Insights on the Impact of Arbuscular Mycorrhizal Symbiosis on Tomato Tolerance to Water Stress[1][OPEN]

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Arbuscular mycorrhizal (AM) fungi, which form symbioses with the roots of the most important crop species, are usually considered biofertilizers, whose exploitation could represent a promising avenue for the development in the future of a more sustainable next-generation agriculture. The best understood function in symbiosis is an improvement in plant mineral nutrient acquisition, as exchange for carbon compounds derived from the photosynthetic process: this can enhance host growth and tolerance to environmental stresses, such as water stress (WS). However, physiological and molecular mechanisms occurring in arbuscular mycorrhiza-colonized plants and directly involved in the mitigation of WS effects need to be further investigated. The main goal of this work is to verify the potential impact of AM symbiosis on the plant response to WS. To this aim, the effect of two AM fungi (*Funneliformis mosseae* and *Rhizophagus intraradices*) on tomato (*Solanum lycopersicum*) under the WS condition was studied. A combined approach, involving ecophysiological, morphometric, biochemical, and molecular analyses, has been used to highlight the mechanisms involved in plant response to WS during AM symbiosis. Gene expression analyses focused on a set of target genes putatively involved in the plant response to drought, and in parallel, we considered the expression changes induced by the imposed stress on a group of fungal genes playing a key role in the water-transport process. Taken together, the results show that AM symbiosis positively affects the tolerance to WS in tomato, with a different plant response depending on the AM fungi species involved.

Climate change is expected to cause more frequent and severe drought in large areas of the planet (Sheffield et al., 2012). Water deficiency has a negative impact on plant growth and productivity, and research efforts are aimed at developing strategies to make agriculture more resilient and to mitigate the effects on crop yield (e.g. through the selection of crop varieties adapted to drought and by improving soil management and irrigation techniques; Campbell, 2012). Under water deficit, plants adapt through physiological responses regulated by changes in gene expression (Ito et al., 2006) and tend to control water status by adjusting root hydraulic conductivity (Parent et al., 2009). A large number of plant stress-responsive genes were identified recently by transcriptomic and proteomic approaches (Hirayama and Shinozaki, 2010; Deshmukh et al., 2014; Liu et al., 2015a). In addition, the role of root-associated microbial communities able to improve plant drought tolerance has been explored in recent years (Marasco et al., 2012; Coleman-Derr and Tringe, 2014, and refs. therein; Nadeem et al., 2014; Timmusk et al., 2014; Rolli et al., 2015). Arbuscular mycorrhizal (AM) fungi form symbioses with the roots of the most important crop species and enhance the mineral nutrition of more than 80% of terrestrial plant species, in exchange for the uptake of carbon compounds derived from the photosynthetic process (Bucher et al., 2014). AM symbiosis is known to improve plant performance under various environmental stresses, such as drought, and to change plant water relations in both well-watered and water-stressed

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conditions (Augé et al., 2001; Ruiz-Lozano, 2003; Ruíz-Lozano and Aroca, 2010). An increased productivity of AM plants in soils under drought also has been reported (Habibzadeh, 2015). The thin AM fungal hyphae can explore soil pores inaccessible to root hairs, thus reaching water sources not available to non-AM plants. Furthermore, hyphal water transport to the root under drought conditions was demonstrated previously (Khalvati et al., 2005). AM fungi, which facilitate the formation of water-stable aggregates, also can influence soil moisture retention properties (Augé et al., 2001).

In addition to an increase in nutrient and water uptake, different mechanisms have been proposed to be involved in the mitigation of drought stress in mycorrhizal plants; as an example, improved stomatal regulation (for review, see Augé et al., 2015) could enhance water use efficiency and reduce oxidative damage (Ruiz-Lozano, 2003; Wu et al., 2006). Saia et al. (2014) recently demonstrated that, in field conditions, berseem clover (*Trifolium alexandrinum*) AM plants subjected to water deficit can better tolerate the negative impact of drought stress on plant growth in combination with a stimulated N_2 fixation. A regulation of the root hydraulic properties during AM symbiosis also has been reported (Ruiz-Lozano et al., 2009; Bárzana et al., 2012; Sánchez-Romera et al., 2015). Bárzana et al. (2012) demonstrated an ability of AM plants to modulate the switch from apoplastic water flow to a cell-to-cell pathway (which involves the transport of water across membranes and the regulation of aquaporin genes), suggesting that AM fungal colonization may allow plants to better respond to water demands from the shoot upon water stress (WS) conditions. However, previous work suggested that the effects of the AM symbiosis on plant water relations are environment and symbiont specific (for review, see Augé, 2001; Augé et al., 2015). Although the role of AM fungi in plant WS tolerance has been well reported for several plant-AM fungus combinations, the underlying molecular mechanisms are still to be elucidated. The regulation of several genes putatively involved in the response to WS, such as aquaporin genes (Porcel et al., 2006), a gene (*P5CS*) encoding a rate-limiting enzyme of Pro synthesis (Porcel et al., 2004), and a gene (*NCED*) encoding a key enzyme in the abscisic acid (ABA) biosynthesis pathway (Aroca et al., 2008), has already been reported for AM-colonized plants under drought conditions. A potential water transport via AM fungus to the host plant has been suggested on the basis of the gene expression profiles for two functionally characterized fungal aquaporin genes, thus supporting the existence of a direct AM fungus involvement in plant tolerance to drought (Li et al., 2013a, 2013b).

In this study, we analyzed the impact of AM symbiosis on the response to water deficit in tomato (*Solanum lycopersicum*). Two different AM fungi (*Rhizophagus intraradices* and *Funneliformis mosseae*) have been used to verify the species specificity of this interaction. The plant genotype selected for this research was cv San Marzano nano (a cultivar with a determined growth rate), which is economically important for fresh consumption or for canning (http://www.consorziopomodorosanmarzanodop.it/index.php?option=com_content&view=article&id=83&Itemid=74&lang=en), whose aboveground and belowground interactions were investigated recently (Digilio et al., 2012; Battaglia et al., 2013; Cascone et al., 2014). Here, a multidisciplinary approach, involving ecophysiological, morphometric, biochemical, and molecular analyses, was used to verify the plant response to WS during AM symbiosis. Overall, the experimental results show that AM symbiosis positively affects tolerance to WS in tomato and that one of the two tested AM fungi seems to be more efficient in improving the tolerance to WS in this tomato cultivar.

RESULTS AND DISCUSSION

Morphometric Data on Plant Biomass and AM Root Colonization

The impact of a severe WS treatment on tomato growth was verified in the absence or presence of the AM fungus, evaluating stem height and biomass production (in term of fresh weight) in the six considered conditions: AM−, not stressed (NS) plants; AM−, WS plants; AM+, *R. intraradices*-colonized not stressed (RiNS) plants; AM+, *R. intraradices*-colonized water-stressed (RiWS) plants; AM+, *F. mosseae*-colonized not stressed (FmNS) plants; and AM+, *F. mosseae*-colonized water-stressed (FmWS) plants. The beneficial effects of AM symbiosis on plant growth parameters and biomass production under WS conditions have already been reported for several plant species (Augé, 2001; Saia et al., 2014; Wang et al., 2014). In our experiments, under irrigated NS conditions, significantly higher values were detected for AM-inoculated plants than for control plants in the case of root fresh weight of *F. mosseae*-colonized and shoot fresh weight of *R. intraradices*-colonized plants. Significant differences ($P < 0.05$) also were found between plants inoculated with the two different fungi, with greater height and shoot fresh weight values for the plants inoculated with *R. intraradices*. Under WS conditions, greater values of plant height and fresh weight were observed in AM+ plants than in control conditions, with the only exception of plant height in FmNS plants. Indeed, this trend was particularly observed in *R. intraradices*-colonized plants, under both NS and WS conditions, although these results were not always statistically significant (Table I). In agreement with previous studies (Bárzana et al., 2012; Hazzoumi et al., 2015), root colonization seemed not to be strongly affected by WS independently of the AM fungal species considered here. The proportion of AM root colonization was greater for *R. intraradices*, while the percentage of arbuscules in the colonized portion was significantly greater for *F. mosseae*, both in NS and WS conditions, suggesting a different colonization strategy adopted by the two fungi. In addition, a statistically significant difference was present in the percentage of arbuscules under WS for both fungi

Table I. *Plant biomass data in control and AM+ tomato plants under NS and WS conditions*

Parameters are expressed as mean values of five replicates ± SD. ANOVA was performed comparing all six treatments, and values with different letters in the same column differ significantly following Tukey's HSD test ($P < 0.05$).

Condition	Treatment	Height	Root Fresh Weight	Aerial Part Fresh Weight
		cm	*g*	*g*
NS	Control	22.00 ± 4.20 a,b	2.22 ± 0.38 a,b	6.15 ± 0.88 a,b
	R. intraradices	26.25 ± 6.38 b	4.15 ± 1.35 b,c	9.04 ± 3.02 c
	F. mosseae	19.50 ± 3.87 a	4.66 ± 2.52 c	7.00 ± 0.94 b
WS	Control	18.80 ± 0.84 a	1.30 ± 0.25 a	4.21 ± 0.73 a
	R. intraradices	21.30 ± 6.86 a,b	0.80 ± 0.19 a	4.75 ± 1.30 a
	F. mosseae	21.42 ± 4.62 a,b	1.28 ± 0.31 a	5.22 ± 1.13 a,b

(Supplemental Fig. S1). No root colonization was observed in control plants, regardless of the growth condition.

