Key Role of Boron Compartmentalisation-Related Genes as the Initial Cell Response to Low B in Citrus Genotypes Cultured *In Vitro*

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Abstract. This work compared the expression of the main B transport-related genes (some members of the aquaporin family -*NIP5, TIP5* and *PIP1*- and some efflux-B transporters -*BOR1* and *BOR4*-), and the response of some physiological parameters in two citrus species [*Citrus macrophylla* W. (CM) and *Citrus aurantium* L. (CA)] under moderate and low boron (B) conditions. Seedlings were cultured “in vitro” in media supplemented with 50 or 0 μM H_3BO_3. *NIP5, BOR1* and *PIP1* expressions were enhanced by low B levels in both genotypes. *TIP5* was down-regulated in the roots and leaves of the CA0 seedlings, and in the roots of CM0. *BOR4* also lowered in the roots of both species at 0 μM H_3BO_3. Consequently, citrus species showed a common tolerance mechanism to low B conditions based on the synergism among transport channel NIP5, non-selective aquaporin PIP1 and transporter BOR1, and the impairment of genes *TIP5* and *BOR4* related with tolerance responses to B-toxic conditions. However, the CA genotype displayed low B symptoms earlier than CM (reduced plant biomass, length, relative growth rate and chlorophyll content). Proline concentration was higher in CM0 than in CA0 leaves, while the latter also enhanced malonaldehyde content. Although both plants had similar B concentrations, they differed in B content and B partitioning fractions. Whereas the CA genotype was more affected by lack of B treatment as more B was needed inside the cell, the more minimal need of cell B in CM favoured its allocation in the insoluble fraction and allowed growth in this genotype. In conclusion, B compartmentalisation seems critical in tolerance to low B level in citrus.

Additional key words: BOR transporters, citrus rootstocks, NIP5 transport channel; PIP1 aquaporin

Introduction

Boron (B) is one of the eight essential micronutrients required in the physiological functions that are important for the normal growth and development of all higher plants (Frommer and Von Wiren, 2002). This element participates in cell wall structure formation through the borate-diol bonding of two rhamnogalacturonan II molecules, and is also involved in root elongation, carbohydrate metabolism, phenol accumulation, pollen-tube growth and integrity of cell membranes (O´Neill et al., 2004). Citrus is a sensitive crop to B deficiency and low B levels in plants cause major disorders that lead to loss of yields (Liu et al., 2013).

B deficiency is commonly found in acidic and sandy soils that are poor in silt and clays, where boric acid (BA) is easily leached below root zones by rainfall or irrigation (Shorrocks, 1997). Root growth is more sensitive to low B levels than shoot growth as the most rapid response to this stress is root elongation inhibition or cessation in the main and lateral roots (Mei et al., 2011). B deficiency symptoms start from developing parts and can cause deformations and translucent spots on leaves, early death of young shoots from the apex to the base, and emergence of multiple buds (Xiao et al., 2007). Mature leaves acquire corky split veins, stems become brittle, and gum may appear from cracked twigs. In *Citrus*, severe B deprivation conditions generate blister-like pockets of gum on the white albedo of fruits, and sometimes in the central core, which arise from dark brown spots on rind. Fruit may be somewhat misshapen with a lumpy surface, which means loss of yields and poor fruit quality (Shorrocks, 1997).

B-efficient genotypes are those able to grow well in soils in which others are adversely affected by B deficiency (Rerkasem and Jamjod, 1997). For the B-efficiency mechanism, a genotype may be efficient or inefficient due to differences in either B uptake from the medium, B utilisation within the plant, or even both. After entering roots, B is transported through xylem vessels, and is bound mostly to cell wall structures (insoluble B pool) or accumulates in apoplastic fluids (soluble B pool), while another soluble portion enters cells (Liu et al., 2013). In plants, B homeostasis seems related to the synergic regulation of the several genes involved in B uptake, transport and partitioning in aerial parts (Hajiboland et al., 2013).

In line with this, the first B transporter identified in a biological system was *Arabidopsis thaliana* BOR1, an essential efflux-type B transporter for efficient xylem loading of B (Takano et al., 2002). This gene is expressed mainly in root pericycle cells

and its overexpression enhances root-to-shoot B translocation, probably as borate, under B-limiting conditions (Miwa et al., 2006). When the complete *A. thaliana* genome was analysed, six sequences had a high homology with *AtBOR1* (Frommer and von Wiren, 2002). Of these, only *AtBOR4* has been characterised (Miwa et al., 2007), and its overexpression markedly improves *A. thaliana* growth under high B conditions through B efflux. In *Brassica napus*, *BnBOR1;1c* and *BnBOR1;2a* are up-regulated in roots under low B stress, but no differences in their expression have been found between B-efficient and B-inefficient cultivars in low or sufficient B environments (Sun et al., 2012). In *Oryza sativa*, OsBOR1 loads B into the xylem and also participates in absorbing this element in roots (Nakagasa et al., 2007). In fruit crops, VvBOR1 and CmBOR1 have been recently characterised as B transporters in *Vitis vinifera* and *Citrus macrophylla* Wester, respectively (Perez-Castro et al., 2012; Cañon et al., 2013).

