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Involvement of the *def-1* Mutation in the Response of Tomato Plants to Arbuscular Mycorrhizal Symbiosis Under Well-Watered and Drought Conditions

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Jasmonic acid (JA) and arbuscular mycorrhizal (AM) symbioses are known to protect plants against abiotic and biotic stresses, but are also involved in the regulation of root hydraulic conductance (L). The objective of this experiment was to elucidate the role of JA in the water relations and hormonal regulation of AM plants under drought by using tomato plants defective in the synthesis of JA (*def-1*). Our results showed that JA is involved in the uptake and transport of water through its effect on both physiological parameters (stomatal conductance and L) and molecular parameters, mainly by controlling the expression and abundance of aquaporins. We observed that *def-1* plants increased the expression of seven plant aquaporin genes under well-watered conditions in the absence of AM fungus, which partly explain the increment of L by this mutation under well-watered conditions. In addition, the effects of the AM symbiosis on plants were modified by the *def-1* mutation, with the expression of some aquaporins and plant hormone concentration being disturbed. On the other hand, methyl salicylate (MeSA) content was increased in non-mycorrhizal *def-1* plants, suggesting that MeSA and JA can act together in the regulation of L. In a complementary experiment, it was found that exogenous MeSA increased L, confirming our hypothesis. Likewise, we confirmed that JA, ABA and SA are hormones involved in plant mechanisms to cope with stressful situations, their concentrations being controlled by the AM symbiosis. In conclusion, under well-watered conditions, the *def-1* mutation mimics the effects of AM symbiosis, but under drought conditions the *def-1* mutation changed the effects of the AM symbiosis on plants.

Keywords: Aquaporins • Arbuscular mycorrhizal symbiosis • Drought • Jasmonic acid • Root hydraulic properties • *Solanum lycopersicum*.

Abbreviations: AM, arbuscular mycorrhiza; AMF, arbuscular mycorrhiza fungi; ANOVA, analysis of variance; AQP, aquaporin; ELISA, enzyme-linked immunosorbent assay; g_s, stomatal conductance; HPFM, hydraulic conductance flow meter; HPLC-ESI-MS, HPLC–electrospray ionization-mass spectrometry; JA, jasmonic acid; JA-Ile, JA-isoleucine; L, root hydraulic conductance; MeJA, methyl jasmonate; MeSA, methyl salicylate; MRM, multiple reaction monitoring; OPDA, 12-oxophytodienoic acid; PIP, plasma membrane intrinsic protein; q-RT-PCR, quantitative real-time PCR; RWC, relative water content; SA, salicylic acid; WC, water consumption; WT, wild type.

Introduction

Jasmonic acid (JA) is a hormone implicated in the physiological development of plants (Santino et al. 2013, Wasternack and Hause 2013), although its role as a protective agent against abiotic and biotic stresses has also been studied for many years. For example, exogenous applications of methyl jasmonate (MeJA; a derivate of JA) improved the tolerance against several abiotic stresses (Abdala et al. 2003). In particular, Anjum et al. (2011) observed that MeJA-treated plants were more resistant against drought conditions, enhancing their antioxidant response and their leaf relative water content (RWC). Abdala et al. (2003) also found that tomato roots increased the content of JA and its derivates after exposure to salt stress.

On the other hand, JA is also involved in the establishment of arbuscular mycorrhizal (AM) symbiosis, which is a widespread association between most plants and fungi from the phylum Glomeromycota (Smith and Read 2008). It is known that JA or its derivatives are released by plants, taking part in the recognition between AM fungi (AMF) and host roots in early stages of AM symbiosis (Hause et al. 2002). Although its role in the rate of colonization by AMF in the plant roots is not clear yet (Hause et al. 2002, Leon-Morcillo et al. 2012), it has been found that AM symbiosis increased JA levels of the host plant (Y. Li et al. 2013, Mandal et al. 2013). In addition, AM symbiosis makes plants more tolerant of stress conditions, such as drought (Ruiz-Lozano et al. 2009, Abbaspour et al. 2011, Doubkova et al. 2013), which is achieved by regulating both stomatal conductance (g_s) and root water uptake capacity (Zaman-Allah et al. 2011). For this reason, several studies have been focused on AMF regulation of root hydraulic

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conductance (L), under both well-watered and drought conditions (Bárzana et al. 2014). L provides information about the plant's capacity to take up soil water and it can change depending on the environmental conditions (Aroca et al. 2007, Ruiz-Lozano et al. 2009, Vandeleur et al. 2009, Bárzana et al. 2014). L is also determined by the morphology of the roots and by the pathways that water can follow inside roots (Sutka et al. 2011).

Water can go through either the apoplastic pathway or the cell to cell pathway, this latter route being that in which aquaporins (AQPs), water channel proteins, are involved (Steudle and Peterson 1998). Although there are different kinds of plant AQPs, those most implicated in regulating root water transport are known as PIPs (plasma membrane intrinsic proteins) which are located mainly in the plasma membrane (Fetter et al. 2004, Zelazny et al. 2007, Maurel et al. 2008), and are divided into two phylogenic subgroups, PIP1s and PIP2s. PIP2s are known to have more transport water capacity than PIP1s, but PIP1s may increase the activity of PIP2s by forming heterotetramers (Fetter et al. 2004). Three AM fungal AQP genes have been found in *Rhizophagus irregularis*, which is one of the AMF best able to take up water from the soil (Marulanda et al. 2003). The first is called *GintAQP1* and, in spite of not transporting water, its function seems to be related to compensating for AQP expression of the host plant, or in unstressed mycelium (Aroca et al. 2009, El-Mesbahi et al. 2012). Most recently, *GintAQPF1* and *GintAQPF2* were isolated by T. Li et al. (2013). These AQPs increased their expression under drought stress. Apparently, all of these AQPs could explain why AM plants usually have improved water uptake capacity under drought conditions. Although the effect of AM symbiosis on L is not consistent (Ruiz-Lozano et al. 2009, Bárzana et al. 2012), it has been observed that AM roots show a decrease in abundance and expression of PIPs compared with non-AM roots (Benabdellah et al. 2009, Bárzana et al. 2014).

It has been found recently that after exogenous MeJA application, an increase of L and up-regulation of some AQPs were observed under hydroponic conditions. Such an increase of L was as a result of changes in ABA and calcium concentrations caused by MeJA application (Sánchez-Romera et al. 2014). There are other hormones involved in L regulation, such as salicylic acid (SA) which is known to be antagonistic to JA (Proietti et al. 2013) and inhibits L (Boursiac et al. 2008a), and IAA which is implicated in reducing L and the expression of genes encoding several AQPs (Peret et al. 2012) as well as being required for normal AM colonization (Hanlon and Coenen 2011).

The aim of the present study was to investigate the involvement of internal JA contents in the regulatory activity of AMF on root hydraulic properties including L and AQPs, under both well-watered and drought stress conditions. The objective was focused on long-lasting effects of drought, where AM symbiosis can have a more positive impact (Ruiz-Lozano et al. 1995). For that, tomato plants deficient in JA synthesis (*def-1*; Howe et al. 1996) and wild-type (WT) plants were grown, without AMF or inoculated with the AMF *R. irregularis* under two water regimes (well-watered or drought). AMF colonization, L, g_s, daily rate of root water consumption (WC), AQP expression and abundance, and hormonal contents were determined. Based on the results from the main experiment, it was decided to carry out an experiment in which different concentrations of methylsalicylate (MeSA) were added to hydroponic grown tomato plants to corroborate the positive correlation found between L and MeSA root contents.

Results

Mycorrhizal colonization

After 11 weeks of plant growth and having subjected half of the plants to a period of 10 d of water deficit (drought), AM root length colonization was determined. Uninoculated plants did not exhibit any mycorrhizal colonization. Inoculated plants displayed a percentage of root length colonization of around 60%, with no significant differences regardless of water regime or genotype (**Supplemetnary Fig. S1**).

Root hydraulic conductance and water consumption

L was determined by the HPFM (hydraulic conductance flow meter) method. *def-1* plants showed different behavior of L depending on the water regime and AM inoculation (**Fig. 1a**). Under well-watered conditions, *def-1* plants had higher L than WT plants, but only in non-AM plants; whereas under drought conditions no significant differences in L between *def-1* and WT plants were observed (**Fig. 1a**). L of AM plants was always higher than L of non-AM plants, except for *def-1* plants under well-watered conditions where a similar L value was recorded (**Fig. 1a**). Analysis of variance (ANOVA) showed that L was significantly up-regulated by AM ($P < 0.01$) and the *def-1* genotype ($P < 0.05$; **Table 1**). Drought treatment had no effect on L (**Table 1**).