Impact of Mycorrhizal Symbiosis on Tomato Physiological Performance

The experimental approach used in this study allowed us to follow the dynamics of physiological parameters until a severe WS level was reached in tomato plants, with leaf water potential (Ψleaf) and gas exchange (g_s) values comparable with those reported by Secchi et al. (2012). Both parameters, as well as the chlorophyll content index (CCI), were always measured at the same time (10 AM to 1 PM) for the whole duration of the experiment, from the moment of treatment imposition (0 d after treatment [DAT]) until severe stress conditions were seen (16 or 20 DAT). No significant variations were observed in Ψ_{leaf} values (from −0.2 to −0.4 MPa) of control and AM+ tomato plants maintained in NS conditions during the whole experimental period (Fig. 1A). Under the WS condition, Ψ_{leaf} showed a decreasing trend without significant differences among all conditions until 11 DAT, while later on, Ψ_{leaf} values decreased progressively in AM conditions, with significant differences with respect to control plants, reaching severe WS levels at 16 DAT, with values of about −1.3 MPa (Fig. 1A). Conversely, in control plants, Ψ_{leaf} values were higher until 16 DAT, while later they decreased steeply, and AM plants reached severe WS status (about −1.3 MPa) about 4 d after AM+ plants (20 DAT). This different timing in the achievement of severe WS status can be explained

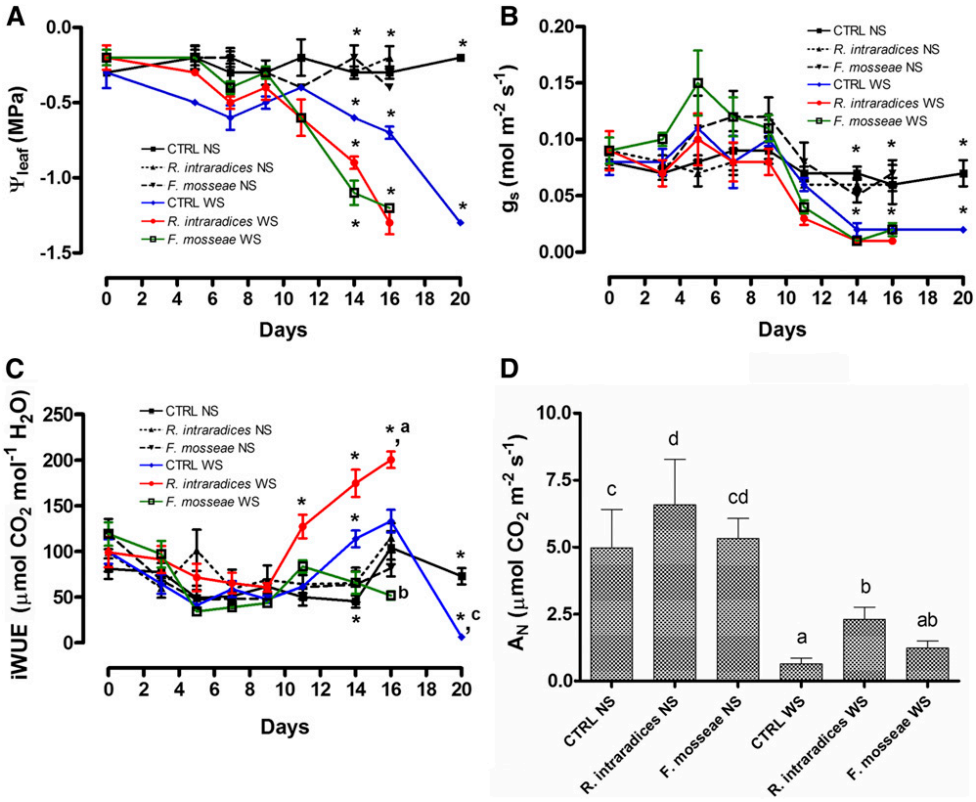

Figure 1. Trends of Ψ_{leaf} and g_s in AM− and AM+ plants subjected to WS. A to C, Dynamic changes in Ψ_{leaf} (MPa; A), g_s (mol water m^{-2} s^{-1}; B), and iWUE (μmol CO_2 mol^{-1} water; C) levels were assessed in AM− (control [CTRL]) and AM+ (*R. intraradices* and *F. mosseae*) plants during the treatment period. D, A_N (μmol CO_2 m^{-2} s^{-1}) was measured at the end of the experiment in AM− and AM+ plants either irrigated (NS) or subjected to WS. Asterisks and lowercase letters denote significant differences attested by Tukey's honestly significant difference (HSD) test ($P < 0.05$) for each experimental day, except for 20 DAT (control NS and WS), for which we used the two-tailed Student's *t* test. Data are expressed as means ± SE ($n = 5$).

by the presence of AM fungi that, in a limited environment such as a pot system, have to share the same source of water and nutrients with the host plant, since they have no access to other sources as in open-field conditions. In addition, in potted inoculated plants, AM-colonized roots can reach a maximum level of exploration of the substrate earlier than noninoculated plants (Berruti et al., 2016). Differences from previous literature data, showing that Ψ_{leaf} either did not differ between AM and noncolonized plants under drought (Augé et al., 2001) or was higher (less negative) in the presence of the AM fungus (Porcel et al., 2004; Khalvati et al., 2005; Asrar et al., 2012), could be due to diverse experimental designs, growth conditions, and plant/fungus combinations. Indeed, it was reported recently that the cooperation in AM interactions is related to the partners involved in the symbiosis and depends on several factors, including environmental conditions, the acquisition of surplus resources, and functional diversity (Walder and van der Heijden, 2015).

Considering g_s, a relatively constant trend was observed in NS conditions as well as for the intrinsic water use efficiency (iWUE) index (Fig. 1C). By contrast, a progressively decreasing trend in g_s levels was measured in WS plants after 9 DAT (Fig. 1B). Since water use efficiency is often considered a variable parameter in AM plants exposed to WS (Asrar et al., 2012; Doubková et al., 2013), to overcome this constraint and obtain a more precise indication of stress effects, we chose to analyze the iWUE, as reported by Schultz and Stoll (2010). During the whole duration of WS treatment, iWUE in RiWS plants followed a progressively increasing trend statistically different from that recorded for control and FmWS plants. Specifically, iWUE showed an increasing trend in *R. intraradices*-colonized plants from 11 DAT up to the severe WS level status (at 16 DAT), with values significantly greater than those measured in either *F. mosseae*-colonized and control plants (Fig. 1C). In *F. mosseae*-colonized plants, iWUE decreased slightly over time up to 16 DAT, when it reached values significantly lower than in control and *R. intraradices*-colonized plants. Finally, iWUE in control plants followed an increasing trend up to 16 DAT (Fig. 1C), when Ψ_{leaf} levels were higher (−0.7 MPa) than those registered in samples from AM+ plants (−1.3 MPa; Fig. 1A). Later on, when a severe WS stress was finally achieved at 20 DAT, control plants showed a strong decrease in iWUE values, significantly lower than in AM conditions (16 DAT; Fig. 1C). The iWUE trends observed at the end of the experimental period were in accordance with net photosynthetic rate (AN) values. AM+ tomato plants showed a significantly greater A_N not only upon drought but also under irrigation (NS), especially in the case of *R. intraradices*-colonized plants (Fig. 1D). Measurements of the CCI in NS plants, at the end of the experiment, showed a significant difference between the two AM fungi, since CCI values were higher in RiNS plants than in FmNS plants and slightly higher than in control NS plants (Supplemental Fig. S2). The results obtained from Ψ_{leaf} and g_s measurements showed the positive effect of AM fungal symbiosis on these parameters in NS plants. In addition, significant differences could be seen in terms of either the trends (e.g. Ψ_{leaf} and g_s) or the extent (CCI) of these parameters in the presence of a specific AM fungal species, thus confirming that the plant response also depends on the plant/AM fungal species association, as suggested previously by Augé et al. (2015). Under WS conditions, an increased PSII efficiency promoted by AM symbiosis was reported in stressed plants (Bárzana et al., 2012; Ruiz-Lozano et al., 2016), while Hazzoumi et al. (2015) demonstrated an increased chlorophyll pigment level in basil (*Ocimum gratissimum*) colonized by an AM fungus. The positive impact of AM symbiosis on WS tolerance was further supported by the A_N and, consequently, iWUE values obtained in this work. Although the experimental condition (laboratory and pots) could be a limiting factor, our data suggest that *R. intraradices* is a better candidate for improving tomato tolerance upon water shortage conditions, at least for the cultivar considered in this study.

