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Ethylene sensitivity and relative air humidity regulate root hydraulic properties in tomato plants

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Main conclusion

The effect of ethylene and its precursor ACC on root hydraulic properties, including aquaporin expression and abundance, is modulated by relative air humidity and plant sensitivity to ethylene.

Abstract

Relative air humidity (RH) is a main factor contributing to water balance in plants. Ethylene (ET) is known to be involved in the regulation of root water uptake and stomatal opening, although its role on plant water balance under different RH is not very well understood. We studied, at the physiological, hormonal and molecular levels (aquaporins expression, abundance and phosphorylation state) the plant responses to exogenous 1-aminocyclopropane-1-carboxylic acid (ACC; precursor of ET) and 2-aminoisobutyric acid (AIB; inhibitor of ET biosynthesis), after 24 hours of application to the roots of tomato wild type (WT) plants and its ET-insensitive *never ripe* (*nr*) mutant, at two RH levels: regular (50%) and close to saturation RH. Highest RH induced an increase of root hydraulic conductivity (L_{p_o}) of non-treated WT plants, and the opposite effect in *nr* mutants. The treatment with ACC reduced L_{p_o} in WT plants at low RH and in *nr* plants at high RH. The application of AIB increased L_{p_o} only in *nr* plants at high RH. In untreated plants, the RH treatment changed the abundance and phosphorylation of aquaporins that affected differently both genotypes according to their ET sensitivity. We show that RH is critical in regulating root hydraulic properties, and that L_{p_o} is affected by the plant sensitivity to ET, and possibly to ACC, by regulating aquaporins expression and their phosphorylation status. These results incorporate the relationship between RH and ET in the response of L_{p_o} to environmental changes.

Keywords: ACC, aquaporins, ethylene, phytohormones, relative humidity, root hydraulic conductivity.

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Authors Contribution

MC-P and RA conceived and designed research. MC-P, PI, SM and AMZ conducted experiments. RA, JMR-L and JMG-M contributed new reagents and analytical tools. MC-P and RA analyzed data. MC-P and RA wrote the manuscript. All authors read and approved the manuscript.

Introduction

Plant transpiration rates are directly related to the difference between the water vapor pressure at the atmosphere and inside the leaves (Hsiao 1973). Water vapor saturation around the leaves stimulates stomatal opening, at the time that the transpiration rates decrease proportionally to the amount of humidity in the air (Rawson et al. 1977). Plant hormones, such as abscisic acid (ABA) and ethylene (ET), are directly involved in the closure and opening of stomata (Pierik et al. 2006; Chen, Dodd et al. 2013), interplaying both at low and high RH (Arve and Torre 2015). ET is related with stomata regulation as an ABA antagonist, especially under drought conditions (Tanaka et al. 2005; Wilkinson and Davies 2010).. In higher plants, ET is biosynthesized via the conversion of S-adenosyl- L-methionine into 1-aminocyclopropane-1-carboxylic acid (ACC) (Adams and Yang, 1979). ACC is catalyzed by ACC synthase (ACS), encoded by a multigene family often considered to be the rate-limiting step in ET synthesis (Boller et al. 1979, Kende 1993), and further oxidized to ET by ACC oxidase (ACO), a small family of genes (Ververedis and John 1991; Kende 1993) that is considered to be less rigorously controlled (Wang et al. 2002). On the other hand, recent findings have suggested that ACC could act as a signaling molecule to regulate plant development and growth, independently of ethylene (revised in Van de Poel and Van Der Straeten 2014), and this could be also affecting how plants regulate water transport along their tissues.

How hormones control gas exchange and transpiration in plants have a direct effect on their regulation of water uptake. Root water uptake capacity in plants is driven by the combination of the transpiration stream and the activity of aquaporins in the root cells (Aroca et al. 2012). Aquaporins are membrane intrinsic proteins present in every membrane of all living organisms which facilitate the passage of water and other small solutes through them, always following and osmotic gradient and hence facilitating the

symplastic water pathway (revised in Maurel et al. 2015). Plant aquaporins are classified into several subfamilies, with the plasma membrane intrinsic proteins (PIP) as the ones thought to control the overall root water uptake capacity (Maurel et al. 2015).