In addition, the daily rate of root WC (**Fig. 1b**) was clearly decreased by drought treatment (**Fig 1b**; **Table 1**). Also, the effect of AM symbiosis on WC depended on the plant genotype (**Table 1**). AM plants had a higher WC than non-AM WT plants under both well-watered and drought conditions, but a lower and equal WC than *def-1* plants under well-watered and drought conditions, respectively (**Fig. 1b**).

Stomatal conductance

g_s was reduced by drought (**Table 1**; **Fig. 1c**), except in non-AM *def-1* plants (**Fig. 1c**). Under well-watered conditions, no significant diferences among treatments were observed (**Fig. 1c**). However, under drought conditions, AM symbiosis increased g_s in WT plants, but decreased it in *def-1* plants. Also, *def-1* plants showed higher g_s than WT plants when not inoculated under drought conditions (**Fig. 1c**).

Plant *PIP* and fungal aquaporin gene expression

The expression of nine plant *PIP* genes was analysed in roots of *Solanum lycorpersicum* by quantitative real-time PCR (q-RT-PCR). More precisely, three *SlPIP1* genes and six *SlPIP2* genes were analyzed. The expression of the nine *PIP* genes tested was up-regulated in *def-1* plants when colonized by AMF under well-watered

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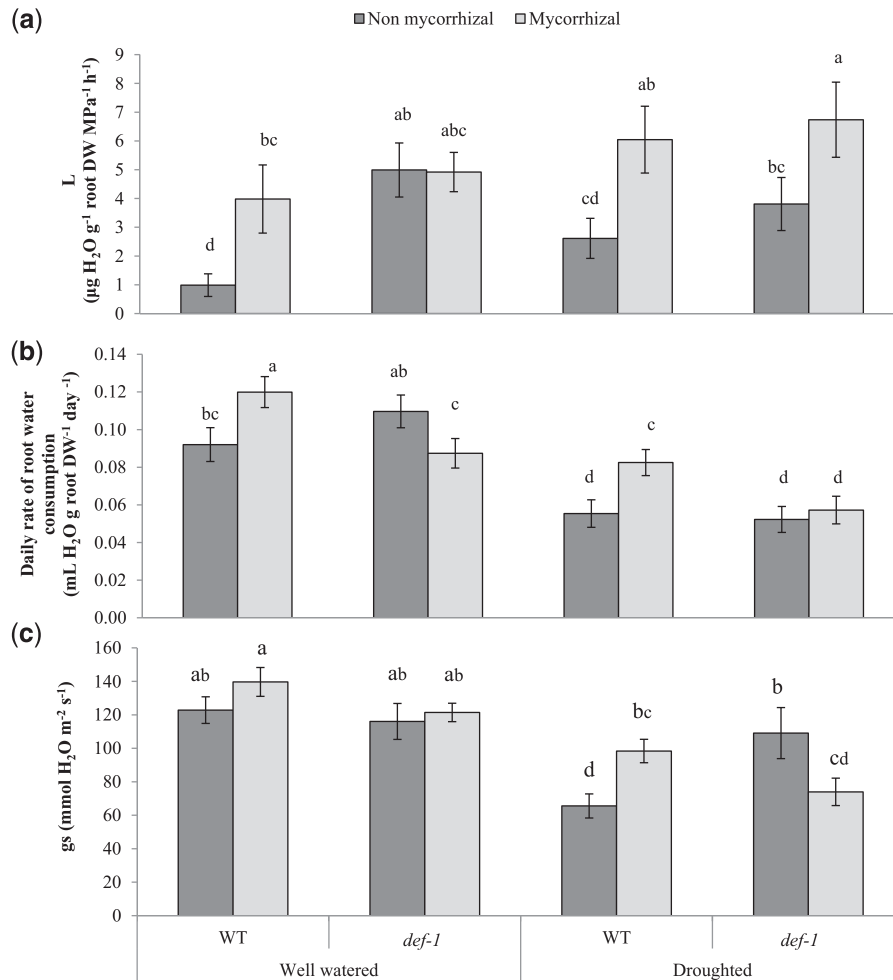

Fig. 1 (a) Root hydraulic conductivity (L) ($n = 6$), (b) relative water content (RWC) ($n = 6$) and (c) stomatal conductance (g_s) ($n = 10$) of WT and *def-1* tomato plants. Plants uninoculated (dark bars) or inoculated with *Rhizophagus irregularis* (gray bars) were grown under well-watered conditions or were subjected to drought conditions for 10 d. Bars represent the mean ± SE. Different letters indicate significant differences ($P < 0.05$) after ANOVA and LSD test.

conditions (**Figs. 2, 3**). The same behavior was observed for *def-1* mutants without AM symbiosis for seven of the nine genes, the exceptions being *SlPIP1;5* and *SlPIP2;3* (**Figs. 2, 3**). Drought treatment down-regulated the expression of all nine *PIP* genes in *def-1* plants colonized by AMF, and only of the *SlPIP2;8* and *SlPIP2;9* genes in non-colonized plants (**Figs. 2, 3**). In contrast, drought treatment up-regulated the expression of *SlPIP1;7* in uncolonized *def-1* plants (**Fig. 2c**). Drought treatment did not cause any effect on the expression of *PIP* genes in WT plants, resulted in down-regulation of *SlPIP2;1* and *SlPIP2;6* in AM plants (**Fig. 3a, d**). Therefore, drought and genotype treatments had a significant effect on the expression of the nine *PIP* genes analyzed, while AM symbiosis only affected the expression of *SlPIP1;7*, *SlPIP2;4* and *SlPIP2;6* (**Table 1**).

The expression of three fungal AQP genes from *R. irregularis* was analyzed by quantitative real-time PCR (q-RT-PCR) (**Fig. 4**). No significant differences in the expression of *GintAQPF2* were observed. However, the expression of *GintAQP1* and *GintAQPF1* was up-regulated in *def-1* roots under well-watered conditions (**Fig. 4**).

Plant PIP protein abundance

We analyzed the abundance of PIP1, PIP2 and PIP2 phosphorylated at Ser280 (PIP2PH) by enzyme-linked immunosorbent assay (ELISA). PIP1 abundance was not significantly affected by either AMF or water regimen. However, ANOVA showed that the genotype factor had an effect on the abundance of PIP1 (**Table 1**; **Fig. 5a**), with *def-1* roots being less abundant.

Under well-watered conditions, uninoculated WT roots had the highest PIP2PH abundance (**Fig. 5c**). However, the abundance of PIP2 was the same in WT and *def-1* roots, being reduced by the presence of AMF (**Table 1**; **Fig. 5b**). On the other hand, the drought treatment reduced PIP2PH abundance in all roots, except non-AM *def-1* roots. Nevertheless, in general, all AM roots had lower PIP2PH abundance than non-AM roots (**Table 1**; **Fig. 5c**).

Additionally, after studying statistical regression between L and the amount of each of these AQPs, a negative linear regression was observed between L and the abundance of PIP1 and PIP2PH (**Fig. 6a, b**).

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Table 1 Significance of sources of variation after three-way ANOVAs for the following parameters: root hydraulic conductivity (L), relative water content (RWC), stomatal conductance (g_s), expression of *SlPIP* isoform genes, PIP abundance, ABA, MeSA, IAA and SA content

	AM	WR	Genotype	AM×WR	AM×genotype	WR×genotype	AM×WR×genotype
L	**	NS	*	NS	NS	NS	NS
WC	NS	***	NS	NS	**	NS	NS
g_s	NS	***	NS	NS	**	NS	*
SlPIP1;1	NS	**	NS	**	NS	NS	NS
SlPIP1;7	*	***	***	*	*	***	NS
SlPIP2;1	NS	**	NS	NS	NS	NS	**
SlPIP2;4	NS	***	NS	NS	NS	NS	NS
SlPIP2;6	NS	0	NS	**	NS	NS	NS
SlPIP2;8	NS	NS	NS	**	NS	*	NS
SlPIP2;9	NS	NS	NS	***	NS	**	NS
PIP1	NS	NS	**	NS	NS	NS	NS
PIP2	***	*	NS	NS	NS	NS	NS
PIP2PH	**	***	*	NS	NS	NS	NS
ABA	***	***	NS	*	**	NS	NS
MeSA	***	NS	***	NS	NS	NS	*
IAA	***	NS	NS	*	**	NS	NS
SA	**	***	NS	*	NS	**	*

The sources of variance were AM symbiosis (AM), water regime (WR) and genotype, as well as their interactions.
*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, NS, non-significant effect.