Stomatal Density and Expression of Genes Involved in Stomatal Development

In addition to the regulation of opening/closing of the stomata, plants may control their g_s rates by varying stomatal density when new leaves are produced (Casson and Gray, 2008). Several environmental stimuli (e.g. light, CO_2, and soil temperature) have been reported to affect stomatal density and size (Casson and Hetherington, 2010; Rogiers et al., 2011). Here, we determined the number of stomata in mature leaves from AM+ and control NS plants, demonstrating that mycorrhizal symbiosis can influence this parameter, especially in *R. intraradices*-inoculated plants. Stomatal density in this condition was almost twice that of control and *F. mosseae*-inoculated plants (Fig. 2, A and B). Greater stomatal density was observed in grapevine (*Vitis vinifera*) plants infected with the *Grapevine rupestris stem pitting associated virus* (GRSPaV) than in GRSPaV-free plants (Pantaleo et al., 2016). Interestingly, the GRSPaV infection can modify the ecophysiological parameters of infected plants and the adaptation to WS through regulating the expression of several microRNAs. Since a high stomatal density can increase plant capacity to absorb CO_2, the impact of AM symbiosis on this parameter is further confirmed by the statistically greater A_N rates recorded here in AM+ plants under both NS and WS conditions at the end of the experiment. Moreover, A_N data also are correlated directly with iWUE values calculated in the same plants. We also evaluated the same plants for the expression of the tomato genes homologous to those involved in the regulation of stomatal development in Arabidopsis: *LeEPFL9*, also known as *STOMAGEN* (Sugano et al., 2010), and genes encoding EPF1 and EPF2, two intercellular signaling factors that function as antagonists of *LeEPFL9*, since they act as negative regulators of stomatal development (Lee et al., 2015).

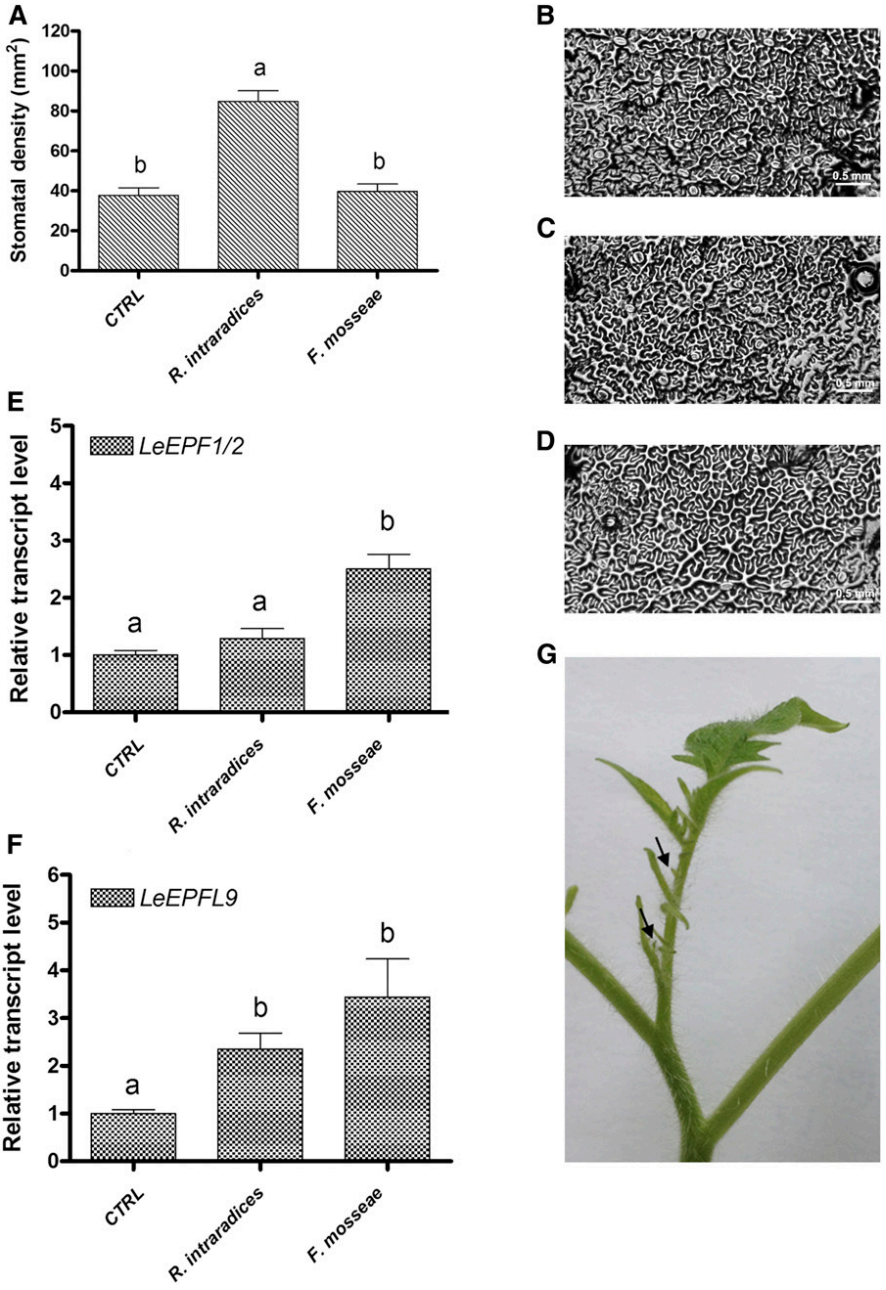

Figure 2. Stomatal density and gene expression analysis in developing leaves collected from irrigated (NS) plants. A, Values of stomatal density in control (CTRL) and AM fungi-colonized plants ($n = 5$). B, Microscope image of stomata present on a leaf imprint obtained from an *R. intraradices*-colonized tomato. C, Microscope image of stomata present on a leaf imprint obtained from an *F. mosseae*-colonized tomato. D, Microscope image of stomata present on a leaf imprint obtained from a control tomato. E and F, Quantitative real-time PCR (RT-qPCR) quantification (values represent means of three biological replicates) of *LeEPFL9* (E) and *LeEPF1/2* (F) transcriptional rates in developing leaves of control and AM fungi-colonized plants. G, New developing leaves in tomato plantlets. Arrows show examples of developing leaves (less than 5 mm, about 1 week from emergence) collected for RNA extraction. Lowercase letters above the bars denote significant differences attested by Tukey's HSD test ($P < 0.05$). Data are expressed as means ± SE.

In the developing leaves of tomato, these genes were modulated significantly only in the presence of AM symbiosis (Fig. 2, C and D). *LeEPFL9* transcript levels were well correlated with the increased stomatal density of *R. intraradices*-colonized plants, since they underwent a significant activation in comparison with control plants, while *LeEPF1/2* expression levels did not change. Conversely, in *F. mosseae*-colonized plants, where *LeEPFL9* expression was slightly higher than in *R. intraradices*-colonized plants, *LeEPF1/2* transcripts increased significantly more than in both control and *R. intraradices*-colonized plants. Although a functional demonstration is not provided here, these transcriptional results, together with the physiological data discussed above, could support a diverse regulation of stomata development promoted by a specific AM fungus.

ABA Content and the Expression of ABA-Responsive Genes

To complete the physiological characterization of the mycorrhizal tomato response to drought, the concentration of active ABA was quantified. The plant hormone ABA is a chemical signal involved in the plant response to various environmental stresses. Under salinity and WS conditions, the production of ABA induces

stomatal closure, limiting water loss (Lim et al., 2015a). A role for ABA in the mechanisms by which AM symbiosis influences stomata conductance under drought stress was suggested recently (Ludwig-Müller, 2010; Ruiz-Lozano et al., 2016). In our work, in the roots of irrigated plants, active ABA was always below the detection limit independently of the time of the experiment and the presence of AM fungi (Fig. 3A). Under severe WS conditions (−1.3 MPa), tomato AM+ plants showed significant lower levels of ABA in roots compared with control plants, in agreement with what was reported for *Knautia arvensis* (Doubková et al., 2013). ABA concentration also was higher in FmWS than in RiWS plants (Fig. 3A). A similar situation was observed in the leaves (Fig. 3B), where ABA increased significantly only in WS samples, particularly in control plants, reaching values up to 4 times higher than AM+ plants. Also in the case of leaves, ABA concentration was slightly higher in FmWS than in RiWS plants (Fig. 3B). It has been suggested that nonmycorrhizal plants probably faced more intense drought stress than mycorrhizal plants and produce/accumulate more ABA. Additionally, ABA has been shown to regulate AM fungal colonization in tomato (Herrera-Medina et al., 2007; Charpentier et al., 2014).

