-W_f)/t\times A$, where t is the time in seconds and A is the leaf area in m^2. Leaf area was calculated as follows: Leaves of a whole plant were detached and scanned (hp scanjet 5550c, Hewlett Packard, Palo Alto, CA, USA). The corresponding images were analyzed with Adobe Photoshop CS (Adobe Systems, San Jose, CA, USA).

Proline Content

Free proline was extracted from 1 g of fresh leaves [10]. Proline was estimated by spectrophotometric analysis at 515 nm of the ninhydrin reaction, according to Bates et al. [8].

ABA Content

ABA was measured on 250 mg of frozen root and leaf tissues that were immersed in 2 ml distilled water and incubated 24 h at 4°C in the dark [5]. Quantitative analysis was performed on crude aqueous extracts using an enzyme-linked immunosorbent assay based on monoclonal antibody DBPA-1 [67], which proved to be highly specific for (S)-*cis*,*trans*-ABA. The procedure of Walker-Simmons [68] was followed. Briefly, microtitration plates were coated overnight at 4°C with bovine serum albumin–ABA [11] and then rinsed three times with 50 mM Tris–HCl (pH=7.8), 1 mM $MgCl_2$, 150 mM NaCl, 0.1% (*p*/*v*) BSA, and 0.05% (*v*/*v*) Tween 20. After that, 200 μl of the overnight-incubated samples and standards with the antibody were added, and plates were incubated at room temperature during 150 min. Then, plates were rinsed again as described above and incubated 2 h at room temperature with Anti-rat IgG (1:1,000). Finally, plates were rinsed again, and *p*-nitrophenyl phosphate was added. Plates were incubated until the absorbance at 405 nm of the non-ABA sample was 1. Absorbance was inversely proportional to the amount of ABA. Three independent samples (n=3) were assayed for each treatment. All sample results were the average of three serial dilutions within the linear range of the ABA standard curve.

Northern Blot Analysis

Total ribonucleic acid (RNA) was isolated from tomato roots and leaves by phenol/chloroform extraction followed by standard precipitation with lithium chloride. Northern blot with *Slnced* [13] (accession Z97215), *Sldhn* (accession CK615624), and the aquaporin genes *SlPIP1-4*, *SlPIP1-5*, *SlPIP2-1*, and *SlPIP2-3* [63] (accessions AF218774, X73848, BI929127, and AW224678, respectively) were carried out as previously described [48]. Hybridizations were conducted overnight at 65°C under standard conditions [58]. After washing twice for 5 min at room temperature in 2× salt–sodium citrate (SSC) and 0.1% sodium dodecyl sulfate (SDS) and twice for 15 min at 65°C with 0.5× SSC and 0.1% SDS, membranes were exposed to the phosphorimager. The amount of ribosomal RNA (rRNA) in the membranes was quantified using Quantity One software (BioRad, Hemel Hempstead, UK) after ethidium bromide staining. Next, the signals on the phosphorimager were analyzed and quantified using the same software. Transcript accumulation levels for each gene probe (in arbitrary units) were divided by the corresponding amount of rRNA in the membrane (also in arbitrary units). Each quantification of signals on screens and of rRNA in the membranes was repeated three times, and the average value for each was used for normalization. The normalized gene expression values were used to establish the percentage of relative up- or downregulation of gene expression between treatments. Northern blot analyses were repeated two times with a different set of plants, and the results obtained were similar. We show representative results for each Northern blot.

Results

Mycorrhizal Colonization and Shoot Dry Weight

Uninoculated control plants from the two tomato genotypes did not show mycorrhizal colonization. The wild-type plants inoculated with *G. intraradices* showed more than 40% root mycorrhizal colonization, and no drought effect was observed in this variable. The application of exogenous ABA significantly reduced the percentage of mycorrhization to 23% under well-watered conditions, but when ABA was added in combination with drought stress, the percentage of mycorrhization remained at more than 40%. In *sitiens* plants, the level of root colonization was lower, ranging from 29% in plants cultivated under well-watered conditions and in the absence of ABA to about 14% for plants subjected to drought. The application of exogenous ABA did not significantly change the percentage of root colonization in *sitiens* plants (data not shown).

In relation to plant development, wild-type plants benefited from inoculation with *G. intraradices* and increased shoot biomass by 22% when cultivated under well-watered conditions and by 27% when subjected to

drought stress, as compared to non-AM plants (Fig. 1). The exogenous addition of ABA to wild-type plants had no additional effect on plant biomass production either under well-watered or drought stress conditions. By contrast, in relation to *sitiens* plants, there was no mycorrhizal colonization effect when they were cultivated under well-watered conditions. However, the application of ABA enhanced plant biomass production by more than 84% in both non-AM plants and in plants colonized by *G. intraradices* (Fig. 1). When *sitiens* plants were subjected to drought stress, both the inoculation with *G. intraradices* and the application of ABA enhanced plant growth by 79% and 106%, respectively, as compared to non-AM plants and plants not supplied with ABA. Under drought conditions, there was a cumulative effect of mycorrhization and ABA application since mycorrhizal plants treated with ABA showed a 165% increase in biomass production as compared to uninoculated control plants in the absence of ABA. In any case, *sitiens* plants grew less than the corresponding wild-type plants under all the conditions tested in this study.

Leaf Water Potential and Relative Water Content

Leaf water potential in wild-type and *sitiens* tomato plants was not affected by the colonization with *G. intraradices* or the application of ABA (data not shown). In both plant cultivars, this variable only declined as a consequence of drought. Shoot water potential showed similar values in control and mycorrhizal plants regardless of ABA addition (data not shown).

The RWC of wild-type plants was also unaffected by mycorrhization or by the addition of ABA both under well-watered and drought stress conditions (Fig. 2). By contrast, in wild-type plants, drought decreased RWC of AM and non-AM plants in a similar way. In wild-type plants colonized by *G. intraradices*, the application of ABA raised RWC to values similar to those for well-watered plants. *Sitiens* plants cultivated under well-watered conditions were also unaffected in terms of this variable by mycorrhization or by the addition of ABA. On the other hand, when *sitiens* plants were subjected to drought stress, colonization by *G. intraradices* decreased RWC as compared to non-AM plants. The application of ABA to *sitiens* plants subjected to drought stress raised RWC in both AM and non-AM plants to similar levels.

