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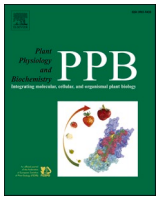

Role of ABA in the adaptive response of Arabidopsis plants to long-term boron toxicity treatment

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ABSTRACT

Boron (B) toxicity causes impairments in several plant metabolic and physiological processes. Under conditions of excessive B availability, this micronutrient is passively transported through the transpiration stream and accumulates in leaves, causing the development of necrotic regions in leaf tips. Some plants have developed adaptive mechanisms to minimize the toxic effects of excessive B accumulation in their tissues. Thus, for instance, in Arabidopsis it has been described an ABA-dependent decrease in the transpiration rate that would restrict B accumulation in aerial plant tissues in response to short-term B toxicity, this effect being mediated by *AtNCED3* (which encodes a key enzyme for ABA biosynthesis). The present work aimed to study the possible involvement of ABA in the adjustment of plant water balance and B homeostasis during the adaptive response of Arabidopsis to prolonged B toxicity. For this purpose, Arabidopsis wild-type and the ABA-deficient *nced3-2* mutant plants were subjected to B toxicity for 7 days. We show that ABA-dependent stomatal closure is determinant for the adjustment of plant water relations under conditions of prolonged B toxicity. Results suggest that, in addition to the *AtNCED3* gene, the *AtNCED5* gene could also be involved in this ABA-dependent stomatal closure. Finally, our results also indicate the possible role of endogenous root ABA content in the mechanism of active efflux of B via BOR4 (efflux-type B transporter) from the root to the external environment under excess B conditions.

1. Introduction

Although the role of boron (B) in plant physiology has been a recent subject of discussion (Lewis, 2019; González-Fontes, 2020; Wimmer et al., 2020), its requirement for plant development was first established at the beginning of the 20th century (Warington, 1923). In fact, it is well known that B is essential for the maintenance and stability of the cell-wall matrix due to its capacity to cross-link two rhamnogalacturonan II molecules (Kobayashi et al., 1996; Bolaños et al., 2004; O'Neill et al., 2004; Bolaños et al., 2023).

Plants take up B from the medium in the form of boric acid, a small, uncharged molecule that can diffuse freely across the plasma membrane by simple diffusion (Brown et al., 2002). Therefore, under conditions of adequate or excessive B availability, root cells take up this micronutrient and load it into the xylem through a passive process that mainly involves diffusion of B across the lipid bilayer and, to a lesser extent, facilitated transport through MIP (major intrinsic protein) channels (Tanaka and Fujiwara, 2008). However, when its supply is limited, plants use several B channels and transporters to satisfy their B requirements. Thus, for instance, the coordinated action of the B channel NIP5; 1 for uptake and the B transporter BOR1 for xylem loading is key for efficient B transport from soil solution to xylem under limited B availability in *Arabidopsis thaliana* (Takano et al., 2008; Reid, 2014). Once B has reached the xylem, it is transported to the shoot along with the transpiration stream,

Abbreviations: ABA, abscisic acid; LWC, leaf water content; NCED, enzyme 9-cis-epoxycarotenoid dioxygenase; PIP, plasma membrane intrinsic protein; RT-PCR, real-time PCR.

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which is driven by the water potential gradient resulting from the soil-plant-atmosphere continuum (Shelp et al., 1995).

Boron has a rather narrow range of optimal concentrations, so controlling its availability in soils and irrigation water is crucial for agricultural production. In particular, toxicity problems often occur on agricultural lands located in arid/semiarid regions with low rainfall and high-water evaporation where this nutrient accumulates in the upper layers of the soil (Reid, 2007b; Camacho-Cristóbal et al., 2008; Landi et al., 2019; Brdar-Jokanović, 2020). Moreover, this stress has currently been aggravated by the use of intensive agricultural techniques and by irrigation with salt-rich water in irrigated areas of arid and semi-arid environments (Cervilla et al., 2007; Bonilla and González-Fontes, 2011; Aquea et al., 2012).