The membrane intrinsic proteins (MIPs), family, also known as aquaporins, act as channels for water and/or small non-charged molecules, and are comprised of four subfamilies; nodulin 26-like intrinsic proteins (NIPs), plasma membrane intrinsic proteins (PIPs), tonoplast intrinsic proteins (TIPs), small basic intrinsic proteins (SIPs) and the uncharacterised X intrinsic proteins (XIPs) (Chaumont et al., 2000; Johanson et al., 2001; Danielson and Johanson 2008). In the NIP subfamily, *AtNIP5;1* encodes a BA channel that facilitates B influx to root cells in *A. thaliana* and *B. napus* (Tanako et al., 2006; Sun et al., 2012). It is localised to the plasma membrane on the outer side of epidermal, cortical, and endodermal cells in roots. NIP5;1 is required for B uptake from the root surface, and its accumulation is regulated in response to B deprivation (Tanako et al., 2006). In *A. thaliana*, the overexpression of *AtNIP5;1* results in root elongation under B-deficient conditions, which improves short-term B uptake (Kato et al., 2009). The vacuolar compartmentalisation of B has been related to *AtTIP5;1* activity, an aquaporin family member localised in the cell tonoplast membrane (Pang et al., 2010). Under B-toxic conditions, *AtTIP5;1* overexpression has been reported to lower the cytoplasmic B concentration by accumulating B in the vacuole and, therefore, confers cell tolerance to toxic B levels (Pang et al., 2010). Finally, there is some evidence that PIPs aquaporins are also involved in plant permeability to BA (Dordas et al., 2000; Martinez-Ballesta et al., 2008).

Moreover, plants subjected to different stresses accumulate the biochemical compounds related with oxidative stress and osmoprotective role, such as malonaldehyde

or proline (Delauney 1993; Mittler 2002; Gimeno et al., 2012). Recently, the induction of an efficient antioxidant system through proline accumulation in citrus grown under B toxicity has been described (Martinez-Cuenca et al., 2015).

Citrus rootstocks can vastly affect scion tolerance to B stress as they control B uptake processes from roots and also its transport to shoots (Sheng et al., 2009). It is likely that the expression of the genes involved in such processes differs among the citrus species used as rootstocks. Thus a comparison of the behaviour of these species when cultured under extreme B availability conditions could provide insight into the mechanisms that determine *Citrus* rootstocks' tolerance to low B availability. For this purpose, seedlings of *C. macrophylla* Wester (CM) and *C. aurantium* L. (Sour orange, CA), B-efficient and B-inefficient genotypes, respectively (Mei et al., 2011; Martinez-Cuenca et al., 2015), were cultured in media with moderate B-level and no supplemented-B to study: (a) plant response to low B level; (b) uptake, transport and B partitioning under the above-mentioned conditions; (c) the expression level of the main genes that control these processes.

Materials and Methods

Plant Material and Treatments

Seeds of *C. macrophylla* W. (CM) and *C. aurantium* L. (CA) were sterilised for 5 min in a 2% commercial bleach solution prior to seed coat removal, and were rinsed 3 times with sterilised deionised water (d-H_2O). Seeds were germinated on media that contained distilled d-H_2O water with 0.4% bacteriological agar (Scharlau, Sentmenat, Spain, pH 6.0). Media were autoclaved for 20 min at 120ºC and then distributed in 150 x 25 mm tubes at a rate of 40 mL per tube. Seeds (one seed per tube) were placed in media culture tubes and maintained in a growth chamber for 20 days (Sanyo MCR-350H, Sanyo Electric Biochemical Co, Japan) at 20-22/26-28ºC night/day temperatures, 80% relative humidity, and 250 $\mu mol \cdot m^{-2} \cdot s^{-1}$ photosynthetic photon flux density for 16 $h \cdot day^{-1}$.

Seedlings with a single shoot were selected for uniformity (10-15 cm high) and were transferred individually after removing cotyledons in plastic 50 mL tubes that contained a basic nutrient solution [1.5 mM $Ca(NO_3)_2$, 1.5 mM KNO_3, 1 mM $MgSO_4$, 1.2 mM H_3PO_4, 20 µM Fe-EDDHA, 7.65 µM $ZnSO_4 \cdot 7H_2O$, 54.4 µM $MnSO_4 \cdot H_2O$, 0.55 µM MoO_3, 0.5 µM $CuSO_4 \cdot 5H_2O$] and 50 or 0 µM H_3BO_3. All the media were supplemented with 0.25%

bacteriological agar (Scharlau, pH 6.0) and were then autoclaved as described above. Seedlings were transferred to new culture media every 20 days to refresh nutrient solution and to ensure adequate oxygen supply to roots. Plants were kept under the same growth chamber conditions as previously described until harvesting time.