Phosphorylation of aquaporins is among the most important mechanisms that promotes water transport in cells (Prak et al. 2008; Horie et al. 2011), although the knowledge of the contribution of each aquaporin to the cell water transport is quite limited and will depend on the PIP protein localization and/or PIPs trafficking to the plasma membrane (Zelazny et al. 2007). How the presence of ET and the application of ACC to plant roots may affect protein abundance and phosphorylation state of aquaporins has not been documented yet. The different studies on the role of ET and other phytohormones on root water uptake regulation have produced contradictory results according to the hormones concentration applied, the time of exposure, or simply to the different responses of plant species to hormones. Hence, water uptake measured as root hydraulic conductivity (L_{p_o}), was found to increase in the presence of ET in poplar (Kamaluddin and Zwiazek 2002) and tamarack (Islam et al. 2003), while Li et al. (2009) found a negative effect of ET on the L_{p_o} of *Medicago falcata* plants. Also, little is known on the effect of ET on aquaporin expression, with just a few studies showing changes in aquaporin expression related to floral and petal development (Chervin et al. 2008; Ma et al. 2008; Xue et al. 2009), and with latex yield (Tungngoen et al. 2009).

In this study, we have used tomato as a reference species as it has high impact in agriculture and is one of the main crops produced around the world. Also, the current availability of several tomato mutants allows their use for a better understanding of plant water relations. Tomato never ripe (*nr*) plants come from a spontaneous, naturally occurring, ET-insensitive mutation from a single base substitution in the N-terminal coding region of the *Le-ETR3* gene (Wilkinson et al. 1995), leading to delayed fruit

ripening (Lanahan et al. 1994) and with some residual ET responsiveness. The objectives of the present study are: 1) to determine if the responses of stomatal conductance and root hydraulic conductivity to the air RH are dependent on the plant sensitivity to ethylene, 2) to determine if the application of external ACC causes a response on L_{p_0} that is directly related with the accumulation of ethylene in the plant tissues, and if this is affected by the saturation of the leaf surrounding air, and 3) to determine if the external application of 2-aminoisobutyric acid (AIB), which blocks ET biosynthesis (Satoh and Esashi 1982), modifies the response of L_{p_0} to RH depending on the plant sensitivity to ET. We hypothesized that: 1) under saturated conditions, the plant will open the stomata and this will contribute to the accumulation of ethylene within the plant tissues, and this will affect water transport through its effect on aquaporin expression and phosphorylation state, 2) ethylene insensitive *nr* mutants will show different physiological and molecular responses to the application of ACC and to the ethylene accumulation within the plant tissues that is going to affect the water transport at the whole plant level, and 3) AIB will block the signal of ET and hence will affect L_{p_0} and *gs*.

Materials and Methods

Plant material and growth conditions

Seeds of *Solanum lycopersicum* cv. Pearson WT (Accession # LA0012, Tomato Genetics Resource Centre (TGRC), CA) and *Solanum lycopersicum* cv. Pearson *never ripe* (Accession # LA0162, TGRC, CA) were germinated in wet vermiculite for ten days. They were later transferred into an aerated hydroponic system with 80% (Sánchez-Romera et al. 2014) mineral solution (Hewitt 1952), in a controlled-environment growth chamber at 22/18 °C, 50% relative humidity, 18/6 day:night

photoperiod and a photosynthetic photon flux density of $350\text{--}400\ \mu\text{mol m}^{-2}\text{ s}^{-1}$. After 2 weeks, the plants were subjected to a combination of chemical and relative humidity treatments applied at the same time for 24 hours (starting three hours after the lights turned on within the growth chamber). We used a split-pot system in order to apply the treatments as: 1) Plants were divided in three groups: the first group was left as untreated treated (control treatment), a second group was treated with 0.1 mM ACC that was applied to the nutrient solution, a third group was treated with 0.1 mM AIB . 2) Within each tray, corresponding to the different chemical treatments, half of the plants were covered with a cylindrical transparent plastic tube to generate saturated humidity conditions (close to 100 %) and the other half were left to regular growth chamber humidity (50%). By this way, we had the roots of the plants of each genotype subjected to the same chemical treatment, and within each of the chemical treatment, we had the shoot of the plants subjected to different relative humidity regime.