Plant species in which B is relatively immobile via phloem tissue exhibit chlorosis and necrosis in the marginal region of mature leaves when exposed to excess B (Roessner et al., 2006; Camacho-Cristóbal et al., 2008). This is because B passively moves to the shoot along with the transpiration stream and accumulates at the leaf margins (Brown and Shelp, 1997; Macho-Rivero et al., 2017). Toxic B concentration exerts different physiological effects during both vegetative and reproductive growth of vascular plants (Reid, 2007b; Camacho-Cristóbal et al., 2008; Herrera-Rodríguez et al., 2010; Landi et al., 2019; Brdar-Jokanović, 2020). Some plants have developed adaptive mechanisms against the harmful effects caused by B toxicity. For instance, it has been shown that tolerant genotypes possess mechanisms to actively excrete this element from the root to the external environment, thus minimizing the toxic effects of its excessive accumulation in their cells. In this regard, different B transporters involved in tolerance to B toxicity have been identified, such as the B efflux transporters in Arabidopsis (BOR4; Miwa et al., 2007, 2014), *Citrus macrophylla* (BOR4; Martínez-Cuenca et al., 2015), maize (RTE3; Chatterjee et al., 2017; Mamani-Huarcaya et al., 2022), and in barley and wheat (BOR2; Reid, 2007a, b; Sutton et al., 2007).

Boron toxicity induces the expression of ABA-related genes and represses others encoding aquaporin water channels in Arabidopsis, so it has been suggested that this stress limits water uptake to restrict the entry of more B into the root (Aquea et al., 2012). Likewise, recent studies in Arabidopsis have described that short-term (24–48 h) B toxicity leads to a rapid decrease (i) in transpiration rate mediated by AtNCED3 through the control of leaf ABA contents (Macho-Rivero et al., 2017) and (ii) in the transcript levels of several genes encoding PIP-type aquaporins, which may decrease cell-to-cell water movement in plant tissues and, consequently, water flow to the shoot (Macho-Rivero et al., 2018). All these changes in the water balance of plants subjected to short-term B toxicity could be mechanisms to prevent excess B accumulation in plant tissues (Macho-Rivero et al., 2018). However, whether these events facilitate the adaptation of Arabidopsis to prolonged B-toxicity stress remains unknown. The aim of the present work was to study the adaptive response of Arabidopsis to long-term B toxicity. In particular, we analyzed the effects of B toxicity treatment for 7 d on several physiological aspects regarding gas exchange parameters, B homeostasis, and water balance in Arabidopsis plants. Furthermore, to explore the role of ABA in the response of Arabidopsis to long-term B toxicity, these studies were extended using the ABA-deficient *nced3-2* mutant, which is impaired in stomatal function (Iuchi et al., 2001; Ruggiero et al., 2004; Macho-Rivero et al., 2017). The strong correlation between *NCED3* gene expression and ABA levels, already contrasted in the literature (Iuchi et al., 2001; Ruggiero et al., 2004; Urano et al., 2009; Hwang et al., 2010; Frey et al., 2012; Zhang et al., 2015; Macho-Rivero et al., 2017; among others), together with the characteristics of the *nced3-2* mutant, namely lower ABA content and higher stomatal conductance than wild-type plants, make it a very appropriate tool for the research carried out in this work. We demonstrated that leaf ABA levels are crucial for the control of stomatal closure and the maintenance of leaf water content under prolonged B toxicity. Furthermore, unlike in the short-term stress response (Macho-Rivero et al., 2017), our results suggest the possible contribution of genes other than *NCED3* in the increase of leaf ABA levels under prolonged B toxicity. Finally, we provide evidence suggesting that endogenous root ABA levels might be involved in the induction of root *BOR4* expression and B efflux rate under long-term B toxicity.

2. Materials and methods

2.1. Plant material and growth conditions

Arabidopsis thaliana ecotype Col-0 (wild-type) and the ABA-deficient *nced3-2* mutant (Urano et al., 2009; Macho-Rivero et al., 2017) were used in this work. The *nced3* mutant used in this study (*nced3-2*) is a knockout mutant line carrying a T-DNA insertion in the NCED3 coding region (Urano et al., 2009; Frey et al., 2012). Individual homozygous mutants were back-crossed twice with the wild-type to reduce the number of undesired insertional mutations, and the genotype of the F2 individuals was checked by PCR as described in Macho-Rivero et al. (2017).

Seeds were surface-sterilized with 75% (v/v) ethanol for 5 min, then 2% (w/v) sodium hypochlorite solution for 5 min and, finally, washed six times with sterile water. Sterile seeds were sown on square (12 × 12 cm) plates containing 40 ml of sterile ½ MS culture medium buffered with 2 mM MES (M0254, Duchefa Biochimie) to pH 5.7 and solidified with 1% (w/v) Phytagel. After incubation at 4 °C for 3 d in darkness to promote and synchronize germination, the plates were transferred to a growth chamber in a vertical orientation with a light/dark regime of 10/14 h, 22 °C, 65% relative humidity, and a light intensity of 120–150 μmol $m^{-2}{\cdot}s^{-1}$ of photosynthetically active radiation (artificial illumination was provided by white fluorescent tubes). After 20 d of growth, seedlings were carefully removed from the solid medium, washed with deionized water, and immediately transferred to 7.5-l plastic containers with a 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 for 8 d in a growth chamber with a light/dark regime of 10/14 h, 22 °C, 65% relative humidity and 250 μmol $m^{-2}{\cdot}s^{-1}$ of photosynthetically active radiation at plant height (artificial illumination was provided by sodium-vapor lamps). The medium was renewed once during this period.

