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Boron Toxicity Reduces Water Transport from Root to Shoot in Arabidopsis Plants. Evidence for a Reduced Transpiration Rate and Expression of Major PIP Aquaporin Genes

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Regular Paper

Toxic boron (B) concentrations cause impairments in several plant metabolic and physiological processes. Recently we reported that B toxicity led to a decrease in the transpiration rate of Arabidopsis plants in an ABA-dependent process within 24 h, which could indicate the occurrence of an adjustment of whole-plant water relations in response to this stress. Since plasma membrane intrinsic protein (PIP) aquaporins are key components influencing the water balance of plants because of their involvement in root water uptake and tissue hydraulic conductance, the aim of the present work was to study the effects of B toxicity on these important parameters affecting plant water status over a longer period of time. For this purpose, transpiration rate, water transport to the shoot and transcript levels of genes encoding four major PIP aquaporins were measured in Arabidopsis plants treated or not with a toxic B concentration. Our results indicate that, during the first 24 h of B toxicity, increased shoot ABA content would play a key role in reducing stomatal conductance, transpiration rate and, consequently, the water transport to the shoot. These physiological responses to B toxicity were maintained for up to 48 h of B toxicity despite shoot ABA content returning to control levels. In addition, B toxicity also caused the down-regulation of several genes encoding root and shoot aquaporins, which could reduce the cell to cell movement of water in plant tissues and, consequently, the water flux to shoot. All these changes in the water balance of plants under B toxicity could be a mechanism to prevent excess B accumulation in plant tissues.

Keywords: Abscisic acid • Aquaporin • Arabidopsis • Boron toxicity • Transpiration • Water transport.

Abbreviations: ICP-MS, inductively coupled plasma-mass spectrometry; MIP, major intrinsic protein; PIP, plasma membrane intrinsic protein; RT-PCR, real-time PCR; q_{leaf}, relative water flux to the shoot; R_{leaf}, deuterium content of the rosette leaves.

Introduction

Boron (B) is an essential nutrient for normal growth of vascular plants that has a very narrow range of optimal concentrations. Therefore, controlling B availability in soil and irrigation water is crucial for crop yield and quality (Brown et al. 2002, Goldbach and Wimmer 2007, Camacho-Cristóbal et al. 2008). Thus, for instance, a toxic B concentration produces negative physiological effects such as reduced root growth, decreased Chl content or lowered photosynthetic rate (Nable et al. 1997, Landi et al. 2012). High levels of B can be naturally present due to the chemical composition of the soil or can be incorporated by anthropogenic activities such as chemical fertilizer application. B toxicity is a major problem that can affect crop yields in arid and semi-arid areas of the world, since the processes of evaporation are greatly favored in these areas and, consequently, B tends to accumulate in the upper layers of the soil (Reid 2007).

B uptake by root cells and xylem loading can be carried out by three different molecular mechanisms, depending on B availability in the medium: (i) passive diffusion across the lipid bilayer; (ii) facilitated transport by major intrinsic protein (MIP) channels; and (iii) active transport mediated via BOR transporters (Takano et al. 2008). Under conditions of adequate or excessive B availability, this nutrient is absorbed by root cells and loaded into xylem through a passive process that involves mostly B diffusion across the lipid bilayer—due to the high permeability of lipid bilayers to boric acid—and, to a lesser extent, facilitated permeation via MIP channels (Brown et al. 2002, Dannel et al. 2002, Tanaka and Fujiwara 2008). Once B has reached the xylem, it is transported to the shoot along with the transpiration stream which is driven by the gradient in water potential resulting from leaf surface transpiration (Shelp et al. 1995).

Interestingly, in a recent study it has been reported that B toxicity leads to a rapid decrease in transpiration rate in Arabidopsis plants, which could be a mechanism to limit the transport of excess B from the root to the shoot (Macho-Rivero et al. 2017). This result would indicate the occurrence of an

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adjustment of whole-plant water relations to restrict B accumulation in plant tissues in response to B toxicity. In addition to transpiration, aquaporins have also been identified as a key component influencing plant water status. Aquaporins are intrinsic membrane proteins that have been shown to function as channels to facilitate transmembrane water transport. Thus, aquaporins play a key role in processes affecting water balance of plants such as root water uptake and tissue hydraulic conductance (Chaumont and Tyerman 2014, Maurel et al. 2015, Moshelion et al. 2015). Vascular plants are characterized by a large number of genes encoding aquaporin proteins, which have been classified into the following subfamilies: PIPs (plasma membrane intrinsic proteins), TIPs (tonoplast intrinsic proteins), NIPs (nodulin 26-like intrinsic proteins) and SIPs (small basic intrinsic proteins) (Johanson et al. 2001, Maurel et al. 2008). The PIPs represent the most abundant aquaporins in the plasma membrane and are divided into two types, PIP1 and PIP2. There are five PIP1 isoforms and eight PIP2 isoforms in *Arabidopsis thaliana*. In general, PIP1 proteins have a low capacity for water transport when they are expressed in heterologous systems which, however, may be different in planta. Instead, all plant PIP2 proteins have a high efficiency for water transport in heterologous cells as well (Maurel et al. 2008, Kumar et al. 2014). In addition to water, some aquaporins can also transport a variety of small neutral molecules such as glycerol (Biela et al. 1999), urea (Eckert et al. 1999), ammonia (Holm et al. 2005), boric acid (Takano et al. 2006), CO_2 (Uehlein et al. 2003) or H_2O_2 (Bienert et al. 2007).