RNA Extraction and Real-time (RT)-PCR Analysis

Total plant RNA was extracted from approximately 0.1 g of frozen (-80ºC) leaf and root tissues pooled from 10 plants using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). To remove contaminating genomic DNA, RNA samples were treated with RNase-free DNase (Qiagen) following the manufacturer's instructions. RNA quality and concentration were assessed in an ND-1000 full spectrum UV-Vis spectrophotometer (Nanodrop Technologies, Thermo Fisher Scientific, Delaware, USA). RT-PCR reactions were run in a LightCycler 2.0 instrument (Roche, Diagnostics GmbH, Mannheim, Germany) equipped with version 4.0 of the Light Cycler Software. Reverse transcriptase reactions contained 2.5 units of MultiScribe Reverse Transcriptase (Applied Biosystems, Roche Molecular Systems, New Yersey, USA), 1 unit of RNase inhibitor (Applied Biosystems), 2 µL of LC Fast Start DNA Master PLUS SYBR Green I (Roche), 25 ng of total RNA, and 250 nM of the specific forward and reverse primers in a total volume of 10 µL. The PCR programme was run at 48ºC for 30 min, 95ºC for 10 min, followed by 45 cycles at 95ºC for 2 s, 58ºC for 8 s and 72ºC for 8 s. Fluorescent intensity data were acquired in the 72ºC extension step and were transformed into relative mRNA values using a 10-fold dilution series of an RNA sample as a standard curve. The relative mRNA levels were then normalised to total RNA amounts, as previously described (Bustin, 2002). Actin was utilised as the reference gene. The specificity of the amplification reactions was assessed by post-amplification dissociation curves and by sequencing the reaction product. At least three independent RNA extractions and real-time reactions with three technical replicates per sample were performed. The putative *BOR4* and *TIP5* genes were identified by a homology search in the clementine genome full-length Phytozome v9.0 database (Goodstein et al., 2012, Martinez-Cuenca et al., 2015). The expression levels of the *NIP5*, *BOR1* and *PIP1* genes were evaluated using the forward and reverse primers described by other authors (Rodriguez-Gamir et al., 2011; An et al., 2012; Cañon et al.,

2013). Details of the forward and reverse primers employed in the RT-PCR are listed in Table 1. The gene-predicted products ranged from 75 to 156 bp.

Plant Growth

Seedlings were removed from culture tubes at different time points (0, 10, 15, 30 and 50 days). An additional group of plants was maintained for 100 days to evaluate the effect of B-deficiency stress on plant growth. After harvesting, plants were rinsed with d-H_2O and divided into leaves, stems and roots. Stem and root lengths were determined with a measuring rule. Organs (shoot and root) were fresh-weighed, dried in a forced draft oven at 70ºC for 48 h and weighed again.

The relative growth rate (RGR) was calculated 50 days after transplanting as:

$$\text{RGR}\ (\text{mg}\cdot\text{mg}^{-1}\cdot\text{day}^{-1}) = \frac{\text{Ln}\ DW_{50} - \text{Ln}\ DW_0}{t_{50} - t_0}$$

where DW_0 and DW_{50} are the dry mass of the leaf, stem and root tissues of the plants at the transplanting (t_0) and harvesting (t_{50}) times, respectively.

Concentration of Total B, Soluble and Insoluble B Fractions

The B concentration was measured in plants 50 days after treatment. Fresh plant biomass (roots and leaves) was split into two subsamples (0.5 g) and used for the total and B fractions (soluble in water and in organic solvents and insoluble). Stem samples were employed only for the total B determinations. The total B concentration was measured according to Mei et al. (2011). Briefly, dried samples (70ºC for 48 h) were placed in a muffle furnace at 550ºC for 12 h. Recovered ashes were dissolved in 0.1 N HCl (Hiperpur Panreac, B<1 ppb) to a final volume of 5 mL and kept at 4ºC until further analysed. B fractions were measured on the fresh biomass according to Liu et al. (2013). Samples were frozen and ground into fine powder in liquid nitrogen (N_2) in a mortar. The powder was then homogenised with 10 volumes of ice-cold water and centrifuged at 1,000 rpm for 10 min. The precipitate was washed with 10 volumes of ice-cold water and re-centrifuged. The combined supernatants were defined as a water-soluble B fraction, which is the B found mainly in the free space of cells. The residue was washed 3 times with 10

volumes of 80% ethanol, once with 10 volumes of the methanol: chloroform mixture (1:1, v/v) and once with 10 volumes of acetone to extract the B inside the protoplast, where it appeared to be linked to organic molecules. The insoluble pellet was defined as cell wall-bound B, which was dried, weighed and ashed similarly to the total B samples. The B concentration in the total organ and fractions was determined by inductively coupled plasma atomic emission spectroscopy (ICP-AES iCAP 6000, Thermo Scientific, Waltham, MA USA).

Leaf Chl Concentration

The leaf Chl concentration was measured in accordance with Moran and Porath (1980). The samples taken from the two youngest fully expanded leaves per plant were separately ground in a mortar in N_2. Then fresh material (0.05 g) was incubated in 4 mL N,N-dimethylformamide at 4ºC for 72 h and centrifuged for 15 min at 4,000 rpm and 4ºC (Eppendorf Centrifuge 5810R, AG, Hamburg, Germany). The supernatant was left for 1 h in the presence of Na_2SO_4 and its absorbance was measured spectrophotometrically at 664 and 647 nm (Lambda 25, PerkinElmer, Shelton, CT, USA). The average value of the two leaves was considered representative of each plant.

