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Influence of salinity on pip gene expression in citrus roots and its relationship with root hydraulic conductance, transpiration and chloride exclusion from leaves

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ABSTRACT

The present work studies the effect of salinity on PIP aquaporins gene expression in citrus roots and its relationship with root hydraulic conductance (Kr), transpiration rate (E) and chloride transport to leaves. To this end, ten-month-old seedlings of Cleopatra mandarin (CM), Carrizo citrange (CC) and *Poncirus trifoliata* (PT) were tested.

No effect was detected of salt treatments on PIP1 and PIP2 aquaporin mRNA transcript abundances from citrus roots, although PIP1 expression in CM roots was lower than in CC and PT.

The lowest Kr and E values were detected in CM, whereas PT had the highest. CC seedlings presented intermediate values for these parameters. Addition of $HgCl_2$ to either control or salt solution (200 mM NaCl) led to a decrease in Kr and E, thus implying aquaporin involvement. By contrast, salinity strongly reduced Kr and E in all plants, with this effect being unrelated to aquaporin activity.

In salinized seedlings, E values appear to be related with Cl^- concentration in leaves. Thus, CM seedlings treated with 80 mM NaCl presented a lower Cl^- uptake by leaves than PT, whereas this trend was intermediate in CC. Moreover, Hg^{2+} treatments significantly reduced leaf Cl^- concentration in salt stressed plants, probably through the reduction of E.

We can conclude that differences among genotypes in PIP1 expression affect Cl^- exclusion from leaves, probably due to effects on water movement. Nevertheless, long-term salt treatments did not affect PIP expression in citrus plants, but reduced root hydraulic conductance and transpiration.

1. Introduction

Citrus is a salt-sensitive crop (Maas and Hoffman, 1977), which, even at moderate salinities, suffers physiological disorders that stunt plant growth and significantly reduce yield. In these plants, the main salts causing injury are chlorides (Bañuls and Primo-Millo, 1992), and the salt tolerance of some citrus rootstocks is determined by their chloride exclusion capacity (Ream and Furr, 1976; Walker et al., 1983; Bañuls and Primo-Millo, 1995), which is the ability to restrict chloride uptake and/or transport from roots to leaves (Flowers and Colmer, 2008). It has been proposed that chloride absorption in citrus depends to a great extent upon water use (Moya et al., 2003). In addition, plant water status is disrupted by salinity due to osmotic stress, which hampers water uptake. These alterations trigger specific mechanisms controlling cell osmotic adjustment and water loss (Walker et al., 1983), which lead to altered gas exchange parameters (Walker et al., 1982; Lloyd et al., 1987, 1990).

Reduced root water uptake capacity in plants grown under saline conditions has also been linked to a decrease in root hydraulic conductance (Zekri and Parsons, 1989; Joly, 1989) as a result of lowered PIP aquaporin activity (Martinez-Ballesta et al., 2003). Among aquaporins (AQPs), the sub-family of plasma-membrane intrinsic proteins (PIPs) appear to play a critical role in controlling water transport through plant tissues, regulating the transcellular pathway (Tyerman et al., 2002; Maurel et al., 2008). PIPs are further divided into sub-groups PIP1 and PIP2 (Chaumont et al., 2000). In general, AQPs can be blocked by mercury ions, resulting in decreased water permeability of the membranes (Maggio and Joly, 1995; Martinez-Ballesta et al., 2003).

Salt stress has been shown to decrease PIP aquaporin gene expression in some plants (Martinez-Ballesta et al., 2003; Boursiac et al., 2005), although this effect has not been demonstrated in others (Suga et al., 2002; Diédhiou et al., 2009).

Since, in citrus, salinity tolerance depends on Cl^- exclusion (Walker et al., 1983; Bañuls et al., 1997), Cl^- uptake is linked to water use (Moya et al., 2003), and, furthermore, water transport

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through the plant greatly depends on AQPs (Tyerman et al., 2002; Maurel et al., 2008), then the hypothesis tested here was whether AQPs influence salinity tolerance in citrus. Thus, the aim of this work was to determine the effect of salinity on PIP aquaporin gene expression in roots and its influence on root hydraulic conductance (Kr), transpiration rate (E) and chloride transport to leaves. The genotypes used in this study – Cleopatra mandarin (*Citrus reshni* Hort ex Tan.), Carrizo citrange (hybrid of *C. sinensis* (L.) Osbeck × *Poncirus trifoliata* (L.) Raf.) and *P. trifoliata* – were chosen on the basis of other reports, as PT presents higher transpiration (Syvertsen and Graham, 1985) and root conductivity (Syvertsen and Graham, 1985; Zekri and Parsons, 1989) than CM, whereas the trend is intermediate in CC. Moreover, the studied plants differ in their salt tolerance, since PT and CC are considered salt-sensitive rootstocks, while CM is a relatively salt-tolerant rootstock, due to its ability to exclude Cl^- ions (Walker et al., 1983).

2. Materials and methods

2.1. Plant material and growth conditions

Ten-month-old seedlings of Cleopatra mandarin (CM), Carrizo citrange (CC) and *Poncirus trifoliata* (PT) were used in this experiment. Plants were grown under glasshouse conditions with supplementary light (50 μmol m^{-2} s^{-1}, 400–700 nm) to extend the photoperiod to 16 h. Temperatures ranged between 16 and 18 °C at night and 24 and 28 °C by day. Relative humidity was maintained at approximately 80%.