The aim of this work was to study the effects of B toxicity on the water balance of plants. In a previous study, we reported that B toxicity for 24 h leads to a decrease in the transpiration rate of Arabidopsis plants in an ABA-dependent process (Macho-Rivero et al. 2017), which could result in a reduced water transport from root to shoot. Therefore, in the present study, we analyzed the effects of B toxicity on the kinetics of water relocation to the shoot in Arabidopsis plants by using deuterium as a tracer. We demonstrated a reduced water flux to the shoot under B toxicity conditions, which correlated not only with a drop in the transpiration rate (Macho-Rivero et al. 2017), but also with the down-regulation of several genes encoding root and shoot aquaporins. Finally, we provide evidence suggesting that all these changes caused by B toxicity in the water balance of plants could be a mechanism to prevent excess B accumulation in plant tissues.

Results

Effects of B toxicity on transpiration rate, stomatal conductance and endogenous ABA levels

The effects of B toxicity on stomatal conductance, transpiration rate and endogenous shoot ABA levels were measured in Arabidopsis plants after 24 and 48 h treatment with or without 5 mM boric acid (**Fig. 1**). B toxicity caused a clear decrease in both transpiration rate and stomatal conductance values, this effect being more marked after 24 h of treatment (**Fig. 1A, B**).

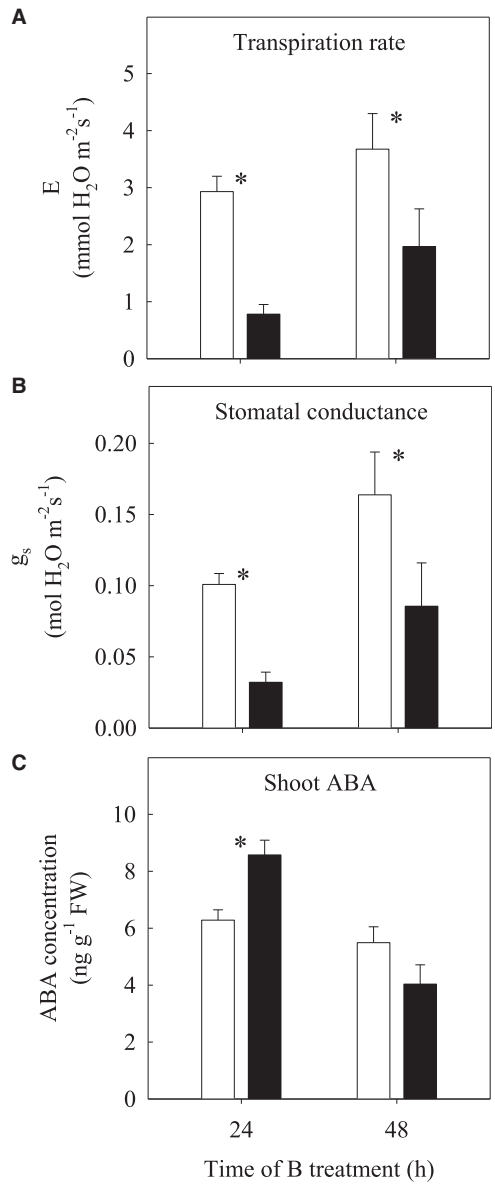

Fig. 1 Transpiration rate (A), stomatal conductance (B) and shoot ABA concentration (C) in Arabidopsis plants grown under control (10 µM B, open squares) or B toxicity (5 mM B, filled squares) conditions for 24 and 48 h. Results are given as the mean ± SD from the analysis of five different plants for each treatment. Asterisks indicate statistically significant differences between treatments at each time according to Student's *t*-test ($P < 0.05$).

Interestingly, B toxicity treatment for 24 h caused a significant increase in shoot ABA contents in comparison with the control treatment, a fact that was not observed after 48 h of B treatments (**Fig. 1C**).

B toxicity reduces relative water flux from root to shoot

The effects of B toxicity on relative water flux to the shoot q_{leaf} were analyzed by using deuterium as a tracer (Da Ines et al. 2010). For this purpose, Arabidopsis plants were subjected or not to B toxicity in deuterated media as described in the