Hormone content

The root contents of hormones [12-oxophytodienoic acid (OPDA), JA, JA-isoleucine (JA-Ile), ABA, IAA, SA and MeSA] were determined. First, we corroborated that *def-1* roots showed a lower content of JA-related metabolites (JA, OPDA and JA-Ile) under well-watered conditions in the absence of AMF than WT roots (**Supplementary Fig. S2**). Curiously, *def-1* roots without AM inoculation had a similar amount of JA, JA-Ile and OPDA as AM WT roots under well-watered conditions (**Supplementary Fig. S2**). Nevertheless, OPDA and JA-Ile concentrations (**Supplementary Fig. S2**) increased in *def-1* roots when they were subjected to drought. This was not observed for JA. AM symbiosis kept the OPDA content constant regardless of the type of plant and irrigation conditions (**Supplementary Fig. S2**). JA content was highest in unstressed WT roots, but this content was reduced by AM symbiosis, drought and *def-1* mutation (**Supplementary Fig. S2**). JA-Ile content was noticeably regulated by the AM symbiosis which increased it, except in unstressed WT roots, whose level was really high (**Supplementary Fig. S2**). Drought reduced JA-Ile in WT plants in contrast to its increase in *def-1* plants, always without AM inoculation. However, since lower amounts of JA derivate compounds (probably basal levels) were found, these data should be taken with caution.

Similar to JA and its derivates, ABA was reduced by *def-1* mutation to the levels of that in AM WT roots under well-watered conditions (**Fig. 7a**). Drought treatment raised the ABA concentration drastically in both genotypes, but to a lesser extent in AM plants. In addition, *def-1* roots had a higher content of IAA, SA and MeSA than WT plants under well-watered conditions in the absence of AMF. However, these concentrations changed when plants were subjected to drought, because *def-1* roots had a lower SA content than WT plants and similar IAA and MeSA contents top WT plants, always in the absence of AMF (**Fig. 7**).

The presence of AMF increased the concentration of IAA and MeSA in all roots except in those of *def-1* under well-watered conditions (**Fig. 7b, d**). Therefore, we analyzed the expression of the SA methyl transferase gene (*SlSAMT1*). However the expression of this gene only was up-regulated by AM symbiosis in *def-1* roots under well-watered conditions (**Supplementary Fig. S3**). On the other hand, SA contents were increased by AM symbiosis in both kinds of plants, but only under drought conditions (**Fig. 7c**). The linear regression analysis showed that there was a positive correlation between IAA and L and between MeSA and L. Instead, the PIP1 abundance exhibited a negative correlation with MeSA content (**Fig. 6**).

In view of this positive correlation between L and MeSA, we decided to run another experiment where tomato plants, which were grown under hydroponic conditions, were subjected to two exogenous MeSA levels (10 and 100 μM during 4 h; **Fig. 8**). It was found that exogenous MeSA increased L, mostly under the lower concentration used and for short times periods, supporting the results of the linear regression found between MeSA and L (**Figs. 6e, 8**).

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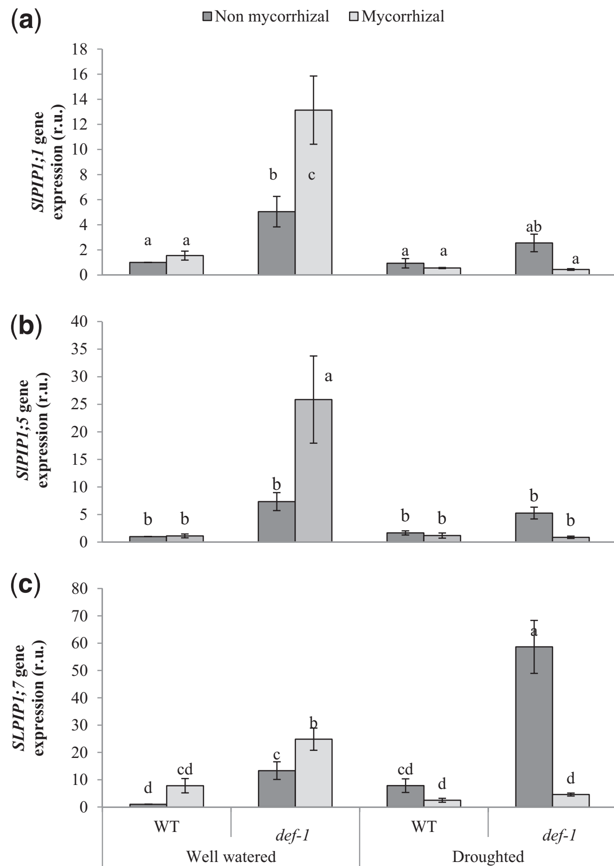

Fig. 2 Relative expression of (a) *SlPIP1;1*, (b) *SlPIP1;5* and (c) *SlPIP1;7* genes determined by q-RT-PCR in WT and *def-1* tomato roots. Plants uninoculated (dark bars) or inoculated with *Rhizophagus irregularis* (gray bars) were grown under well-watered conditions or were subjected to drought conditions for 10 d. Bars represent the mean ± SE. Different letters indicate significant differences ($P < 0.05$) after ANOVA and LSD test ($n = 6$).

Discussion

The mechanisms by which plants regulate their ability to absorb and transport water are still poorly understood. It is known that the interaction between different plant hormones and the presence of AMF affect the ability of plants to take up water under optimal and stressed conditions (Aroca et al. 2008a, Bárzana et al. 2014). The aim of this work was to obtain new insights into the effects of the interaction between JA and AMF on root hydraulic conductance (ability to take up water), in plants growing under well-watered or drought-stressed conditions. For this purpose, we used the tomato mutant plants (*def-1*), which are defective in JA biosynthesis (Howe et al. 1996, Howe and Ryan 1999, O'Donnell et al. 2003, Glas et al. 2014, Grinberg-Yaari et al. 2015). Previously, we conducted an experiment adding exogenous MeJA to AM and non-AM bean plants (Sánchez-Romera et al. 2016) and found that MeJA and AM symbiosis avoided the inhibition of L caused by drought. However exogenous applications most frequently did not match physiological levels and also could cause side effects. Therefore, the use here of mutant plants defective in JA biosynthesis will cast some light on the involvement of JA in regulating L in AM plants.

Effect of *def-1* mutation on root hydraulic properties

There is little information about the possible role of JA in the regulation of root hydraulic properties, although several studies found that exogenous JA improved plant stress tolerance (Lee et al. 1996, Walia et al. 2007, Anjum et al. 2011). For example, Lee et al. (1996) observed that plants treated with MeJA increased their RWC and root bleeding rate under cold conditions, and Anjum et al. (2011) noted that MeJA-treated plants were more resistant against drought. In general, it was observed that MeJA application provided benefits to plants under well-watered and drought conditions (Anjum et al. 2011). In support of this hypothesis, a previous study by our research group found that L increased in different plant species (tomato, bean and Arabidopsis) under hydroponic conditions after exogenous applications of MeJA (Sánchez-Romera et al. 2014).

Here we used mutant plants whose JA response is affected by impairment of the JA biosynthesis pathway, called *def-1* (Howe et al. 1996, Howe and Ryan 1999, O'Donnell et al. 2003, Glas et al. 2014, Grinberg-Yaari et al. 2015), and we found that these plants had higher L than WT plants under well-watered conditions. Therefore, these results contrast with those obtained previously (Sánchez-Romera et al. 2014), where *def-1* plants had lower L values than WT plants and this effect was counteracted by MeJA application. These differences in L behavior between the two studies could be caused by the conditions in which the plants were grown, because in the present work the plants were grown in soil and, in previous studies, plants were grown under hydroponic conditions. We should thus consider the changes on root anatomy due to the growing conditions. For example, Matsuo et al. (2009) analyzed three different genotypes of rice grown under hydroponic and soil conditions, and the plants with the highest L value under hydroponics were not the same as that under soil conditions. This was linked to variations in the maturation of the exodermal root cells associated with the medium in which the root had been cultivated (Matsuo et al. 2009, Meyer et al. 2009), affecting the water absorption properties of these roots (Hachez et al. 2012). Hence, the results obtained under hydroponic conditions could not always be extrapolated to plants growing on soil. Another consideration could be the method used to determine L here which was different from that in Sánchez-Romera et al. (2014), who used the free exudation and pressure chamber methods. These methods are considered optimal for plants grown under hydroponic conditions, where exogenous compounds are added, but in our case we decided to use a HPFM because we can measure L without manipulating roots, avoiding their damage.