To better understand the role of ABA in our conditions, the expression of ABA-related genes was analyzed. The expression of the ABA biosynthetic gene *LeNCED1* was low and constant in the root of NS plants (Fig. 3C), without significant changes due to the presence of AM symbionts. Under WS, a strong increase in the transcription of this gene was observed in control plants, while in AM-colonized plants, *LeNCED1* was only up-regulated in FmWS plants, whereas it did not undergo significant expression changes in roots from the RiWS plants (Fig. 3C). *LeNCED1* expression correlated with ABA contents measured in the root of control and AM+ plants. Ruiz-Lozano et al. (2016) analyzed the expression of the same gene, showing a lack of regulation by drought regardless of the AM colonization, a discrepancy that could be due to the different system and experimental conditions adopted. In tomato leaves, the same gene had a similar expression pattern in well-watered conditions, but it underwent a different trend upon drought (Fig. 3D). Although slightly induced in CTRL leaves, the gene was significantly up-regulated in AM+ samples, especially in FmWS plants (Fig. 3D). However, the expression pattern of *LeNCED1* did not mirror the ABA content measured in leaves. This point could be further investigated, considering that it was suggested recently that, in well-watered conditions, the ABA synthesized in the leaves can be transported to the roots, where it might act as a signal to promote root growth (McAdam et al., 2016). To further investigate the molecular differences occurring in AM+ and control samples upon the different conditions, other stress-responsive (*LeTAS14*, a dehydrin [DHN]-encoding gene) and ABA-responsive (*LePYL9*, *LePP2C*, and *LeSnR2K*, encoding one of the PYR-like ABA receptors, a phosphatase 2C protein, and a Ser/Thr kinase protein, respectively) genes were analyzed in tomato leaves (Fig. 4).

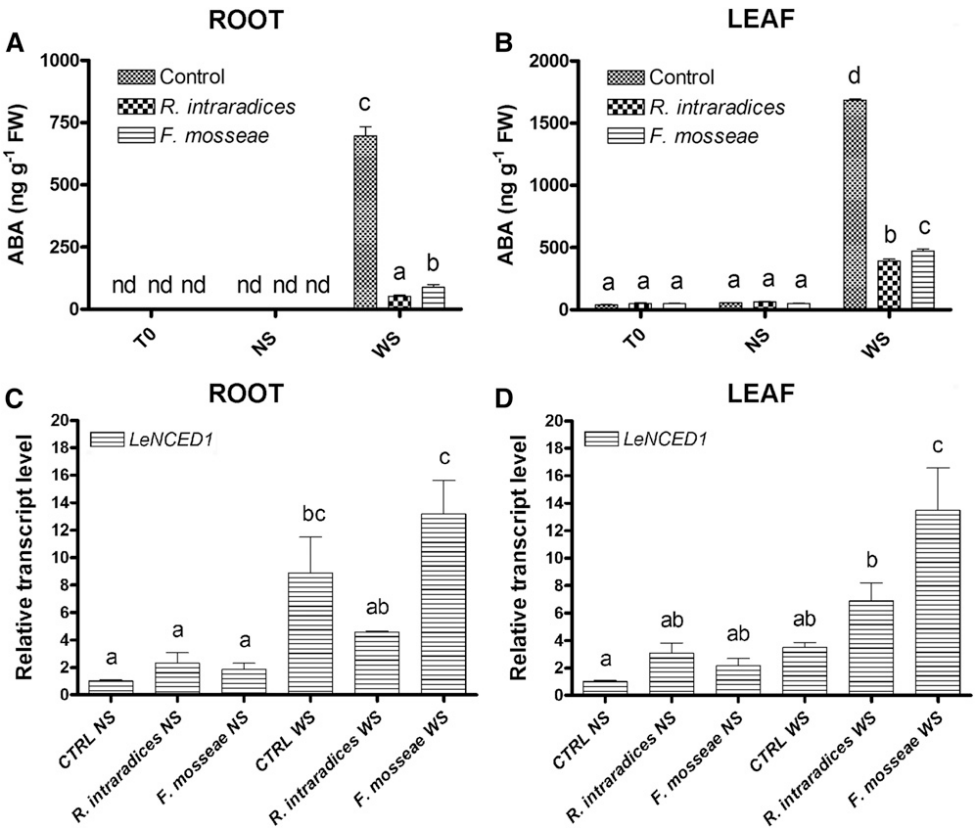

Figure 3. Analysis of endogenous ABA levels. A and B, Measurements of root (A) and leaf (B) active ABA content at the beginning (0 DAT; T0) and end of the experiment (NS and WS at 20 DAT) in AM− (Control) and AM+ (*R. intraradices* and *F. mosseae*) tomatoes. FW, Fresh weight; nd, not detected. C and D, Expression changes of the ABA biosynthetic gene *LeNCED1* in AM− and AM+ plants under NS and WS conditions in root (C) and leaf (D) samples. CTRL, Control. Lowercase letters above the bars denote significant differences attested by Tukey's HSD test ($P < 0.05$). Data are expressed as means ± SE ($n = 3$).

The transcriptional levels of *LeTAS14*, which encodes a DHN that accumulates in response to mannitol, NaCl, or ABA (Sacco et al., 2013), underwent significant increases only when drought occurred and expression levels were much higher in AM+ plants (Fig. 4A). DHNs accumulate to high amounts in vegetative tissues when plants are exposed to various stresses related to cellular dehydration (e.g. drought, osmotic stress, salinity, and temperature), and a positive correlation between DHN gene expression or protein accumulation and plant stress tolerance was reported (Hanin et al., 2011).

Among the genes strictly related to ABA signaling mechanisms, *LePYL9* encodes one of the components of the ABA receptor family in tomato, recently characterized by González-Guzmán et al. (2014). Its gene sequence was identical to that of the *LePYL1* gene, previously shown to have a higher transcriptional level in the presence of exogenous ABA treatment and dehydration with respect to other PYR-like receptors in tomato (Sun et al., 2011). However, in our experiments, *LePYL9* transcripts did not show any specific regulation upon WS in target plants independently of the presence of the AM fungus (Fig. 4B). In parallel, a significant down-regulation of *LeSNR2K2;4*, encoding a protein kinase of the tomato SnRK2 family, typically involved in the ABA signaling transduction core pathway (Sun et al., 2011), was observed in the presence of the fungus both in NS and WS conditions, where transcript levels were comparable with each other, as well as in control plants under WS (Fig. 4C). *LePP2C1* was the only component of this group of genes involved in the ABA signaling cascade that was overexpressed upon WS, but only in the leaves of control plants (Fig. 4D). The high ABA content in the leaves of control plants could serve as a triggering signal to induce a negative feedback regulation of the ABA perception components (PYL9 and SnRK2;4) by promoting the activation of PP2C enzymes. These enzymes are known to block the intracellular ABA signaling response by inhibiting SnRK2 kinases (Umezawa et al., 2009). On the contrary, in AM+ plants upon both NS and WS conditions, and in control plants upon NS conditions, no significant differences in endogenous ABA levels were detected, a result supported by the absence of transcriptional changes in the selected genes of the ABA core signaling pathway. In woody plants, such as grapevine, it has been demonstrated that stomatal closure can be driven both by active (ABA-mediated) and/or passive (hydraulics-mediated) mechanisms (Tombesi et al., 2015). Our results, showing that ABA concentrations remain low in AM+ plants, raise the question of whether an involvement of hydraulics-mediated mechanisms in the regulation of stomatal closing under WS could be proposed for mycorrhizal tomato too.

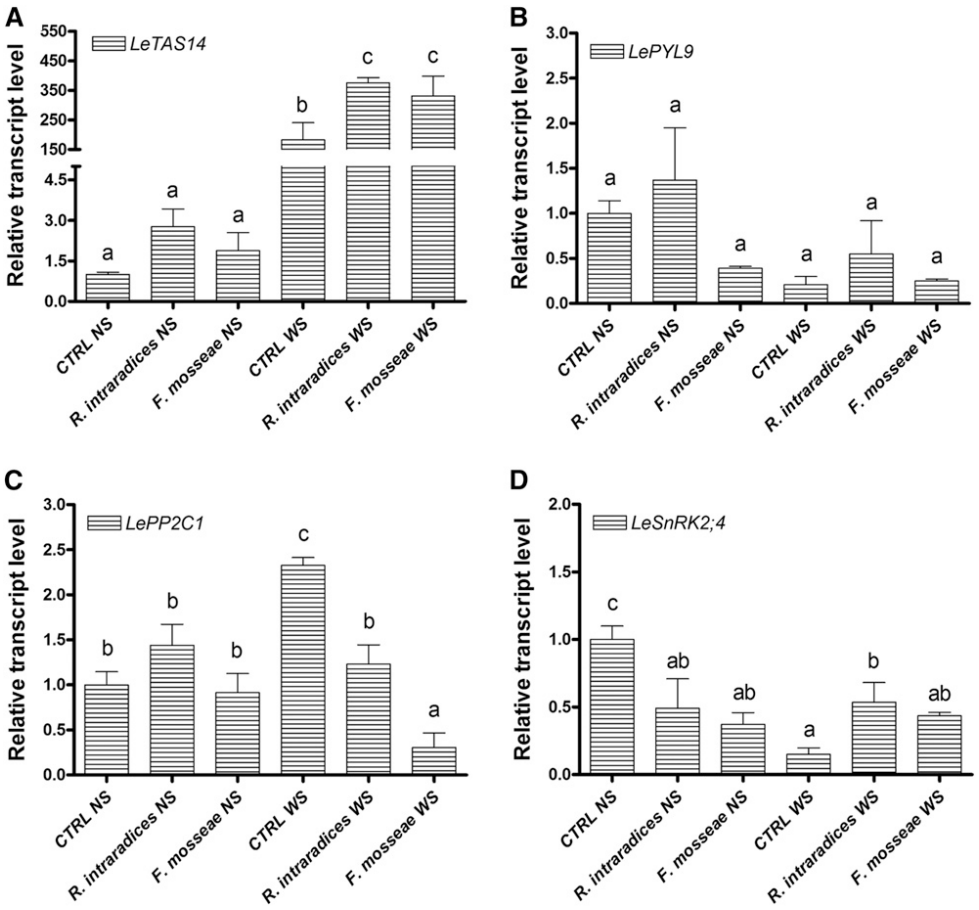

Figure 4. Expression changes of ABA-responsive genes in leaves of AM− and AM+ plants under NS and WS conditions. RT-qPCR analysis is shown for the DHN *LeTAS14* (A) and the ABA-related genes *LePYL9*, *LePP2C1*, and *LeSnR2K2;4* (B–D, respectively). CTRL, Control. Lowercase letters above the bars denote significant differences attested by Tukey's HSD test ($P < 0.05$). Data are expressed as means ± SE ($n = 3$).