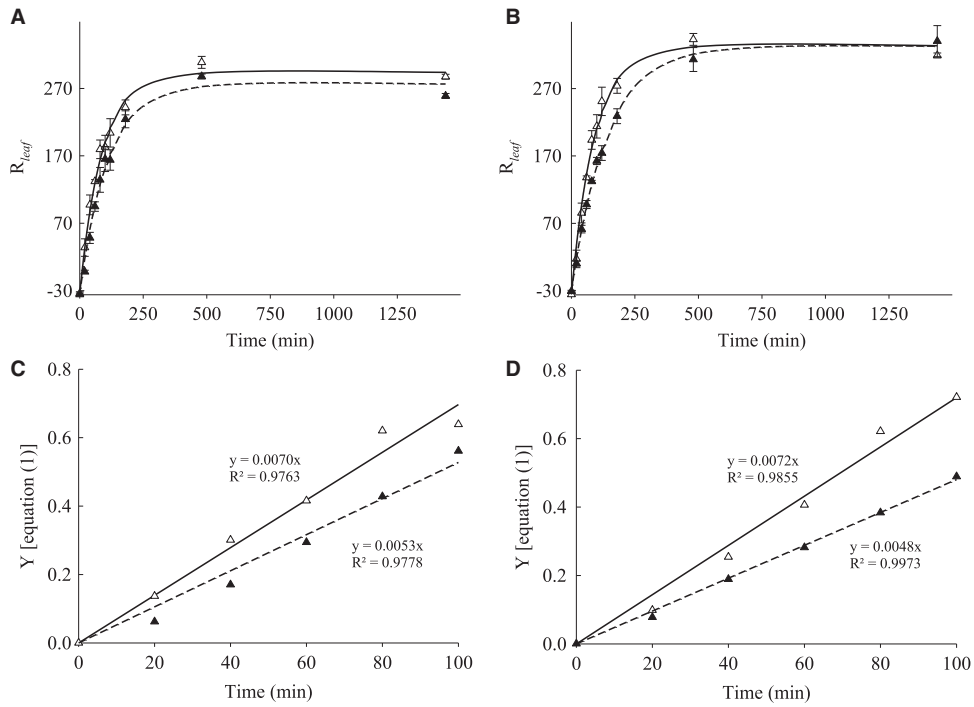

Fig. 2 Deuterium uptake curves (A, B) and relative water flux to the shoot (q_{leaf}) (C, D) in Arabidopsis plants grown in a deuterated hydroponic medium under control (10 μM B; open triangles and continuous lines) or B toxicity (5 mM B; filled triangles and dotted lines) conditions for 24 (A, C) and 48 h (B, D). (A, B) The increase in the measured leaf deuterium content R_{leaf} (open and filled triangles, $n = 6 \pm SD$) was fitted (continuous and dotted lines) using the equation $\{R_{leaf}(t) = R_{leaf,SS}[1 - \exp(-aq_{leaf}t)] + R_{leaf,0}\exp(-aq_{leaf}t)\}$ described in Da Ines et al. (2010). (C, D) q_{leaf} was inferred from the regression analysis of the initial uptake phase (0–100 min) of the fitted curves based on equation (1) $y = 1/a \cdot ln \cdot \left(\frac{R_{leaf,0}-R_{leaf,SS}}{R_{leaf}(t)-R_{leaf,SS}}\right) = q_{leaf} \cdot t$ described in Da Ines et al. (2010) to determine q_{leaf} as the slope of this linear equation. The experiments were independently repeated with similar results.

Materials and Methods, and the kinetics of deuterium increase in the aerial parts were recorded during a short period of time (24 h) after enhancing the deuterium content in the source water of a hydroponic culture. The change in deuterium content of the rosette leaves R_{leaf} was modeled as a function of q_{leaf}, the initial $R_{leaf,0}$ and the new, enhanced steady-state $R_{leaf,SS}$ using the equation $\{R_{leaf}(t) = R_{leaf,SS}[1 - \exp(-aq_{leaf}t)] + R_{leaf,0}\exp(-aq_{leaf}t)\}$ described in Da Ines et al. (2010) (**Fig. 2A, B**). The relative water flux q_{leaf} was derived as a fitting parameter and could be easily inferred from the regression analysis of the initial uptake phase (0–100 min) of the modeled curves using equation $1/a \cdot \ln\left(\frac{R_{leaf,0}-R_{leaf,SS}}{R_{leaf}(t)-R_{leaf,SS}}\right) = q_{leaf} \cdot t$ described in Da Ines et al. (2010) (**Fig. 2C, D**).

Interestingly, B toxicity reduced q_{leaf} of Arabidopsis plants after 24 h (about 25%, $P < 0.05$) and 48 h (about 33%; $P < 0.001$) of treatment when compared with the control condition (**Fig. 2C, D**).

B toxicity decreases the transcript levels of several aquaporins

Experiments were conducted to elucidate whether the reduction in q_{leaf} caused by B toxicity was related to changes in the expression levels of several aquaporins involved in water transport. Thus, both shoot and root transcript levels of *PIP1;1*, *PIP1;2*, *PIP2;1* and *PIP2;2*, which encode about 80% of the total PIP aquaporins present in these tissues of Arabidopsis plants grown in similar conditions to those in this study (Monneuse et al. 2011), were analyzed by quantitative real-time PCR (RT-PCR) in plants treated or not with B toxicity for 24 and 48 h (**Figs. 3, 4**). In shoots, B toxicity treatment led to a significant decrease in the transcript levels of all analyzed *PIP* genes (with the exception of *PIP1;1* after 48 h of B treatment) in comparison with the control treatment (**Fig. 3**). However, in roots, B toxicity only significantly decreased the transcript levels of *PIP2;1* after 24 and 48 h of the treatment, and *PIP2;2* and *PIP1;2* after 48 h of treatment when compared with the control condition (**Fig. 4**).

The expression patterns within plant tissues of *PIP1;1*, *PIP1;2*, *PIP2;1* and *PIP2;2* genes were analyzed histochemically by using PIP::GUS (β-glucuronidase) transgenic reporter lines. *PIP1;2*, *PIP2;1* and *PIP2;2* genes were strongly transcribed throughout roots and leaves, with the highest levels in vascular tissues (**Fig. 5**; Da Ines et al. 2010, Postaire et al. 2010). PIP1;1::GUS activity also shows a strong signal throughout roots; however, accumulation of PIP1;1::GUS signal in leaves was only observed in the leaf margin (**Fig. 5**). According to the quantitative RT-PCR analysis, decreased PIP1;2::GUS, PIP2;1::GUS and PIP2;2::GUS activities were observed in roots and, especially, in leaves of Arabidopsis plants after 48 h of B toxicity treatment (**Fig. 5**).