Nevertheless, our results point to a hormonal counterbalance in *def-1* plants that induces an increase in L under well-watered conditions, and this effect is reversed in the presence of AM fungi. It is known that JA can have a beneficial or detrimental effect depending on its concentration in the plant (Reinbothe et al. 2009, Wasternack and Hause 2013). Previous

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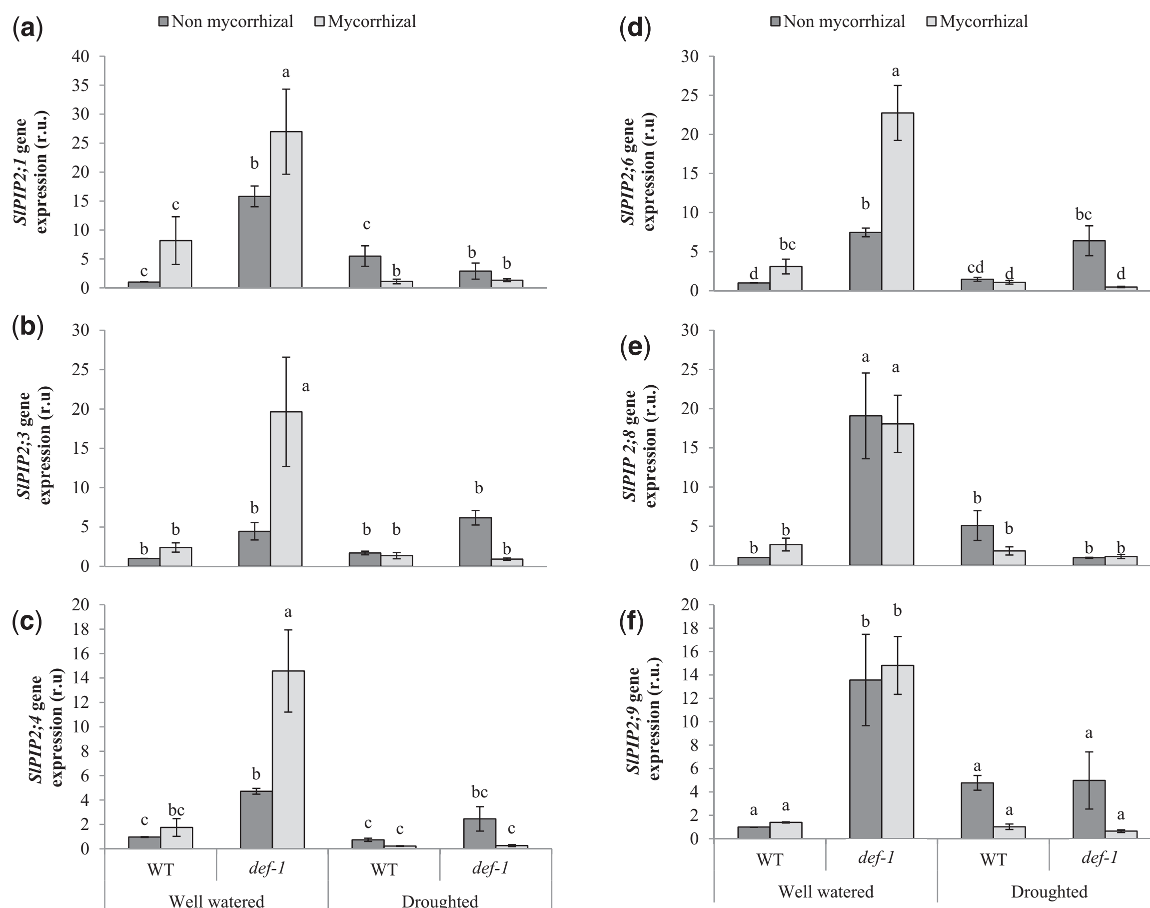

Fig. 3 Relative expression of *SlPIP2;1*, *SlPIP2;3*, *SlPIP2;4*, *SlPIP2;6*, *SlPIP2;8* and *SlPIP2;9* genes determined by q-RT-PCR in WT and *def-1* tomato roots. Plants uninoculated (dark bars) or noculated with *Rhizophagus irregularis* (gray bars) were grown under well-watered conditions or were exposed to drought conditions. Bars represent the mean ± SE. Different letters indicate significant differences ($P < 0.05$) after ANOVA and LSD test ($n = 6$).

studies showed the effect of MeJA applications in the short term (24 h) on L (Sánchez-Romera et al. 2014). However *def-1* plants are always defective in JA, so other hormones have to cope with this situation. Our results show that the increase of L in *def-1* plants could be provoked by an increase of MeSA concentration in these plants. The experiment under hydroponic conditions supports this hypothesis, since MeSA-treated plants showed higher L. Boursiac et al. (2008a) found that SA inhibited L; however, it is possible that a derivate of it (MeSA) could have an opposite effect. More studies on the role of SA and their derivates in L are needed to obtain a solid conclusion.

Involvement of JA in aquaporin regulation

Sánchez-Romera et al. (2014) observed an increase in the abundance of PIP2PH proteins in MeJA-treated bean plants; here we observed that WT plants had a greater abundance of PIP2PH with respect to *def-1* plants under well-watered conditions and this could be due to the higher content of JA in roots of WT plants. In any case, in the present study such increasing protein levels were not accompanied by higher L. In contrast, L and the abundance of AQPs were not affected by the plant genotype under drought conditions. Therefore, it is possible that JA is involved in the regulation of the synthesis of AQPs and their post-translational modifications, specifically by regulating the concentration of AQPs in their active state (phosphorylated; Azad et al. 2008) under well-watered conditions. However, we found a negative correlation between L and PIP2PH ($P < 0.005$) and PIP1 ($P < 0.02$) abundances. Therefore, protein abundance did not explain the increase of L caused by *def-1* mutation. Similarly, some studies have found a negative correlation between L and the transcript abundance of some *PIP2* genes (Sutka et al. 2011, Calvo-Polanco et al. 2016). So, different PIP subcellular localizations could also cause this negative correlation (Boursiac et al. 2005, Boursiac et al. 2008b). Thus, it is possible that the presence of higher amounts of PIP1 proteins caused retention of these proteins in internal membranes.

Our results showed an increase in gene expressions of seven out of nine *PIP* AQP genes in *def-1* plants under well-watered conditions, and their expression was further increased by AM symbiosis. These data help to understand the increase of L in *def-1* plants under well-watered conditions. However, the expression of the remaining *PIP* AQP genes did not change in *def-1* plants. In the same way, Sánchez-Romera et al. (2014) noted that not all AQPs responded in the same way to the exogenous

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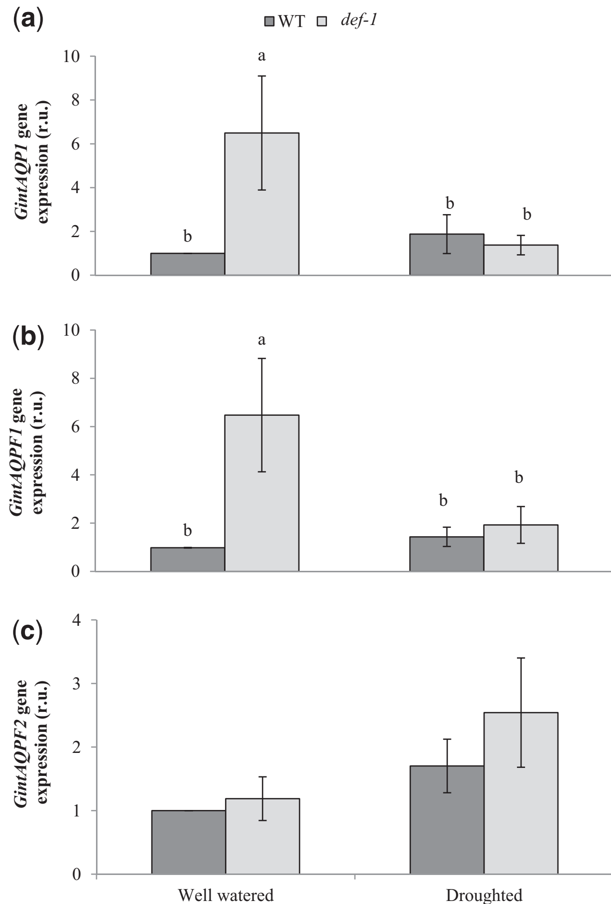

Fig. 4 Relative expression of (a) *GintAQP1*, (b) *GintAQPF1* and (c) *GintAQPF2* in WT tomato roots and *def-1* tomato roots under well-watered or drought conditions. Bars represent the mean ± SE (n = 6). Different letters indicate significant differences among treatments (P < 0.05) after ANOVA and LSD tests.