Transpiration Rate

Wild-type plants cultivated under well-watered conditions showed a similar transpiration rate regardless of ABA or AM treatments (Fig. 3). Drought stress decreased the transpiration of wild-type plants to similar extents in AM and non-AM plants regardless of the application of ABA.

Sitiens plants showed a higher transpiration rate than wild-type plants, mainly the non-AM plants. It is surprising to note that when *sitiens* plants were subjected to drought, they showed higher transpiration rates than under well-watered conditions. This effect was remarkable in non-AM plants. Nevertheless, the addition of ABA to *sitiens* plants decreased the transpiration rate in AM and non-AM plants regardless of the water regime (Fig. 3).

Proline Content

The accumulation of proline in wild-type plants cultivated under well-watered conditions was not affected by mycorrhization (Fig. 4). The exogenous application of

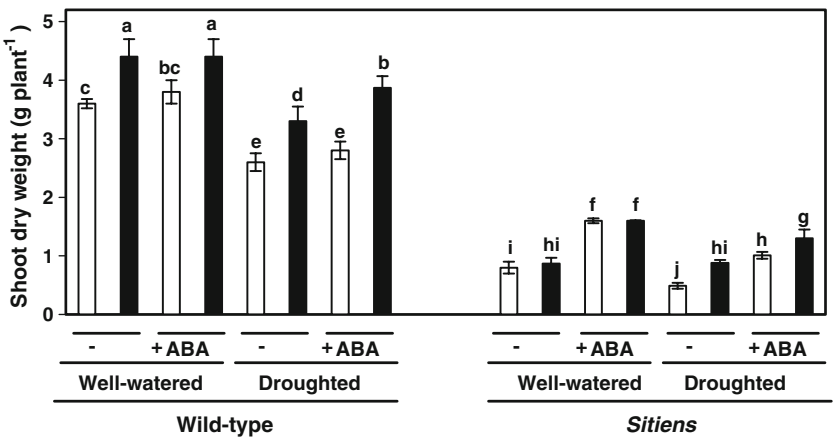

Figure 1 Effect of mycorrhizal fungi colonization, water regime, and exogenous application of ABA on shoot dry weight (g plant^{-1}) in tomato plants (wild type and the *sitiens* mutant) after 52 days of culture. *White bars* represent noninoculated controls plants, and *black bars* represent plants inoculated with *G. intraradices*. Plants were cultivated under well-watered conditions or subjected to drought stress with or without addition of exogenous ABA. *Bars* represent means plus standard error (n=5). Means followed by the same letter are not significantly different (P<0.05) as determined by Duncan's multiple-range test

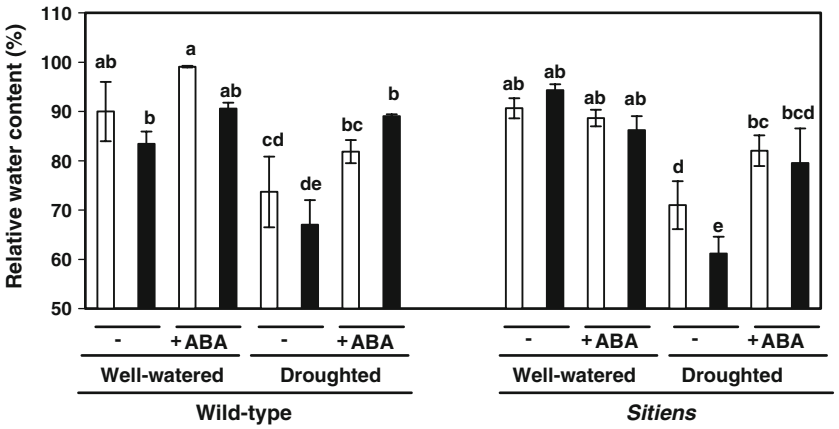

Figure 2 Effect of mycorrhizal fungi colonization, water regime, and exogenous application of ABA on RWC (%) in tomato plants (wild type and the *sitiens* mutant) after 52 days of culture. See legend for Fig. 1 (n=5)

ABA only increased the accumulation of proline in AM plants. When wild-type plants were subjected to drought stress, they accumulated much more proline, with mainly non-AM plants increasing proline content by 142% as compared to those under well-watered conditions. The increase in proline content in AM plants was 112%. Under drought stress, the addition of ABA did not further increase the accumulation of proline.

Sitiens plants accumulated more proline than wild-type plants, even under well-watered conditions, but no differences between AM and non-AM plants were observed. The application of ABA to these well-watered plants decreased the accumulation of proline by more than 50% in AM and non-AM plants. Drought stress only enhanced the accumulation of proline in non-AM plants supplied with ABA as compared to the well-watered plants (40% increase). By contrast, for plants colonized by *G. intrarradices*, the combination of drought plus ABA resulted in a lower proline content as compared to the same treatment in the absence of drought and ABA (52% reduction).

ABA Content

The ABA content in root and leaf tissues at the end of the experiment was not affected either by mycorrhizal inoculation or by the exogenous addition of ABA (data not shown). Only differences between both plant genotypes were observed. The values in roots ranged from 25 ng ABA g^{-1} DW in *sitiens* plants to 340 ng ABA g^{-1} DW in wild-type plants. In leaves, ABA ranged from 79 ng ABA g^{-1} DW in *sitiens* plants to 843 ng ABA g^{-1} DW in wild-type plants.

Gene Expression

The expression of six plant genes was analyzed by Northern blot in both root and shoot tissues of the two plant cultivars under the various growing conditions tested in this work. This means the wide range of experimental treatments generated many different gene expressions. Therefore, only increases of more than 50% or reductions of more than 50% in gene expression will be highlighted.