Proline Concentration

The free proline concentration in leaves was determined according to Delauney (1993). Fresh tissue was ground in a mortar in N_2. Sample material (0.1 g) was homogenised (Vortex) for 1 min in 1.5 mL of sulphosalicylic acid (3%), centrifuged at 14,000 rpm for 5 min (Eppendorf Centrifuge 5810R, AG, Hamburg, Germany) and the supernatant was stored at 4ºC. A 0.2-mL aliquot was added with 0.5 mL of sulphosalicylic acid (3%), 0.7 mL of reactive ninhydrin acid reagent [ninhydrin, phosphoric acid 6 M, glacial acetic acid 60%] and 0.6 mL of glacial acetic acid (99%) to be then incubated in a bath at 100ºC for 1 h (Thermostatic Bath BD, Bunsen SA, Humanes, Spain). Samples were cooled in an ice bath for 15 min and absorbance was measured spectrophotometrically at 520 nm (SmartSpec Plus, Bio-Rad, West Berkeley, California, USA).

Malondialdehyde (MDA) concentration

Lipid peroxidation in leaves was determined by measuring the MDA concentration according to Hodges et al. (1999). The frozen sample material (0.1 g) was homogenised (Polytron PT 3100, Lucerne, Switzerland) at 10,000 rpm for 1 min in 5 mL of cold ethanol (80%), centrifuged at 3,000 rpm and 4ºC for 30 min (Eppendorf Centrifuge 5810R, AG, Hamburg, Germany) and the supernatant was stored at 4ºC: (a) an aliquot (1 mL) was combined with an equal volume of a 20% trichloroacetic solution (TCA), while (b) another aliquot (1 mL) was combined with an equal volume of a 0.5% thiobarbituric acid (TBA) and 20% TCA solution. Test tubes were covered with glass marbles to avoid evaporation and were incubated at 90ºC in a water bath for 1 h (Thermostatic Bath BD, Bunsen SA, Humanes, Spain). Afterwards, samples were cooled in an ice bath for 15 min and centrifuged at 1,000 rpm for 15 min at 4ºC (Eppendorf Centrifuge 5810R, AG, Hamburg, Germany). The absorbance of both supernatants was measured spectrophotometrically (SmartSpec Plus, Bio-Rad, West Berkeley, California, USA). The supernatant (a) was measured at 532 and 600 nm using 20% TCA as a blank. The supernatant (b) was measured at 440, 532 and 600 nm by taking a solution of 0.5% TBA and 20% TCA as a blank. The MDA concentration was calculated with the MDA extinction coefficient of 155 $mM^{-1} \cdot cm^{-1}$ according to the following equations:

$$A = [Abs532_b - Abs600_b - (Abs532_a - Abs600_a)]$$

$$B = [(Abs440_b - Abs600_b) \times 0.0571]$$

$$\text{MDA equivalents } (nmol \cdot mL^{-1}) = (A - B/155000) \times 10^6$$

Statistical Analyses

For the statistical analyses, the values of the growth parameters (biomass and length) are the mean and the standard deviation of six independent plants per treatment. Values from other analyses are the mean and the standard deviation of six independent groups of ten plants per treatment. Data were submitted to an analysis of variance (ANOVA) with Statgraphics Plus, version 5.1 (Statistical Graphics, Englewood Cliffs, NJ, USA) prior to testing for normality and homogeneity. When the ANOVA showed a statistical effect, means were separated by least significant differences (LSD) at $P < 0.05$.

Results

Gene Expression Analysis

The expression analysis of genes *NIP5, BOR1, PIP1, TIP5* and *BOR4* was determined from the total RNA of root and leaf extracts (Figs. 1 and 2). The expression level of gene *NIP5* was similar in the 50 µM H_3BO_3-treated plants of both species, and significantly increased in the roots of both the 0 µM H_3BO_3-treated genotypes (around 75%), while no effect on the *NIP5* transcript level was recorded when analysed in leaves (Fig. 1). The 0 µM treatment enhanced *CmBOR1* expression in both species and plant organs compared to their respective 50 µM-treated seedlings (more than 58% and 71% in the roots, and more than 77% and 25% in the leaves, of the CM and CA plants, respectively; Fig. 1). Interestingly, the CM species presented the greatest *BOR1* transcript abundance in roots, independently of B treatment. The 0 µM treatment raised the *PIP1* expression level in the leaves of both species (217% and 101% in CM and CA, respectively), and also in the roots of the CM genotype (61% increase, Fig. 1).

The CA seedlings presented the highest *TIP5* gene expression level in all B treatments and organs (Fig. 2). No addition of B to culture media reduced the transcript abundance of this protein in the roots of both genotypes (41% and 47% in CM and CA, respectively) and in the leaves of the CA0 plants (s 49% decrease). The CM50 seedlings presented a higher BOR4 transcript level in roots than the CA50 ones (Fig. 2). BOR4 expression significantly reduced with the 0 µM treatment in the roots of both genotypes (41% and 47% in CM and CA, respectively), but no effect was observed when analysed in leaves.