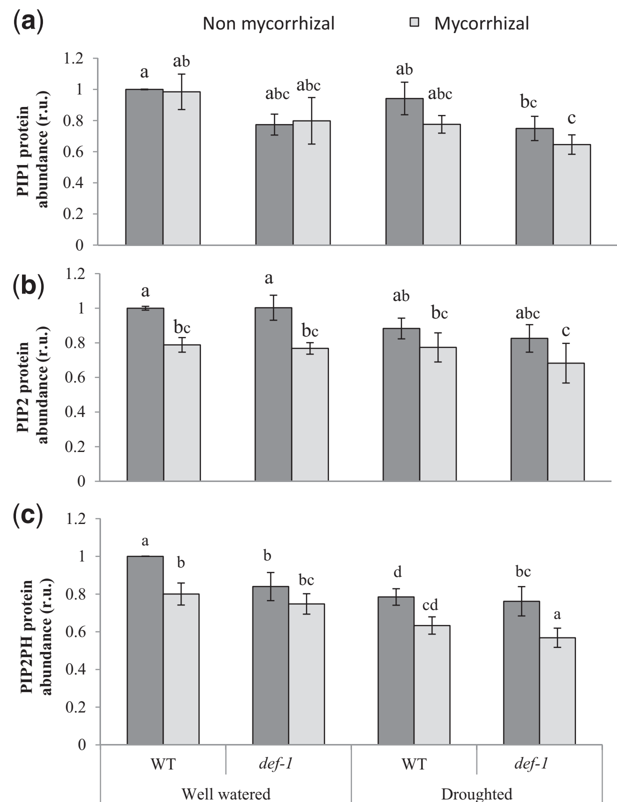

Fig. 5 Protein abundance of (a) PIP1s, (b) PIP2s and (c) PIP2 proteins phosphorylated at Ser280 (PIP2PH) in WT and *def-1* tomato roots. Plants were uninoculated (dark bars) or inoculated by *Rhizophagus irregularis* (gray bars) and were grown under well-watered or drought conditions. Bars represent the mean ± SE. Different letters indicate significant differences (P < 0.05) after ANOVA and LSD tests (n = 6).

application of MeJA. Although most AQPs decreased their expression, others increased it or did not change. Therefore, it was concluded that JA negatively regulates the expression of some AQPs and positively regulates the abundance of others. In addition, previous studies have shown that *PIP* gene expression is not always followed by an increaae in the amount of protein (Aroca et al. 2005, Marulanda et al. 2010, Muries et al. 2011).

Effects of *def-1* mutation on the response to AM symbiosis

There are several studies dealing with the involvement of JA in the establishment and development of AM symbiosis. On the one hand, there are studies where it has been observed that AM plants have a higher level of MeJA than non-AM plants (Hause et al. 2002). On the other hand, some JA-deficient mutant plants had more AM colonization (Herrera-Medina et al. 2008, Leon-Morcillo et al. 2012) while in another case these JA mutant plants showed less mycorrhizal colonization (Isayenkov et al. 2005). Consequently, according to our outcomes, we do not support any of these findings because the fungal colonization rate did not show differences between the two types of plants, nor was it affected by the water regimes used. Similarly, Gutjahr et al. (2015) did not find any difference in AM colonization rate between WT and JA-deficient mutant rice plants. Such results could be have resulted from the fact that we checked the AM colonization after 11 weeks of growth, and JA could be more involved in the initial phases of colonization (Hause et al. 2002).

More outstanding was the presence of AMF during the drought period, since both AM plants (WT and *def-1*) exhibited opposite behaviors to regulate g_s and to cope with drought conditions. Similarly, the higher water uptake capacity largely observed in AM plants (Fig. 1b; Marulanda et al. 2003, Khalvati et al. 2005, Ruth et al. 2011) was abolished in *def-1* plants. So, JA could be implicated in the water uptake capacity of AM plants since JA may also increase L (Sánchez-Romera et al. 2014).

On the other hand, we have found that the expression of *GintAQPF1* and *GintAQP1* was increased in *def-1* AM plants grown under well-watered conditions, so *def-1* could regulate its expression. In contrast, we could not observe any difference in gene expression of fungal AQPs caused by drought treatment as had been observed by T. Li et al. (2013) and El-Mesbahi et al. (2012). As happens with JA effects on AM establishment, the role of *R. irregularis* in plant AQP regulation is not clear yet due to results showing contradictory effects. Therefore, some studies found an increase in AQP gene expression in AM plants

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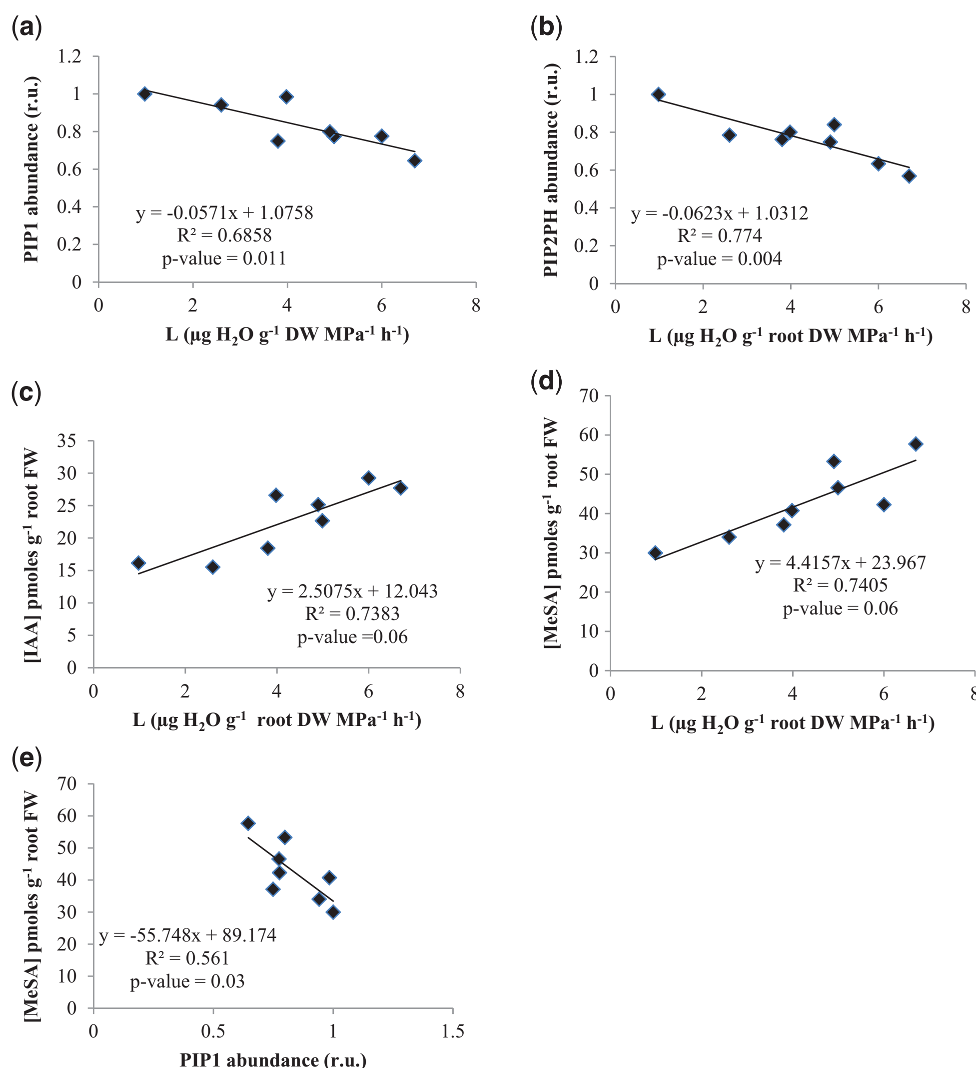

Fig. 6 Representation of a linear regression model established between different parameters. (a) PIP1 abundance–L, (b) PIP2PH abundance–L, (c) IAA content–L, (d) MeSA content–L and (e) MeSA content–PIP1 abundance.

under non-stressed conditions (Uehlein et al. 2007, Aroca et al. 2008a, Alguacil et al. 2009, El-Mesbahi et al. 2012) and other studies found opposite results (Ouziad et al. 2006, Aroca et al. 2008b, Ruiz-Lozano et al. 2009). Recently, Bárzana et al. (2014) checked the expression of 31 plant AQPs in maize roots and they observed that 13 of them were down-regulated and only three up-regulated in AM plants under well-watered conditions. In our work it was observed that seven out of nine genes increased their expression in AM *def-1* plants under well-watered conditions, indicating that *def-1* gene function could be involved in up-regulation of *PIP* genes by AM symbiosis in tomato plants. In fact, AM symbiosis did not change the expression of any *PIP* gene in WT plants, although L was increased. This L increase could be caused by the hyphal absorption of water.