After 24h of chemical and humidity treatments application, physiological measurements were carried out between 3-6 h after the lights at the growth chamber were turned on. ,

Stomatal conductance (gs) and root hydraulic conductivity (Lp_o)

Stomatal conductance was determined after 24 hours of the application of the treatments in eight plants (n=8) per treatment combination. Stomatal conductance was measured in the last fully developed mature leaves with a portable AP4 Porometer (Delta-T Devices Ltd, Cambridge) three hours after the lights were turned on within the growth chamber at around $400\ \mu\text{mol m}^{-2}\text{ s}^{-1}$ light intensity.

Root hydraulic conductivity (Lp_o) was determined in eight detached roots per treatment combination (n=8), 24 h after the application of the different treatments, just

after the g_s measurements. Plant shoots were cut 2 cm above the root collar, just below the cotyledons, and the exudates collected with a silicone tube after one hour. The exudates collected for the fifteen first minutes were discarded to avoid phloem contamination. A cryoscopic osmometer (Osmomat 030, GonotecGmbH, Berlin) was used to determine the osmolarity of the exuded sap and the nutrient solution. Osmotic root hydraulic conductivity was calculated as $Lp_o = J_v / \Delta\Psi_s$, where J_v is the exuded sap flow rate per root volume and $\Delta\Psi_s$ the osmotic potential difference between the nutrient solution and the exuded sap (Aroca et al. 2006).

Root aquaporin-PIP expression analysis

Root aquaporins expression was determined as described in Calvo-Polanco et al. (2014a) in roots of three plants per treatment combination (n=3) for nine tomato PIP genes: *SlPIP1;1* [Tigr.No.:TC175784], *SlPIP1;2* [Tigr.No.:TC171321], *SlPIP1;5* [Tigr.No.:TC178447], *SlPIP1;7* [Tigr.No.:TC170092], *SlPIP2;1* [Tigr. No.:TC170322], *SlPIP2;4* [Tigr.No.:TC181456], *SlPIP2;6* [Tigr.No.:TC175989], *SlPIP2;8* [Tigr.No.:TC180270] and *SlPIP2;9* [Tigr.No.:TC173223], with the primers described at the Supplemental Table S1 (Sade et al. 2009). RNA integrity and quality were tested by gel electrophoresis as well as measuring 260/230 and 260/280 ratios in NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific). The expression of the different aquaporins was determined using a real time quantitative PCR (iCycler-Bio-Rad, Hercules, CA) as in Calvo-Polanco et al. (2014a). We used actin-specific primers after testing different housekeeping genes (actin, tubulin and ubiquitin) for standardization (Karlova et al. 2011). Three different root RNA samples were repeated three times and samples without cDNA were used as negative controls in all the PCR reactions.

Microsomes preparation and ELISA analysis

Microsomes were isolated as described in Calvo-Polanco et al. (2014a, b). We used, as primary antibodies (at a dilution of 1:2000), the two that recognize several PIP1 and PIP2, three antibodies that recognize the phosphorylation of PIP2 proteins at their C-terminal region at Serine 280 (PIP2₂₈₀), Serine 283 (PIP2₂₈₃) and both at Serine 280 and 283 (PIP2_{280/283}). We also used two antibodies that recognized the PIP2 proteins at their loop B either phosphorylated (PIP2LP) or not (PIP2L) at Serine 126 (Aroca et al. 2005; Bárzana et al. 2014).