Seedling Growth

Plant growth (organ length and dry weight) was evaluated at different times during a 100-day treatment period under the 50 and 0 µM H_3BO_3 conditions. Both the 50 µM H_3BO_3-treated genotypes showed a similar root length, but CM had a longer stem than CA (Fig. 3). Under low B conditions, organ length in both species was affected differently. The CA0 seedlings showed shorter stems than the CA50 ones 30 days after treatment began, and a slightly shorter root length at harvesting time. However, no significant differences in organ length were observed between the CM0- and CM50-treated seedlings.

In relation to dry weight biomass, some differences were found when compared to the 50 µM H_3BO_3-treated plants (Fig. 4). CA50 presented higher dry matter in roots and a lower one in stems than CM50, while no differences in leaf biomass between genotypes were found. When plants were grown in the 0 µM supplemented media, the dry biomass of the CA seedlings was markedly lower 30 days after the experiment began until harvesting time. Thus the dry weight in the roots, stems and leaves of the CA0 plants at the end of the experiment was 41%, 35% and 31% lower, respectively, than in CA50 plants. The B-non supplemented media also reduced the biomass in the CM plants, but did so to a lesser extent than in the CA ones. The CM0 seedlings showed 23% and 24% reduced root and stem dry weight compared with the CM50 ones, while no effect was observed in the biomass of the CM0 leaves by the end of the experiment.

Despite the RGR having a similar value in the 50 µM H_3BO_3-treated genotypes (Fig. 5), the 0 µM supplemented media lowered this ratio in both species 50 days after the experiment began. This reduction became more marked in the CA0 plants than in the CM0 ones (23% and 13%, respectively) compared to their corresponding CA50 and CM50 ones.

B Concentration and Total B Content

Both species presented a similar total B concentration in the roots and leaves of the plants grown under B sufficient conditions (Table 2), but the same could not be stated for this concentration in stems (lower in the CM50 seedlings than in the CA50 ones). As expected, no addition of B to culture media led to a lower B concentration in the 0 µM H_3BO_3-treated genotypes compared to the 50 µM H_3BO_3-treated plants. Leaves were dominant B accumulation sites and showed the most markedly lowered B concentration compared to other plant organs. Interestingly, this decrease was similar in the leaves and roots of both genotypes (around 35% and 10%, respectively). The accumulation of B in the stems of the CA0 plants also lowered by 32% compared to CA50, but no significant effect was recorded compared to the CM plants.

Regarding B content, leaves were the preferential organ for B accumulation in plants. Of the two genotypes grown under 50 µM treatment, CM had a higher B content in leaves and a lower one in roots than CA, while no differences were found in stems. The 0 µM treatment reduced the B content in all the plant organs and in both genotypes. This

reduction in leaves, stems and roots was more marked in CA0 (55%, 54% and 37%, respectively) than in CM0 (45%, 31% and 28%, respectively) compared with their corresponding CA50 and CM50 plants.

B Partitioning

Table 3 shows B partitioning in the soluble (water and organic solvents) and insoluble forms in roots and leaves. Insoluble B was the main fraction of B in CM50 and CA50 roots (63% and 55%, respectively). The second B accumulation fraction in CM roots was found in the organic solvents fraction (around 24%), with the last one in water-soluble B (13%). This tendency was the opposite in the CA50 roots, which respectively showed 21% and 24% accumulation in organic solvents and water fractions. In leaves, both the 50 µM H_3BO_3-treated genotypes showed a similar B partitioning pattern. Most B was allocated to the organic solvents fraction (within the 63-58% range), followed by the insoluble fraction (within the 20-32% range), and lastly by the water soluble fraction (below 17%).

No addition of B to culture media had a different effect on B partitioning in both genotypes. The CM0 plants showed increased insoluble B allocated toroots and leaves (14% and 27%, respectively), and reduced soluble B in the organic solvents fraction (14% and 21%, respectively) and water-soluble B in leaves (8%). In the CA0 plants, the water-soluble B proportion increased by 12% in roots, but slightly decreased in leaves compared to their CA50-treated seedlings. Regarding soluble B in organic solvents, the proportion of B located in this fraction lowered by 9% in roots, but no effect was observed in leaves. Interestingly, insoluble B distribution did not alter in the CA plants treated with the 0 µM supplemented media.

Leaf Chl Content, Proline and MDA Concentration in Leaves

The nutrient media lacking B supplement lowered the values of a, b and total Chl content in the leaves of both citrus genotypes (Table 4), which became more marked in the CA0 plants (14%, 30% and 18%, respectively) than in the CM0 ones (7%, 9% and 8%, respectively) compared to their corresponding 50 µM H_3BO_3-treated seedlings. The Chl a/b ratio was similar between both the 50 µM H_3BO_3-treated genotypes. Interestingly, the 0 µM treatment enhanced this parameter in the CA0 leaves, but not in the CM0 ones.