Hormonal regulation by AM symbiosis

Previous studies have shown that several hormones are involved in the establishment and development of AM symbiosis. These include ABA, which is involved in development of symbiosis and in the formation of arbuscules (Herrera-Medina et al. 2007, Martin-Rodriguez et al. 2010, Martin-Rodriguez et al. 2011). However, we could not find a significant increase of ABA in AM roots. Similarly, Chitarra et al. (2016) found a decrease of ABA content1 by AM symbiosis in tomato roots subjected to water stress. In the same way, ABA is known to increase its concentration under stress conditions (De Diego et al. 2013) as in this study. Our results also confirm the interaction between JA and ABA. This interaction was observed previously in regulation of stomata (Herde et al. 1997) but it is clear in this work that *def-1* reduces the amounts of both hormones under well-watered conditions.

SA is known to have a negative effect on AM symbiosis (Blilou et al. 1999, Herrera-Medina et al. 2003, Riedel et al. 2008). Nevertheless, it has been observed that SA was accumulated in AM barley roots (Khaosaad et al. 2008). In this study, we obtained a higher SA concentration in AM roots than in non-AM roots under drought conditions. This increase could be due to a defensive response of AM plants to the stress conditions

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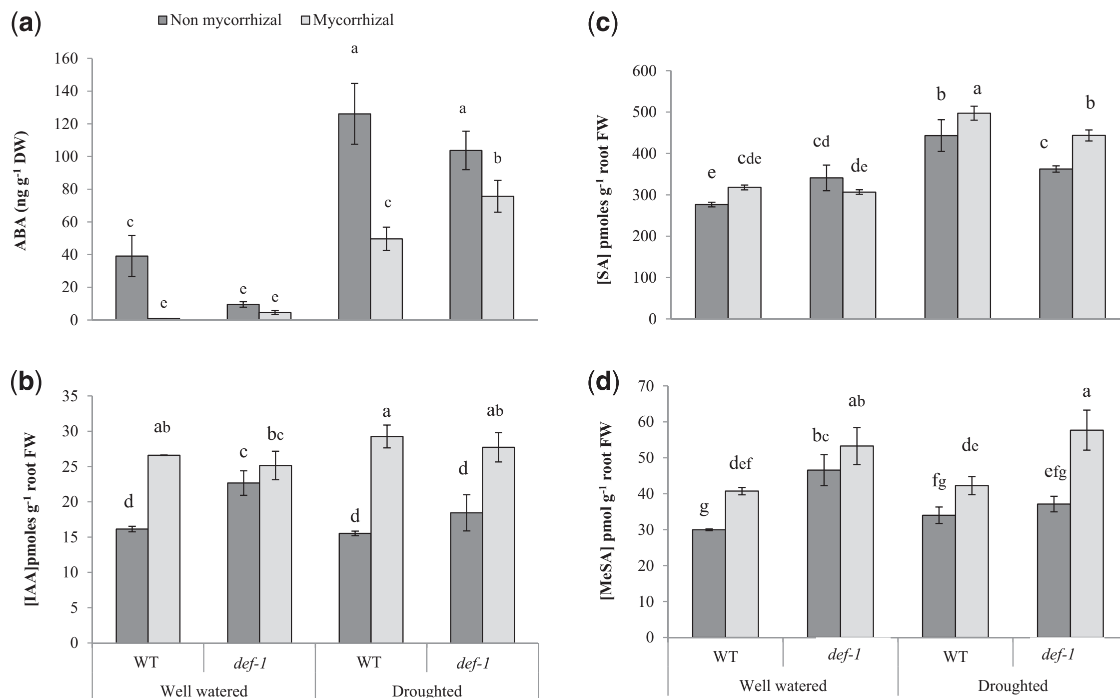
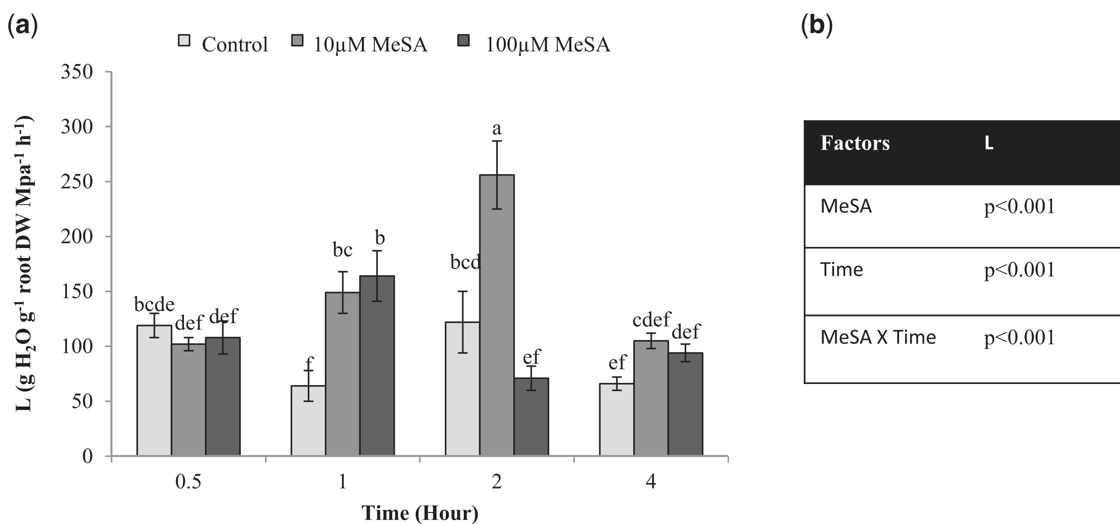


Fig. 7 Root content of (a) ABA, (b) IAA, (c) SA and (d) MeSA in WT and *def-1* plants which were uninoculated (dark bars) or inoculated with *Rhizophagus irregularis* (gray bars), and grown under well-watered or drought conditions. Bars represent the mean ± SE. Different letters indicate significant differences ($P < 0.05$) after ANOVA and LSD test ($n = 4$).



Factors	L
MeSA	p<0.001
Time	p<0.001
MeSA X Time	p<0.001

Fig. 8 (a) Root hydraulic conductivity (L) of *Solanum lycopersicum* plants. Tomato plants were subjected to 10 or 100 µM MeSA during 0.5, 1, 2 and 4 h. L was measured by the free exudation method. Bars represent the mean ± SE. Different letters indicate significant differences ($P < 0.05$) after ANOVA and LSD test ($n = 15$). (b) Multifactor ANOVA table showing the interactions between two factors (MeSA concentrations and exposure time) on L.

rather than due to its implication in symbiotic development. At the same time, higher levels of MeSA were found in AM roots than in non-AM roots. There is little information about the role of AM symbiosis in regulating MeSA levels. Schausberger et al. (2012) found that AM symbiosis diminished MeSA emission in above-ground parts of bean plants. However, Babikova et al. (2013) found that emission of MeSA by above-ground parts of non-infested bean plants only happened when they were interconnected to infested plants via AM mycelia. We tried to determine if the expression of the *SlSAMT1* gene was related to the increase of MeSA levels observed in AM roots. We failed to see such a relationship, probably because the expression of this gene was not previously checked in roots (Tieman et al. 2010). At the same time, in tomato the enzyme benzoic acid/salicylic

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acid carboxyl methyltransferase (BSMT) is also able to synthesize MeSA (Krol et al. 2015). Therefore, the enzyme BSMT could be the cause of the increase in MeSA observed in AM roots. However, its regulation is beyond the objective of the present research. There are also studies under hydroponic conditions which found that IAA reduced the expression of AQP genes and L (Peret et al. 2012). Nevertheless, our outcomes showed a positive relationship between L and IAA concentration. In addition, we observed that this positive correlation between L and IAA is due to the presence of AMF. In fact, it is known that IAA is important for AM symbiosis (Hanlon and Coenen 2011), and in our study the IAA concentration was increased in AM treatments, except in *def-1* AM plants under well-watered conditions.

