UNIVERSIDAD POLITECNICA DE VALENCIA

ivia
instituto valenciano
de investigaciones agrarias

Water-deficit tolerance in citrus is mediated by the down regulation of PIP gene expression in roots

Máster en Producción Vegetal y Ecosistemas Agroforetales

Alumno: Juan Rodríguez Gamir

Directora: Mª Ángeles Forner Giner

Tutor: Manuel Agustí Fonfría

Water-deficit tolerance in citrus is mediated by the down regulation of PIP gene expression in roots

Juan Rodríguez Gamir

ABSTRACT

Water deficit (WD) is a growing problem in agriculture. In citrus crops, genetically-determined characteristics of the rootstock are important factors in plant responses to WD. Aquaporins are involved in regulating the water supply to the plant by mediating water flow through the cell membranes. Recent studies support a direct role for aquaporins in plant water relations and demonstrate their involvement in tolerance to WD. This study investigates the relationship between photosynthetic and water-balance parameters with levels of expression of aquaporins in conditions of moderate WD in the rootstocks *Poncirus trifoliata* (L.) Raf. (PT), Cleopatra Mandarin (*Citrus reshni* Hort. ex Tan.) (CM) and 030115 (a hybrid of the two former rootstocks). Under conditions of WD, the hybrid 030115 drastically reduced aquaporin expression, accompanied by a loss of plant vigour but without reducing the net CO_2 assimilation (A_{CO2}). PT maintained the same level of aquaporin expression under WD as under normal irrigation conditions, but suffered a sharp reduction in A_{CO2}. CM, which has lower expression of aquaporins than PT under both normal irrigation conditions and WD, responded better to water stress conditions than PT. Thus, low levels of aquaporins, or repression of their expression, accompanied by decreased plant vigour resulted in a decrease in plasma membrane permeability, thereby facilitating water retention in the cells under conditions of water stress. This can induce water stress tolerance in citrus rootstocks.

RESUMEN

El déficit hídrico (WD) es un problema creciente en la agricultura. En el caso de los cítricos, las características del patrón, determinadas genéticamente, son un factor importante en la respuesta de la planta al WD. En la regulación del aporte agua a la planta están implicadas las acuaporinas, las cuales regulan el flujo del agua a través de las membranas celulares. Recientes estudios sustentan un papel directo de las acuaporinas en las relaciones hídricas de la planta y demuestran su participación en la tolerancia al WD. En el presente estudio se relacionó la respuesta de parámetros fotosintéticos y relaciones hídricas con los niveles de expresión de acuaporinas en condiciones de WD moderado de los patrones *Poncirus trifoliata* (L.) Raf.(PT), Mandarino Cleopatra (*Citrus reshni* Hort ex Tan.) (CM) y 030115 (híbrido monoembriónico de los dos anteriores). El híbrido 030115 redujo drásticamente su expresión de acuaporinas acompañada de una pérdida de vigor de la planta pero sin reducir su tasa neta de asimilación de CO_2 (A_{CO2}) en condiciones de WD. PT en condiciones de WD mantuvo los mismos niveles de acuaporinas que en condiciones de riego normal y sufrió una fuerte reducción de A_{CO2}. CM, que posee una expresión de acuaporinas inferior a PT tanto en condiciones de riego normal como en condiciones de WD, tuvo mejor respuesta a las condiciones de WD que PT. Por tanto, bajos niveles de acuaporinas o la represión de su expresión acompañada de una pérdida de vigor en la planta se traducen en un descenso de la permeabilidad de las membranas plasmáticas que permite conservar el contenido de agua en las células en situaciones de estrés hídrico. Esto puede inducir tolerancia al estrés hídrico en los patrones de cítricos.

INTRODUCTION

Water stress in citrus reduces stomatal conductance (gs), transpiration rate (E) and net assimilation of CO_2 (A_{CO2}) (Arbona et al. 2005; García-Sánchez et al. 2007). In addition, water relations and tolerance to abiotic stresses vary significantly between rootstock (Syvertsen and Levy 2005). Rootstocks present genetically-determined characteristics that affect plant water relations (Castle and Krezdorn 1975). These features include root system distribution, water and nutrient absorption efficiency (Castle and Krezdorn 1975) and the anatomy of the vascular elements (Vasconcellos and Castle 1994; Rodríguez-Gamir 2010). These characteristics are associated with differences in root hydraulic conductance (Sinclair and Allen 1982; Syvertsen and Graham 1985), which determine the ability of the rootstock to supply water and nutrients to the plant. This ability could be the main factor influencing fruit development in citrus trees, determining the strength of the grafted variety and its tolerance to water stress (Syvertsen and Lloyd 1994; Medina and Machado 1998). Taken together, this information indicates that rootstock characteristics are an important factor influencing plant responses to water deficit (WD) situations.

In many plant species, root hydraulic conductance decreases significantly under WD conditions (Sumner and Boswell 1981; Cruz et al. 1992; North and Nobel 1996; Lo Gullo et al. 1998; Martre et al. 2001; North et al. 2004). Those decreases can be associated with substantial anatomical modifications, such as the development of Casparian bands and suberin lamellae in the exodermis and the endodermis (Enstone and Peterson 1998; North and Nobel 2000) or with a reduction in aquaporin activity (Martre et al. 2001; Siefritz et al. 2002; North et al. 2004). Besides reducing hydraulic conductance, plants have developed various mechanisms to withstand water stress, such as higher root-shoot ratios, fewer and smaller leaves, concentrated solutes or increased activity of oxidative stress enzymes in leaf cells (Lei et al. 2006).

Such flexible, short-term regulation of root water uptake appears to involve aquaporins. Aquaporins are trans-membrane protein channels located in the plasma membrane, tonoplast and other intracellular membranes, which are abundantly expressed in roots

(Javot and Maurel 2002; Tyerman et al. 2002; North et al. 2004). Aquaporins belong to the superfamily of MIPs (major intrinsic proteins) and facilitate the passive flow of water molecules across cell membranes by apparently regulating the transcellular passage of water (Agree et al. 1993; Maurel 1997). Depending on their location, aquaporins in plants are classified as tonoplast intrinsic proteins (TIPs) or intrinsic proteins of the plasma membrane (PIPs) (Maurel 1997), or are assigned to one of two other less-studied sub-families: Nodulin 26-like intrinsic membrane proteins (NIPs) or basic intrinsic membrane proteins (SIPs) (Johanson et al. 2001; Zardoya 2005). Furthermore, the PIPs sub-family is divided into two evolutionary groups based on homologous sequences (PIP1 and PIP2) (Kammerloher et al. 1994), each presenting several isoforms (*e.g.*, PIP1;1) (Kammerloher et al. 1994; Daniels et al. 1994; Weig et al. 1997). Concerning water transport through the plant, PIPs are probably more important than TIPs in regulating water uptake by the roots because the plasma membrane is generally much less permeable to water than the tonoplast (Javot and Maurel 2002). Aquaporins in plants often show specificity of expression in tissues and organs (Tyerman et al. 2002), but the role of aquaporins in the roots and stems is more important in regulating water relations than in other areas of the plant (Smart et al. 2001; Luu and Maurel 2005).