When analysing some stress indicator parameters in leaves (Table 4), the CM50 genotype presented a 1.9-fold higher proline concentration than CA50. When plants were grown in the 0 µM supplemented media, the proline concentration in the leaves of both species significantly rose compared to their corresponding 50 µM H_3BO_3-treated seedlings, and this increase was sharper in the CM0 seedlings than in the CA0 ones (32% and 14%, respectively). The MDA concentration was similar in both the the the 50 µM H_3BO_3-treated genotypes. When measured in the 0 µM H_3BO_3-treated plants, MDA significantly increased in the CA0 plants (by 12%) compared with their CA50. However, this parameter was not apparently affected by the absence of supplemented B in the culture media in the CM0 plants.

Discussion

At the gene expression level, studying the regulation of B uptake, transport and distribution processes may be a determinant to elucidate the underlying mechanisms involved in the differential responses of both citrus species to cope with B deficiency. As a first earlier response to B content in the cultural medium related to BA uptake across the plasma membrane, *NIP5* transcript abundance showed a similar pattern in both genotypes (Fig. 1), and was expressed mainly in roots and induced with the 0 µM supplemented media, which strongly suggests that NIP5 in citrus acts as an AtNIP5;1 in *Arabidopsis* (Takano et al., 2006). Therefore in citrus, NIP5 may have a strong potential for B deficiency tolerance as it efficiently facilitates BA uptake with a limited B supply. Similar behaviour has been previously described by An et al. (2012), who showed a relation between the *NIP5* gene expression in roots and B deficiency tolerance in Fragrant citrus and Carrizo citrange plants. This aptitude for both BA channel and water transport was first described in *A. thaliana*, where its preferential up-regulation in the elongation and hair zones of roots was detected (Takano et al., 2006; Hato et al., 2009). Some authors have identified water channel OsNIP 3;1 as a boron-inducible channel involved in root uptake and B distribution in rice plants (Hanaoka and Fujiwara, 2007). In contrast, its down-expression under B excess conditions has revealed a point of regulation of B uptake in the roots of citrus plants (Martinez-Cuenca et al., 2015). The importance of AtNIP5:1 degradation for plant acclimation to high B conditions has also been suggested (Tanaka et al., 2011).

386 *C. macrophylla*'s response to keep growing under the B-limiting conditions of this
387 experiment (Figs. 3 and 4) indicates that the underlying mechanism that determines
388 tolerance to low B levels may involve other genes related to B transport from roots to
389 leaves, or to other organs (Takano et al., 2006). In this way, we also monitored the
390 expression of the *BOR1* gene (Fig. 1), which has been reported to modulate B transport in
391 plants given its role in loading B into the xylem under B-limiting conditions, which exports B
392 from pericycle cells to the root stelar apoplast, and allows B translocation from roots to
393 shoots (Takano et al., 2002). Both tested *Citrus* species showed a similar *BOR1*
394 expression pattern in response to the external B level. The data on *BOR1* presented
395 herein confirm the previously reported inductive behaviour of this gene in citrus and other
396 plants, such as *A. thaliana* and rice, as a result of B deficiency (Takano et al., 2002;
397 Nakagawa et al., 2007; Cañon et al., 2013). The higher the *BOR1* relative expression in
398 the roots of CM, plus the higher biomass recorded in shoots from the 0 µM treatment,
399 could also indicate that this genotype has a more effective B transport system, and
400 suggest that low B level tolerance may be linked to an inducible B transport mechanism. It
401 has been proposed that *BOR1* expression generates a concentration gradient between
402 root cells and the medium that helps activate other B uptake system components, such as
403 NIP5, and therefore improves B uptake by roots (Miwa and Fujiwara, 2010). The lateral
404 polar localisation of BOR1 and NIP5 proteins to opposite plasma membrane domains
405 might prove important to control the radial transport and homeostasis of B (Takano et al.,
406 2010). A transcriptome analysis conducted into root tips has revealed the induction of the
407 WRKY6 transcription factor under low B conditions (Kasajima et al., 2010), and its likely
408 influence on the CM response should not be ruled out. Contrarily to the *NIP5* pattern,
409 *BOR1* expression has also been up-regulated in the leaves from the 0 µM H_3BO_3-treated
410 plants, and likely helps maintain B supply to newly forming primary cell walls, where B
411 forms an essential component of peptic polysaccharide rhamnogalacturonan II, as
412 described in barley (Sutton et al., 2007).
413 *PIP1* expression also increases under B deprivation in both citrus species,
414 especially in leaves (Fig. 1). This indicates a good response of this protein to enhance the
415 permeability of cells to B in citrus under low B conditions where a rapid boron transporter
416 is also required to, therefore, facilitate the diffusion of small and neutral solutes, such as
417 BA (Gaspar et al., 2003). In this way, when maize *ZmPIP1* is expressed in *Xenopus laevis*
418 oocytes, it leads to a 30% increase in B uptake (Chaumont et al., 2000), which suggests