A goat anti-rat Ig coupled to horseradish peroxidase (Sigma-Aldrich Co., USA) was used as secondary antibody at 1:10,000 for PIP1. Goat anti-rabbit Ig coupled to horseradish peroxidase (Sigma-Aldrich Co., USA) was used as a secondary antibody at 1:10,000 for PIP2 and PIP2₂₈₀, PIP2₂₈₃, PIP2_{280/283}, PIP2L and PIP2LP. Protein quantification was done in three different independent root samples isolated from three different plants per treatment (n=3), repeated three times each. The specificity of the PIP2 and phosphorylated antibodies PIP2₂₈₀, and PIP2_{280/283} is described in Calvo-Polanco et al. (2014b). Also, Aroca et al. (2005) shown that PIP2LP only recognized phosphorylated proteins. The equal loading of proteins in the different treatments was confirmed by staining a gel blot loaded with the same quantities used for the ELISA measured with Coomassie brilliant blue and also by Bradford quantification.

Leaf and root ethylene and ACC content

Eight excised developed leaflets of the last fully developed leaf per treatment (n=8) were enclosed in a 90x15 mm (ODxH) Petri dish (Fisher Scientific SL) and sealed with a pressure sensitive tape (3M) (Fiserova et al. 2008; Calvo-Polanco et al. 2014a). The leaves were left on moist paper (in order to avoid tissue drying) for 2.5 hours. ET was

recovered from the vial with a 1 ml syringe and measured with a gas chromatograph (HP 5890, Hewlett Packard Co, USA) equipped with a Poropak-R column and a hydrogen flame ionization detector. Whole root ET was determined following the same procedure as explained for the leaves but using a 150x15mm Petri Dish. Fresh and dry weight was measured in order to refer ET production to biomass.

The extraction, purification and further analytical determination of ACC, in roots and shoots were carried out in three roots and shoots per treatment combination (n=3) as described in (Mora et al. 2010).

Statistical analysis

The experiment had split-plot design, with the chemical treatments (non-treated Control, ACC and AIB) as the main factor, and the relative air humidity (RH) treatments as the split-plot factor nested to the chemical treatments. The data were analyzed as a three way analysis of variance (ANOVA) using the MIXED procedure in SAS (version 9.2, SAS Institute Inc., NC, USA). Tukey's adjustment was used to determine significant differences between treatments means at $\alpha=0.05$.

Principal Component Analyses was determined using SAS, to discriminate groups among the different Lp_o , g_s , aquaporin expression and protein abundance and phosphorylation state. Pearson correlations were used to determine possible correlations between g_s , Lp_o , aquaporin expression and PIP1 and PIP2 abundance and the phosphorylation forms of PIP2, and root and shoot ET and ACC contents.

Results

Stomatal conductance (g_s) and root hydraulic conductivity (Lp_o) responded different to RH and ACC depending on the ethylene sensitivity

High RH treatment induced the stomatal opening (g_s) in both WT and *nr* plants ($p < .0001$, Supplemental TableS2), but the increase was higher in *nr* plants. ACC treatment diminished stomatal aperture in WT plants at low RH and in *nr* plants at high RH. No effects of AIB on stomatal aperture were observed (Fig. 1A).

L_{p_o} was differently affected by the RH and the chemical treatments in the two genotypes considered (PG x RH x T $p=0.0025$, Supplemental TableS2). In untreated WT plants, high RH significantly reduced L_{p_o} . On the other hand, the application of ACC and AIB reduced L_{p_o} under low RH in WT plants and cause no change under high RH (Fig. 1B). In *nr* plants, L_{p_o} significantly increased with high RH in untreated plants (Fig. 1B). When AIB was applied, there was an increase on L_{p_o} at low RH, while ACC decreased L_{p_o} at high RH (Figure 1B). Also the behavior of L_{p_o} in the two tomato lines was the opposite under low RH compared to high RH (Fig. 1B).

In general, the saturation of the air induced stomatal opening both in WT and *nr* under control conditions, with different responses among genotypes when ACC was applied. Moreover, the sensibility of each genotype to ET determined their L_{p_o} response to the different RH.