After 8 d of hydroponic growth, plants were divided into groups and subjected to different B treatments: 10 μM (control conditions) and 2 mM (B toxicity) boric acid. Plants were maintained with these B treatments for 7 d and then harvested for further analysis. The media were renewed once during this period.

2.2. Gas exchange parameter measurements

Photosynthetic rate (P_n, μmol CO_2 m^{-2} s^{-1}), stomatal conductance (g_s, mol H_2O m^{-2} s^{-1}), transpiration rate (E, mmol H_2O m^{-2} s^{-1}), and intercellular CO_2 concentration (C_i, μmol CO_2 mol air^{-1}) were measured 2 h after the beginning of the photoperiod using a portable infrared gas analyzer (IRGA) (Li 6400, Li-Cor Inc., Lincoln, NE) equipped with an extended chamber (Extended Reach 1 cm Chamber Li 6400–15, Li-Cor Inc.). The air flow rate was adjusted to 250 μmol s^{-1} of air and the irradiance was 250 μmol m^{-2} s^{-1} of photosynthetically active radiation.

2.3. Stomatal aperture index

Epidermal peels were prepared from leaves of plants treated with 10 μM or 2 mM B for 7 d as described in Wu and Zhao (2017) with small modifications. The adaxial epidermal leaf surface was affixed to a strip of laboratory adhesive tape with the abaxial side facing upwards. Subsequently, a thin layer of nail polish was applied to the abaxial surface of the affixed leaf and incubated at room temperature to air dry the nail

polish. Then, a strip of transparent universal adhesive tesa film was gently applied to the dried nail polish area (abaxial surface) and carefully pulled away from the laboratory tape, peeling away the abaxial epidermal cell layer. Imaging of stomata from the adhesive tape with attached epidermis was obtained with a Zeiss Axioskop microscope equipped with an AxioCam digital camera. Image analysis was performed using ImageJ software (https://imagej.nih.gov/ij/). The width and the length of the stomatal aperture were measured, and the stomatal aperture index was calculated as width to length ratio. The stomatal aperture index of at least 25 stomata per plant was calculated, and five plants per treatment were used for statistical analysis.

2.4. Whole-plant water loss rate

The IRGA used to measure leaf transpiration rate had a special clamp with a 1-cm diameter chamber (Arabidopsis Chamber 6400-15, LiCor), which limited its use to large, rosette-expanded leaves. To quantify the total water loss by transpiration of each whole plant, four Arabidopsis plants of each genotype and treatment were removed from the respective hydroponic culture medium and individually placed in a falcon tube (wrapped in aluminum foil) containing 47.5 ml of culture medium. The cap of the falcon tube was drilled to hold the plant in the tube with the roots submerged in the medium. Transpirational water loss of whole plants was determined gravimetrically using a Sartorius CP423S balance. Changes in weight were recorded every hour for a period of 3 h. Background water loss was determined by having an identical setup as for plant analyses, except that the plant was missing (only falcon tube, nutrient solution and drilled cap). Whole-plant water loss rate was expressed on a shoot fresh weight basis.

2.5. Leaf water content

Five fully-expanded leaves of each genotype and treatment were carefully removed from plants and weighed to determine their fresh weights (FW). Next, leaves were dried in an oven at 80 °C for 72 h and weighed again to get their dry weights (DW). Leaf water content (LWC) was determined using the following equation: LWC (%) = [(FW-DW)/FW] x 100.

2.6. B determination

Leaves and roots were ground to a fine powder with liquid nitrogen and aliquots of 200–250 mg were dried in an oven at 80 °C. Dried samples were digested in 250 μl of nitric acid overnight at room temperature and the acidic extracts were bleached with H_2O_2 and incubated at 80 °C for 15 min in a closed vessel with the cap punctured with a fine needle. The resulting acid extracts were diluted to 1 ml with ultrapure water and the contents of B were determined by the azomethine-H method as described by Beato et al. (2010).