419 that this aquaporin is involved in transporting B to enter cells. Moreover, the up-regulation
420 of this protein could be conditioned by an altered lipid composition in the membrane as a
421 result of low B levels (Dordas et al., 2000).
422 Another remarkable effect was the lower putative *BOR4* expression level in the
423 roots of both genotypes (29% and 51% in CM and CA, respectively) with the 0 µM
424 treatment, but this response was not observed in leaves (Fig. 2). The opposite response of
425 BOR4 (down-regulated) to the BOR1 (up-regulated) protein under low B conditions
426 suggests that both proteins might perform different activities. In fact the *BOR4* gene has
427 been reported to codify for a transporter to allow an active B efflux across the plasma
428 membrane at high B levels, and its overexpression might encourage any excess B being
429 pumped out of the cell to alleviate B toxicity stress in the cytoplasm (Miwa and Fujiwara,
430 2010). To support this notion, high B-tolerant barley and wheat cultivars are able to
431 maintain low B concentrations in shoots and roots by the active B efflux mediated by the
432 overexpression of paralogues *Hv-BOR2* and *Ta-BOR2* (*Reid*, 2007). BOR4 has been
433 localised on the outer side of plasma membranes on the distal sides of epidermal cells,
434 which is strategically important for effective B export from roots to soil to prevent B
435 concentrations in the xylem and to limit symplast B accumulation (Miwa et al., 2007).
436 However, its down-regulation in the 0 µM roots suggests an adaptive response mechanism
437 to avoid the B efflux from the symplast to soil solution, and to prevent B from being
438 transported out of the cell against the natural element gradient as a result of B being
439 absent in the external medium (Miwa et al., 2014). As far as we know, this is the first time
440 that this behaviour has been observed in citrus under low B conditions.
441 Boron deficiency stress also impaired the expression level of the gene that coded
442 for the TIP5 transporter in the CA genotype (a drop of around 47% in roots and leaves),
443 but to a much lesser extent in the CM (Fig. 2). This transporter has been related to a
444 lowering B concentration in the cytoplasm through its transport across the tonoplast
445 membrane and its compartmentalisation into the vacuole complexed with B-polyols (Pang
446 et al., 2010; Martinez-Cuenca et al., 2015). However, putative *TIP5* gene down-regulation
447 with a low B supply in culture media might be responsible for the preferential allocation of
448 B in the cytoplasm to cover cell functions. This was supported by the highest *TIP5*
449 expression levels observed in the CA in both organs and under both B conditions, and by
450 the highest relative distribution of soluble B in the organic solvents fraction in leaves (58%,
451 similar values to CA50; Table 3). This B fraction is related with the B located inside cells or

452 the protoplast linked to sugars, alcohols and polyhydroxycarbolates, as well as sugar-
453 containing compounds (Makkee et al., 1985). The highest protoplast B proportion to total B
454 in the CA0 plants suggested that more B in this fraction could be involved in maintaining a
455 normal plasma membrane structure (Liu et al., 2013). In contrast, the very low B organic
456 fraction concentration recorded in the CM0 leaves and the less damage to the biomass in
457 these plants suggest that this genotype is likely to be more tolerant to low B conditions
458 than CA as its 'critical level' of soluble B was lower than that in CA. Accordingly, CM
459 accumulated a lower B content in roots than CA with a sufficient B supply, which suggests
460 that the former requires much less B than the latter. This was supported by the slighter
461 reduction noted in the B content in all the plant organs as a result of the 0 µM treatment in
462 the CM plants (a decrease ranging from 45% to 28%) than in the CA ones (a drop ranging
463 from 55% to 37%) compared with their corresponding 50 µM H_3BO_3-treated plants (Table
464 2). This behaviour was also observed when the B-efficient citrange rootstock was
465 compared with the B-inefficient trifoliate orange (Liu et al., 2013).
466 In addition to this, the relative B distribution in the CM0 plants significantly increased
467 in the B insoluble fraction, which represents the B bound to the cell walls linked to peptic
468 polysaccharides (Liu et al., 2013). The minimum B level inside the cell in CM0 implied the
469 possibility of water-soluble B being distributed and located preferentially in the cell wall.
470 This would allow CM plants to maintain the integrity of cell membranes and to continue
471 growing at low B levels in the external medium. As B is required for cell wall structural
472 integrity, the relatively higher proportion of B allocated to the cell wall under B-limited
473 conditions has been associated with B efficiency (Pan et al., 2012). We speculated that the
474 direct function of B in cross-linking and fixing proteins in the membrane, and the drop
475 expected in cell wall tensile strength as a result of a low B concentration in the cell wall
476 fraction in the CA0 seedlings, would cause a mechanical signal to be rapidly transferred to
477 the plasma membrane (Camacho-Cristobal, 2011). This would alter the physical
478 stabilisation of membranes and/or microdomains due to subsequent changes in protein
479 conformation, interaction and functional integrity (Kasajima et al., 2010; Liu et al., 2013).
480 As cell wall-bound B could not be apparently remobilised and water-soluble B was the only
481 fraction that could be re-translocated in the phloem, water-soluble B fraction accumulation
482 in the CA0 roots suggests that the translocation system of B from roots to shoots could be
483 depressed in some way, which would limit CA growth under low B conditions. This is
484 probably further evidence that the differential distribution of B fractions can constitute

485 another factor of B deficiency tolerance in citrus, as reported in other plants (Pan et al.,
486 2012).