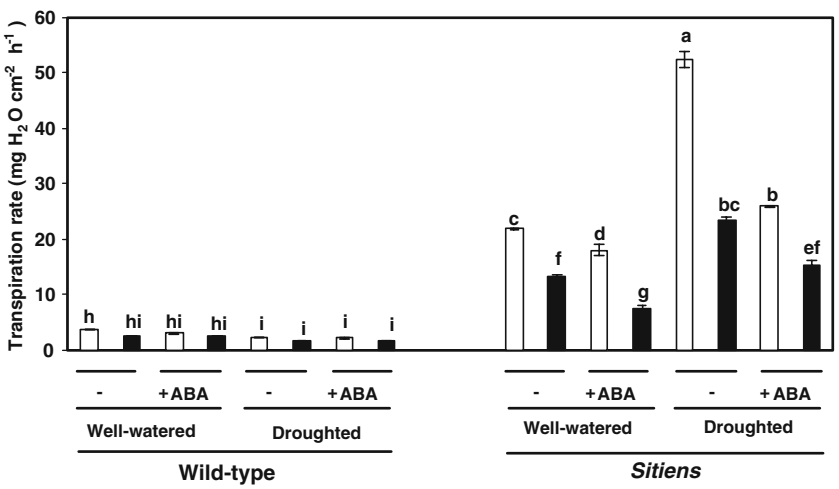

Figure 3 Effect of mycorrhizal fungi colonization, water regime, and exogenous application of ABA on transpiration rate (mg H_2O cm^{-2} h^{-1}) in tomato plants (wild type and the *sitiens* mutant) after 52 days of culture. See legend for Fig. 1 (n=4)

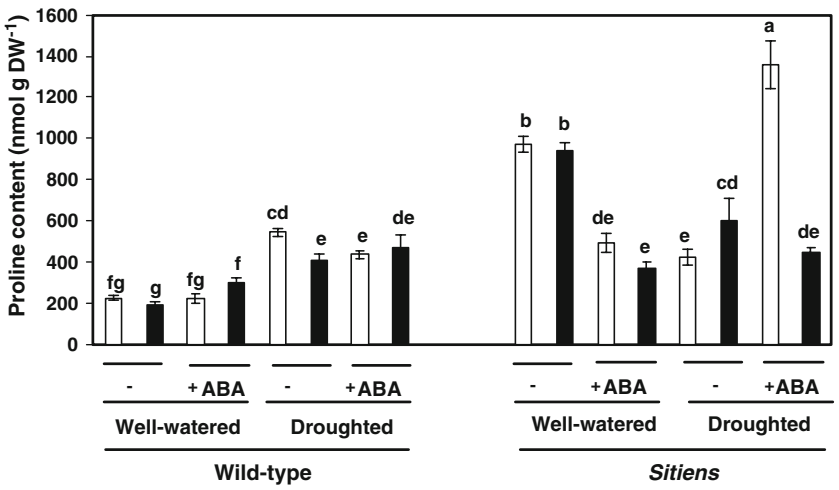

Figure 4 Effect of mycorrhizal fungi colonization, water regime, and exogenous application of ABA on proline content (nmol proline g DW^{-1}) in tomato plants (wild type and the *sitiens* mutant) after 52 days of culture. See legend for Fig. 1 (n=5)

Slnced Gene in Wild-type Plants

In root tissues, inoculation with *G. intraradices* induced the expression of *Slnced* gene by 77% (line 2 vs line 1) when plants were cultivated under well-watered conditions (Fig. 5), but this did not occur when plants were subjected to drought stress (line 6 vs line 5). The application of exogenous ABA enhanced the expression of this gene by 192% in non-AM plants grown under well-watered conditions (line 3 vs line 1). Drought stress increased the expression of the *Slnced* gene in non-AM plant roots by 150% (line 5 vs line 1) and in AM plant roots by 95% (line 6 vs line 2).

In shoots, inoculation with *G. intraradices* induced *Slnced* gene expression by 149% (line 2 vs line 1) under well-watered conditions (Fig. 5) but had no effect under drought stress conditions (line 6 vs line 5). The application of exogenous ABA did not significantly affect *Slnced* gene expression in non-AM plants. By contrast, in plants colonized by *G. intraradices* application of ABA decreased gene expression by 66% under well-watered conditions (line 4 vs line 2) but increased gene expression by 53% when plants were subjected to drought stress (line 8 vs line 6). Drought stress decreased *Slnced* gene expression by 50% in AM plant shoots (line 6 vs line 2).

Slnced Gene in *Sitiens* Plants

In roots, inoculation with *G. intraradices* decreased *Slnced* gene expression by 50% when plants were cultivated under well-watered conditions (line 10 vs line 9) but increased gene expression by 184% when plants were subjected to drought stress (line14 vs line 13; Fig. 5). The application of exogenous ABA enhanced gene expression by 125% in AM plants cultivated under well-watered conditions (line 12 vs line 10). Drought stress decreased *Slnced* gene expression by 66% in non-AM plants (line 13 vs line 9),

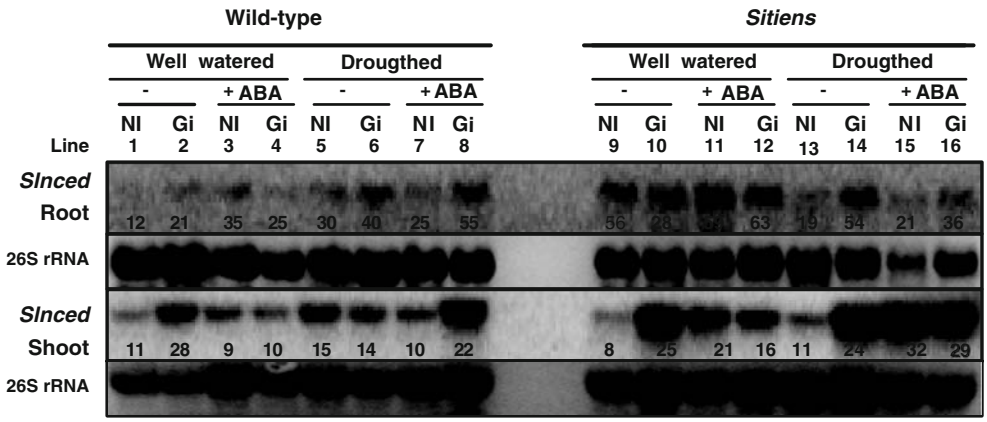

Figure 5 Northern blot of total RNA (15 μg) from tomato roots and shoots (wild type and *sitiens*) using the *Slnced* gene probe (accession Z97215). Treatments are designed as *NI*, noninoculated controls, or *Gi*, plants inoculated with *G. intraradices*. Plants were cultivated under well-watered conditions or subjected to drought stress with or without addition of exogenous ABA. The *lower panels* show the amount of 26S rRNA loaded for each treatment after ethidium bromide staining. Numbers close to each Northern represent the relative gene expression after normalization to rRNA

while in AM plants, a 93% increase was observed (line 14 vs line 10).