2.7. Measurement of B efflux

The time course of B efflux was performed after transferring Arabidopsis plants treated with 2 mM B for 7 days to a B-free solution. Roots were sampled at the indicated times, rinsed in ultrapure water for a few seconds, blotted with paper towels, frozen with liquid nitrogen, and stored at −80 °C until further analyses. Soluble B was extracted twice from 150 to 200 mg of ground plant material with 0.5 ml of high-purity deionized water at 80 °C for 30 min in screw-cap tubes, vortexing vigorously every 10 min. Between each step, the extract was centrifuged (13,000×*g*, 15 min) and the two supernatants were collected and combined in Eppendorf Safe-Lock tubes. Water extracts were then dried completely at 80 °C and the dried extracts were processed as above for B determination.

The B efflux experiment was carried out under high humidity conditions (>85% relative humidity) to minimize the transport of B from roots to shoots via transpiration (Hayes and Reid, 2004).

2.8. RNA isolation, cDNA synthesis and quantitative RT-PCR analyses

For these determinations, five Arabidopsis plants of each genotype treated with 10 μM or 2 mM B for 7 d were harvested. Roots were quickly separated, dried with a paper towel, frozen in liquid nitrogen, and stored at −80 °C until further analyses.

Total root 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 1 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. The relative expression levels were calculated according to the $2^{-\Delta\Delta CT}$ method. The measurement for each sample was repeated twice.

In previous experiments, we checked the expression stability of several reference genes (*EF1*, 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), therefore, the amplicon of Arabidopsis *TON1A* was used as an internal control to normalize all data.

2.9. ABA analysis

The extraction of endogenous leaf ABA content was performed as described by Müller and Munné-Bosch (2011). ABA analysis was carried out by liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) using a Thermo Scientific-Dionex Ultimate 3000 UHPLC coupled to a Q Exactive™ Hybrid Quadrupole-Orbitrap High Resolution Mass Spectrometer. ABA was separated by reversed-phase chromatography using an Acquity UPLC® BEH C18 Column (2.1 × 100 mm, 1.7 μm, Waters, Milford, MA, USA). Solvents A and B consisted of water and methanol, both with 0.1% (v/v) formic acid, respectively. The temperatures of the column and the auto-sampler were regulated to 40 and 4 °C, respectively. The following gradient conditions were used: (flow rate of 300 μL/min) 30% solvent B to 100% solvent B for 6 min, and return to 30% solvent B for 1 min, and then 1 min at 30% solvent B prior to the next injection. The injection volume was 5 μl. The liquid chromatography eluent was then analyzed by ESI-MS/MS using Full MS/ddMs2 scan mode. Mass spectrometric detection was performed using a Q Exactive™. ABA was detected by electrospray ionization in negative mode with a mass resolution of 70, 000. Nitrogen was used as the desolvation gas. Data were acquired over the m/z range from 50 to 750. Electrospray ionization parameters were as follows: sheath gas flow 45 arb (arbitrary units), auxiliary gas flow 15 arb, auxiliary gas heater temperature 350 °C, spray voltage 3.5 kV, capillary temperature 320 °C. ABA quantification was performed using external calibration standards.

2.10. Statistical analyses

Data were compared by one-way analysis of variance (ANOVA). Differences among means were evaluated using Tukey's Honestly Significant Difference test ($P < 0.05$). When indicated, Student's *t-test* was used for comparison between two means ($P < 0.05$). Data are from a representative experiment that was repeated twice with very similar results.

3. Results

3.1. Leaf damage

Fig. 1 shows the rosette phenotype of wild-type and *nced3-2* mutant plants after 7 days of treatment with control (10 μM B) or B toxicity (2 mM B) conditions. Typical symptoms of B toxicity, such as necrotic spots at the tips and margins of mature leaves, were more apparent in the *nced3-2* mutant than in wild-type plants after 7 d of B toxicity treatment (Fig. 1), which suggests a higher sensitivity of the mutant to B toxicity than wild-type plants (Macho-Rivero et al., 2017). In agreement with these results, B toxicity caused a greater decrease in the rosette fresh mass of *nced3-2* mutant than in wild-type plants (Fig. 1).

3.2. Leaf gas exchange parameters and stomatal aperture index

Boron toxicity treatment with 2 mM B for 7 d caused a significant decrease in photosynthetic rate, stomatal conductance, transpiration rate, and stomatal aperture index when compared to control in both genotypes (Fig. 2A, C-E). However, no significant effect of B-toxicity treatments on intercellular CO_2 concentration was observed in any of the two genotypes studied (Fig. 2B). Regardless of the B treatment, the ABA-deficient *nced3-2* mutant had significantly higher values of transpiration rate, stomatal conductance, intercellular CO_2 concentration, and stomatal aperture index than wild-type plants (Fig. 2 B-E; Macho-Rivero et al., 2017), which highlights the importance of endogenous ABA levels in the regulation of stomatal closure.