Plant water relations may be altered by water stress, salinity and/or cold temperatures due to the water potential reduction in the cell or the extracellular environment, which can difficult water absorption and promote water loss from cells. It has been shown that these environmental stimuli can regulate TIPs and PIPs expression at different levels (Hachez et al. 2006). Moreover, recent studies support a direct role of PIPs in plant water relations and demonstrate their involvement in water stress tolerance (Peng et al. 2007). Studies relating aquaporin gene expression to water stress are contradictory. Some studies indicate that expression of PIPs is typically reduced in roots during water stress (Smart et al. 2001; Suga et al. 2002), others have revealed no change (Morillon and The Versailles 2002; Galmés 2007) while others indicate expression increases (Kirch et al. 2000; Galmés et al. 2007).

In addition to varying in response to water deficiency, water channel activity, may vary with the time of day (Henzler et al. 1999; Tsuda and Tyree 2000), with root development

(Barrowclough et al. 2000; North and Nobel 2000; Martre et al. 2001; Hukin et al. 2002) and in response to other stresses such as salinity (Carvajal et al. 1999; Martinez-Ballesta et al. 2000) or nutritional deficiency (Carvajal et al. 1996; Clarkson et al. 2000).

According to Galmés (2007), most studies conducted to test aquaporin expression in response to environmental stresses lack simultaneous analyses of plant physiological responses to these stresses, such as photosynthesis or stomatal conductance. Moreover, most studies into citrus water stress conditions do not consider the role of aquaporins in their response to this abiotic stress. Thus, our study evaluates responsiveness of photosynthetic parameters and water balance to moderate water stress in the rootstocks *Poncirus trifoliata* (L.) Raf. (PT), Cleopatra Mandarin (*Citrus reshni* Hort ex Tan.) (CM) and 030115 (a mono-embryonic hybrid of the former two rootstocks) and its relation with aquaporin expression.

MATERIAL AND METHODS

Plant Material and growth conditions

The plant materials used in the experiments were 15-month old rootstocks of *Poncirus trifoliata* (PT), Cleopatra mandarin (CM) and the hybrid 030115 (CMxPT).

Seeds of PT and CM rootstocks were harvested from the mother seed trees held in the germplasm collection at Instituto Valenciano de Investigaciones Agrarias (IVIA), Valencia, Spain. Seeds were sown on 55x40 cm trays containing a mixture of peat and siliceous sand (3:2 vol:vol) in an aphid-proof greenhouse with a cooling system that kept temperatures between 15 ºC and 18 ºC and 80% relative humidity. Plants were grown with supplementary light (<50 mol m-2 s-1, 400-700 nm) to extend the photoperiod to 16 h. Five-month old plants were transplanted into 3 lt pots with peat.

The hybrid 030115 was obtained through a breeding program of targeted hybridizations, specifically focused on the development of new rootstocks, initiated by J. B. Forner in 1974 at

IVIA (Forner-Giner et al. 2003). This hybrid, because it is mono-embryonic, cannot be propagated by seed, hampering their reproduction. However, *in vitro* propagation produces sufficient plant numbers for experimentation. For this propagation, young apical shoots were collected from mother plants in April - May and subjected to a sterilization process in a laminar flow. The material was pre-washed with alcohol, disinfected with sodium hypochlorite solution (0.425% active chlorine) for 10-12 minutes, followed by three washes with sterile distilled water for durations of 5, 10 and 15 minutes. After the sterilization process, plant buds were transferred to test tubes containing nutrient medium based on the DKW (Driver and Kuniyuki, 1984) mineral solution. The cultures were maintained under controlled conditions at temperature of 22-24 ºC and a 16 h light / 8 h darkness photoperiod throughout. In the multiplication phase, buds were transferred every two weeks to fresh DKW medium, but with an additional 0.5 mg/L of benzyladenine (BA) and 0.01 mg/L indole butyric acid (IBA) to promote shoot elongation. Cultures were maintained in the growth room for 8-10 days, until they reached a height of 6-8 cm. Elongated shoots of good quality were prepared by cutting the base to be transferred to soil. In the rooting and acclimatization phase, cuttings prepared from *in vitro* cultures were planted in a substrate composed of peat and perlite and placed under conditions of high humidity in acclimatization tunnels inside the greenhouse. Under these conditions the plants developed a root system in 15-20 days. Following this, they were transplanted and transferred to natural environmental conditions for growth.

All rootstocks used in the experiment were grafted to the citrus variety 'Valencia Late' (*C. sinensis* (L.) Osb) when the plant was six-months old. All plants were of uniform size at the beginning of the experiment. The experiments were conducted at IVIA (39.28 N - 0.22 W) during Summer and early Fall, with a photoperiod of between 10.5-15.5 daylight hours. The plants were maintained in a glass greenhouse with a constant temperature of 24 ± 1 º C and relative humidity of 80%.

Six control plants of each rootstock were watered normally [PT(c), MC(c) and 030115(c)] and another six plants with deficit irrigation [PT(wd), MC(wd) and 030115 (wd)]. The 36 plants

were randomly distributed in six rows with six plants per row and surrounded by a guard row not included in the experiment.

The plants were contained individually in 3-liter pots with peat and were watered every seven days with the following nutrient solution: 3 mM $Ca(NO_3)_2$, 3 mM KNO_3, 2 mM $MgSO_4$, 2.3 mM H_3PO_4, 17.9 mM Fe-EDDHA and micronutrients as described by Hoagland and Arnon (1950). The pH of the nutrient solution was adjusted to 6.0 with 1 M KOH or 1 M H_2SO_4. The plants were kept in the greenhouse for eight weeks until some of the plants showed slight symptoms of wilting. The dose of irrigation for the control plants (normal irrigation) was 300 ml/week. The plants under water deficit were received a dose of 50 ml/week.

Weekly transpiration (T_w) and total transpiration of the whole plant (T_{Total})

To measure transpiration of the whole plant after each irrigation weekly (T_w), the pots were covered with a sheet of plastic containing a hole of similar diameter to that of the plant stem through which the plant protruded. This system prevented the evaporation of the substrate and reduced the possibility of anaerobic conditions affecting the health of the root. Previously it was found that the results of this measurement system did not differ from those obtained by placing the pot in a polypropylene bag sealed tightly around the base of the stem. The weekly transpiration of each plant was calculated as the difference between the weight of the watered pot (after draining) and the weight of the pot before watering the following week. The total transpiration of the plant throughout the trial (T_{Total}) was also calculated by adding the T_w values of the eight weeks of the experiment.

Photosynthesis and related parameters

The net CO_2 assimilation (A_{CO2}), transpiration (E), stomatal conductance (gs) and substomatal CO_2 concentration (Ci) were measured fortnightly with a portable photosynthesis system (CIRAS-2) (PP-systems, Hitchin, UK), equipped with an external source of light to maintain a constant PAR of 900 mmol m^{-2} s^{-1}. Previously, a saturation curve of A_{CO2} as a function of PAR was determined, coinciding this value with the saturation point of the curve in agreement with Syvertsen (1984). The measurements were made in the

morning from 10:00 am to 12:00 pm and it was found that during this interval, the time of measurement did not interfere with the results. Parameters were measured from fully expanded leaves from the central part of each plant.