In conclusion, regardless of the soil water status, the fungal colonization capacity was not affected by *def-1* mutation. Under well-watered conditions, *def-1* mutation increased L, as did AM symbiosis. The increase of L by *def-1* mutation could be caused by an increase in MeSA. Also, *def-1* mutation was involved in regulation of AQP abundance and gene expression under well-watered conditions, its effect being enhanced by AM symbiosis. However, the well-documented increase of water consumption by AM symbiosis was abolished by *def-1* mutation, this gene being fundamental for such AM effects. On the other hand, *def-1* plants under well-watered conditions behave similarly to WT AM plants, indicating that AM symbiosis effects could be mediated in part by the *def-1* gene under well-watered conditions.

Materials and Methods

Experimental design

Main experiment. Plants of *S. lycopersicum* were used in an experiment following a combined factorial design with three factors: (i) a biological factor, with plants inoculated or not with the AMF *R. irregularis* (isolate BEG 121); (ii) a genotype factor formed by WT plants (*Castle mart* genotype) and mutant plants which are deficient in JA (*def-1*) in the same background genotype as WT plants; and (iii) an abiotic factor, in which plants were grown under well-watered conditions or a water deficit period (drought). Finally, we had eight different treatments with 10 replicates in each treatment. Six replicates of each treatment were used for the measurement of L, and the remaining four replicates were frozen in liquid nitrogen immediately after harvest for later use in molecular and biochemical determinations. For all treatments, physiological measurements and the collection of plant samples were carried out 3 h after sunrise, in order to avoid diurnal fluctuations in plant processes. The experiment was repeated twice with similar results. Only the results of one experiment are shown.

Second experiment. Tomato seeds were sown in sterile vermiculite for 1 week and then seedlings were transplanted to aerated 8 liter containers filled with a 80% nutrient solution (Aroca 2006). The nutrient solution was replaced every 7 d. The plants were grown for 1 month. Measurements of L were done 0.5, 1, 2 and 4 h after MeSA (10 and 100 μM) applications. L was measured by the free exudation method (Aroca 2006).

Growth conditions

The first experiment lasted 11 weeks from seed germination and was conducted under greenhouse conditions with temperatures from 19 to 25 °C, 16/8 h light/dark, a relative humidity of 50–60% and a photosynthetic photon flux density of 800 μE m^{-2} s^{-1}, as measured with a light meter (LICOR model LI-188B). Before the beginning of drought treatment, all plants received 10 ml of 80% Hewitt's nutrient solution (Hewitt 1952). The original soil had a pH of 8.2 [measured in water 1 : 5 (w/v)], 1.5% organic matter and nutrient concentrations (g kg^{-1}) as follows: N, 1.9; P, 1.0; and K, 6.9. Soil moisture was controlled gravimetrically and water was supplied daily to maintain soil at field capacity before starting the water deficit period (drought). Ten days before harvest, half of the plants were subjected to drought (60% of field capacity) while the other half were maintained at field capacity until harvest.

Symbiotic colonization

The percentage of mycorrhizal root length colonization of nine roots of each treatment 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). The extent of mycorrhizal colonization was calculated according to the gridline intersect method (Giovannetti and Mosse 1980).

Stomatal conductance

Stomatal conductance was measured with a porometer system (Porometer AP4; Delta-T Devices Ltd.) following the manufacturer's instructions. Stomatal conductance measurements in tomato plants were taken in the apical leaflet of the last fully developed leaf from 10 different plants from each treatment. The measurements were taken 3 h after sunrise during the last 2 d before the harvest.

Daily water consumption

Daily water consumption of each plant was controlled during the last 10 d. The amount of water to be supplied to each pot per day to maintain constant the percentage of soil moisture was recorded. A gravimetric method was used. Moisture percentages were adjusted by weighing each pot and reaching the required amount of water that corresponded to that percentage [1,150 g for well-watered plants and 1,050 g for the drought-treated plants (60% of field capacity)].

Root hydraulic conductance

Root hydraulic conductance was calculated using an HPFM (Dynamax, Inc.) in the AM experiment. Detached roots were connected to the HPFM and water was pressurized into the root from 0 to 0.5 MPa in the transient mode to calculate root hydraulic conductance per plant (Kr). L was determined by dividing Kr by the root dry weight (Calvo-Polanco et al. 2014).

In the case of the hydroponic experiment, L was determined by the free exudation method (Sánchez-Romera et al. 2014). The plants were exuding for 1 h, then these exudates were collected and weighed. Also, the root dry weight of each plant was determined after incubation for 2 d at 75 °C. The osmolarity of the exuded sap was determined using a cryoscopic osmometer (Osmomat 030, Gonotec Gmbh). L was calculated as: $L = Jv/\Delta\Psi s$, where Jv is the exuded sap flow rate expressed on a root dry weight basis and $\Delta\Psi s$ is the osmotic potential gradient between the exuded sap and the solution.

Molecular determinations

Quantitative real-time PCR. Expression analyses of the selected *PIP* genes were carried out by q-RT-PCR, using an iCycler (Bio-Rad) (Benabdellah et al. 2009). Total RNA was isolated from roots by a phenol/chloroform extraction method followed by precipitation with LiCl (Kay et al. 1987). DNase treatment of total RNA and reverse transcription were done following Qiagen's protocol (Quantitect Reverse Transcription KIT Cat#205311). The PCR mix contained 1 μl of cDNA (100 ng μl^{-1}), 10.5 μl of Master Mix (Bio-Rad Laboratories S.A.), 8.6 μl of deionized water and 0.45 μl of each primer pair (1 μM). Root AQP expression were determined for nine tomato PIP genes, *SlPIP1;1* (Tigr. No.:TC175784), *SlPIP1;5* (Tigr. No. TC178447), *SlPIP1;7* (Tigr. No. TC170092), *SlPIP2;1* (Tigr. No. TC170322), SlPIP2;3 (Tigr. No. TC174068) *SlPIP2;4* (Tigr. No. TC181456), *SlPIP2;6* (Tigr. No. TC175989), *SlPIP2;8* (Tigr. No. TC180270) and *SlPIP2;9* (Tigr. No. TC173223) as described in Sade et al. (2009). The PCR program consisted of a 3 min incubation at 95°C to activate the hot-start recombinant Taq DNA polymerase, followed by 31 cycles of 30 s at 94°C, 30 s at

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annealing temperature and 30 s at 72°C. Annealing temperatures were 58°C for *SlPIP1;1*, *SlPIP1;5*, *SlPIP 1;7*, *SlPIP 2;1*, *SlPIP 2;4* and *SlPIP2;6*, 55°C for *SlPIP2;9* and 57°C for *SlPIP 2;8*.

The relative abundances of all transcripts were calculated by using the $2^{-\Delta\Delta ct}$ method (Livak and Schmittgen 2001). Expression analyses were carried out in three independent biological RNA samples with each one from a different pool of three different root samples, and were repeated twice for each RNA sample. All standardization was carried out based on the expression of the *S. lycopersicum* ubiquitin and elongation factor genes in each sample. A list of primers used is showed in **Supplementary Table S1**.

For fungal AQPs, the analyzed genes were: *GintAQP1* (accession No. FJ861239) described by Aroca et al. (2009), and *GintAQPF1* (accession No. JQ412059) and *GintAQPF2* (accession No. JQ412060) described by T. Li et al. (2013). The primers used to amplify fungal genes were described previously (Aroca et al. 2009, T. Li et al. 2013). The PCR program consisted of a 3 min incubation at 95°C to activate the hot-start recombinant Taq DNA polymerase, 40 cycles of 30 s at 94°C, followed by annealing temperature and then 30 s at 72 °C. Annealing temperatures were 60°C (30 s) for *GintAQP1*, 55°C (50 s) for *GintAQPF1* and 58 °C (30 s) for *GintAQPF2*. Standardization was carried out based on the expression of the 18S rRNA and elongation factor genes (Aroca et al. 2009, Tamayo et al. 2016).

Finally, the expression of the salicylic acid methyl transferase gene (*SlSAMT1*) was analyzed by q-RT-PCR as described above, following the protocol and primers of Tieman et al. (2010).

Microsome isolation and enzyme-linked immunosorbent Assay (ELISA). Microsomal membranes were isolated from roots as described by Sánchez-Romera et al. (2014).