In shoots, the inoculation with *G. intraradices* induced *Slnced* gene expression both under well-watered conditions (by 212%, line 10 vs line 9) and under drought stress (by 118%, line 14 vs line 13). The application of ABA enhanced gene expression only in non-AM plants. The increase was 162% under well-watered conditions (line 11 vs line 9) and 191% under drought stress conditions (line 15 vs line 13). No significant induction of this gene by drought stress was observed in *sitiens* plants regardless of mycorrhizal presence (line 13 vs line 9 or line 14 vs line 10).

Sldhn Gene in Wild-type Plants

The expression of the *Sldhn* gene was induced in plant roots subjected to drought stress (lines 5 to 8) as opposed to those grown under well-watered conditions (lines 1 to 4; Fig. 6). The application of exogenous ABA, however, slightly induced the expression of the gene in well-watered plant roots (lines 3 and 4). Under drought stress, the application of ABA also induced *Sldhn* gene expression but only very slightly (20% in non-AM plants, line 7 vs line 5, and 30% in AM plants, line 8 vs line 6). The inoculation with *G. intraradices* did not induce the expression of this gene.

In shoots, the expression of the *Sldhn* gene was lower than in roots, while plants inoculated with *G. intraradices* showed the highest levels of gene expression in shoots (lines 2, 4, 6, and 8).

Sldhn Gene in *Sitiens* Plants

The expression of this gene in *sitiens* plant roots was only clearly detected in plants colonized by *G. intraradices* and subjected to drought (lines 14 and 16; Fig. 6).

In shoots, the expression of the *Sldhn* gene was only clearly detected in non-AM plants subjected to stress and supplied with exogenous ABA (line 15).

SlPIP1-4 Gene in Wild-type Plants

In roots, the inoculation with *G. intraradices* did not significantly affect the expression of the *SlPIP1-4* gene either under well-watered (line 2 vs line 1) or drought stress conditions (line 6 vs line 5; Fig. 7). The application of exogenous ABA enhanced the expression of this gene by 83% in non-AM plants grown under well-watered conditions (line 3 vs line 1) and by 183% in AM plants (line 4 vs line 2). However, this impact of ABA was not visible under drought stress. Drought stress more than doubled the expression of the *SlPIP1-4* gene both in AM plant roots (line 6 vs line 2) and non-AM plant roots (line 5 vs line 1).

In shoots, the expression of this *PIP* gene was considerably lower than in roots, and no effects were observed in wild-type plants.

SlPIP1-4 Gene in *Sitiens* Plants

In roots, the expression of this gene was differentially affected by the inoculation with *G. intraradices* depending on the type of water regime (Fig. 7). Under well-watered conditions, *G. intraradices* inhibited the expression of the *SlPIP1-4* gene by 53% (line 10 vs line 9), while under drought stress conditions, *G. intraradices* enhanced the expression of the gene by 100% (line 14 vs line 13). Under well-watered conditions, the application of exogenous ABA inhibited the expression of *SlPIP1-4* by 53% in non-AM plants (line 11 vs line 9) and had no effect in AM plants (line 12 vs line 10). By contrast, under drought stress conditions, ABA application to non-AM plants did not change *SlPIP1-4* gene expression (line 15 vs line 13), but the application to AM plants inhibited the expression of the *SlPIP1-4* gene by 50% (line 16 vs line 14). Drought stress inhibited the expression of this gene by 80% in non-AM plants (line 13 vs line 9) but had no effect in AM plants (line 14 vs line 10).

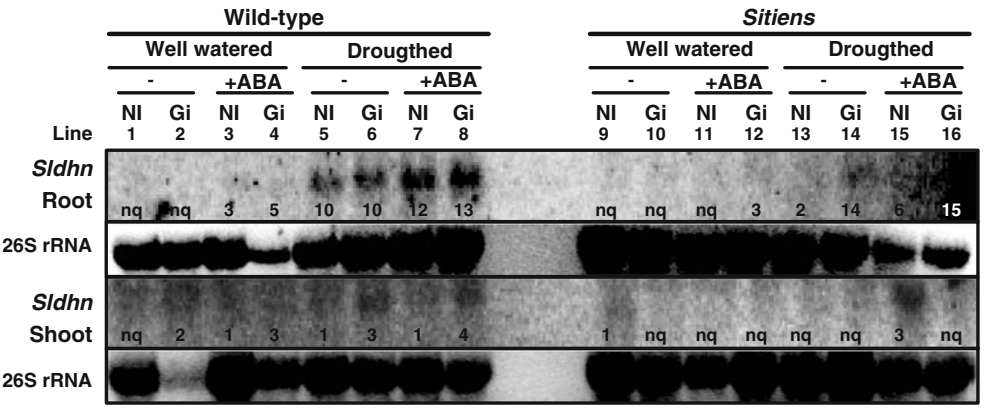

Figure 6 Northern blot of total RNA (15 µg) from tomato roots and shoots (wild type and *sitiens*) using the *Sldhn* gene probe (accession CK615624). See legend for Fig. 5. *nq* means not quantifiable

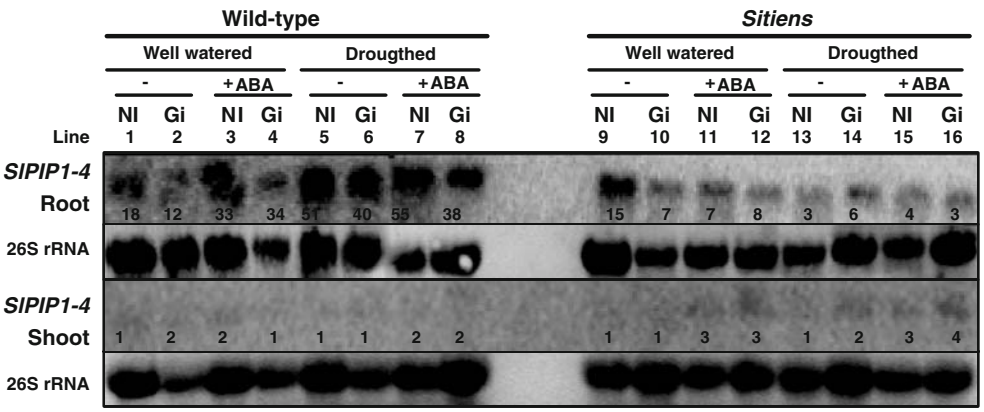

Figure 7 Northern blot of total RNA (15 μg) from tomato roots and shoots (wild type and *sitiens*) using the *SlPIP1-4* gene probe (accession AF218774). See legend for Fig. 5

As in wild-type plants, the expression of the *SlPIP1-4* gene in *sitiens* plant shoots was considerably lower than in roots.