On the last day of the experiment, SPAD values relating to chlorophyll concentration, were measured in all leaves of each plant with SPAD portable equipment (Minolta Co., Osaka, Japan).

Measures of biomass

When the experiment was completed (57 days), plants were uprooted and separated into taproot, fine roots, stem and leaves. All plant parts were washed and weighed. Part of the fine roots of each plant was frozen immediately with liquid nitrogen and stored at -80 º C for subsequent RNA extraction. To avoid the influence of time of day in the expression of aquaporins (Henzler *et al*. 1999), all plants were uprooted simultaneously between 10:00 am and 11:00 am.

All plant fractions were dried in a forced-draft oven at 60 ºC for 48 hours and re-weighed. The dry weight of shoots (S) (the sum of the dry weight of stem and leaves), the root dry weight (R) (the sum of the dry weight of fine roots (FR) and the taproot) and the ratios S/R and S/FR, were calculated.

Cloning of PIPs

Specific primers were designed to amplify conserved sequences to detect PIP1 and PIP2. Amplified fragments were 156 and 137 bp in length respectively, corresponding to each open reading frame (ORF). Reverse transcription and PCR reactions were carried out using the SuperScript III one-step RT-PCR system with Platinum Taq DNA polymerase kit (Invitrogen, Renfrewshire, UK) following the manufacturer's instructions. Amplified products were cloned into a pTZ57R/T vector (MBI Fermentas) to generate pTZ/PIP-1 and pTZ/PIP-2. After sequencing, plasmids cloned in plus-strand polarity were selected.

Northern Blot Analysis

Total RNA was extracted from roots according to the protocol described by Ancillo et al. (2007). Total RNA (12 μg per lane) were electrophoresed on 1% (W/v) denaturing formaldehyde-agarose gels and transferred by capillarity to nylon membranes (Sambrook et al. 1989). Membranes (Roche, Basel, Switzerland) were air-dried and the nucleic acids were covalently linked by UV cross-linking (700x100 J/cm2). The pTZ plasmids were linearized with the restriction enzyme Xba I and used to synthesize digoxigenin-labelled riboprobes as described previously by Más et al. (1993). Pre-hybridisations and hybridisations with the digoxigenin-labelled RNA probes were conducted as described by Pallás et al. (1998). Chemi-luminescent detection, with CSPD reagent (Roche, Basel, Switzeland) as the substrate, was performed as recommended by the manufacturer. Films were exposed for 15-30 minutes before development.

RESULTS

Effect of water stress on parameters related to gas exchange

Figure 1 shows the course of the percentage of net CO_2 assimilation (A_{CO2}), transpiration (E) and stomatal conductance (gs) of rootstocks PT, CM and 030115 under WD with respect to the values of these parameters in their respective controls throughout the experiment. In PT, A_{CO2} was significantly lower in stressed plants than in control plants from day 28 until the end of the experiment. In CM, A_{CO2} was lower in stressed plants than in control plants only at the end of the experiment (day 56). However, the rootstock 030115 showed no significant differences in A_{CO2} values between control plants and stressed plants throughout the entire experiment despite of the reduction in E and gs compared to controls from day 42 of the experiment. Nevertheless, the patterns of E and gs parallel that of A_{CO2} in PT(wd) and CM(wd).

The ANOVA performed to compare the values of A_{CO2} measured at the end of the experiment (day 56) among the three rootstocks studied (Table 1) showed that A_{CO2} value was significantly higher in PT(c) than in MC(c) and 030115(c). A_{CO2} value from 030115(wd) presented no significant differences with those of 030115(c) or MC(c) and was statistically-significant higher than MC(wd) and PT(wd) values.

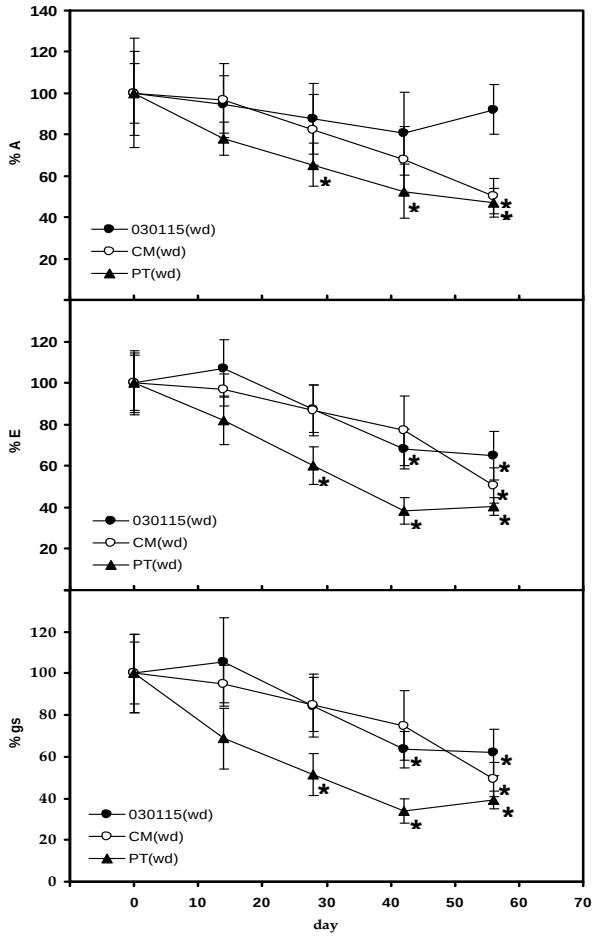


Fig. 1. Patterns of percentage net CO_2 assimilation (A_{CO2}), transpiration (E) and stomatal conductance (gs) of PT(wd), CM (wd) and 030115(wd) with respect to their controls. Vertical bars indicate the standard error of six independent measurements. Asterisks indicate significant differences with their respective controls (100%) at P> 0.95.

Although water stress treatment caused a decrease in gs in rootstocks of PT(wd) and CM(wd) (Table 1) it did not change sub-stomatal CO_2 concentration (Ci) in these two rootstocks. However, rootstock 030115 presented significantly lower Ci in stressed plants than in controls.

The SPAD values, associated with chlorophyll content, were significantly lower in PT(wd) and CM(wd) compared to their respective controls, whereas there was no significant difference between those of 030115(c) and 030115(wd).

Table 1. Values of whole plant total transpiration throughout the experiment (T_{Total}), percentage of T_{Total} with respect to controls, and net CO_2 assimilation (A), transpiration (E), stomatal conductance (gs), sub-stomatal CO_2 concentration (Ci) and SPAD values measured 56 days after the start of the test (n=6) in PT(c), PT(wd), CM(c), CM(wd), 030115(c) and 030115(wd). Means with different letters indicate statistical differences at P>0.95.