Root PIP expression of nr plants was more responsive to high RH than WT plants

Using the different expression of aquaporins, we could estimate the different contributions to L_{p_o} using PCA analyses (Fig. 2). We found that the expression of *SLIP1;1*, *SLIP1;5* and *SLIP2;6* had higher contribution to the variance of the data and that *SLIP2;1*, *SLIP2;3*, *SLIP2;8*, and *SLIP2;9*, grouped together with the whole L_{p_o} in the experimentation (Fig. 2).

At the treatment level, only the expression of *SLIP1;1* and *SLIP1;5* were significant affected by the different treatments applied (0.028, <0.0001 respectively,

Supplemental Table S2), and both of them were negatively correlated with Lp_o under low RH conditions (Table 1). However, the expression of *SIPIP1;1* increased only in *nr* plants with higher RH under control conditions and when AIB was applied (Fig. 3A). On the other hand, the expression of *SIPIP1;5* was only affected by the AIB treatment, being lower with higher RH in WT, and higher with the increase of RH in *nr* plants (Fig. 3B). The expression of *SIPIP2;1* and *SIPIP2;6* were positively correlated with Lp_o only under high RH conditions for both genotypes (Table 1). So it is possible that under different RH regimes, different PIP aquaporins could regulate Lp_o . Expression of *SIPIP2;1* followed the same trend that Lp_o under control conditions, with lower expression at high RH for WT and higher expression for *nr*, and also reducing its expression when ACC was applied (Fig. 3C). In the case of *SIPIP2;6*, its expression was only related with Lp_o in *nr* plants, with higher values at high RH (Fig. 3D). The expression of *SIPIP2;8* was significantly higher in *nr* plants under control conditions at high RH (Fig. 3E). *SIPIP2;9* expression was inhibited by ACC treatment in both genotypes and under both RH treatments (Fig. 3F).

As a general trend, the expression of aquaporins were more sensitive to the change of RH in the ET-*nr* mutants, where we could detect an increase of the expression of *SIPIP1;1*, *SIPIP2;1*, *SIPIP2;6* and *SIPIP2;1* at high RH, at the same time that an increase of Lp_o is reported. This will show the relevance of ET as a regulator of aquaporin expression in order to balance root water uptake.

Root PIP protein abundance and phosphorylation is more responsive to RH in nr plants than in WT ones

PCA analyses showed how all the phosphorylated proteins grouped closely with Lp_o , while the abundance of PIP2 and PIP2L (being the same protein detected by two different antibodies) showed the higher variability in the data and did not group with Lp_o (Fig. 2).

When taking into account the different treatments, we could observe a general and significant effect of the RH treatment on the abundance and phosphorylation state of all the proteins studied except for PIP2₂₈₃ (Supplemental TableS2). Pearson correlations analyses showed that this effect of RH influences also the relation of these proteins with Lp_o , with no correlation at high RH, while all the abundance and phosphorylation state of the PIP1 and PIP2 proteins (except PIP2₂₈₀) were positively correlated with Lp_o under regular RH (Table 1).

On the other hand and under control conditions only, increases on the abundance of PIP1, PIP2, PIP₂₈₀, PIP2_{280/283}, PIP2L and PIP2LP due to high RH were only observed in *nr* plants (Fig. 4 and 5). The application of ACC resulted in different responses according to the genotype studied. In WT plants, there was an increase of PIP1, PIP2, PIP2_{280/283}, PIP₂₈₀, PIP2L and PIP2LP that was independent of the RH, but the abundance of PIP2₂₈₀ was higher with the high RH (Fig. 4). In *nr* plants, ACC application increased the abundance of PIP1 at high RH and that of PIP2_{280/283}, PIP2L and PIP2LP at low RH (Fig.4 and 5). The treatment with AIB was clearly affected by the RH especially in WT plants, with increases in PIP1, PIP2, PIP2₂₈₀, PIP2₂₈₃, PIP2L and PIP2LP abundance at high RH (Fig.4 and 5). In *nr* plants AIB had no effects on PIP protein abundance (Fig. 4 and 5).