SlPIP1-5 Gene in Wild-type Plants

In roots, the inoculation with *G. intraradices* induced the expression of the *SlPIP1-5* gene by 157% when plants were cultivated under well-watered conditions (line 2 vs line 1) but not when plants were subjected to drought stress (line 6 vs line 5; Fig. 8). The application of exogenous ABA enhanced the expression of this gene by 88% in non-AM plants grown under well-watered conditions (line 3 vs line 1), but AM plants did not show a significant change in gene expression (line 4 vs line 2). In plants subjected to drought stress, the application of ABA enhanced the expression of the *SlPIP1-5* gene (by 80% in non-AM plants, line 7 vs line 5 and by 50% in AM plants, line 8 vs line 6). Drought stress enhanced the expression of this gene by 159% in non-AM plants (line 5 vs line 1), while in AM plant roots, it had no effect on gene expression (line 6 vs line 2).

In shoots, the inoculation with *G. intraradices* induced *SlPIP1-5* gene expression by 56% (line 2 vs line 1) under well-watered conditions but had no significant effect under drought stress conditions (line 6 vs line 5). Exogenous ABA application or drought stress did not significantly affect *SlPIP1-5* gene expression.

SlPIP1-5 Gene in *Sitiens* Plants

In roots, the expression of this gene was unaffected by inoculation with *G. intraradices* regardless of the water regime (Fig. 8). Under well-watered conditions, the application of exogenous ABA did not significantly change *SlPIP1-5* gene expression. By contrast, under drought stress conditions, the application of ABA enhanced *SlPIP1-5* gene expression by 134% in non-AM plants (line 15 vs line 13). No effect was observed in AM plants (line 16 vs line 14). Drought stress inhibited the expression of this gene by 50% in both non-AM plants (line 13 vs line 9) and AM plants (line 14 vs line 10).

As in wild-type plants, *SlPIP1-5* gene expression in *sitiens* plant shoots was induced by 54% following the inoculation with *G. intraradices* but only under well-watered conditions (line 10 vs line 9). In shoots, the application of exogenous ABA enhanced *SlPIP1-5* gene expression by 107% in non-AM plants both under well-watered conditions (line 11 vs line 9) and under drought stress (line 15 vs line 13). Drought stress did not affect *SlPIP1-5* gene expression in shoots.

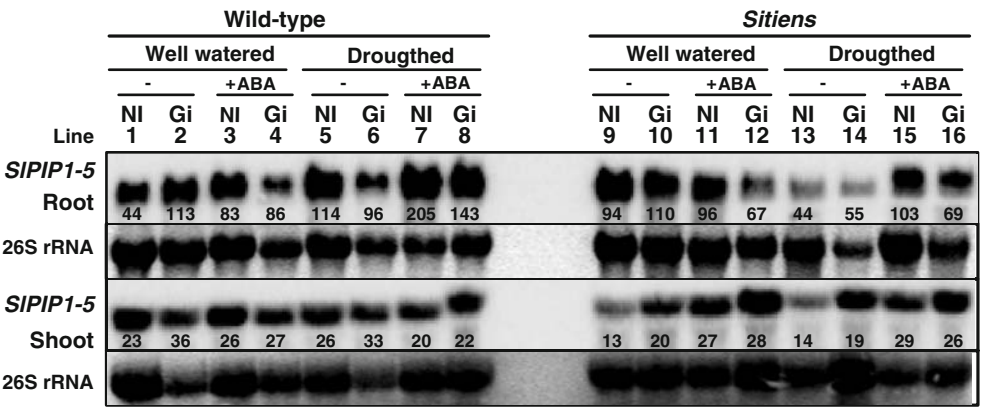

Figure 8 Northern blot of total RNA (15 μg) from tomato roots and shoots (wild type and *sitiens*) using the *SlPIP1-5* gene probe (accession X73848). See legend for Fig. 5

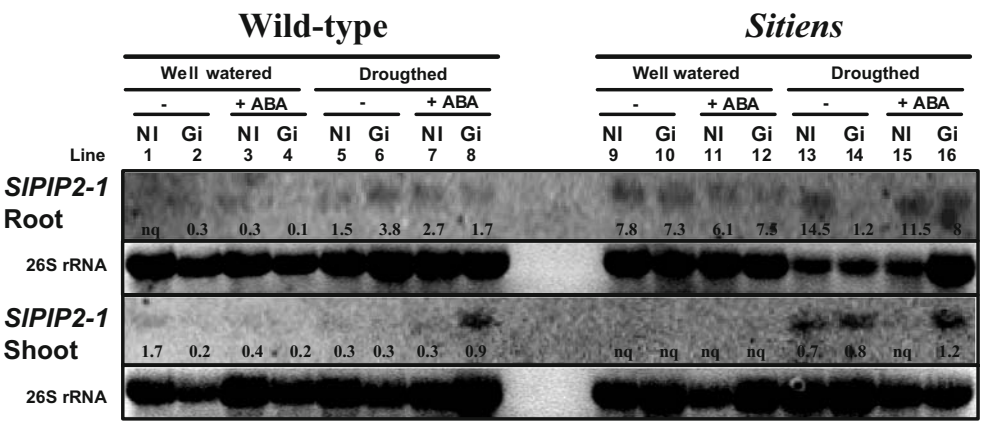

Figure 9 Northern blot of total RNA (15 μg) from tomato roots and shoots (wild type and sitiens) using the *SlPIP2-1* gene probe (Accession BI929127). See legend for Fig. 5. *nq* means not quantifiable

SlPIP2-1 Gene in Wild-type Plants

SlPIP2-1 gene expression in well-watered plant roots was detected only to a very slight degree (Fig. 9). Only plants subjected to drought stress exhibited *SlPIP2-1* gene expression. Thus, drought stress enhanced the expression of this gene in both non-AM plant roots (line 5 vs line 1) and AM plant roots (line 6 vs line 2).