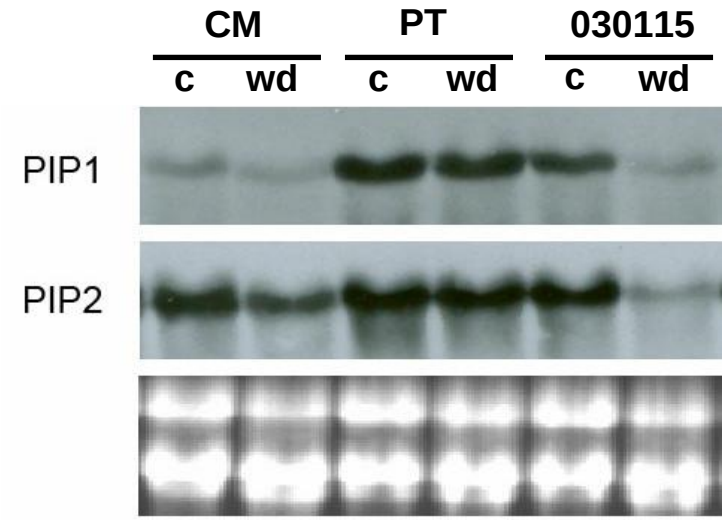
	T_{Total} g_{H2O}	% T_{Total} respect to the control	A µmol CO_2*m^{-2}*s^{-1}	E mmol H_2O*m^{-2}*s^{-1}	gs mol CO_2*m^{-2}*s^{-1}	C_i ppm	SPAD
PT(c)	1678,02 ± 174,24 b		5,83 ± 0,41 a	1,59 ± 0,21 a	0,066 ± 0,007 a	189,89 ± 14,28 a	56,95 ± 15,06 a
PT(wd)	808,07 ± 46,16 cd	48,16 ± 2,75 b	2,47 ± 0,17 c	0,62 ± 0,08 c	0,026 ± 0,004 c	194,47 ± 14,62 a	42,78 ± 21,63 cd
CM(c)	1048,67 ± 50,57 c		4,69 ± 0,41 b	1,22 ± 0,14 b	0,051 ± 0,007 b	172,46 ± 19,23 a	47,15 ± 19,23 bc
CM(wd)	602,64 ± 27,12 d	57,47 ± 2,59 a	2,35 ± 0,20 c	0,61 ± 0,07 c	0,025 ± 0,004 c	197,06 ± 21,98 a	37,30 ± 26,14 d
030115(c)	2053,65 ± 136,88 a		4,35 ± 0,47 b	1,07 ± 0,09 b	0,042 ± 0,005 b	189,55 ± 14,25 a	57,92 ± 19,32 a
030115(wd)	809,00 ± 68,21 cd	39,39 ± 3,32 c	4,01 ± 0,43 b	0,69 ± 0,06 c	0,028 ± 0,003 c	116,16 ± 8,73 b	51,75 ± 14,35 ab

Reduced irrigation resulted in a smaller, statistically-significant value of T_{Total} in the three rootstocks under water stress conditions with respect to their respective controls (Table 1). Moreover, this reduction was significantly greater in rootstock 030115(wd) (60.6% reduction with respect to 030115(c)) than in PT(wd) (51.8% reduction with respect to PT(c)) and CM(wd) (42.5% reduction compared to CM(c)). However, there were no significant differences in T_{total} values between rootstocks PT(wd), CM(wd) and 030115(wd).

Effect of water stress on gene expression

In order to study the gene expression levels of PIP1 and PIP2 total RNA was extracted from the fine roots of control plants and plants subjected to WD. The expression levels of PIP2 mRNA were similar in plants PT(c), 030115(c), and MC(c), while the levels of PIP1 in CM(c) were much lower than in PT(c) and 030115(c) (Fig. 2). Expression of mRNA aquaporins in response to WD was very different in the three rootstocks studied: in PT, water deficiency did not change aquaporin expression; in CM, there was a slight repression in the expression of PIP2; whereas in rootstock 030115 there was a drastic decrease in the levels of mRNAs from both, PIP1 and PIP2, under WD treatment.

Fig. 2. Northern blot analysis of PIP1 and PIP2 expression in well-watered (c) and water-stressed (wd) plants. A representative ethidium bromide-stained gel is shown to demonstrate approximately equal loading of intact samples.



Effect of water stress on biomass and water relations

Water stress treatment did not significantly alter the root dry weight (R) in any of the rootstocks studied (Table 2). However, there was a significant reduction in dry weight of scions (S) grafted on PT and 030115 under water stress conditions, compared to their controls. This reduction in aerial parts under water stress conditions caused a significant reduction in the ratio S/R in PT(wd) and 030115(wd) versus PT(c) and 030115(c), respectively. In CM, the S/R ratio did not change with water stress.

The value of whole plant weekly transpiration (T_w) (measured gravimetrically) obtained on the last week of the experiment was divided by the value of the dry weight of fine roots (FR) to calculate the amount of water supplied to the plant per unit of fine root dry weight during the last week of the experiment (T_w/FR) (Table 2). This parameter, at least in control plants, may be related to the hydraulic conductance of the plant and, more specifically, to the hydraulic conductance of the root system since in citrus there is a positive correlation between whole plant transpiration and root hydraulic conductance (Syvertsen and Graham 1985).

Table 2. Values of R (root dry weight), S (dry weight of aerial part) and ratios S/R, S/FR (dry weight of aerial part/dry weight of fine roots), T_w/FR (whole plant transpiration during the last week of the experiment/dry weight of fine roots) and percentage of Tw/FR relative to controls (n=6) in PT (c), PT(wd), CM(c), CM(wd), 030115(c) and 030115(wd). Means with different letters indicate statistical differences at P> 0.95

	R (g)	S (g)	S/R	S/FR	Tw/FR ($gH2O*gfine\text{-}roots^{-1}*week^{-1}$)	% T_w/FR respect to the control
PT(c)	7,33 ± 1,11 a	11,74 ± 1,69 b	1,67 ± 0,20 ab	4,24 ± 0,53 a	77,4 ± 13,3 a	
PT(wd)	6,75 ± 0,58 a	7,12 ± 0,41 c	1,08 ± 0,09 cd	2,94 ± 0,18 b	38,3 ± 3,9 b	49,5 ± 5,1 b
CM(c)	8,25 ± 1,10 a	6,80 ±0,60 c	0,87 ± 0,10 d	2,29 ± 0,24 b	37,6 ± 4,7 b	
CM(wd)	8,36 ± 0,84 a	6,57 ± 0,65 c	0,78 ± 0,01 d	2,52 ± 0,20 b	24,6 ± 1,3 b	65,4 ± 3,4 a
030115(c)	9,20 ± 1,37 a	17,66 ± 2,31 a	1,97 ± 0,11 a	4,59 ± 0,59 a	73,6 ± 12,6 a	
030115(wd)	7,65 ± 0,79 a	10,18 ± 0,51 bc	1,37 ± 0,10 bc	2,67 ± 0,09 b	24,8 ± 3,2 b	33,7 ± 4,3 c

In control plants, the T_w/FR value in PT and 030115 was almost double that of MC, which implies that CM plants needed twice the root system of PT or 030115 in order to provide the same amount of water. This directly influences the S/R ratio since, as shown in ANOVAs conducted for the parameters T_w/FR, S/R and S/FR, a higher value of T_w/FR implied higher values of S/R and S/FR in control plants. This was confirmed by a linear regression analysis of the S/FR ratio, dependent on the T_w/FR value in control plants (Fig 3.A), resulting in a regression whose intercept is significant at P>95% and slope significant at P>99% with r^2=0.77. This correlation demonstrates the increasing S/FR ratio in control plants, coincident with greater capacity for water uptake per unit of dry weight of fine roots. The lower values of these two parameters in the rootstock CM(c) are shown in Fig 3.A.