3.3. Whole-plant water loss rate, leaf water content (LWC), and endogenous ABA content

In both genotypes, the whole-plant water loss rate was reduced by B toxicity in a similar way to the transpiration rate measured in individual leaves (Fig. 3A and 2D). Furthermore, consistent with the results observed for transpiration rate, *nced3-2* mutant plants showed higher rates of whole-plant water loss than wild-type plants under control and B toxicity treatments (Fig. 2D and 3A).

Under control conditions, the ABA-deficient mutant *nced3-2* showed a lower LWC than wild-type plants (Fig. 3B), which can be explained by its higher stomatal conductance and water loss rate by transpiration (Fig. 2C and 3A). In wild-type plants, B toxicity treatment did not affect LWC levels when compared to control treatment; however, a significant reduction of LWC was observed in the *nced3-2* mutant grown with excess B as compared to the control (Fig. 3B).

Leaf and root ABA contents were analyzed in wild-type and *nced3-2* mutant plants treated or not with B toxicity for 7 d (Fig. 3C and D). B toxicity led to an increase in the leaf ABA content in both genotypes in comparison to control treatments (Fig. 3C), a fact that was not observed in roots, where B toxicity caused a slight increase in ABA content only in wild-type plants (Fig. 3D). However, as would be expected, the endogenous ABA concentration in the *nced3-2* mutant was significantly lower than that of the wild-type under both control and B toxicity conditions (Fig. 3C and D). These results are in line with the established importance of the *NCED3* gene in the control of endogenous ABA levels in Arabidopsis plants (Iuchi et al., 2001; Macho-Rivero et al., 2017).

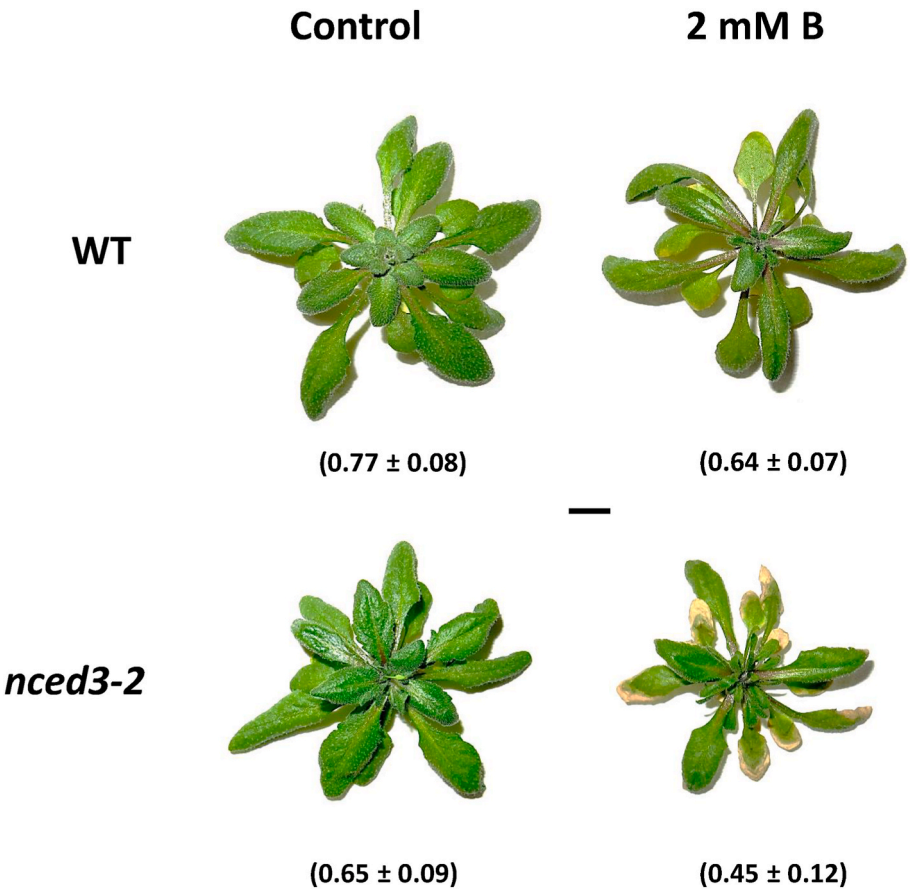

Fig. 1. Effects of B toxicity on the rosette morphology of wild-type and *nced3-2* Arabidopsis plants after 7 d of treatment. Arabidopsis plants were grown for 4 weeks with 10 μM B as described in Material and Methods. After that, plants were transferred to the control medium (10 μM B) or B-toxicity medium (2 mM B) for 7 d. Rosette fresh weight (g) is shown in brackets as mean ± SD from the analysis of five different plants for each genotype and treatment. Scale bar 1 cm.