Pro and Hydrogen Peroxide Contents and Superoxide Dismutase Activity Assay

To counteract drought stress effects, plants produce molecules involved in osmoregulation, including Pro, which is the most widely distributed osmolyte in plants (Szabados and Savouré, 2010). In our experiment, Pro concentrations increased significantly as a function of WS, with values ranging from 1.09 to 2.63 μmol g^{-1} fresh weight in leaves of NS plants to 18.63 to 30.53 μmol g^{-1} fresh weight in leaves of WS plants. Higher values of Pro were detected in WS plants grown in the presence of the fungus, in particular *F. mosseae*, compared with control plants (Fig. 5A). In the presence of AM symbiosis and upon WS conditions, the content of Pro was reported to be either higher (Ruíz-Sánchez et al., 2011; Abbaspour et al., 2012; Yooyongwech et al., 2013) or lower (Wu and Xia, 2006; Bhosale and Shinde, 2011; Zou et al., 2013; Wang et al., 2014; Hazzoumi et al., 2015) than in control plants. Our results, showing a higher content of Pro in AM+ plants during WS with respect to control plants, could suggest a better resistance of colonized plants to WS. Indeed, Pro accumulation induced by drought was observed in several plant species, leading to the hypothesis that this increase could promote drought tolerance, although it was proposed recently that Pro metabolism and turnover, rather than simply Pro accumulation, function to maintain growth during water limitation (Bhaskara et al., 2015).

Reactive oxygen species, among them H_2O_2, also have been correlated with plant responses to several stresses, including drought, and as a counterpart, antioxidant enzymes, such as SOD, are considered important actors in plant defense against oxidative stresses caused by various biotic and abiotic factors. In this study, H_2O_2 levels and SOD enzymatic activity were assessed to investigate the effect of AM symbiosis on stress-avoiding mechanisms. Although H_2O_2 levels increased sharply as a function of WS in both control and mycorrhizal tomato plants, they were significantly lower in the presence of AM fungi (Fig. 5B). In different plant-AM systems (Wu et al., 2006; Fouad et al., 2014), H_2O_2 levels increased under WS conditions in both AM-colonized plants and control plants, although this increment was always lower in the former. This trend was confirmed overall by the patterns of SOD isozymes (i.e. multiple forms of enzymes, putatively manganese-SOD, iron-SOD, and copper/zinc-SOD) that were followed during the WS treatment using the in-gel activity staining technique. Four different isozymes were clearly detected in all the samples (data not shown). A general involvement of SOD activity in response to WS seemed to be present regardless of the presence of the AM fungi, and the isozyme corresponding to band 3 mainly responded in mycorrhizal plants independently of the fungal species (Fig. 5C). This form putatively belongs to a manganese-SOD, since its activity was not inhibited by H_2O_2 treatment during the in-gel activity staining (data not shown). The significant changes observed in

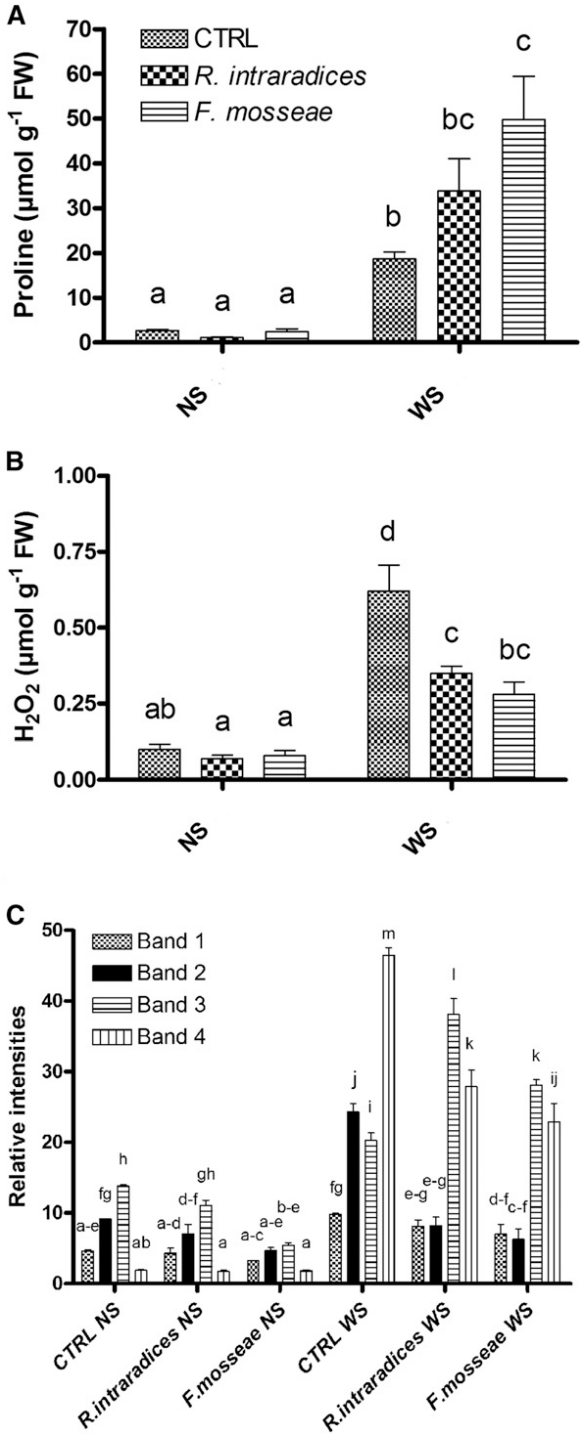

Figure 5. Analysis of Pro and hydrogen peroxide (H_2O_2) contents and superoxide dismutase (SOD) activity. Quantification of Pro (A) and H_2O_2 (B) contents (both expressed as μmol g^{-1} fresh weight [FW]) and measurement of SOD enzymatic activity (C) are shown in AM− (control [CTRL]) and AM+ (*R. intraradices* and *F. mosseae*) tomatoes under NS and WS conditions. Bars (bands 1–4) represent quantifications of different SOD isozymes. Lowercase letters above the bars indicate significant differences attested by Tukey's HSD test ($P < 0.05$). Data are expressed as means ± SE ($n = 3$).

SOD activity under WS in the arbuscular mycorrhiza-inoculated plants with respect to the control plants (Fig. 5C) are in agreement with previous observations (Ruiz-Lozano, 2003; Wu et al., 2006), where an increased SOD activity was observed in the leaves of AM+ plants under WS, supporting the role of this enzyme category in facing the excess of reactive oxygen species upon drought.