In deficient plants (Fig 3.B), linear regression of the S/FR ratio was also significant, but with r^2 = 0.26. The low value of r^2 suggests that other factors such as stomatal conductance, water availability or that anatomical modifications of root cells can also affect the relationship found between T_w/FR and S/FR in control plants. However, statistical comparison of the two regression lines indicated that both the slope and the intercept showed no significant differences between the two treatments. Therefore, the relationship between T_w/FR and S/FR in control plants and stressed plants is the same.

Values of PT(c) and 030115(c) had a similar distribution in the regression analysis performed on control plants (the two rootstocks had higher values of T_w/FR and S/FR than CM(c)) (Fig. 3.A), whereas for water deficient plants, rootstock 030115(wd) presented values of T_w/FR

and S/FR similar to those of CM(wd) (Fig. 3.B). This implies a change in the relationship between T_w/FR and S/FR in rootstock 030115 on exposure to water deficiency.

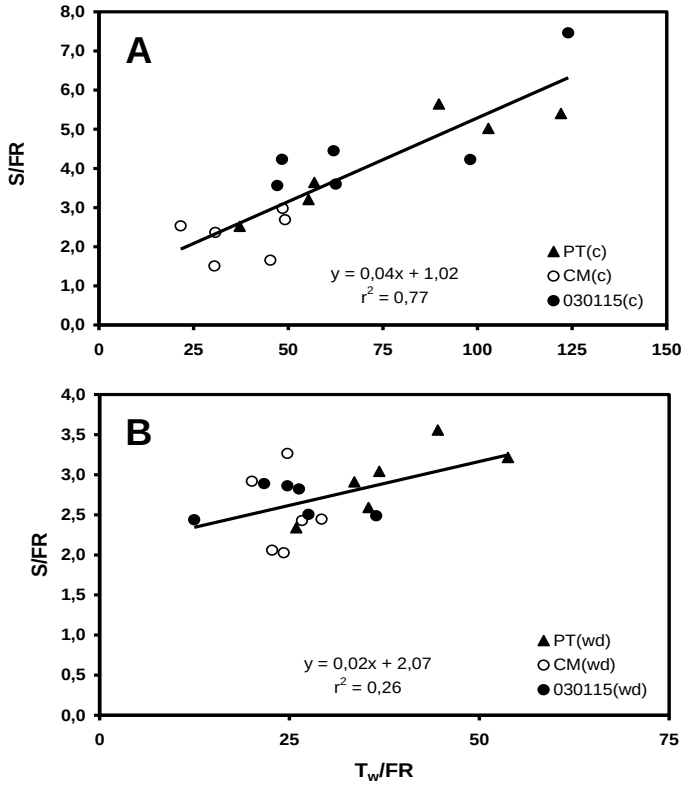


Fig. 3 Linear regression of the ratio S/FR (dry weight of the aerial part/dry weight of fine roots) against the function of weekly water transported per unit of fine root dry weight in the last week of the experiment (Tw/FR) in plant controls (Fig. 3A) and water-stressed plants (Fig 3.B). In Figure 3A the intercept is significant at P>0.95 and the slope at P>0.99. In Figure 3B the intercept is significant at P>0.99 and the slope at P>0.95. The two regression lines do not differ significantly either in slope or constant.

DISCUSSION

In this study, gene expression of aquaporins under conditions of water deficiency in three citrus rootstocks with different physiological responses to this abiotic stress, were evaluated. Of the three rootstocks studied, only 030115 did not reduce its A_{CO2} that additionally coincided with a dramatical decreased of aquaporin expression under WD.

The increased WD tolerance found in 030115 could be related to the down-regulation of PIP1 and PIP2 mRNAs transcription in the fine roots of this rootstock under water stress, leading to a decrease in membrane permeability to water. This would preserve water content in the cells and, thus, maintain the proper water balance of the plant (Yamada et al. 1995;

Johansson et al. 1998; Smart et al. 2001; Suga et al. 2002; Aharon et al. 2003). This translates into lower water flow through the plant because repressed expression of aquaporins is associated with decreases in the hydraulic conductance of plant organs (Henzler et al. 1999; Clarkson et al. 2000; North et al. 2004), which is related to the parameter T_w/FR. T_w/FR was reduced in the three rootstocks studied under conditions of water deficit. However, this reduction was much greater in rootstock 030115 (wd) (77.7% reduction with respect to its control) than in PT(wd) (50.5% reduction with respect to its control) and MC(wd) (34.5% reduction with respect to its control). The sharp reduction in T_w/FR in rootstock 030115, probably a result of repressed expression of aquaporins, is in agreement with studies conducted on complete root systems or on specific areas of roots that suggest aquaporins can account for between 60% and 80% of the hydraulic conductance of roots (Maggio and Joly 1995; Wan and Zwiazek 1999; Barrowclough et al. 2000; Matre 2001).

Rootstock CM has historically been characterized as moderately tolerant to water stress (Romero 2006; Gimeno 2009). In this study, CM showed a better response to moderate WD than PT. The levels of aquaporin expression also explain the greater water stress tolerance of CM than PT, since CM under normal water supply has lower expression of aquaporins than PT(c) and 030115(c). In addition, that CM has hydraulic characteristics allowing it to tolerate water deficiency is reinforced by the fact that CM was the only rootstock in the study that did not show an altered S/R ratio in response to water deficit. Additionally, the value of T_w/FR under conditions of normal irrigation was significantly lower in CM(c) than in PT(c) and 030115(c), which may be related to lower expression of aquaporins in CM under these conditions. The T_w/FR value in MC(c) was 51.42% lower than in PT(c). This is consistent with other studies in which CM presented root hydraulic conductance 50% lower than that of PT (Syvertsen and Graham 1985; Zekri and Parsons 1989).