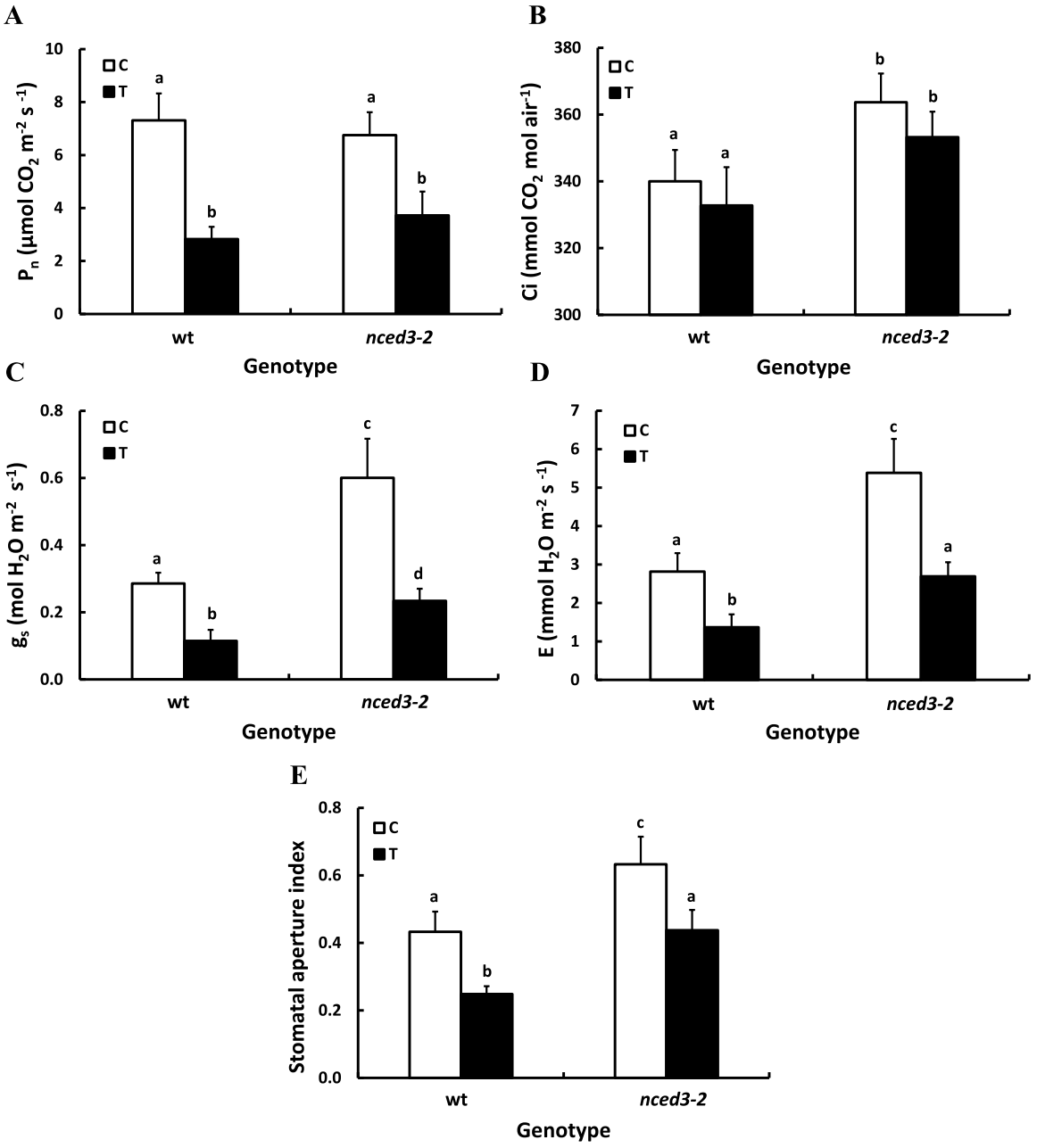

Fig. 2. Photosynthetic rate (A), intercellular CO_2 concentration (B), stomatal conductance (C), transpiration rate (D), and stomatal aperture index (E) in wild-type and *nced3-2* Arabidopsis plants grown under control (10 µM B, open squares) or B toxicity (2 mM B, filled squares) conditions for 7 d. Results are given as mean ± SD from the analysis of five different plants for each genotype and treatment. Different letters indicate statistically significant differences between genotypes and treatments according to ANOVA with Tukey's HSD test ($P < 0.05$).