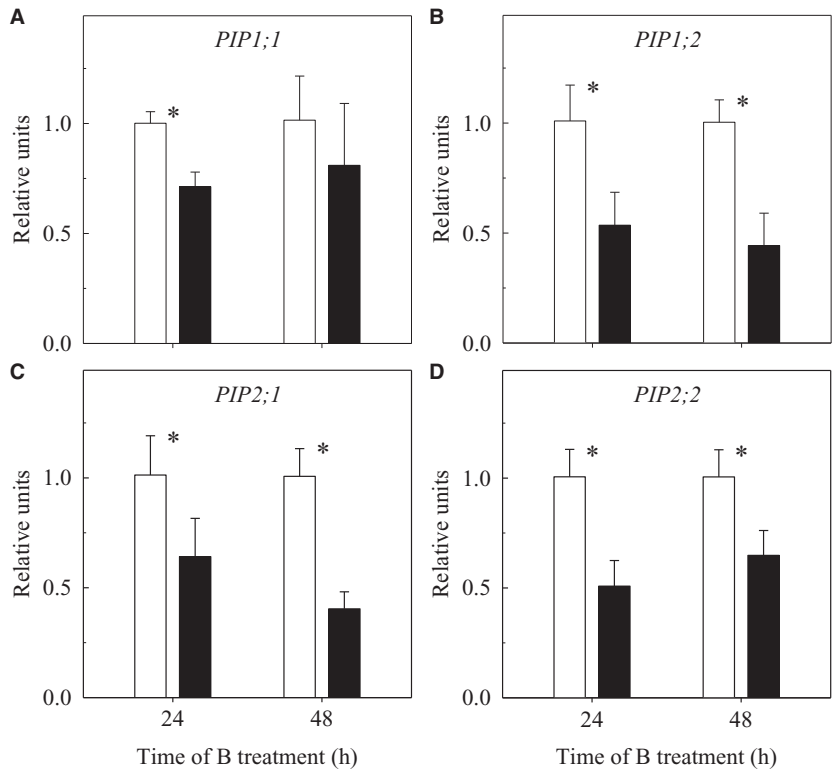

Fig. 3 Quantitative RT-PCR analysis of *PIP1;1* (A), *PIP1;2* (B), *PIP2;1* (C) and *PIP2;2* (D) transcript levels in Arabidopsis shoots after treatment with 10 μM B (open squares) or 5 mM B (filles squares) for 24 and 48 h. Results are given as the mean ± SD from the analysis of five different plants for each treatment. Asterisks indicate statistically significant differences between treatments at each time according to Student's *t*-test ($P < 0.05$).

Effect of B toxicity on shoot B concentration and B transporter gene expression

Typical B toxicity symptoms such as leaf marginal or tip chlorosis/necrosis have been described to be a consequence of the B accumulation in these leaf areas (Roessner et al. 2006, Camacho-Cristóbal et al. 2008). Therefore, experiments were performed to study how B toxicity affects shoot B concentrations and the rate of B transport to the shoot. Thus, Arabidopsis plants were grown in a medium containing 10 μM (control condition) or 5 mM (B toxicity) boric acid enriched with ^{10}B for 24 and 48 h. After that, the levels of ^{10}B and ^{11}B in the rosette leaves were measured by inductively coupled plasma-mass spectrometry (ICP-MS). Under control conditions, shoot B concentration and the rate of ^{10}B transport to the shoot remained steady throughout the 48 h of treatment (**Fig. 6**). As expected, shoot B contents and ^{10}B transport to the shoot significantly increased in B toxicity-treated plants when compared with the respective control plants (**Fig. 6**). However, it is interesting to highlight that the rate of ^{10}B transport to the shoot decreased from 24 h until the end of the B toxicity treatment (**Fig. 6B**).

The transcript levels of three B transport-related genes were also measured in Arabidopsis plants under control and B toxicity conditions. The transcript level of the B efflux transporter *BOR1* gene was significanly affected only after 24 h of B toxicity treatment (**Fig. 7A**). However, the transcript level of another gene encoding a B efflux transporter, *BOR4*, was significantly stimulated by the presence of 5 mM B (**Fig. 7B**). Conversely, the transcript level of *NIP5;1*, which encodes a B channel protein, notably decreased in plants subjected to B toxicity when compared with control plants (**Fig. 7C**).

Discussion

Water balance of plants depends on several factors, such as (i) the water uptake by roots; (ii) the water transport in vascular tissues; and (iii) the water loss by transpiration. Many abiotic stresses modify the water status of plants by affecting one or several of the above-cited parameters (Aroca et al. 2012). For instance, B deficiency affects the water balance of plants by decreasing the water transport to the shoot due to damage to the xylem structure (Wimmer and Eichert 2013). It has also been described that salinity stress results in a reduction in root water permeability (root hydraulic conductivity) that modifies the water status in many plants species (Martínez-Ballesta et al. 2003, Boursiac et al. 2005, Aroca et al. 2012). In the present work, we show that B toxicity strongly reduces q_{leaf} in Arabidopsis plants after 24 and 48 h of treatment (**Fig. 2C, D**), which could be a consequence of the reduced transpiration rate observed under this stress condition. Since B toxicity also led to a significant decrease in the stomatal conductance and transpiration rate of Arabidopsis plants (**Fig. 1**; Macho-Rivero et al. 2017), these results support the occurrence of a positive relationship between transpiration rate and q_{leaf} in Arabidopsis plants.