The tolerance of 030115 to water deficiency involved a change in behaviour with respect to its ability to supply water. As shown in Figure 3.A, under normal irrigation conditions, the relationship for rootstock 030115 between T_w/FR and the ratio S/FR is very similar to that found for these parameters in rootstock PT. However, under WD, the relationship between these two parameters for rootstock 030115 is similar to that for rootstock CM (Fig 3.B), for which, as stated above, low expression of aquaporins allows greater tolerance to water stress

than PT. This change in behaviour of 030115 can be attributed to the repression of aquaporins that regulate water supply to the aerial part of the plant. This is reinforced by the fact that, despite both PT and 030115 decreasing their S/FR ratios to ones similar to that of CM, 030115 was the rootstock which had a greater decrease in S/FR and T_{Total} under water stress conditions (Table 1, Table 2). Given that the relationship between S/FR and T_w/FR (Fig. 3) is the same under normal irrigation and WD conditions even though the values of T_w/FR are much lower under conditions of water stress, this implies that the ratio S/R is equilibrated in the plant depending on the ability of the rootstock to provide water. This is in agreement, firstly, with the positive correlation in citrus between the ratio S/R and hydraulic conductance reported by Syvertsen and Graham (1985) and, secondly, with changes in hydraulic conductance under water deficit conditions (Sumner and Boswell 1981; Cruz et al. 1992; North and Nobel 1996; Lo Gullo et al. 1998; Martre et al. 2001; North et al. 2004). Moreover, further reduction of total plant growth in water deficit conditions occurred in 030115. This result is in accordance with that obtained by Aharon (2003) where the over-expression of PIPs resulted in increased plant vigour. In the present work, the greatest reduction in growth was observed in plants that reduced the expression of PIPs.

Rootstock 030115 did not demonstrate a reduction in A_{CO2} values throughout the entire experiment. Despite this, E and gs values of this rootstock decreased from 42 days after the start of the trial. The reduction of E and gs may also be a consequence of repressed aquaporin expression, since they regulate the transport of water to the aerial component of the plant. The reduction in gs resulted in a statistically-significant decrease in Ci (Table 1). Reductions in gs, Ci and E, could lead to lowered A_{CO2}, since the transport of water in the leaves is functionally related to their photosynthetic capacity (Aasamaa et al. 2001; Brodribb and Holbrook 2003; Franks 2006). Leaves with high photosynthetic capacity need a large supply of water (Voicu and Zwiazek 2009). However, citrus (C3 plants) are characterized by low maximum rates of CO_2 absorption (<12 μmol CO_2 m^{-2} s^{-1}) compared to other C3 plants (20-30 μmol CO_2 m^{-2} s^{-1}) (Jifon and Syvertsen 2003). So it is possible that the reduction of available CO_2, due to stomatal closure, does not cause a decrease in A_{CO2} in rootstock 030115 under WD conditions. In addition, 030115, unlike PT and CM, showed no differences in SPAD values between leaves of control and water-stressed plants, indicating that WD had a lower overall negative effect.

The A_{CO2} reduction in PT(wd) and CM(wd) paralleled reductions in E and gs. This could indicate that stomatal factors were responsible for reducing A_{CO2}. However, PT and CM, unlike 030115, showed no significant differences between control plants and stressed plants in C_i values on day 56 (Table 1). The fact that PT(wd) and CM(wd) have the same availability of CO_2 as their controls suggests that non-stomatal factors are responsible for the reduction of A_{CO2} in rootstocks PT and CM under WD conditions. García-Sánchez et al. (2007) also attributed an A_{CO2} reduction in CM, subjected to water stress, to non-stomatal factors. Both PT and CM decreased their A_{CO2} values under conditions of water stress, without drastically altering aquaporin expression, unlike the pattern of 030115. This supports the fact that regulation of water content in the plant by controlling the expression of aquaporins ensures an adequate water status in order to maintain A_{CO2} levels. According to Aharon et al. (2003), plants can better tolerate water stress if the total integrated amount of symplastic water transport via plasma membrane aquaporins is reduced during the stress.

Gimeno et al. (2009) who studied the effect of water deficiency in CM with the use of microarrays, found that only one gene coding for aquaporin was altered in roots under WD. The expression of this gene, which showed high homology with *Arabidopsis* PIP2;4, was down regulated in CM under water stress treatments. The result found by these authors is consistent with our findings (Fig. 2).

In *Arabidopsis thaliana* (L.) Heynh. Ecotype *Columbia* (Col-0) (Alexandersson et al. 2005), *Agave deserti* Engelm. (Agavaceae) (North et al. 2004), *Opuntia acanthocarpa* var. *Ganderi* C.B. Wolf (Cactaceae) (Martre *et al*. 2001), *Nicotiana glauca* (Graham) (Smart et al. 2001) and radish (*Raphanus sativus* L. cv Tokinashi-daikon) (Suga et al. 2002) a reduction in the activity of aquaporins in roots under conditions of water deficiency has been observed. However, in rice (*Oryza sativa* L.) (Lu and Neumann 1999), the activity of water channels in roots under water deficient conditions actually has been found to increase.

In *A. thaliana*, the PIPs repressed under conditions of water stress are those with high levels of expression in roots when irrigation conditions are normal, whilst PIPs that increased or maintained their expression under WD have a very low expression in roots (Alexandersson et al. 2005). Moreover, the re-opening of water channels is apparently crucial in *A. thaliana*

for the recovery of water absorption following reirrigation (Matre et al. 2002; Alexander et al. 2005).

Studies relating to aquaporins in *O. acanthocarpa*, *A. deserti* and *O. sativa* have been made by inhibiting the water transport using mercuric chloride (Martínez-Ballesta 2003). In *A. deserti* and *O. acanthocarpa*, the addition of $HgCl_2$ reduced aquaporin activity under WD with respect to activity in humid conditions. These studies raise the question of whether reducing the activity of aquaporins by water stress is due to down-regulation of aquaporins, to the closing or narrowing the opening of existing water channels, or to the aquaporin desensitization to $HgCl_2$ in a manner unrelated to water transport (North et al. 2004). In addition, the permeability of PIPs can be regulated post-transcriptionally via phosphorylation (Johansson et al. 1996; Johansson et al. 1998) and by the cytosolic pH (Tourna-Roux et al. 2003). In this work, as in those of Alexander et al. (2005), Smart et al. (2001) and Suga et al. (2002), we show a marked repression in aquaporin expression under conditions of water stress. However, this repression is determined by the genotype, since only one rootstock of the three studied demonstrated this effect.

The theory that tolerance to water deficiency involves the repression of aquaporin expression is reinforced by work done on transgenic tobacco plants that over-express the *Arabidopsis* gene PIP1;2. PIP over-expression in transgenic tobacco plants under water stress was shown to have a negative effect (Aharon et al. 2003), with earlier wilting in transgenic plants ascribed to water loss due to the increased permeability of their cell membranes.

In our study we conclude that the low expression of aquaporins in CM and its down regulation at transcriptional level in 030115 results in decreased permeability of plasma membranes. This facilitates water maintenance in the cells and adequate levels of photosynthesis in plants under water stress. Thus, low levels of aquaporins or repression of their expression, accompanied by a loss of vigour, in roots, could be a mechanism of water stress tolerance in citrus.