Expression Changes of Plant Stress-Responsive Genes

The expression of genes encoding proteins involved in different plant-response pathways, such as in response to biotic stresses, was assessed. In particular, two tomato genes (*LeLOXC* and *LeLOXD*), encoding different lipoxygenase isoforms that participate in the synthesis of jasmonic acid, the germacrene C synthase gene (*LeGCS*), which encodes a protein involved in the biosynthesis of terpenoids (a major class of VOCs in plants), and the hydroperoxide lyase gene (*LeHPL*), producing stress-inducible compounds such as green leaf volatile, were considered (Fig. 6). Among them, it is worth noting the significant overexpression of *LeLOXD* in the leaves of AM-colonized plants under WS conditions (Fig. 6B). Plant lipoxygenase (LOX) enzymes are known to be involved both in processes associated with development and in response to biotic and abiotic stresses (Digilio et al., 2010; Hu et al., 2013; Lim et al., 2015b), including drought stress (Sofo et al., 2004). In many plants including tomato, LOXs are encoded by a multigene family (Mariutto et al., 2011), and the individual LOX isoforms not only are differentially regulated but also can have distinct functions (Hu et al., 2011; Yang et al., 2012). Recently, a transcriptomic study performed on leaves of mycorrhizal tomato plants has shown the activation of a mycorrhiza-inducible LOX gene, suggesting a role for this gene in priming defense mechanisms (Cervantes-Gámez et al., 2016). To assess the positive relationship between the stress response and this category of enzymes, we focused our attention on two main LOX genes (*LeLOXC* and *LeLOXD*) that have already been reported to have different expression patterns under environmental stimuli (Fig. 6, A and B). In particular, *LeLOXD* was overexpressed in response to wounding, pathogen infection, jasmonate, and systemin (Heitz et al., 1997; Digilio et al., 2010; Hu et al., 2011). It has been suggested that *LeLOXD* plays a role as a component of the octadecanoid defense signaling pathway and that it may act as a regulator in response to various stresses (Hu et al., 2013). Why this enzyme is differentially regulated by drought in the presence of AM symbiosis remains unknown. Nevertheless, on the basis of the

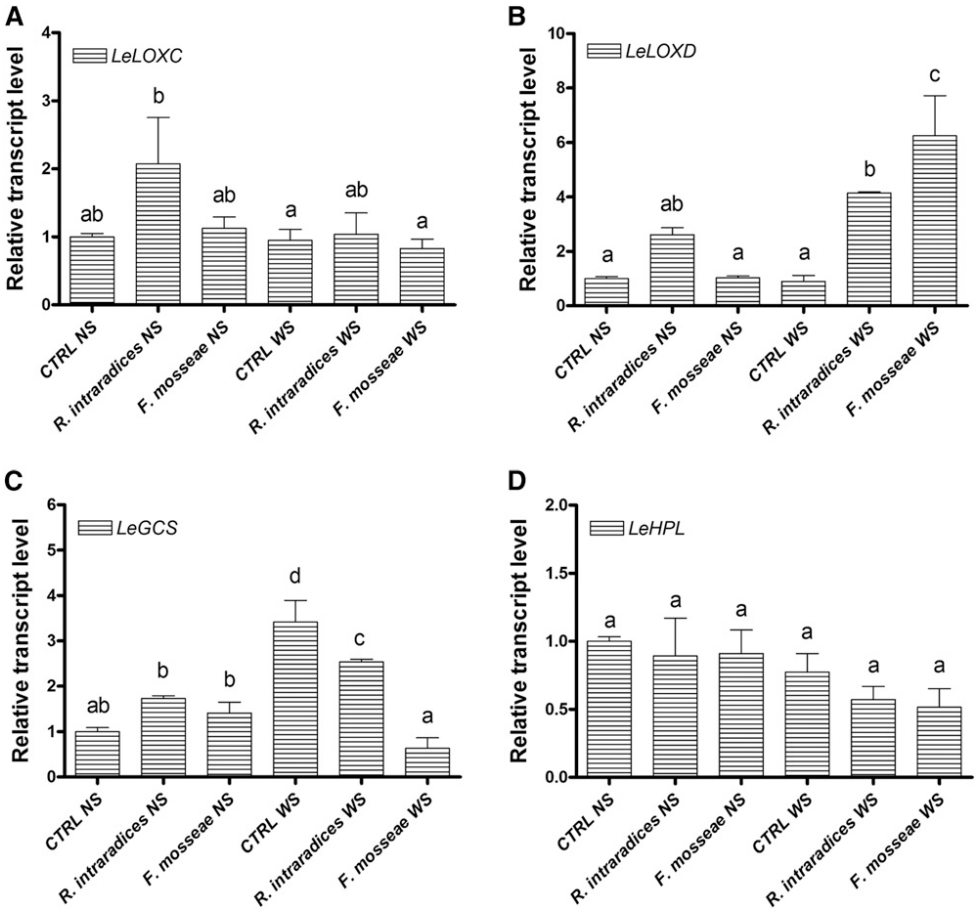

Figure 6. Expression changes of stress-dependent genes in leaves of AM− and AM+ plants under NS and WS conditions. RT-qPCR analysis is shown for *LeLOXC* (A), *LeLOXD* (B), *LeGCS* (C), and *LeHPL* (D). CTRL, Control. Lowercase letters above the bars denote significant differences attested by Tukey's HSD test ($P < 0.05$). Data are expressed as means ± SE ($n = 3$).

results of this study, we could hypothesize that AM symbiosis can help the plant to react against WS by inducing a LOXD-mediated pathway. Among the other selected genes involved in defense responses, only *LeGCS* was positively affected by WS in both control and RiWS plants (Fig. 6C), while the expression profiles of *LeLOXC* (Fig. 6A) and *LeHPL* (Fig. 6D) seemed not to be strongly affected by the different growth conditions, although *LeLOXC* expression was higher in RiNS plants than in all other conditions.

Expression Changes of Plant and Fungal Aquaporin Genes in Roots

The physiological responses of plants to drought stress also are regulated by the expression of genes encoding aquaporin proteins (AQPs), which control water movement through the plant in different physiological conditions, and several studies identified a crucial role for these proteins during WS (Maurel at al., 2015, and refs. therein). In addition, AM symbiosis may regulate the expression of AQP genes to improve root hydraulic conductivity as well as the plant water status and tolerance under water deficiency (Porcel et al., 2006; Aroca et al., 2007, 2012; Uehlein et al., 2007; Ruiz-Lozano et al., 2009; Giovannetti et al., 2012; Bárzana et al., 2014). In plants, there are currently five major subfamilies of AQPs divided on the basis of their sequence similarity. In this work, we focused on three tomato AQP genes belonging to three different subfamilies (NIPs, NOD26-like intrinsic proteins; PIPs, plasma membrane intrinsic proteins; and TIPs, tonoplast intrinsic proteins) that have been reported to be expressed mainly or only in roots (Reuscher et al., 2013). Our results indicated that, in irrigated (NS) conditions, all the selected genes showed an increasing trend in roots from AM+ plants, and, in particular, expression values for the three aquaporin genes were significantly different in *R. intraradices*-colonized plants compared with control plants (Fig. 7, A–C). WS treatment affected the transcriptional pattern of these aquaporin genes differently: *LeNIP3;1* was overexpressed in AM+ plants, particularly in those inoculated with *F. mosseae*; conversely, *LePIP1;1* and *LeTIP2;3* were down-regulated in both AM+ and control plants (Fig. 7, A–C). Recently, it was reported that a NIP aquaporin gene (*LjNIP1*) is expressed specifically in the arbuscule-containing cells in mycorrhizal roots of *Lotus japonicus* (Giovannetti et al., 2012). The observation that *LeTIP2.3* and *LePIP1.1* were less expressed in AM-colonized plants under severe WS is