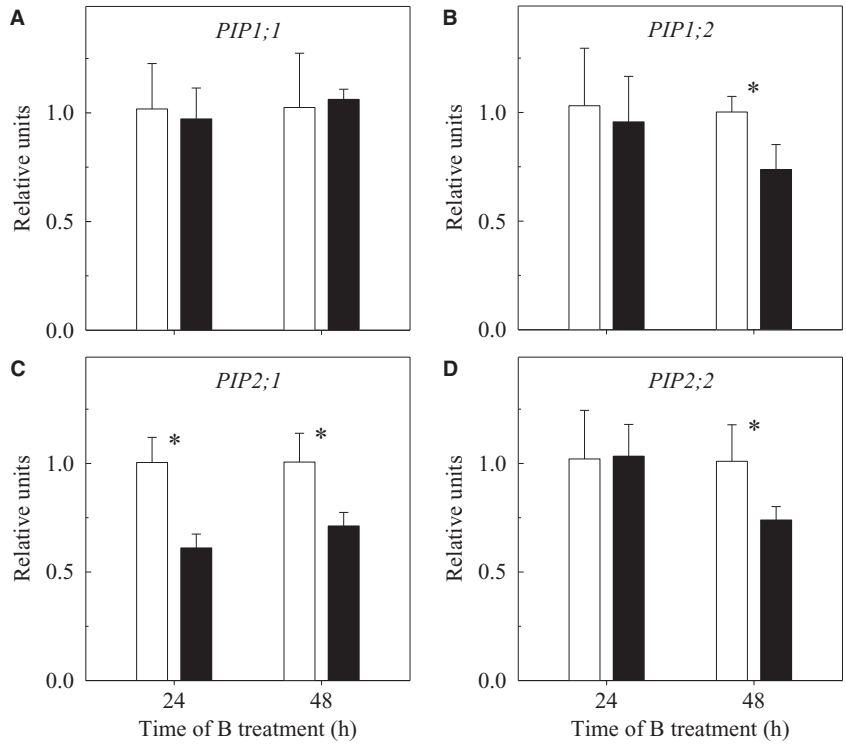

Fig. 4 Quantitative RT-PCR analysis of *PIP1;1* (A), *PIP1;2* (B), *PIP2;1* (C) and *PIP2;2* (D) transcript levels in Arabidopsis roots after treatment with 10 μM B (open squares) or 5 mM B (filled squares) for 24 and 48 h. Results are given as the mean ± SD from the analysis of five different plants for each treatment. Asterisks indicate statistically significant differences between treatments at each time according to Student's *t*-test ($P < 0.05$).

It is well known that ABA is synthesized de novo in response to abiotic stresses and induces a series of downstream processes leading to stress tolerance. For instance, increased ABA content in leaves induces stomatal closure to reduce transpiration under drought conditions (Wilkinson and Davies 2002, Kollist et al. 2014). Interestingly, B toxicity for 24 h caused an increase in shoot ABA content (**Fig. 1C;** Macho-Rivero et al. 2017) that correlated with a decrease in stomatal conductance, transpiration rate and q_{leaf} in Arabidopsis plants (**Figs. 1A, B, 2C**). This physiological response to B toxicity (reduced stomatal conductance, transpiration rate and q_{leaf}) was also observed after 48 h of B toxicity treatment (**Figs. 1A, B, 2D**) despite shoot ABA content returning to control levels (**Fig. 1C**). These results seem to indicate that the transient shoot ABA increase observed after 24 h of B toxicity treatment triggers a physiological response that is maintained at least up to 48 h of treatment.

The reduced water flux to shoot q_{leaf} under B toxicity could be explained not only by a lower transpiration rate (as above discussed), but also by a drop in water supply from roots to the rosette and/or a reduction in hydraulic exchange between leaf cells (Da Ines et al. 2010). Plasma membrane aquaporins have been shown to facilitate transmembrane water transport and, hence, they could be involved in water uptake as well as in routes of long-distance transport and distribution of water within tissues (Maurel et al. 2008, Chaumont and Tyerman 2014, Maurel et al. 2015). Thus, aquaporins could also influence the B-dependent plant water relations. The transcript level of most aquaporin-encoding genes decreases in response to several abiotic stresses such as drought and cold (Jang et al. 2004, Alexandersson et al. 2005). Previous studies have shown that B toxicity also led to a decrease in the expression of several *PIP* genes in Arabidopsis roots (Aquea et al. 2012). In the present work, a decrease in the transcript levels of several root and, in particular, shoot *PIP* genes was observed in Arabidopsis plants after 24 and 48 h of B toxicity (**Figs. 3–5**), which could result in a repressed uptake and/or distribution of water in both roots and leaves and, consequently, in a reduced q_{leaf} (**Fig. 2C, D**). Accordingly, it has been described that AtPIP1;2 significantly contributes to the hydraulic conductivity in both roots and rosette and therefore represents a key component of whole-plant hydraulics (Postaire et al. 2010). Moreover, a reduced q_{leaf} has been demonstrated in *pip2;1* and *pip2;2* knockout Arabidopsis mutants in comparison with the wild type despite their similar transpiration rates (Da Ines et al. 2010), which highlights the key role of these *PIP* genes in the water balance of plants. In this regard, repression of *PIP1;2*, *PIP2;1* and *PIP2;2* transcription under B toxicity was stronger in shoots than in roots (**Figs. 3–5**), the organ that first senses this stress. This result is consistent with the higher accumulation of B in shoots than in roots observed when Arabidopsis plants are exposed to B toxicity (Sakamoto et al. 2011, Macho-Rivero 2015, Mosa et al. 2016). Although further studies are required to elucidate whether these facts are directly related or not, our results seem to suggest that excessive B accumulation in the shoot itself or the occurrence of a signaling cascade triggered by excess B could be involved in shoot repression of these *PIP* genes.