As in the expression of PIP aquaporin genes, ET-sensitive mutants under control conditions were affected in their protein content and phosphorylation with increasing RH. When AIB was applied, the same effect could be shown in WT plants and these

two effects give an important insight into the role to ET in PIP aquaporins abundance and phosphorylation state.

ACC and AIB treatments were effective in regulating ET contents independently of RH

The application of ACC induced higher concentrations of ACC and ethylene in the leaves of *nr* plants only, being these values higher under low RH (Supplemental Fig. S1). In roots, ACC accumulated both in WT and *nr* plants, with higher values under low RH, because of ACC application (Supplemental Fig.S1). ET accumulation was dependent on the genotype studied and on the chemical treatments applied (Supplemental TableS2). Hence, ET significantly increased in the roots of WT and *nr* when ACC was applied, but with higher accumulation in WT plants (Supplemental Fig.S1). Pearson correlations analyses showed a positive correlation among Lp_o and the root ET only at low RH (0.575, $p=0.003$).

Since ACC treatment caused a big increase of ET and ACC (Supplemental Fig.S1), and in order to detect the effects of RH and AIB on the amount of both compounds, graphs without ACC treatment were analyzed (Fig. 6). The chemical treatment with AIB increased ACC leaf concentrations of plants under low RH to the same values of plants under high RH values, both in WT and *nr* genotypes (Fig. 6A). While in roots, ACC content significantly increased in WT plants independently of the RH treatment (Fig.6B). At the same time root ET rates were reduced in both genotypes independently of the RH (Fig. 6D), providing the efficiency of the AIB treatment on reducing the ET in plant roots. In leaves, no effect of AIB on ET rate was observed (Fig. 6C).

The ACC and AIB chemical treatments had a big effect on the root and shoot ET content of WT and *nr* genotypes independent of the RH considered.

Discussion

Saturated relative air humidity increased stomatal conductance in WT and *nr* plants independently of the application of ACC and AIB. Plants growing under high RH and under well-watered conditions have been previously shown to have larger stomatal aperture (Rawson et al. 1977). In our case, we found a marginal role of ET on *gs* over a 24h treatment, although direct relations between ET and *gs* have been found at high RH over longer periods of time in tomato (Arve and Torre 2015). The ACC treatment in *nr* plants had an effect on stomatal closure at high RH that was reverted by the AIB treatment. This stomatal closure was related with higher amounts of ACC and ET in the leaves of *nr* as compared with WT plants (Supplemental Fig.S1) Even though *nr* plants are known to block the ET perception, they could be more sensitive to certain ACC concentrations within the tissues than WT plants. This could partially explain the stomatal conductance values in *nr* plants at the ACC and high RH treatments, since *nr* plants treated with ACC also had higher ACC and ET contents in their leaves than WT plants. However, the modulation of ET in these mutants could occur at the perception level, as tomato plants responses to ET are mediated by a family of six receptors isoforms (Sl-ETR1-6) that bind ET with high affinity (O'Malley et al. 2005). The subfamily of receptors I (SL-ETR1-3), to which *nr* mutation belongs, is supposed to play a major role in ET signaling in different aspects of tomato development and not just at the ripening level (Whitelaw et al. 2002; Wang et al. 2006), while subfamily II (Sl-ETR4, 5 and 6) have a predominant role in controlling fruit ripening. As in the case of the ET receptors in Arabidopsis, all of them could be contributing to ET signaling and may overlap functionally (even though they do not need to be entirely redundant).

However, more information will be needed to completely elucidate the role of tomato receptors in the response of these plants to ET.