3.4. Transcript levels of NCED3 and NCED5

The expression levels of two ABA biosynthetic genes (*NCED3* and *NCED5*) were analyzed in the leaves and roots of Arabidopsis plants subjected to prolonged B toxicity or not (Fig. 4). An increase in *NCED3* transcript levels was observed in both leaves and roots of wild-type plants after B-toxicity treatment (Fig. 4A), which correlates with their endogenous ABA levels (Fig. 3C and D). As expected, *NCED3* transcripts were not detected in the *nced3-2* mutant (data not shown; Hwang et al., 2010; Macho-Rivero et al., 2017).

B toxicity also led to an increase in the *NCED5* transcript level in leaves of wild-type and *nced3-2* mutant plants (Fig. 4B), thus suggesting a possible contribution of this gene to leaf ABA production in response to prolonged B toxicity. In contrast to leaves, B toxicity did not increase *NCED5* expression in roots of both wild-type and *nced3-2* mutant plants (Fig. 4C).

3.5. Transcript levels of major aquaporins

Experiments were conducted to analyze the effect of B toxicity on the expression levels of the major aquaporins (*PIP1;1*, *PIP1;2*, *PIP2;1* and *PIP2;2*) involved in Arabidopsis water transport (Monneuse et al., 2011). Boron toxicity led to a significant decrease in the root transcript levels of all analyzed *PIP* genes when compared to the control treatment in both wild-type and *nced3-2* mutant plants (Fig. 5), which suggests that endogenous ABA synthesis via NCED3 is not involved in the drop of root PIP transcript levels under prolonged B toxicity.