It has long been known that B toxicity tolerance in plants is related to the ability to restrict B accumulation in both roots

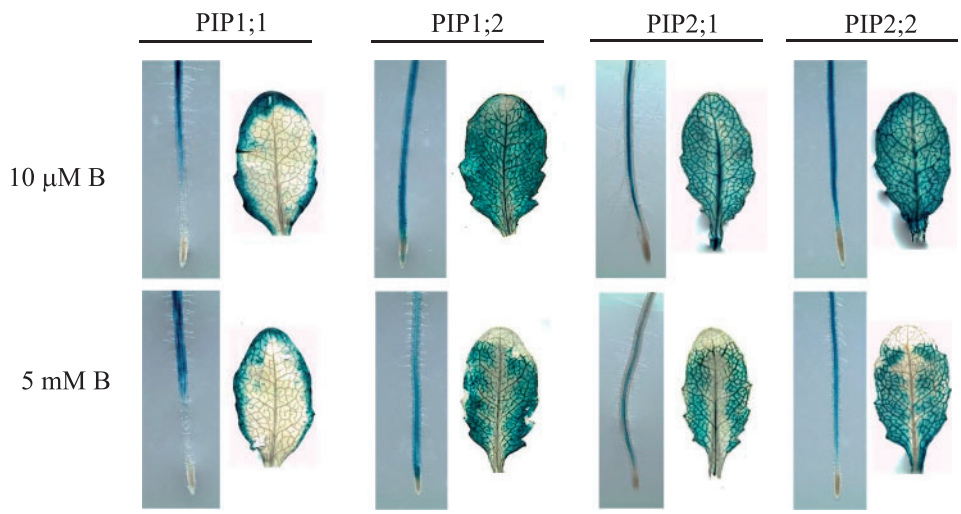

Fig. 5 Histochemical localization of PIP1;1::GUS, PIP1;2::GUS, PIP2;1::GUS and PIP2; 2::GUS activity in roots and leaves of Arabidopsis plants grown hydroponically with 10 μM B for 3 weeks and then treated with 10 μM B (control) or 5 mM B (B toxicity) for 48 h. Due to the strong activity in roots, the glucuronidase assay in this organ was limited to 45 min; however, leaves were stained overnight. Images are representative individuals of two independent experiments with at least six plants examined for each experiment. Representative images were chosen for each B treatment.

and shoots (Reid 2007, Sutton et al. 2007). In agreement with this, the transcript level of *NIP5;1*—a B channel protein located in plasma membranes of root hair and cortex cells that is required for efficient B uptake in Arabidopsis (Takano et al. 2006)—decreased under B toxicity (**Fig. 7C**). Consequently, it has been described that *NIP5;1* mRNA degradation is important for plant acclimation to excess B conditions (Tanaka et al. 2011). Moreover, despite the fact that the transcript level of *BOR1*—an efflux-type B transporter for xylem loading (Takano et al. 2002)—was not affected after 48 h of B toxicity (**Fig. 7A**), it is well documented that endocytosis and degradation of BOR1 are increased under B toxicity conditions (Takano et al. 2005). These data would suggest a mechanism to limit B entry into the root and B transport to shoots under this stress condition. In addition, the decreased expression of several *PIP* genes observed in Arabidopsis roots and, in particular, shoots under B toxicity (**Figs. 3–5**) could also contribute to this mechanism to try to prevent excessive B accumulation in roots since some PIP members may have a role in B permeability. Thus, for instance, the expression of a maize *PIP1* in *Xenopus laevis* oocytes resulted in a 30% increase in the B permeability of the oocytes (Dordas et al. 2000), and expression of *Hv-PIP1;3* and *Hv-PIP1;4* from barley increased the sensitivity of yeast cells to B (Fitzpatrick and Reid 2009). However, the physiological relevance of this mechanism would be very limited because, under conditions of excessive B availability, B uptake by roots is mediated through a passive process that involves mostly B diffusion across the lipid bilayer (Brown et al. 2002, Tanaka and Fujiwara 2008). Interestingly, B exclusion from roots under B toxicity conditions has also been proposed to be a mechanism to prevent excess B accumulation in roots. Thus, in transgenic plants overexpressing the B efflux transporter BOR4, root and leaf B contents were lower than those of wild-type plants grown in B toxicity (Miwa et al. 2007). This fact suggests that this B efflux transporter could play a crucial role in the mechanism of B exclusion from roots under B toxicity conditions (Miwa et al. 2007, Miwa et al. 2014). Accordingly, an increase in the transcript level of the *BOR4* gene under B toxicity in Arabidopsis roots was observed (**Fig. 7B**). After being loaded into the xylem, B is transported to the shoot in a process mediated by the transpiration stream (Brown and Shelp 1997, Nable et al. 1997). Previous studies have shown a direct relationship between the rate of transpiration and B accumulation in leaves (Pfeffer et al. 1999, Dannel et al. 2000, Ben-Gal and Shani 2002). Therefore, the decrease in transpiration rate and q_{leaf} observed under B toxicity (**Figs. 1A, 2C, D**) could be a mechanism to limit the transport of excess B from the roots to the shoots under this stress condition (Macho-Rivero et al. 2017). Consequently, the rate of ^{10}B transport to the shoot was lower after 48 h compared with 24 h of B toxicity treatment (**Fig. 6B**).