487 Consequently for the CA genotype, more major perturbations were recorded during
488 several metabolic and physiological processes under the 0 µM treatment than for CM. The
489 CA0 genotype presented markedly impaired root growth, which was noted 20 days after
490 transplanting until the end of the experiment (Fig. 4). This was reflected by the more
491 markedly lowered RGR in the CA than in the CM plants grown for 50 days without B (23%
492 and 13%, respectively) compared with their 50 µM H_3BO_3-treated plants (Fig. 5), and
493 agrees with what has been previously reported for other citrus species (Sheng et al.,
494 2009). A remarkable reduction in the stem and root lengths for the CA seedlings grown
495 with the 0 µM treatment (23% and 12% decrease, respectively) was recorded and
496 compared with the 50 µM H_3BO_3-treated plants, but not in the CM ones (Fig. 3). Despite
497 stunted root growth being a typical symptom in the plants grown under B stress conditions,
498 likely due to cell wall plasticity loss (*Kato* et al., 2009; *Sheng* et al., 2009; *Mei* et al., 2011),
499 it was not evidenced in the CM seedlings (Figs. 3 and 4), which seemed capable of
500 maintaining their growth index despite B being absent in the external medium. This
501 aptitude has already been described to confer resistance to B deficiency in citrus and other
502 species (Mei et al., 2011; Yang et al., 2013a).

503 In addition to growth, the 0 µM treatment also affected other stress indicators, such
504 as Chl proline and MDA (Table 4). Chl (*a, b* and *total*) contents were altered in the leaves
505 of both genotypes at low B levels, as reported for other citrus species (Sheng et al., 2009).
506 However, the CA genotype displayed more marked changes in Chl as a result of B
507 deficiency than CM. The higher Chl a/b ratio noted in the CA0 leaves was noteworthy. This
508 effect, which was not evident in the CM leaves, has been related to changes in chloroplast
509 structure (Ohtuska et al., 1997), repression of photosynthetic enzymes (Han et al., 2008)
510 and diminished photosystem II efficiency, which ultimately implies photosynthetic damage
511 (Sheng et al., 2009; Herrera-Rodriguez et al., 2010). Moreover, B effects on the proline
512 concentration were studied in leaves because this element usually accumulates in
513 response to several abiotic stresses (Table 4). The possible roles attributed to proline
514 include balance of osmotic pressure, preservation of enzymes in the cytoplasm,
515 detoxification of reactive oxygen species (ROS) and protection of membranes against lipid
516 peroxidation (Delauney and Verna, 1993). Although the 0 µM treatment increased the
517 proline concentration in both genotypes, the higher proline levels reached by CM0 than by

CA0 (31.5% and 14.0% increase, respectively, related to the 50 µM H_3BO_3-treated plants) suggest an enhanced protective response of leaves in the former genotype. This capacity has also been described in citrus and other plants submitted to B stress (Pandey, 2013; Yang et al., 2013b). Finally, low B conditions also led to slight MDA accumulation in the CA genotype leaves, while the CM seedlings did not reflect this effect (Table 4). This indicates that CA is more susceptible to oxidative stress as a result of B deficiency than CM. Oxidative damage due to excess ROS production has been described under both deficient and excess B supplies (Han et al., 2008; Pandey, 2013). The involvement of ROS species as a signal for cell wall structure disturbance should not be ruled out as they build up until they reach a toxic level, unless B is re-supplied and the cell wall structure is restored (Koshiba et al., 2009). Low proline levels have also been related with a rise in MDA concentration in organs under excessive B conditions and, consequently, with enhanced oxidative damage, as other authors have reported (Keles et al., 2004; Molassiotis et al., 2006). This behaviour coincides with that observed in the citrus genotypes studied herein. Thus CA0 presented lower proline levels in leaves than CM0, but the opposite occurred with the MDA concentration. This indicates differences between citrus genotypes in antioxidant system efficiency, which is able to cope with the ROS species generated as a result of B deficiency.

In conclusion, both the studied citrus species presented a common mechanism of B deficiency tolerance based on the collaborative action of several genes involved in B transport. Thus under the low B availability conditions in the external medium, channel NIP5, transporter BOR1 and non-selective aquaporin PIP1 were up-regulated, and their combined activities could improve B uptake, transport and distribution in low B plants. However, the CA plants were markedly affected by the 0 µM treatment (growth reduction, Chl content, MDA, etc.), which suggests B is required more inside the cell in this genotype than in CM, and would likely cover physiological functions. Accordingly, the *TIP5* gene, which transports B inside the vacuole, is seriously impaired. The minimum need for B inside the cell allows CM to locate B in the cell wall and to keep growing despite low B conditions. The BOR4 down-regulation in the 0 µM H_3BO_3-treated roots suggests an adaptive response mechanism to avoid the efflux of B from the symplast to soil solution against the concentration gradient.

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