Plants were grown individually in 3 L pots filled with coarse sand. All plants were irrigated every 3 d until the beginning of the experiments 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 μM Fe-EDDHA, 46.25 μM H_3BO_3, 54.4 μM $MnSO_4{\cdot}H_2O$, 7.65 μM $ZnSO_4{\cdot}7H_2O$, 0.55 μM MoO_3 and 0.5 μM $CuSO_4{\cdot}5H_2O$. Nutrient solution pH was adjusted to 6.0 with 1 M KOH or 1 M H_2SO_4. Approximately one L of solution per pot was used in each watering event. Excess solution drained out of the pot, thereby avoiding salt accumulation in the sand.

Plants growing as a single shoot were selected for uniformity of size at the beginning of the experimental treatments.

2.2. Physiological determinations

Leaf transpiration rates (E) were determined as described by Rodríguez-Gamir et al. (2010).

Root hydraulic conductance (Kr) was measured by the high pressure flow meter (HPFM) method using the Dynamax flow meter (Dynamax, Inc. Houston, USA) according to Tyree et al. (1995). The procedure followed was the one adapted to citrus plants by Rodríguez-Gamir et al. (2010).

2.3. Analysis of PIP expression

Available PIP1 and PIP2 citrus sequences from the Citrus Functional Genomic Project EST database (http://bioinfo.ibmcp.upv.es/genomics/cfgpDB) were separately aligned in search of the most conserved region in order to design specific subfamily probes. Reverse transcription and PCR reactions were carried out using specific primers and the SuperScript III one-step RT-PCR system with a Platinum Taq DNA polymerase kit (Invitrogen, Renfrewshire, UK) following the manufacturer's instructions. Amplified products (156 bp and 137 bp length for the specific probe for PIP1 and PIP2 respectively) were cloned into a pTZ57R/T vector (MBI Fermentas) to generate pTZ/PiP-1 and pTZ/PiP-2.

RNA from roots was extracted according to Ancillo et al. (2007). Electrophoresis, blotting, digoxigenin-labeling and hybridizations were conducted according to Pallás et al. (1998). Chemiluminescent detection with CSPD reagent (Roche, Basel, Switzerland) as substrate, was performed as recommended by the manufacturer. Films were exposed for 30–60 min before development.

2.4. Harvesting and chemical analysis

At the end of the experiments, ten mature leaves from the mid-stem zone of each plant were harvested, washed and weighed. The fractions were then dried in a forced-draft oven at 65 °C for 48 h and re-weighed. For analysis of Cl^-, leaves were crushed in a hammer-mill and stored at room temperature. Chloride was determined by silver ion-titration (Gilliam, 1971) with a Corning 926 chloridometer (Corning, Halstead, Essex, UK).

2.5. Experimental design

Two independent experiments were performed as follows:

Experiment 1: CM, CC and PT seedlings were given standard solution to which either 0 (control) or 80 mM of NaCl were added. Treatments were applied for 30 days.

Experiment 2: CC seedlings were treated with the standard nutrient solution alone (control) or containing either 0.05 mM $HgCl_2$, 200 mM NaCl or 200 mM NaCl + 0.05 mM $HgCl_2$. Treatments were maintained for 14 days.

At the end of the experiments Kr, E, and [Cl^-] in leaves were measured. Roots were collected for RNA extraction and analysis of PIP aquaporin gene expression.

Sets of six plants per genotype and treatment were used for the different determinations, except for PIP transcript abundances, which were analyzed in roots from three plants. Plants were randomized over the experimental area and analyzed individually. A row of plants, not included in the experiment, was placed around the perimeter as a buffer row.

Parameters were statistically tested by analyses of variance and averages were compared with a Duncan test ($P \leq 0.05$). Statistical analyses were performed with Statgraphics Plus version 5.1 (Statistical Graphics, Englewood Cliffs, NJ).

Table 1
Root hydraulic conductance by unit root weight (Kr), transpiration rate (E) and Cl^- concentration in leaves of Cleopatra mandarin (CM), Carrizo citrange (CC) and *Poncirus trifoliata* (PT) seedlings treated with 0 (control) or 80 mM NaCl for 30 days.

Plant species	Treatment	Kr × 10^{-6} (kgs^{-1} Mpa^{-1} g^{-1})	E (mmol H_2O m^{-2} s^{-1})	[Cl^-] leaves (mg g^{-1} DW)
CM	Control	1.86^{c}	1.16^{c}	3.05^{e}
CM	80 mM NaCl	0.21^{d}	0.44^{e}	20.04^{c}
CC	Control	9.7^{b}	1.62^{b}	10.55^{d}
CC	80 mM NaCl	3.7^{c}	0.83^{d}	40.33^{b}
PT	Control	54.67^{a}	1.93^{a}	8.40^{d}
PT	80 mM NaCl	20.92^{b}	1.31^{bc}	52.73^{a}

Means ($n = 6$) within a column with the same letter are not significantly different (Duncan's test, $P \leq 0.05$).