In conclusion, this study provides evidence that several parameters are involved in the control of water balance of *A. thaliana* plants under B toxicity. Our results indicate that, during the first 24 h of B toxicity treatment, increased shoot ABA content would play a key role in reducing stomatal conductance and transpiration rate, which greatly contributes to a reduction in the water relocation from root to shoot in Arabidopsis plants. The ABA-dependent response to B toxicity was maintained until 48 h of B toxicity treatment despite shoot ABA content returning to control levels. Moreover, B toxicity seems to cause a decrease in tissue hydraulic conductance mediated by the down-regulation of several genes encoding root and shoot aquaporins, which would also contribute to the reduction in the water flux to the shoot. Finally, all these changes in the water balance of plants under B toxicity could be a mechanism to prevent excess B accumulation in plant tissues.

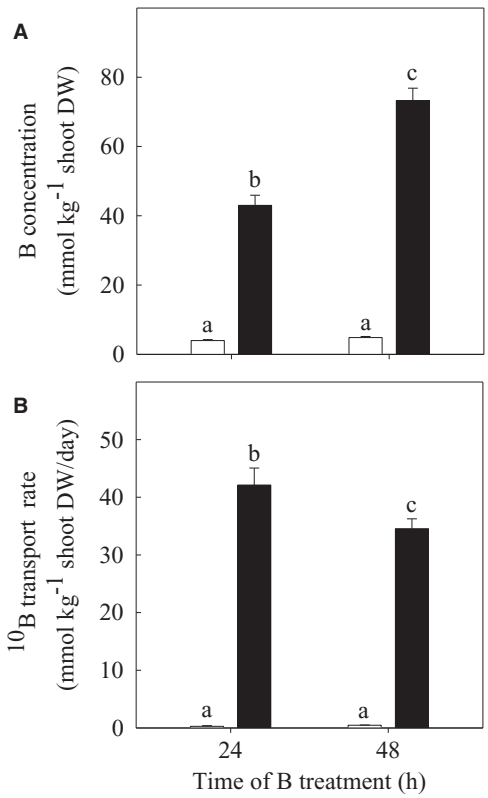

Fig. 6 Total shoot B concentration (A) and rate of ^{10}B transport to the shoot (B) in Arabidopsis plants after treatment with 10 μM B (open squares) or 5 mM B (filled squares) for 24 and 48 h. Results are given as the mean ± SD from the analysis of five different plants for each treatment. Different letters indicate statistically significant differences between treatments according to ANOVA with Tukey's HSD test ($P < 0.05$).

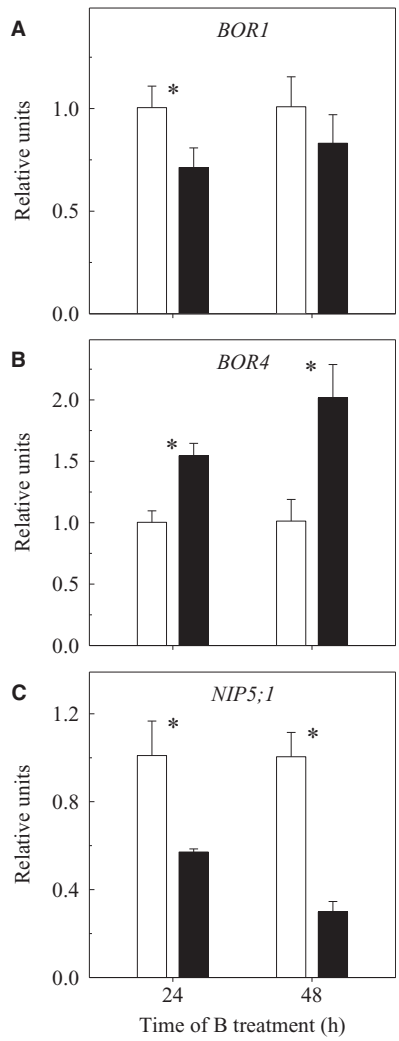

Fig. 7 Quantitative RT-PCR analysis of *BOR1* (A), *BOR4* (B) and *NIP5;1* (C) transcript levels in Arabidopsis roots after treatment with 10 μM B (open squares) or 5 mM B (filled squares) for 24 and 48 h. Results are given as the mean ± SD from the analysis of five different plants for each treatment. Asterisks indicate statistically significant differences between treatments at each time according to Student's *t*-test ($P < 0.05$).

Materials and Methods

Plant material and growth conditions

Seeds of *A. thaliana* (Col-0) were germinated in trays filled with peat and watered every other day with high purity deionized water for a week. Subsequently, seedlings were removed, washed with deionized water and immediately transferred to 8 liter plastic containers with a complete nutrient solution containing 1 mM KNO_3, 1 mM $Ca(NO_3)_2$, 0.5 mM $MgSO_4$, 0.75 mM KH_2PO_4, 12.5 μM FeNa-EDTA, 12.5 μM NaCl, 2.5 μM $MnCl_2$, 0.5 μM $ZnSO_4$, 0.25 μM $CuSO_4$, 0.125 μM Na_2MoO_4, 0.05 μM $CoCl_2$ and 10 μM H_3BO_3 (pH 5.7 adjusted with KOH). Plants were grown hydroponically under an 8 h light/16 h dark regime (150 μmol m^{-2} s^{-1} of photosynthetically active radiation at plant height), 22°C and 65% relative humidity. After 30 d of growth, plants were divided into two groups and subjected to either B toxicity treatment (5 mM boric acid) or control conditions (10 μM boric acid). Plants were maintained with these B treatments and harvested at the indicated times. Under these growth conditions, plants of both B treatments remained in the vegetative stage throughout the experiments. Nutrient solutions in hydroponic cultures were aerated by air pumps and completely renewed twice a week.