In shoots, *SlPIP2-1* gene expression was even lower than in roots, and only plants inoculated with *G. intraradices* and subjected to drought as well as ABA addition (line 8) showed a clear gene expression signal.

SlPIP2-1 Gene in *Sitiens* Plants

In *sitiens* plant roots cultivated under well-watered conditions, neither the inoculation with *G. intraradices* nor the application of exogenous ABA had a significant effect on *SlPIP2-1* gene expression (Fig. 9). By contrast, under drought stress, the inoculation with *G. intraradices* inhibited *SlPIP2-1* gene expression by 91% (line 14 vs line 13). The application of exogenous ABA under drought stress conditions enhanced gene expression only in AM plants (a 566% increase, line 16 vs line 14). Drought stress itself enhanced *SlPIP2-1* gene expression by 86% in non-AM plants (line 13 vs line 9) and decreased it by 83% in AM plants (line 14 vs line 10).

In shoots, *SlPIP2-1* gene expression was not detected under well-watered conditions. Thus, drought stress considerably enhanced the expression of this gene under all treatment regimes except in non-AM plants supplied with ABA (line 15). The application of exogenous ABA inhibited gene expression in non-AM plants (line 15 vs line 13) but increased it by 50% in AM plants (line 16 vs line 14).

SlPIP2-3 Gene in Wild-type Plants

In roots, *SlPIP2-3* gene expression was not affected by the inoculation with *G. intraradices*, the application of exogenous ABA, or drought stress (Fig. 10).

In shoots, the application of exogenous ABA to plants colonized by *G. intraradices* and subjected to drought

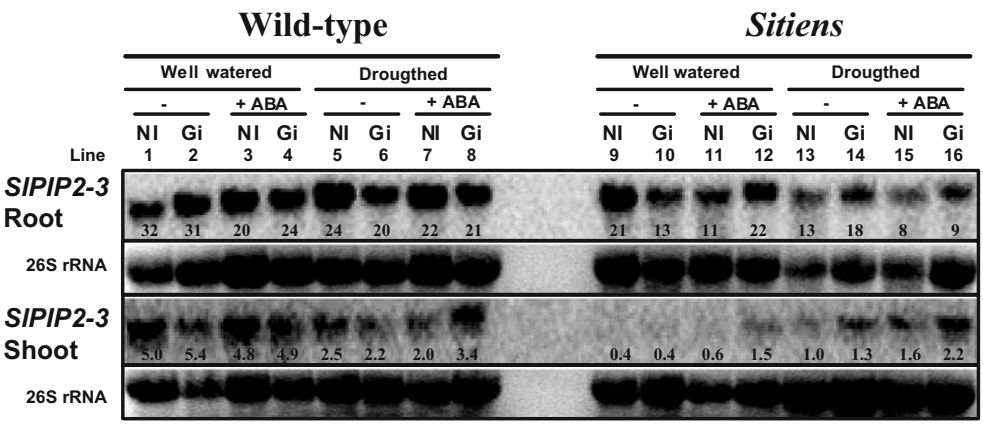

Figure 10 Northern blot of total RNA (15 μg) from tomato roots and shoots (wild type and sitiens) using the *SlPIP2-3* gene probe (accession AW224678). See legend for Fig. 5

stress enhanced *SlPIP2-3* gene expression by 54% (line 8 vs line 6). Drought stress decreased the expression of this gene by 50% in non-AM plants (line 5 vs line 1) and by 60% in AM plants (line 6 vs line 2).

SlPIP2-3 Gene in *Sitiens* Plants

In roots, the expression of this gene was not significantly affected by the inoculation with *G. intraradices* (Fig. 10). Under well-watered conditions, the application of exogenous ABA enhanced the expression of *SlPIP2-3* by 70% in AM plants (line 12 vs line 10) but decreased it by nearly 50% in non-AM plants (line 11 vs line 9). Under drought stress conditions, ABA had the opposite effect on AM plants as AM plants inhibited *SlPIP2-3* gene expression by 50% (line 16 vs line 14). Drought stress did not have a significant effect on the expression of this gene in *sitiens* plant roots.

SlPIP2-3 gene expression in *sitiens* plant shoots was lower than in roots. Under well-watered conditions, we observed an increase of 275% in *SlPIP2-3* gene expression in AM plants due to exogenous ABA application (line 12 vs line 10). When plants were subjected to drought stress, the application of ABA enhanced gene expression by 60% in non-AM plants (line 15 vs line 13) and by 120% in AM plants (line 16 vs line 14). Finally, drought stress had an important influence on *SlPIP2-3* gene expression in *sitiens* plant shoots, showing an increase of 150% in non-AM plants (line 13 vs line 9) and 225% in AM plants (line 14 vs line 10).

Discussion

Plant Growth and Physiology

The plant hormone ABA plays a major role in plant responses to a range of stresses, particularly those with a dehydration component [73]. ABA regulates plant water status through guard cells and growth as well as by induction of genes that encode enzymes and other proteins involved in cellular dehydration tolerance [12, 71]. Thus, ABA constitutes the most important stress signal transduction pathway among all the plant responses to stresses [73]. In this study, we have used an ABA-defective tomato mutant together with its near-isogenic wild-type parent to study the relationship between the stress hormone ABA and the induction of drought stress tolerance by AM symbiosis. It has been found that *sitiens* plant roots do not synthesize increasing amounts of ABA under stress and that *sitiens* does not export significant amounts of ABA in the xylem even in dry soil [26].