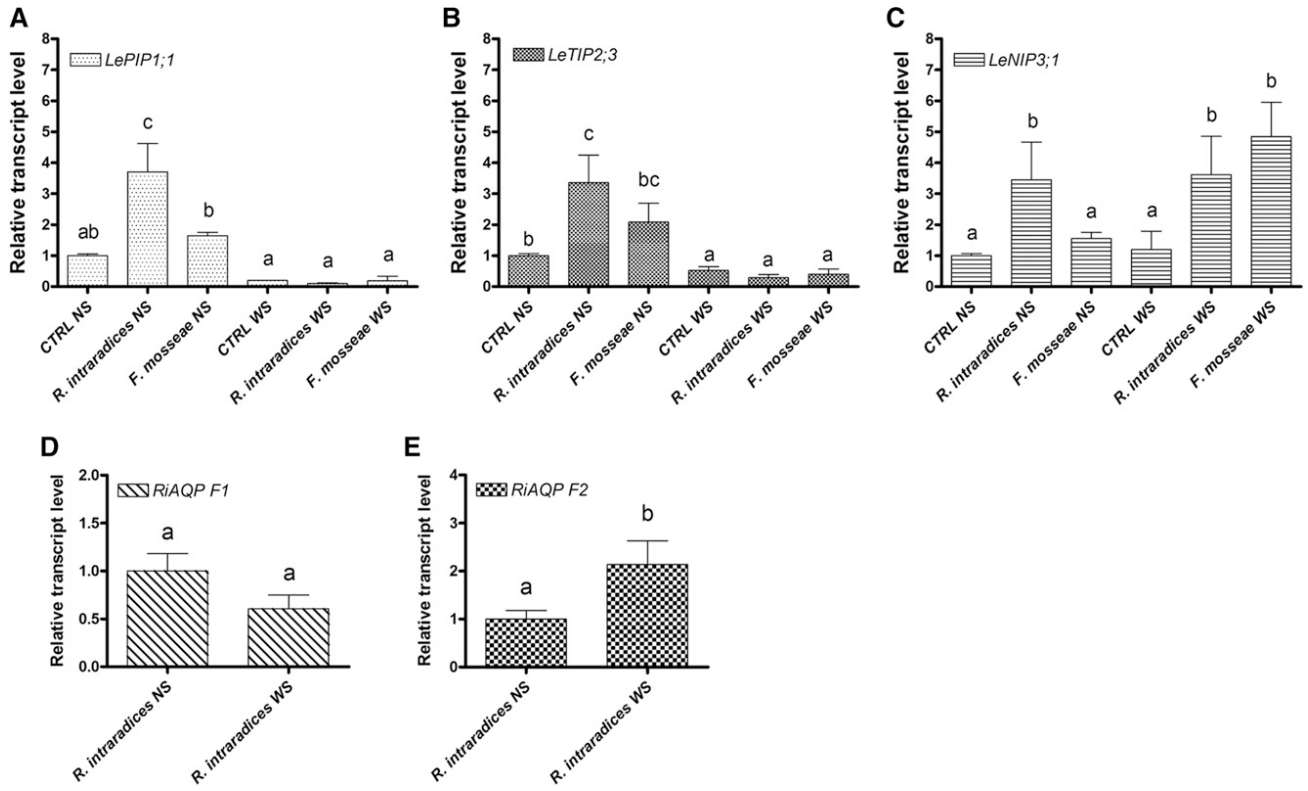

Figure 7. Transcript levels of plant and fungal aquaporin genes in roots. RT-qPCR analysis is shown for *LePIP1;1* (A), *LeTIP2;3* (B), and *LeNIP3;1* (C) genes in control (CTRL) and AM fungi-colonized tomato roots under NS and WS conditions and for *RiAQPF1* (D) and *RiAQPF2* (E) genes in NS and WS roots. Lowercase letters above the bars denote significant differences attested by Tukey's HSD test ($P < 0.05$) for A to C and by two-tailed Student's *t* test for D and E. Data are expressed as means ± SE ($n = 3$).

in agreement with data reported for maize (*Zea mays*), where the expression of some AQPs was up-regulated under short-term drought stress but simultaneously down-regulated under sustained drought, while others were unaffected or up-regulated (Bárzana et al., 2014). Since AM fungal aquaporin also can have a role in drought tolerance during AM symbiosis (Aroca et al., 2009; Li et al., 2013a, 2013b), the expression of two *R. intraradices* AQP genes (*RiAQPF1* and *RiAQPF2*; Fig. 7, D and E) also was evaluated. *RiAQPF2* was up-regulated significantly (Fig. 7E) under WS, suggesting its putative involvement in response to drought. A possible role in AQP-mediated water transport from the AM fungi to the host plant was proposed previously by Li et al. (2013b) in a study where both these fungal genes were shown to be activated in maize arbuscule-containing cells under drought induction using polyethylene glycol.

The simultaneous induction of both fungal and plant AQP genes in our experiments is another confirmation that the two symbionts strictly cooperate to regulate the mycorrhizal drought stress response, and it is also in agreement with the enhanced physiological performance (especially in terms of higher iWUE level) observed upon WS. A similar cross talk between specific categories of fungal and plant genes involved in the stress response has been described, for instance, in the case of mitogen-activated protein kinase (*MAPK*) genes in a recent work on mycorrhizal soybean (*Glycine max*) roots (Liu et al., 2015b). Those authors suggested that the interaction between fungal MAPKs and soybean MAPKs could lead to the soybean response to drought, thereby regulating the fungal growth and the physiological status of the plant.

CONCLUSION

Water deficit is one of the major limiting factors for crop production, and several studies have been performed to verify its effect on tomato growth, yield, secondary metabolite production, and gene expression (Nuruddin et al., 2003; Sánchez-Rodríguez et al., 2011; Giannakoula and Ilias, 2013; Sacco et al., 2013). Previous work also studied the relationship between tomato and AM fungi under WS (Dell'Amico et al., 2002; Subramanian et al., 2006; Aroca et al., 2008; Wang et al., 2014; Ruiz-Lozano et al., 2016). The discrepancy among data already available could be due to the use of different plant varieties and fungal species/isolates as well as to different growth and drought stress conditions (Augé et al., 2015). The results obtained in this work, using two fungal symbionts, support that the plant response to WS is influenced by the specific plant/fungus combination and explain, at least in part, the controversial results obtained so far.

In summary, our findings indicated that the colonization of tomato roots by AM fungi can significantly improve tomato tolerance to WS. The mechanisms are multifaceted (Fig. 8), particularly with the association between *R. intraradices* and tomato 'San Marzano nano', showing an improved iWUE and A_N rate. Nonetheless, new questions are raised regarding the role of ABA in regulating stomata closing under WS during AM symbiosis, an aspect that still requires investigation. As a consequence of the results obtained here, *R. intraradices* has been selected for further experiments focused on assessing the impact of AM symbiosis on the tomato response to the combined action of WS and biotic stresses (i.e. insects and nematodes).

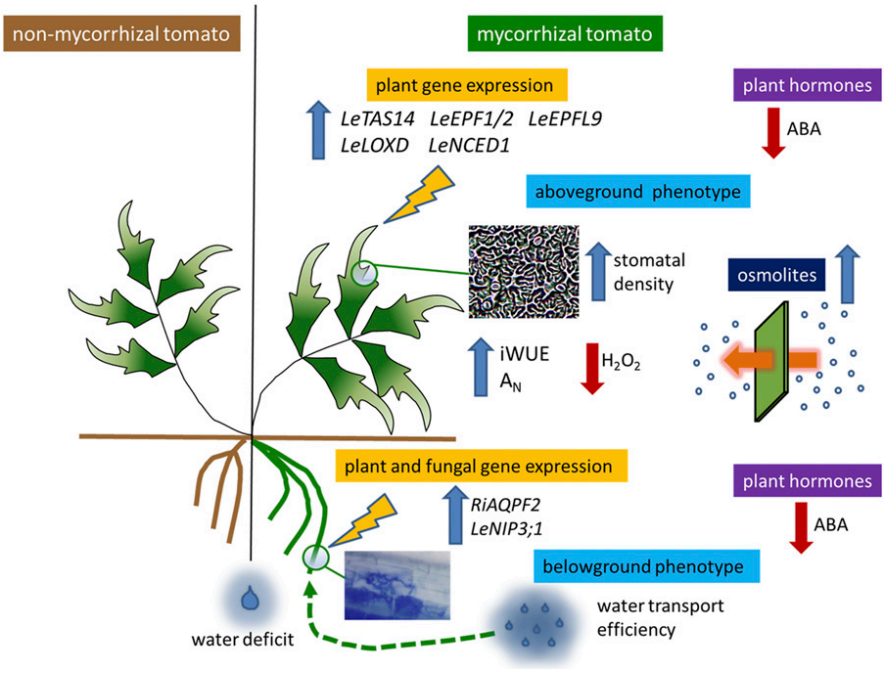

Figure 8. Biochemical, physiological, molecular, and phenotypic changes induced by AM symbiosis in tomato under water stress. The blue arrows indicate increase/up-regulation, while the red arrows indicate decrease/down-regulation, with respect to control nonmycorrhizal tomato.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Tomato (*Solanum lycopersicum* 'San Marzano nano') seeds were surface sterilized in sodium hypochlorite for 20 min, washed five times in sterile water, and germinated on wet paper. Seedlings were then moved to pots containing a mixture of quartz sand (50%), sterile pumice (20%), and an inoculum (30%) of either *Funneliformis mosseae* (formerly *Glomus mosseae*; BEG 12) or *Rhizophagus intraradices* (FR 121), both purchased from MycAgro. For noninoculated plants, sterile inoculum carrier only (30%) was used instead of the specific inoculum. Plants were grown in controlled conditions, with a temperature of 23°C/21°C ± 1°C day/night, 16/8-h light/dark photoperiod, and relative humidity of 65% ± 10%. From transplanting to the beginning of the experiment (after about 6 to 7 weeks), all the plants were watered twice per week with tap water and, once per week, with a modified Long-Ashton nutrient solution (Hewitt, 1966) containing 300 μM inorganic phosphate. A total of 24 plants for each of the three considered conditions (control plants, *R. intraradices*-colonized plants, and *F. mosseae*-colonized plants) were used to determine the role of AM symbiosis under WS. Out of 72 plants, 36 were used as controls (irrigated or NS) and maintained in a well-watered state (at container capacity). The remaining 36 plants were subjected to a WS treatment. For the WS treatment, irrigation was withheld about 6 to 7 weeks after fungal inoculation, and severe WS was achieved in about 3 weeks (petiole water potential less than −1 MPa). To allow the collection of data from a sufficient number of replicates, five randomly chosen NS plants and five WS plants for each condition were subjected to the physiological measurements. The experiment was repeated using 12 plants, six NS and six WS, for each condition (36 plants total).

Assessment of Plant Biomass, Growth, Stomatal Density, and Symbiosis Development

At the end of the experiments, plants were harvested and plant height, stem diameter, and fresh weight were recorded. Sixty randomly chosen 1-cm-long root segments were stained with 0.1% Cotton Blue in lactic acid, and fungal colonization was quantified according with the Trouvelot system (Trouvelot et al., 1986) using MYCOCALC software, while the remaining root systems were stored at −80°C until molecular analysis. Root segments, obtained from at least five plants, were analyzed. Fungal colonization also was assessed before starting the WS treatment, although in this case the colonization was not quantified.