REFERENCES

Agre P, Sasaki S, Chrispeels MJ (1993) Aquaporins: a family of water channel proteins. Am J Physiol 261:F461

Aharon R, Shahak Y, Wininger S, Bendov R, Kapulnik Y, Galili G (2003) Overexpression of a plasma membrane aquaporin in transgenic tobacco improves plant vigor under favorable growth conditions but not under drought or salt stress. Plant Cell 15:439–447

Alexandersson E, Fraysse L, Sjövall-Larsen S, Gustavsson S, Fellert M, Karlsson M, Johanson U, Kjellbom P (2005) Whole gene family expression and drought stress regulation of aquaporins. Plant Mol Biol (2005) 59:469–484

Ancillo G, Gadea J, Forment J, Guerri J, Navarro L (2007) Class prediction of closely related plant varieties using gene expression profiling. J Exp Bot 58:1927–1933

Asamaa K and Sõber A (2001) Hydraulic conductance and stomatal sensitivity to changes of leaf water status in six deciduous tree species. Biol Plant 44:65-73

Arbona V, Iglesias DJ, Jacas, Primo-Millo E, Talon M, Gomez-Cadenas A (2005) Hydrogel substrate amendment alleviates drought effects on young citrus plants. Plant Soil 270:73-82

Barrowclough DE, Peterson CA, Steudle E (2000) Radial hydraulic conductivity along developing onion roots. J Exp Bot 51:547-557

Brodribb TJ, Holbrook NM (2003) Changes in leaf hydraulic conductance during leaf shedding in seasonally dry tropical forest. New Phytol 158:295-303

Carvajal M, Cooke DT, Clarkson DT (1996) Responses of wheat plants to nutrient deprivation may involve the regulation of water-channel function. Planta 199:372-381

Carvajal M, Martínez V, Alcaraz CF (1999) Physiological function of water channels as affected by salinity in roots of paprika pepper. Physiol Plant 105:95-101.

Castle WS, Krezdorn AH (1975) Effect of citrus rootstocks on root distribution and leaf mineral content of Orlando Tangelo trees. J Am Soc Hortic Sci 100:1–4.

Clarkson DT, Carvajal M, Henzler T, Waterhouse RN, Smyth AJ, Cooke DT, Steudle E (2000) Root hydraulic conductance: diurnal aquaporin expression and the effects of nutrient stress. J Exp Bot 51:61-70

Cruz RT, Jordan WR, Drew MC (1992) Structural changes and associated reduction of hydraulic conductance in roots of *Sorghum bicolor* L. following exposure to water deficit. Plant Physiol 99:203-212

Daniels MJ, Mirkov TE, Chrispeels MJ (1994) The plasma membrane of *Arabidopsis thaliana* contains a mercury-insensitive aquaporin that is a homolog of the tonoplast water channel protein TIP. Plant Physiol. 106:1325-1333

Driver S, Kuniyuki S (1984) In vitro propagation of Paradox walnut rootstock. HortScience 19:507-509

Enstone DE, Peterson CA (1998) Effects of exposure to humid air on epidermal viability and suberin deposition in maize (*Zea mays* L.) roots. Plant Cell Environ 21:837-844

Forner-Giner MA, Alcaide A, Primo-Millo E, Forner JB (2003) Performance of "Navelina" orange on 14 rootstocks in Northern Valencia (Spain). Sci Hortic-Amsterdam 98:223-232

Franks PJ (2006) Higher rates of leaf gas exchange are associated with higher leaf hydrodynamic pressure gradients. Plant Cell Environ 29:584-593

Galmés J, Pou A, Alsina MM, Tomás M, Medrano H, Flexas J (2007) Aquaporin expression in response to different water stress intensities and recovery in Richter-110 (*Vitis* sp.): relationship with ecophysiological status. Planta 226:671-681

García-Sánchez F, Syvertsen JP, Gimeno V, Botía P, Perez-Perez JG (2007) Responses to flooding and drought stress by two citrus rootstock seedlings with different water-use efficiency. Physiol Plant 130:532-542

Gimeno J, Gadea J, Forment J, Pérez-Valle J, Santiago J, Martínez-Godoy MA, Yenush L, Bellés JM, Brumós J, Colmenero-Flores JM, Talón M, Serrano R (2009) Shared and novel molecular responses of mandarin to drought. Plant Mol Biol 70:403-409

Hachez C, Zelazny E, Chaumont F (2006) Modulating the expression of aquaporin genes in planta: a key to understand their physiological functions ? Biochim Biophys Acta 1758:1142-1156

Henzler T, Waterhouse RN, Smyth AJ, Carvajal M, Cooke DT, Schäffner AR, Steudle E, Clarkson DT (1999) Diurnal variations in hydraulic conductivity and root pressure can be correlated with the expression of putative aquaporins in the roots of *Lotus japonicus.* Planta 210:50-60

Hoagland DR, Arnon DI (1950) The water culture method for growing plants without soil. Calif Agric Exp Sta Circular 347:1-32

Hukin D, Doering-Saad C, Thomas CR, Pritchard J (2002) Sensitivity of cell hydraulic conductivity to mercury is coincident with symplasmic isolation and expression of plasmamlemma aquaporin genes in growing maize roots. Planta 215:1047-1056

Javot H, Maurel C (2002) The role of aquaporins in root water uptake. Ann Bot 90:301-313

Jifon JL, Syvertsen JP (2003) Moderate shade can increase net gas exchange and reduce photoinhibition in citrus leaves. Tree Physiol 23:119-127

Johansson I, Larsson C, Ek B, Kjellbom P (1996) The major integral proteins of spinach leaf plasma membranes are putative aquaporins and are phosphorylated in response to Ca^{2+} and apoplastic water potential. Plant Cell 8:1181–1191

Johansson I, Karlsson M, Shukla VK, Chrispeels MJ, Larsson C, Kjellbom P (1998) Water transport activity of the plasma membrane aquaporin PM28A is regulated by phosphorylation. Plant Cell 10:451–459.

Johanson U, Karlsson M, Johansson I, Gustavsson S, Sjövall S, Fraysse L, Weig AR, Kjellborm P (2001) The complete set of genes encoding major intrinsic proteins in *Arabidopsis* provides a framework for a new nomenclature for major intrinsic proteins in plants. Plant Physiol 126:1358-1369

Kammerloher W, Fischer U, Piechottka GP and Schäffner AR (1994) Water channels in the plant plasma membrane cloned by immunoselection from a mammalian expression system. Plant J 6:187-199

Kirch HH, Vera-Estrella R, Golldack D, Quigley F, Michalowski CB, Barkla BJ, Bohnert HJ (2000) Expression of water channel proteins in *Mesembryanthemum crystallinum* Plant Physiol 123:111-124

Lei YB, Yin CY, Li CY (2006) Differences in some morphological, physiological, and biochemical, responses to drought stress in two contrasting populations of *Populus przewalskii*. Physiol Plant 127:182-191

Lo Gullo MA, Nardini A, Salleo S, Tyree MT (1998) Changes in root hydraulic conductance (K_R) of *Olea oleaster* seedlings following drought stress and irrigation. New Phytol 140:25-31

Luu DT, Maurel C (2005) Aquaporins in a challenging environment: molecular gears for adjusting plant water status. Plant Cell Environ 28:85-96