Although it has been suggested that a high level of exogenous ABA can inhibit plant growth under non-stressful conditions, an increased ABA content is beneficial for plants under environmental stress as a result of ABA-induced changes at the cellular and whole-plant levels [71]. However, whether ABA reduces, enhances, or maintains plant growth during drought is still not clear [41, 51]. Results obtained in this study indicate that under all the conditions tested, *sitiens* plants grew considerably less than its corresponding wild-type plants, a finding that is in line with previous studies of that plant genotype [45]. The wild-type plants did not benefit from ABA addition either under well-watered or drought stress conditions. By contrast, the ABA-defective *sitiens* plants treated with ABA clearly enhanced plant growth both under well-watered and drought stress conditions. Furthermore, the addition of ABA had a cumulative effect in relation to that of the inoculation with *G. intraradices* only for *sitiens* plants when subjected to drought stress. Our results agree with those of Mäkelä et al. [41] and Sharp et al. [62] who found that low ABA (*flacca* or *sitiens* plants, respectively) limited plant growth even in the absence of stress. The addition of ABA restored the growth rate to close to that of wild-type tomatoes, enabling us to conclude in this study that ABA functions as a growth promoter rather than an inhibitor [41].

Despite these effects of ABA on plant growth, it is curious that the ABA content in plants at harvest time did not show significant differences between treatment regimes, even in those that received exogenous ABA. However, bearing in mind that the oxidative degradation of ABA to phaseic acid and dihydrophaseic acid can be extremely rapid [75], it is almost impossible to predict the percentage of externally applied ABA that reaches its destination and remains active. For instance, Zhang et al. [74] found that after 24 h of feeding maize leaves with ABA, only 8% remains unmodified. Factors such as redistribution of ABA within plant tissues and exudation of ABA from the roots to the rhizosphere may decrease the internal plant ABA concentration as well [25]. The effects of ABA on different aspects of plant physiology are, in any case, highlighted in this study. ABA could affect the different treatments simply by changing its internal location or by moving from apoplastic to symplastic zones, rather than by increasing its internal concentration levels [16, 70].

In this study, we have observed that the effects of mycorrhization or application of exogenous ABA on plant water status such as shoot water potential or RWC are limited. Only *sitiens* plants, when subjected to drought, exhibited some change in RWC by mycorrhization or exogenous ABA addition. The transpiration rate in wild-type plants was only reduced by drought, with ABA addition or mycorrhization showing no additional effects. By contrast, in relation to *sitiens* plants, we found an unexpected result since drought stress enhanced their transpiration rates. This effect was remarkable in non-AM

plants, while in AM plants, the increase in transpiration due to drought was lower. These results demonstrate that, as is to be expected, the stomatal behavior of *sitiens* plants is altered [45], probably due to their lack of ABA accumulation under drought stress conditions [26]. It is noticeable that both exogenous ABA application and root colonization by *G. intraradices* prevented the enhanced transpiration rate of *sitiens* plants. These results suggest that the inoculation of *sitiens* plants with *G. intraradices* could compensate to some extent the anomalous induction of transpiration by drought in this plant mutant.

Regarding proline accumulation, our most interesting finding was that in *sitiens* plants subjected to drought, the application of exogenous ABA enhanced the accumulation of proline in non-AM plants but reduced it in AM plants. The regulation of proline accumulation by an ABA-dependent and an ABA-independent (plant dehydration) pathway and their cumulative effects have been described [59]. Our results suggest that, in non-AM plants, both pathways (the water dehydration produced by drought stress and the addition of exogenous ABA) induced the accumulation of proline. On the other hand, in AM plants, the combination of both pathways resulted in lower proline accumulation. Although the precise reason for such an effect is currently not known, the AM symbiosis has been reported to protect host plants from dehydration stress, and these plants may need to accumulate less proline than their non-AM counterparts [49]. These results clearly show that the response of *sitiens* plants to exogenous ABA varies quite significantly according to the AM fungal presence in their roots. Contrasting plant responses to exogenous ABA in AM and non-AM plants have also recently been observed in lettuce plants (Aroca et al., unpublished).

In relation to mycorrhizal colonization, our results show that *sitiens* plants were less colonized by *G. intraradices* than wild-type plants. This is in line with previous results by Herra-Medina et al. [27] who studied the role of ABA during AM symbiosis formation in the same tomato plant cultivars and also with the AM fungus *G. intraradices*. They demonstrated 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.

Gene Expression

Promoter analyses of ABA-induced genes have implicated transcription as a primary mechanism of gene regulation during periods of osmotic stress [12, 16]. For that reason, we have studied the effect of AM symbiosis, exogenous ABA, and drought on the accumulation of messenger RNAs (mRNAs) of several plant genes expected to be modulated by drought and ABA.

The genes selected for this study were the *Slnced* gene that encodes for NCED, the enzyme catalyzing the rate-limiting step in the biosynthesis of ABA [60]. The *Sldhn* gene, encoding for a dehydrin (late embryogenesis abundant [LEA] proteins), was also used since dehydrins accumulate in vegetative plant tissues during periods of water deficit, which reinforced the role of these proteins as desiccation protectants [15]. In addition, it has been shown that the expression of genes encoding LEA proteins is regulated by the levels of ABA in the plants [9, 12]. Finally, four plasma membrane intrinsic protein [PIP] aquaporin-encoding genes were also studied. The role of aquaporins in plants under water deficit conditions has not been fully explored. It is clear, however, that water largely passes through the plasmalemma or the tonoplast thanks to the aquaporin activity [40, 42]. Consequently, a correlation between the expression or activity of aquaporins and the sensitivity or resistance of plants to drought stress is expected [35].

Several of the genes analyzed in this study have shown different regulation patterns in wild-type and *sitiens* plants, indicating that gene expression is modulated by the plant ABA phenotype. In addition, the inoculation of roots with the AM fungus *G. intraradices* modified the behavior of several genes in wild-type and in *sitiens* plants. One example of this is provided by the *Slnced* gene. *Slnced* gene expression was regulated differently by AM symbiosis and drought in wild-type and *sitiens* plant roots. The *Slnced* gene was induced by drought in wild-type plant roots in both AM and non-AM plants. The induction of *nced* genes by drought stress has previously been observed in a variety of plants [30, 54, 65, 69]. By contrast, in ABA-deficient mutant *sitiens*, the expression of this gene was inhibited in non-AM plant roots but was enhanced in AM plant roots. Similarly, AM symbiosis enhanced *Slnced* gene expression in wild-type plant roots grown under well-watered conditions but decreased its expression in well-watered *sitiens* plant roots. Cheng et al. [14] demonstrated in *Arabidopsis* that a minimum level of ABA is required in plant tissues for full induction of a *nced* gene since ABA-deficient mutants accumulated less mRNAs for this gene in response to drought and salt stress treatments.