Stomatal density was measured following the method described by Hopper et al. (2014) on two leaves from each mycorrhizal and nonmycorrhizal plant. Leaf imprints were collected from abaxial lamina of leaves under well-watered conditions, and six counts for each imprint were done using an optical microscope with a 10× objective (Nikon Eclipse 55i).

Ψ_{leaf}, Leaf g_s, and CCI

Ψ_{leaf} was measured on one transpiring leaf per plant over the 20 d of the experiment by collecting the data every 2 to 4 d using a Scholander-type pressure chamber (Soil Moisture Equipment). Measurements of transpiration rate, g_s, and A_N were performed on adult, nonsenescing leaves at the same physiological age (in the middle part of the plant, considering the third to fourth leaf from the shoot apex). iWUE was calculated as the ratio between A_N and g_s. Measurements were taken with an infrared gas analyzer (ADC-LCPro+ system; Analytical Development Company). During measurements, light intensity in the leaf chamber was set at 1,200 μmol photons m^{-2} s^{-1}, temperature was 25°C, and the concentration of CO_2 was maintained at 450 to 470 μL L^{-1}. Measurements were taken between 10 AM and 1 PM. The CCI was determined at the end of the experiment (16 or 20 DAT) using the portable chlorophyll meter SPAD 502 (CCM-200; Opti-Sciences). Readings were collected from the second or third fully developed leaves from the top of five randomly selected tomato plants for each experimental condition.

Analysis of ABA Content

Leaves and roots for ABA analysis were immediately frozen at −80°C after harvest, and ABA concentration was quantified following the method of Siciliano et al. (2015). Briefly, 200 mg of fresh plant tissue, previously ground in a chilled mortar, was homogenized with 80% (v/v) methanol and extracted for 1 h in an ultrasonic bath. The extract was centrifuged at 13,000*g* at 4°C for 5 min, and then the supernatant was filtered through a 0.45-μm membrane filter and analyzed by HPLC-tandem mass spectrometry. To this aim, a 1260 Agilent Technologies system with a binary pump and a vacuum degasser was used. Aliquots (20 μL) were injected on a Kinetex PFP column (100 × 2.1 mm, 2.6 μm; Phenomenex), and ABA was eluted in isocratic conditions with 65:35 (v/v) water:CH_3CN acidified with 0.1% HCOOH under a flow of 200 μL min^{-1} for 5 min. Using an electrospray ion source operating in negative ion mode, samples were introduced into a triple quadrupole mass spectrometer (Varian 310-MS TQ). Analyses were conducted in multiple reaction monitoring mode using two transitions: 263 > 153 (CE 12V) for quantification and 263 > 219 (CE 12V) for monitoring, with 2 mbar argon as collision gas.

In order to quantify ABA concentrations in the samples, the external standard method was used. A standard curve with an original standard of +/− ABA (Sigma-Aldrich; purity of 98.5%) was built, using concentrations ranging from 5 to 1,000 ng mL^{-1}. The limit of detection (LOD) and limit of quantification (LOQ) were determined by the SD of the response (σ) and the slope of calibration curve (S) ratio in accordance with the ICH Harmonized Tripartite Guideline, expressed as LOD = 3.3 σ/S and LOQ = 10 σ/S. Calculated final values were LOD = 0.87 ng g^{-1} and LOQ = 2.90 ng g^{-1}.

Pro Content Analysis, H_2O_2 Determination, and SOD Activity Assay

Analyses were performed on leaves form three biological replicates, each formed by two plants within a treatment.

Pro Content Analysis

The extraction and determination of Pro were performed according to Bates et al. (1973) with slight modifications. Briefly, leaf samples (20 mg) were extracted with ethanol:water (70:30). Five hundred microliters of extract was added to 1 mL of ninhydrin reagent (1% [w/v] ninhydrin in 60% [v/v] glacial acetic acid and 20% [v/v] ethanol) and incubated for 20 min at 95°C. Pro content was measured with a spectrophotometer (EASYSPEC SAFAS UV-Vis) at 520 nm and calculated on the basis of a Pro standard curve (5-2-1-0.5-0.2 mM Pro in 40:60 [v/v] ethanol:water). Data were expressed as μmol g^{-1} fresh weight.

H_2O_2 Analysis

Endogenous H_2O_2 content was determined according to Velikova et al. (2000) with slight modifications. Briefly, leaves (0.25 g) were ground in 3 mL of 5% TCA at 4°C. The homogenate was centrifuged at 12,000*g* for 15 min. Then, 0.5 mL of 10 mM potassium phosphate buffer (pH 7) and 0.75 mL of 1 M potassium iodide were added to a 0.5-mL aliquot of the supernatant, and the differential A_{390} (sample absorbance minus the absorbance of the same supernatant aliquot without potassium iodide) was used to determine the H_2O_2 content against an H_2O_2 standard curve. Data were expressed as μmol g^{-1} fresh weight.

SOD Activity Assay

For the determination of SOD activity, 40 mg of leaves was homogenized in 80 μL of extraction buffer (cold 50 mM potassium phosphate buffer [pH 7] containing 5 mM ascorbic acid, 1 mM EDTA, 1% [w/v] polyvinylpyrrolidone, and 0.1% [v/v] Triton X-100) and centrifuged at 4°C for 10 min at 15,000*g*. The protein concentration in the extract was determined according to Bradford (1976). Bovine serum albumin was used to generate a standard curve. After the protein assay, samples were stored at −20°C until use. Native PAGE was performed at 4°C on 10% polyacrylamide gels with 10% (w/v) glycerol. SOD activity staining was performed by monitoring the inhibition of the photochemical reduction of nitroblue tetrazolium according with the method of Beauchamp and Fridovich (1971) with minor modifications. To this aim, the gel was first soaked in 25 mL of 1.23 mM nitroblue tetrazolium for 15 min, briefly washed in distilled water, then soaked in the dark in 30 mL of 100 mM potassium phosphate buffer (pH 7) containing 28 mM *N,N,N′,N′*-tetramethylethylenediamine and 2.8 × 10^{-2} mM riboflavin for another 15 min. After a brief wash in distilled water, gels were illuminated below a light box for 20 min. Gels were scanned with the Gel-Doc system (Bio-Rad), and the intensities of the SOD isoenzyme bands were evaluated using ImageLab software version 5.2.1 (Bio-Rad) by comparing them with the intensity due to the activity of 400 ng of manganese-SOD from *Escherichia coli* (Sigma-Aldrich S5639) loaded onto the first lane of each gel.

Quantitative Gene Expression Analysis of Leaves and Roots

Expression changes of the target transcripts were quantified on leaf and root samples (three independent biological replicates) by RT-qPCR. Leaves and roots from two plants within each treatment were pooled to form a biological replicate, immediately frozen in liquid nitrogen, and stored at −80°C. Developing leaves (less than 5 mm, about 1 week from emergence; Fig. 2E) were used to determine the expression of genes involved in stomata formation. Total RNA was extracted from each biological replicate using the Spectrum Plant Total RNA extraction kit (Sigma-Aldrich) with slight modifications. RNA quantity was checked using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific), RNA samples were then treated with DNase I (Invitrogen), and genomic DNA contamination was checked before proceeding with cDNA synthesis by PCR using *LeEF*-specific primers of tomato (Supplemental Table S1). First-strand complementary DNA was then synthesized starting from 1 μg of total RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) following the manufacturer's instructions. Reactions were carried out in the StepOnePlus RT-qPCR System (Applied Biosystems), and the SYBR Green method (Power SYBR Green PCR Master Mix; Applied Biosystems) was used to quantify the amplification results. Three biological replicates were run for each experiment. Thermal cycling conditions were as follows: an initial denaturation phase at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. Specific annealing of primers was checked using dissociation kinetics performed at the end of each RT-qPCR run. The expression of tomato target transcripts was quantified after normalization to two reference genes (elongation factor and ubiquitin; *LeEF* and *LeUBI* primers are listed in Supplemental Table S1), while *R. intraradices* elongation factor (*RiEF1*; Supplemental Table S1) was used to normalize the expression of fungal target transcripts. Gene expression data were calculated as expression ratios (relative quantity) to control NS plants. Gene-specific primers are listed in Supplemental Table S1 (Corrado et al., 2007; Weiss and Egea-Cortines, 2009; González-Guerrero et al., 2010; López-Ráez et al., 2010; Li et al., 2013b; Chen et al., 2014). In all experiments, the transcript level of the arbuscular mycorrhiza-inducible phosphate transporter *LePT4* was evaluated as a marker for mycorrhizal functionality, showing a typical expression profile with an overexpression trend in the presence of both the AM fungi, in both WS and NS conditions, with respect to control NS plants (data not shown).

Statistical Analyses

Significant differences among treatments were statistically analyzed by applying a one-way ANOVA test, and Tukey's posthoc test was used for mean separation when ANOVA results were significant ($P < 0.05$). Significant differences of pairwise comparisons were assessed by Student's t test. The SPSS statistical software package (version 22; SPSS) was used to run statistical analyses.

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. AMF colonization in tomato roots.

Supplemental Figure S2. Measurement of chlorophyll content index (CCI, SPAD units).

Supplemental Table S1. List of the primers used in the current work.

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