The RH treatment had a contrasting effect on the Lp_o on both genotypes under control conditions, with WT plants decreasing their Lp_o at high RH, and *nr* plants increasing their Lp_o at high RH, thus the differences between the two tomato lines switched with the RH. The changes on the osmotically determined Lp_o are mainly related with the movement of water through the symplastic pathway (Steudle 2001), closely related with the change in expression of aquaporins and their phosphorylation state. In our study, the PCA analyses (Fig. 2) showed the relationship of Lp_o on the expression and phosphorylation state of aquaporins, with different contributions according to the genotypes studied and especially with the RH treatment applied (Fig. 2). Under control conditions, the expression of *SlPIP2;1* is the only one that follows the same trend as Lp_o for both WT and *nr*. PIP2s aquaporins are usually related with high water transport capacity (Törnroth-Horsefield et al. 2006; Li et al. 2011). The tomato aquaporin *SlPIP2;1* has been described by Li et al. (2016) as a high permeable to water, expressed almost ubiquitously in all major root tissues and participating in the drought stress tolerance of tomato plants, what make it a good candidate to deal with the different RH conditions and treatments in both WT and *nr* mutants in our experiment. However, it was in *nr* mutants under high RH when the expression of the aquaporins changed, giving ET a main role in the downregulation of the *SlPIP1;1*, and an upregulation of *SlPIP1;6* and *SlPIP2;9* at saturated RH.

We found similar trends, as for aquaporin expression, in the abundance and phosphorylation state of aquaporins, with different regulation in WT and *nr* plants and with changes in aquaporins abundance and phosphorylation in ET-insensitive plants. Phosphorylation of aquaporins is proposed as one of the main mechanisms controlling

water through the cell membranes (Johansson et al. 1998; TorNroth-Horsefield et al. 2006). Our results support the idea of the importance of the aquaporin phosphorylation as one of the main factor driving root water transport, as previously reporter for barley under salt stress (Horie et al. 2011), tomato under flooding (Calvo-Polanco et al. 2014a) and other plants under drought (maize-Barzana et al. 2014; tomato-Sanchez-Romera et al. 2016; olive trees-Calvo-Polanco et al. 2016), and its dependence of ET for a proper regulation of these proteins.

In our case, the tight regulation of aquaporins in WT plants lead to a decrease of Lp_o at high RH that correspond with an increase of root and leaf ACC and ET. These results are in concordance with the results reported on ET negatively affecting Lp_o (Li et al. 2009). In the *nr* plants, the strategy was different with no changes in ET content, but lower root ACC contents that induced the expression of aquaporin expression and phosphorylation. ACC has been previously proposed no just as one step-molecule to ET conversion but as one of the signals (Van de Poel and Van Der Straeten 2014) that may affect Lp_o under different stresses (Calvo-Polanco et al. 2014a).

In conclusion, we have studied the effects of ACC, AIB and RH on root water transport in WT and *nr* tomato plants. We have found a different response in Lp_o to different RH conditions, and to the application of AIB in *nr* mutants. These responses have been linked to a fine tune plant hormonal regulation and to the phosphorylation of aquaporins in order to increase root water transport. On the other hand, the *nr* plants were responsive to high RH enhancing Lp_o , which was abolished by ACC application and enhanced by the presence of AIB, showing how the sensitivity to ET can alter water balance in plants. ET responses in plants and the mechanisms involved in those responses is a critical factor in the understanding of plant development and adaptation to changing environmental conditions. Future research in this area should include the

different ET receptors involved as well as a deeper insight into the transpiration processes that are affected by the root responses to ET.

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Table 1. Pearson correlations analyses among protein abundance (PIP1, PIP2, PIP2L), PIP2 phosphorylation at different residues (PIP2₂₈₀, PIP2₂₈₃, PIP2_{280/283}, PIP2LP) and expression with *Lp_o* at low and high RH.