Plates containing 2 µg of microsomes from tomato roots in each well were incubated overnight at 4 °C with coating buffer (0.05 M carbonate–bicarbonate, pH 9.6). The plates were then washed three times for 15 min with TTBS (Tris-buffered-saline with 0.05% Tween-20) and blocked for 1 h at room temperature with 1% bovine serum albumin (BSA) in TTBS, then washed again three times for 10 min with TTBS. After that, the plates were incubated with (1 : 200) dilutions of antibodies raised against the first 26 amino acids of the N-terminal peptide of PvPIP1;3 (accession No. DQ855475) and the last 12 amino acids of the C-terminal peptide of PvPIP2;1 (accession No. AY995195) (Marulanda et al. 2010). Also, an antibody against phosphorylated PIP2 at the C-terminal end of PvPIP2;1 (CAIKALG{pSER}FRSNA) was used. These antibodies recognize several PIPs from different plant species including tomato (Calvo-Polanco et al. 2014). To check the specificity of each PIP2 antibody, we carried out an ELISA test where we cross-reacted each one of the target peptides with each antibody. The results showed that each antibody was very specific to its target protein (Sánchez-Romera et al. 2014). For more details, see Sánchez-Romera et al. (2014).

ABA, OPDA, JA-Ile and JA hormone root content. Hormone extraction and analysis were carried out essentially as described in de Ollas et al. (2015) with slight modifications. Briefly, 0.4 g of frozen plant material was extracted in 5 ml of distilled water after spiking with 50 ng of [$^2H_6$]ABA, dihydrojasmonic acid, [$^2H_3$]*N*-[(−)-jasmonoyl]-isoleucine and [$^2H_5$]*cis*-12-OPDA (Arbona et al. 2010). After centrifugation at 4,000×*g* at 4°C, supernatants were recovered and the pH was adjusted to 3.0 with a 30% acetic acid solution. The acidified water extract was partitioned twice against 3 ml of diethyl ether. The organic layer was recovered and evaporated under vacuum in a centrifuge concentrator (Speed Vac, Jouan). The dry residue was then resuspended in a 10% MeOH solution by gentle sonication. The resulting solution was filtered through regenerated cellulose 0.22 µm membrane syringe filters (Albet S.A.) and directly injected into a UPLC system (Acquity SDS,Waters Corp.). Separations were carried out on a C18 column (Nucleodur C18, 1.8 µm particle size, 50×2.1 mm, Macherey-Nagel) using a MeOH : H_2O (both supplemented with 0.1% acetic acid) gradient at a flow rate of 300 µl min^{-1}. Hormones were quantified with a Quattro LC triple quadrupole mass spectrometer (TQD, Micromass) interfaced to the liquid chromatograph through an orthogonal Z-spray electrospray ion source. Three biological and two technical replicates were performed for each sample.

Root IAA accumulation

IAA root concentrations in tomato plants were determined as explained by Bacaicoa et al. (2011). The concentration of IAA was analyzed in root extracts using HPLC–electrospray ionization-mass spectrometry (HPLC-ESI-MS/MS). The extraction and purification of IAA were carried out using the method described by Dobrev and Kamínek (2002), with some variations. Frozen plant tissue (0.5 g), previously ground to a powder in a mortar with liquid nitrogen, was homogenized with 5 ml of pre-cooled (−20°C) methanol : water (80 : 20, v/v) and 2.5 mM Na diethyldithiocarbamate (DDTC). The deuterium-labeled internal standard {[$^2H_5$]IAA (D-IAA)} was added (100 µl of a stock solution of 400 ng ml^{-1} in methanol) to the extraction medium. After overnight extraction at −20°C, solids were separated by centrifugation at 12,000×*g* for 10 min at 4°C using a Centrikon T-124 centrifuge with an A8.24 rotor (Kontron Instruments) and re-extracted for 1 h with an additional 4 ml of extraction mixture. Supernatants were passed through a Strata C18-E cartridge (3 cm^3, 200 mg) (Phenomenex; Ref. 8B-S001-FBJ), pre-conditioned with 4 ml of methanol followed by 2 ml of extraction medium. After evaporation at 40°C using a Labconco Vortex Evaporator (Labconco Co.), 0.5 ml of 1 M formic acid was added. Then, IAA was extracted successively with two portions of 5 and 4 ml of diethyl ether, and the organic phase was evaporated to dryness. The residue was re-dissolved in 250 µl of methanol : 0.5% acetic acid (40 : 60, v/v). Before the injection in the HPLC-ESI-MS/MS system, the solution was centrifuged at 8,000×*g* for 5 min. IAA was quantified by HPLC-ESI-MS/MS using a high-performance liquid chromatograph (2795 Alliance HT; Waters Co.) coupled to a 3200 Q TRAP LC/MS/MS System (Applied Biosystems/MDS Sciex), equipped with an electrospray interface. A reverse-phase column (Synergi 4 µm Hydro-RP 80A, 150×2 mm; Phenomenex) was used. A linear gradient of methanol and 0.5% acetic acid in water was used: 35% A for 1 min, 35% to 95% A in 9 min, 95% A for 4 min and 95% to 35% A in 1 min, followed by a stabilization time of 5 min. The flow rate was 0.20 ml min^{-1}, the injection volume was 40 µl and the column and sample temperatures were 30 and 20°C, respectively. The detection and quantification of IAA were carried out using multiple reaction monitoring (MRM) in the negative-ion mode, employing multilevel calibration curves with deuterated hormones as internal standards. The source parameters are: curtain gas, 172.37 kPa; GS1, 310.26 kPa; GS2, 413.69 kPa; ion spray voltage, 4,000 V; and temperature, 600°C. Data samples were processed using Analyst 1.4.2 Software from Applied Biosystems/MDS Sciex.

Root MeSA and SA accumulation

SA root concentrations in tomato plants were determined as detailed by Sánchez-Romera et al. (2016). Briefly, 0.2 g of frozen plant tissue (previously ground to a powder in a mortar with liquid nitrogen) was homogenized with 2 ml of MeOH/H_2O/HCOOH (90 : 9 / 1, by vol.). The deuterium-labeled internal standard for each hormone (D-MeSA and D-SA) was added (50 µl of a stock solution of 1,000 ng ml^{-1} in methanol) to the extraction medium. After 1 h of mixing the samples at 2,000 r.p.m. using a Multi Reax shaker (Heidolph), the solids were separated by centrifugation at 12,000×*g* for 15 min. A 0.5 ml aliquot of the supernatant was separated and 0.3 ml of 0.2% acetic acid was added. Before injection in the HPLC-ESI-MS/MS system, the samples were centrifuged at 12,000×*g* for 10 min and the supernatants were introduced into chromatographic vials. The hormones were quantified by HPLC (2795 Alliance HT; Waters) linked to a 3.200 Q TRAP LC/MS/MS system (Applied Biosystems/MDS Sciex), equipped with an electrospray interface, using a reverse-phase column (Synergi 4 µm Hidro-RP 80A, 150×2 mm; Phenomenex). A linear gradient of methanol and 0.2% acetic acid in water was used: 60% A for 3 min, 60% A to 85% A in 9 min, 85% A for 1 min and 85% A to 60% A for 1 min, followed by a stabilization time of 4 min. The flow rate was 0.2 ml min^{-1}, the injection volume was 40 µl and the column and sample temperatures were 30 and 20°C, respectively. The detection and quantification of MeSA were performed by MRM in the positive-ion mode, employing a multilevel calibration graph with deuterated hormones as internal standard. MeSA source parameters were: curtain gas, 20 p.s.i; GS1, 45 p.s.i; GS2, 50 p.s.i; ion spray voltage, 5,000 V; and temperature 500°C. SA detection and quantification were performed by MRM in the negative-ion mode, employing a multilevel calibration graph with deuterated hormones as internal standards. SA source parameters were: curtain gas, 20 p.s.i; GS1, 45 p.s.i; GS2, 50 p.s.i; ion spray voltage, 4,000 V; and temperature,

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500°C. Data samples were processed using Analyst 1.4.2. (Applied Biosystems/MDS Sciex).

Statistical analysis

Experimental data were subjected to multifactorial ANOVA with three factors (biological, genotype and abiotic factors). Post-hoc comparisons with least significant difference (LSD) tests were used to investigate differences between groups. Also, we performed Pearson correlations of all parameters measured checking all possible combinations in order to determine new relationships among factors.

Supplementary data

Supplementary data are available at PCP online.

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Disclosures

The authors have no conflicts of interest to declare.

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