Transpiration rate and stomatal conductance measurements

Transpiration rate (mmol H_2O m^{-2} s^{-1}) and stomatal conductance (mol H_2O m^{-2} s^{-1}) were measured 1 h after the beginning of the photoperiod using a portable infrared gas analyzer (6400 LiCor Biosciences) equipped with an extended chamber (Extended Reach 1 cm Chamber, 6400-15, LiCor). The air flow rate was adjusted to 350 μmol s^{-1} and the irradiance was 150 μmol m^{-2} s^{-1} of photosynthetically active radiation.

ABA analysis

The extraction and UHPLC-MS/MS analysis of endogenous ABA were performed as described by Müller and Munné-Bosch (2011). Deuterium-labeled phytohormone [$^2H_6$]ABA was used as an internal standard.

Analysis of water transport

Arabidopsis plants were grown hydroponically in 15 liter containers in a growth chamber with a light/dark regime of 16 h/8 h, 22°C, 65% relative humidity and 450 μmol m^{-2} s^{-1} of photosynthetically active radiation at plant height. After 20 d of growth, half of the plants was treated with 5 mM boric acid (toxicity) and the other half were grown under control conditions (10 μM boric acid). A 1 ml aliquot of D_2O (90% purity) was added to 15 liters of hydroponic medium after 24 and 48 h of B treatments, and 1 ml samples were withdrawn before adding D_2O (start level) and from the deuterated media at 0 and 40 min, and 2, 3, 8 and 24 h. The heavy water content in the plant rosettes (R_{leaf}) at 0, 20, 40,

60, 80 and 100 min, and 2, 3, 8 and 24 h was also analyzed. For sampling of plants, the whole rosette was cut, quickly placed in glass bottles, frozen with liquid nitrogen to prevent evaporation, and stored at −20°C until water extraction for isotopic analysis as described in Da Ines et al. (2010). The deuterium uptake curves, their modelings and the regression analysis of the initial uptake phase (0–100 min) of the modeled curves to deduce the relative water flux to the shoot (q_{leaf}) were carried out as described in Da Ines et al. (2010). *t*-test was used to analyze the significance of a difference in slopes of q_{leaf} between treatments.

RNA isolation, cDNA synthesis and quantitative RT-PCR analyses

Total RNA was extracted by using Tri-Reagent RNA/DNA/Protein Isolation Reagent (Molecular Research Center) and then treated with RNase-free DNase (Qiagen) according to the manufacturer's instructions. RNA was purified using a RNA Clean & Concentrator column (Zymo Research). A 2 μg aliquot of DNase-treated total RNA was used to prepare cDNA by reverse transcription with M-MLV reverse transcriptase (Biolabs) and oligo$(dT)_{18}$ primers (Bioline), according to the manufacturer's protocol. Gene expression was determined by quantitative RT-PCR (MyiQ real-time PCR detection system, Bio-Rad) by using gene-specific primers (Supplementary Table S1), SensiMix SYBR and a Fluorescein Kit (Bioline) following the manufacturer's instructions.

In previous experiments, we checked the expression stability of several reference genes (*EF1α*, At1g07940; *TON1A*, At3g55000; and *UBQ10*, At4g05320) under B toxicity (Macho-Rivero et al. 2017). *TON1A* was the most stable gene in geNorm analysis (Vandesompele et al. 2002), and therefore the amplicon of Arabidopsis *TON1A* was used as an internal control to normalize all data.

Histochemical analysis

PIP2;1::GUS, PIP2;2::GUS and PIP1;2::GUS reporter lines were described in Da Ines et al. (2010) and Postaire et al. (2010); they contained 2.3, 3.7 and 2.2 kb upstream of the ATG start codon, respectively. The PIP1;1::GUS line was established as described for PIP2;1 and PIP2;2 by Da Ines et al. (2010) harboring a 2.1 kb upstream fragment.

Transgenic reporter lines were grown hydroponically in a medium containing 10 μM boric acid for 3 weeks. The plants were then transferred to a medium containing 10 or 5 mM boric acid for 48 h. For histochemical analysis of GUS reporter enzyme activity, roots and leaves of the reporter lines were incubated at 37°C in a GUS reaction buffer containing 2 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronide in 100 mM sodium phosphate (pH 7.0). GUS staining patterns were analyzed on a Leica S8APO Stereozoom microscope equipped with a digital camera (Leica EC3) and driven by analysis software (LAS EZ; Leica). For each marker line and for each treatment, at least six transgenic plants from two independent experiments were analyzed. Representative plant images were chosen for each B treatment.

Boron determination

Arabidopsis plants were grown hydroponically in a medium containing 10 μM boric acid of natural isotopic abundance (19.9% ^{10}B) for 4 weeks. The plants were then transferred to a medium containing 10 or 5 mM boric acid enriched with ^{10}B (99% ^{10}B, Sigma-Aldrich). Shoots were harvested after 24 and 48 h of incubation and dried at 80 °C in an air incubator. The samples (50 mg) were digested in HNO_3 and the contents of ^{10}B and ^{11}B were measured by ICP-MS.

Supplementary Data

Supplementary data are available at PCP online.

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Disclosures

The authors have no conflicts of interest to declare.

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