	PIP1	PIP2	PIP2 ₂₈₀	PIP2 ₂₈₃	PIP2 _{280/283}	PIP2L	PIP2LP	<i>SIPIP1;1</i>	<i>SIPIP1;5</i>	<i>SIPIP2;1</i>	<i>SIPIP2;3</i>	<i>SIPIP2;6</i>	<i>SIPIP2;8</i>	<i>SIPIP2;9</i>	gs
<i>Lp_o</i> (Low RH)	0.525 0.008*	0.578 0.003*	0.314 0.134	0.549 0.008*	0.550 0.005*	0.485 0.016*	0.533 0.007*	-0.439 0.032*	-0.497 0.013*	-0.057 0.790	0.221 0.311	-0.109 0.637	-0.255 0.252	-0.488 0.040*	0.165 0.342
<i>Lp</i> (High RH)	-0.349 0.095	-0.245 0.272	0.041 0.856	-0.180 0.449	-0.274 0.242	-0.029 0.908	-0.187 0.456	0.230 0.280	0.369 0.076	0.510 0.011*	-0.030 0.899	0.462 0.035*	0.272 0.233	0.186 0.475	0.053 0.761

Values of both genotypes were considered for each RH. Numbers are correlation coefficient and p values. Asterisks mean significant correlation

($p < 0.05$).

Figure Legends

Figure 1. Stomatal conductance (A) and root hydraulic conductivity (L_{p_o}) (B) of *Solanum lycopersicum* wild type (WT) and *never ripe* (*nr*) plants treated with 0, 0.1 mM ACC, or 0.1mM AIB and grown at low RH (50%) or high RH (close to saturation) for 24 hours. Significant differences among treatment means were determined with the Tukey's test and are shown with different letters at 0.05. Means ($n=8$) $\pm$ SE.

Figure 2. Principal component analyses (PCA) run in SAS, accounting for the expression of seven aquaporin tomato genes, PIP1 and PIP2 abundances, PIP2 phosphorylation state, root hydraulic conductivity (L_{p_o}), and stomatal conductance (g_s).

Figure 3. Relative root aquaporin expression of *Solanum lycopersicum* wild type (WT) and *never ripe* (*nr*) plants. The relative expression of aquaporins *SlPIP1;1*(A), *SlPIP1;5*(B), *SlPIP2;1*(C), *SlPIP2;6*(D), *SlPIP2;8*(C) and *SlPIP2;9*(D) is shown in plants treated with 0, 0.1 mM ACC, or 0.1mM AIB and grown at low RH (50%) or high RH (close to saturation) for 24 hours. Significant differences among treatment means were determined with the Tukey's test and are shown with different letters at 0.05. Means ($n=3$) $\pm$ SE.

Figure 4. *Solanum lycopersicum* wild type (WT) and *never ripe* (*nr*) plant roots protein abundance PIP1(A) and PIP2(B), phosphorylated PIP2 protein abundance at Ser-280:PIP2₂₈₀(C) and Ser-283: PIP2₂₈₃(D). Plants were non-treated, or treated with 0.1 mM ACC, or 0.1mM AIB and grown at low RH (50%) or high RH (close to saturation) for 24 hours. Significant differences among treatment means were determined with the Tukey's test and are shown with different letters at 0.05. Means ($n=3$) $\pm$ SE.

Figure 5. *Solanum lycopersicum* wild type (WT) and *never ripe* (*nr*) plant roots protein abundance of phosphorylated PIP2 at Ser-280-283: PIP2_{280/283} (A), and PIP2L abundance (B) and its phosphorylated form PIP2LP (C). Plants were non-treated, or

treated with 0.1 mM ACC, or 0.1mM AIB and grown at low RH (50%) or high RH (close to saturation) for 24 hours. Significant differences among treatment means were determined with the Tukey's test and are shown with different letters at 0.05. Means (n=3) $\pm$ SE.

Figure 6. Leaf and root ACC content (A,B), and leaf and root ethylene rates (C,D), in *Solanum lycopersicum* wild type (WT) and *never ripe* (*nr*) plants treated with 0, or 0.1mM AIB and grown at low RH (50%) or high RH (close to saturation) for 24 hours. Significant differences among treatment means were determined with the Tukey's test and are shown with different letters at 0.05. Means (n=3) $\pm$ SE.