Lu Z, Neumann PM (1999) Water stress inhibits hydraulic conductance and leaf growth in rice seedlings but not the transport of water via mercury-sensitive water channels in the root. Plant Physiol 120:143-151

Maggio A, Joly RJ (1995) Effects of mercuric chloride on the hydraulic conductivity of tomato root systems: evidence of a channel-mediated water pathway. Plant Physiol 109:331-335

Martre P, North GB, Nobel PS (2001) Hidraulic conductance and mercury-sensitive water transport for roots of *Opuntia acanthocarpa* in relation to soil drying and rewetting. Plant Physiol 126:352-362

Martínez-Ballesta MC, Aparicio F, Pallás V, Martínez V, Carvajal M (2003) Influence of saline stress on root hydraulic conductance and PIP expression in Arabidopsis. J Plant Physiol 160:689-697

Martínez-Ballesta MC, Martínez V, Carvajal M (2000) Regulation of water channel activity in whole roots and in protoplasts from roots of melon plants grown under saline conditions. Aust J Plant Physiol 27:685-691

Más P, Sánchez-Navarro JA, Sánchez-Pina MA, Pallás V (1993) Chemiluminescent and colorigenic detection of Cherry leaf roll virus with digoxigeninlabelled RNA probes. J Virol Methods 45 93102. doi:10.1016/0166-0934(93)90143-F

Maurel C (1997) Aquaporins and water permeability of plant membranes. Annu Rev Plant Physiol 48:399-429

Medina CL, Machado FC (1998). Gas exchange and water relations of Valencia orange tree grafted on rangpur lime and *Poncirus trifoliata* submitted to a water deficit. Bragantia, Campinas 57:15–22

Morillon R, Lasalles JP (2002) Water deficit during root development: effects on the growth of roots and osmotic water permeability of isolated root protoplasts. Planta 214:392-399

North GB, Nobel PS (1996) Radial hydraulic conductivity of individual root tissues of *Opuntia ficus-indica* (L.) Miller as soil moisture varies. Ann Bot 77:133-142

North GB, Nobel PS (2000) Heterogeneity in water availability alters cellular development and hydraulic conductivity along roots of a desert succulent. Ann Bot 85:247-255

North GB, Martre P, Nobel PS (2004) Aquaporins account for variations in hydraulic conductance for metabolically active root regions of *Agave desserti* in wet, dry, and rewetted soil. Plant Cell Environ 27:219-228

Pallás V, Más P, Sánchez Navarro JA (1998) Detection of plant RNA viruses by nonisotopic dot-blot hybridization. Methods in Molecular Biology. Clifton, N.J. 81,461-468

Peng Y, Lin W, Cai W, Arora R (2007) Overexpression of a *Panax ginseng* tonoplast aquaporin alters salt tolerance, drought tolerance and cold acclimation ability in transgenic *Arabidopsis* plants. Planta 226:279-740

Rodríguez-Gamir J, Intrigliolo DS, Primo-Millo E, Forner-Giner MA (2010) Relationships between xylem anatomy, root hydraulic conductivity, leaf/root ratio and transpiration in citrus trees on different rootstocks. Physiol Plant 139:159-169

Romero P, Navarro JM, Pérez-Pérez J, García-Sánchez F, Gómez-Gómez A, Porras I, Martínez V, Botía P (2006) Deficit irrigation and rootstock: their effects on water relations, vegetative development, yield, fruit quality and mineral nutrition of Clemenules mandarin. Tree Physiol 26, 1537–1548

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular Cloning: A Laboratory Manual, 2nd Edition, pp. 9.58. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Siefritz F, Tyree MT, Lovisolo C, Schubert A, Kaldenhoff R (2002). PIP1 plasma membrane aquaporins in tobacco: from celular effects to function in plants. Plant Cell 14:869-876

Sinclair TR, Allen LH (1982) Carbon dioxide and water vapour exchange of leaves on field-grown citrus trees. J Exp Bot 33:1166–1175.

Smart LB, Moskal W, Cameron KD, Bennett AB (2001). MIP Genes are down-regulated under drought stress in *Nicotiana glauca*. Plant Cell Physiol 42:686-693

Suga S, Komatsu S, Maeshima M (2002) Aquaporin isoforms responsive to salt and water stresses and phytohormones in radish seedlings. Plant Cell Physiol 43:1229-1237

Sumner ME, Boswell FC (1981) Alleviating nutrient stress. In: Arkin GF, Taylor HM (ewd) Modifying the root environment to reduce crop stress. Amer. Soc. Agr. Eng. Monograph No. 4, St. Joseph, Mich, pp 99-137

Syvertsen JP (1984) Light acclimation in citrus leaves. II. CO_2 assimilation and light, water, and nitrogen use efficiency. J Am Soc Hortic Sci 109:812-817

Syvertsen JP, Graham JH (1985) Hydraulic conductivity of roots, mineral nutrition, and leaf gas exchange of Citrus rootstocks. J. Amer. Soc. Hort. Sci 110:865-869

Syvertsen JP, Levy Y (2005) Salinity interactions with other abiotic and biotic stresses in citrus. HortTech 15:100-103.

Syvertsen, JP, Lloyd JJ (1994) Citrus. In: Schaffer BA, Andersen PC (ewd) Handbook of environmental physiology of fruit crops. Vol. II. Subtropical and tropical crops. CRC Press, Boca Raton, FL, pp 65–99.

Tournaire-Roux C, Sutka M, Javot H, Gout E, Gerbeau P, Luu DT, Bligny R, Maurel C (2003) Nature 425:393-397

Tsuda M, Tyree MT (2000) Plant hydraulic conductance measured by the high pressure flow meter in crop plants. J Exp Bot 51:823-828

Tyerman SD, Niemietz CM, Bramley H (2002) Plant aquaporins: multifunctional water and solute channels with expanding roles. Plant Cell Environ 125:173-194

Vasconcellos LABC, Castle WS (1994) Trunk xylem anatomy of mature healthy and blighted grapefruit trees on several rootstocks. J Am Soc Hortic Sci 119:185–194.

Voicu MC and Zwiazek JJ (2009) Inhibitor studies of leaf lamina hydraulic conductance in trembling aspen (*Populus tremuloides* Michx.) leaves. Tree Physiol 30:193-204

Wan X, Zwiazek JJ (1999) Mercuric chloride effects on root water transport in aspen seedlings. Plant Physiol 121:939-946

Weig A, Deswarte C, Chrispeels MJ (1997) The major intrinsic protein family of Arabidopsis has 23 members that form three distinct groups with functional aquaporins in each group. Plant Physiol 114:1347–1357.

Yamada S, Katsuhara M, Kelly WB, Michalowski CB, Bohnert HJ (1995) A family of transcripts encoding water channel proteins: tissue-specific expression in common ice plant. Plant Cell 7:1129-1142.

Zardoya R (2005) Phylogeny and evolution of the major intrinsic protein family. Biol Cel 97:397-414

Zekri M, Parsons LR (1989) Growth and root hydraulic conductivity of several citrus rootstocks under salt and polyethylene glycol stresses. Physiol Plant 77:99-106