Another example of the differential gene regulation patterns in wild-type and *sitiens* plants is provided by the *Sldhn* gene that is clearly expressed in wild-type plant roots subjected to drought. By contrast, in *sitiens* plants, this gene is induced by drought only in plants colonized by *G. intraradices*. Our results suggest that *Sldhn* gene expression requires a minimum level of ABA that does not occur in *sitiens* plants. Another possible explanation for this effect is based on observations by Holbrook et al. [28] who suggested that the roots of ABA-deficient mutants may be exporting precursors of ABA that may possibly accumulate in mutants blocked at the final step of ABA synthesis, as

happens with sitiens mutants. In fact, *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 drought [38]. This or other accumulated compounds can involve biological activity that could affect the expression of the genes analyzed here. Our data indicate that the AM fungus *G. intrarradices* could, in some way, compensate for such ABA deficiency or accumulation of ABA precursors, thus allowing *sitiens* plants to express this gene.

The negative water potential in drying soils confronts plants with the problem of acquiring sufficient amounts of water [46], a process in which aquaporins are involved [33, 40]. In addition, it has been shown that ABA modulates the expression of some PIP genes in roots and leaves [3, 32, 76]. In this study, we have analyzed the expression of four PIP aquaporin genes, and we have also observed differences in the expression of some of the genes between AM and non-AM plants after ABA or drought treatments depending on the plant ABA genotype. As an example of this, the application of exogenous ABA under well-watered conditions enhanced *SlPIP1-4* gene expression in wild-type plant roots in both AM and non-AM plants. On the other hand, in *sitiens* plants, the application of ABA decreased the gene expression in non-AM roots and did not affect the gene expression of AM roots. In addition, drought doubled *SlPIP1-4* gene expression in wild-type plants roots in both AM and non-AM plants. However, in *sitiens* plants, drought reduced gene expression in non-AM plants and did not change the expression of the gene in AM plants. Similarly, the *SlPIP1-5* gene was induced by drought in wild-type non-AM plant roots and remained unchanged in wild-type AM plants. By contrast, in *sitiens* plants, drought decreased the expression of this gene in both AM and non-AM plant roots. This suggests that the *SlPIP1-5* gene is not only regulated by drought but also by ABA and needs high levels of ABA to induce its gene expression. If the ABA levels are low as in *sitiens* plants, its expression is downregulated by drought. Drought induced *SlPIP2-1* gene expression in wild-type AM and non-AM plant roots. By contrast, in *sitiens* plants, drought enhanced gene expression only in non-AM roots and decreased the expression of the *SlPIP2-1* gene in AM roots. The expression of the *SlPIP2-3* gene was also regulated differently by drought in wild-type and *sitiens* plants. Drought decreased the expression of this gene in wild-type plant shoots in both AM and non-AM plants but induced *SlPIP2-3* gene expression in *sitiens* plant shoots.

The reasons for such differences are currently unknown. The presence of the AM fungus may directly regulate gene expression independently of ABA, as has been seen for a variety of genes (for reviews, see [7, 22]). However, differences in the compartmentation of ABA within cells and tissues or differences in the ABA metabolic rate [25, 70, 73] between AM and non-AM plants could also account for such differential gene expression.

The different effects of AM symbiosis on the expression of PIP aquaporin isoforms under salinity conditions [46] or several osmotic stresses [4] have already been described. These results suggest that AM symbiosis exerts varying degrees of control on aquaporin gene expression, inducing or inhibiting particular genes depending on the nature of the stress [4]. The upward or downward regulation patterns produced by osmotic stresses of mRNAs encoding aquaporins homologues have been described in the roots of many plant species [34]. There are currently two contrasting descriptions of the role of aquaporins in response to dehydration stress [64]. The first is based on evidence that gene expression of some aquaporins is induced under dehydration stress [32], which is predicted to result in greater membrane water permeability and facilitate water transport. The second description is based on the fact that aquaporin expression is downregulated under dehydration stress, which should result in decreased membrane water permeability and may enable cellular water conservation [64] during periods of dehydration stress. Thus, the induction or inhibition of particular aquaporins by AM symbiosis under osmotic stress should result in improved regulation of plant water status and contribute to global plant resistance to stressful conditions [32].

Our results also show that the PIP aquaporin genes analyzed here showed different regulation patterns due to drought stress or ABA application, and this phenomenon was dependent on the plant genotype studied. This agrees with the results produced by Lian et al. [37] who found that PIP genes in rice responded differently to water stress and ABA and suggested that during water deficit, the regulation of PIP genes involves both ABA-dependent and ABA-independent signaling pathways.

In conclusion, this study showed that only wild-type tomato plants responded positively to mycorrhizal inoculation. On the other hand, AM symbiosis was not observed to have any effect on plant development in *sitiens* plants grown under well-watered conditions. The application of ABA to *sitiens* plants clearly enhanced plant growth both under well-watered and drought stress conditions. Furthermore, in *sitiens* plants subjected to drought stress, the addition of ABA had a cumulative effect in relation to that of inoculation with *G. intraradices*. The stomatal behavior of *sitiens* plants is altered, as evidenced by their enhanced transpiration rate under drought conditions, but the inoculation of these plants with *G. intraradices* could compensate for such an alteration to a certain extent, allowing these plants to grow better under drought stress conditions. Most of the genes analyzed in this study showed different regulation patterns in wild-type and *sitiens* plants, suggesting that their expression is modulated by the plant ABA

phenotype. In addition, colonization of roots with the AM fungus *G. intraradices* regulated the behavior of these genes differently in wild-type and *sitiens* plants, which could explain the difference in the impact of the symbiosis on each plant ABA phenotype. This also suggests that the effects of the AM symbiosis on plant responses and resistance to water deficit are mediated by the plant ABA phenotype.

Acknowledgments This work was financed by CICYT-FEDER (Project AGL2005-01237). R. Aroca was financed by the Spanish Ministry of Education and Science throughout the Juan de la Cierva program. We thank Dr. I. Taylor (University of Nottingham, UK), Dr. A. Downie (University of Kentucky Lexington, USA), and Dr. H. Shiota (Yokohama City University, Japan) for providing us with the probes for NCED, dehydrin, and PIP aquaporin genes, respectively. We thank Michael O'Shea for correcting the English text.

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