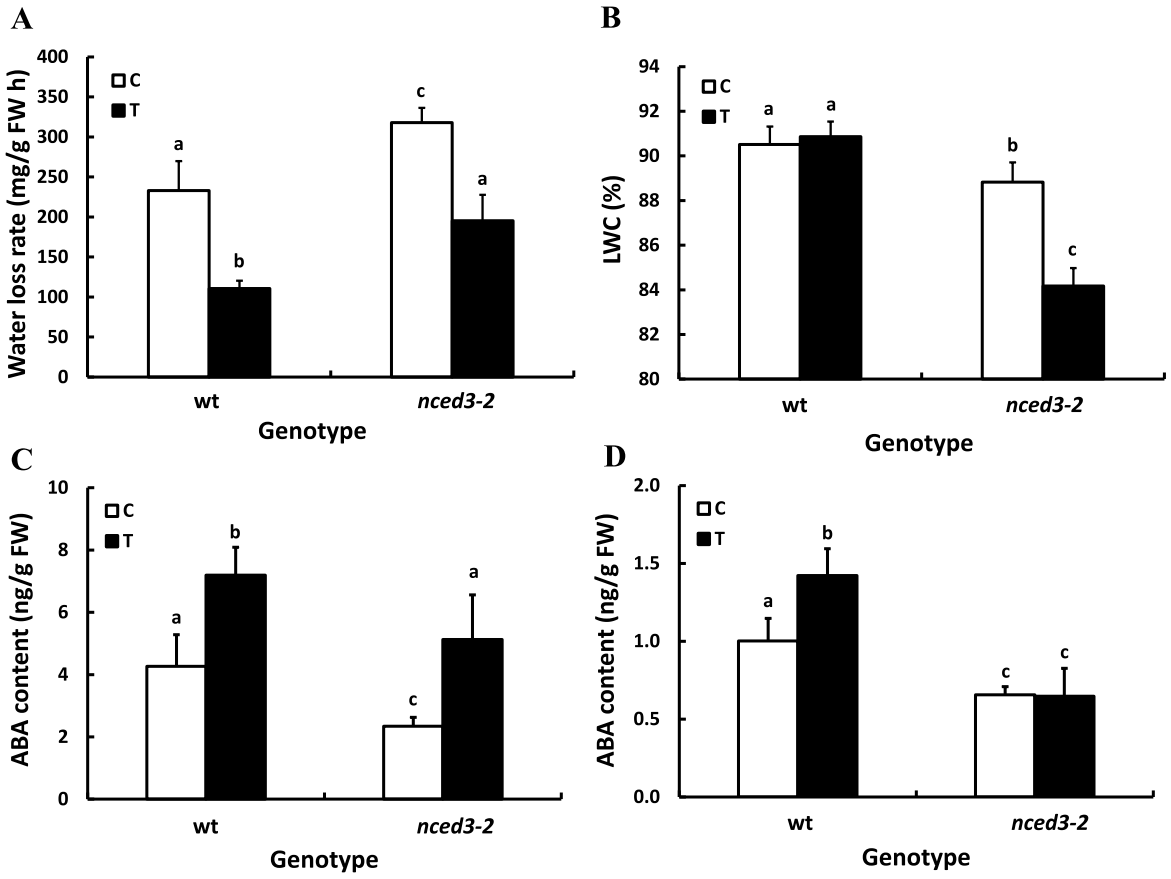

Fig. 3. Water loss rate (A), leaf water content (B), leaf ABA content (C), and root ABA content (D) in wild-type and *nced3-2* Arabidopsis plants grown under control (10 μM B, open squares) or B toxicity (2 mM B, filled squares) conditions for 7 d. Results are given as mean ± SD from the analysis of five different plants for each genotype and treatment. Different letters indicate statistically significant differences between genotypes and treatments according to ANOVA with Tukey's HSD test ($P < 0.05$).

3.6. Tissue B concentration, B efflux rate, and B transporter genes expression

Under control conditions, root and shoot B concentrations were very similar in both genotypes (Fig. 6A and B). As expected, B content in roots and leaves increased after 7 d of B toxicity treatment in both wild-type and *nced3-2* mutant plants (Fig. 6A and B). However, it is interesting to highlight that the ABA-deficient *nced3-2* mutant had a greater shoot B concentration than the wild-type under B-toxicity treatment (Fig. 6A). Regardless of genotype and B treatment, B content in leaves was always higher than in roots, indicating that most of the B taken up by roots is transported to shoots through the transpiration stream and accumulates at the end of the transpiration (Roessner et al., 2006; Camacho-Cristóbal et al., 2008).

A short-term B efflux assay was conducted after transferring Arabidopsis wild-type and *nced3-2* mutant plants treated with 2 mM B for 7 days to a solution not containing B. Concentration of soluble B in roots was measured at each indicated time (Fig. 6C). A decrease of about 50% of the starting soluble B was observed within the first 15 min of the experiment in root of wild-type plants. However, soluble B concentration in the roots of the *nced3-2* mutant was reduced to about 90% and 70% after 15 and 30 min, respectively, from the onset of the experiment (Fig. 6C).

The transcript levels of three B transport-related genes were also measured in Arabidopsis roots under control and B toxicity conditions. The transcript levels of *BOR1* (B efflux transporter for xylem loading) and *NIP5;1* (B channel protein for efficient B uptake in roots under B limitation) notably decreased in wild-type and *nced3-2* mutant plants subjected to B toxicity when compared with control plants (Fig. 7A and B). However, the transcript level of another gene encoding a B efflux transporter, *BOR4*, was significantly stimulated in wild-type plants whereas it remained unaffected in *nced3-2* mutant plants under B toxicity (Fig. 7C), which correlates with the B efflux rates observed in both genotypes (Fig. 6C).

4. Discussion

One of the typical symptoms of B toxicity in Arabidopsis is the appearance of chlorotic and/or necrotic spots at the leaf margins and tips. These symptoms of toxicity occur because, after being uptaken by roots, B is transported to shoots through the xylem driven by the transpiration stream and is accumulated in older leaves without being retranslocated via the phloem. For this reason, the severity of toxicity symptoms is closely related to the B content in leaves (Reid, 2014; Princi et al., 2016; Landi et al., 2019). This is evident when comparing the visible symptoms of prolonged B toxicity in the leaves of wild-type and *nced3-2* mutant plants. Thus, this mutant showed greater damage in the margins of mature leaves in comparison to the wild-type (Fig. 1; Macho-Rivero et al., 2017), which correlated with its higher leaf B accumulation (Fig. 6A; Macho-Rivero et al., 2017). Interestingly, the ABA-deficient *nced3-2* mutant had higher stomatal conductance and transpiration rate than wild-type plants (Fig. 2C and D; Iuchi et al., 2001; Macho-Rivero et al., 2017), which highlights the importance of transpiration rate in the B leaf accumulation under B toxicity (Ben-Gal and Shani, 2002; Macho-Rivero et al., 2017, 2018).

It is well known that B toxicity negatively affects several parameters

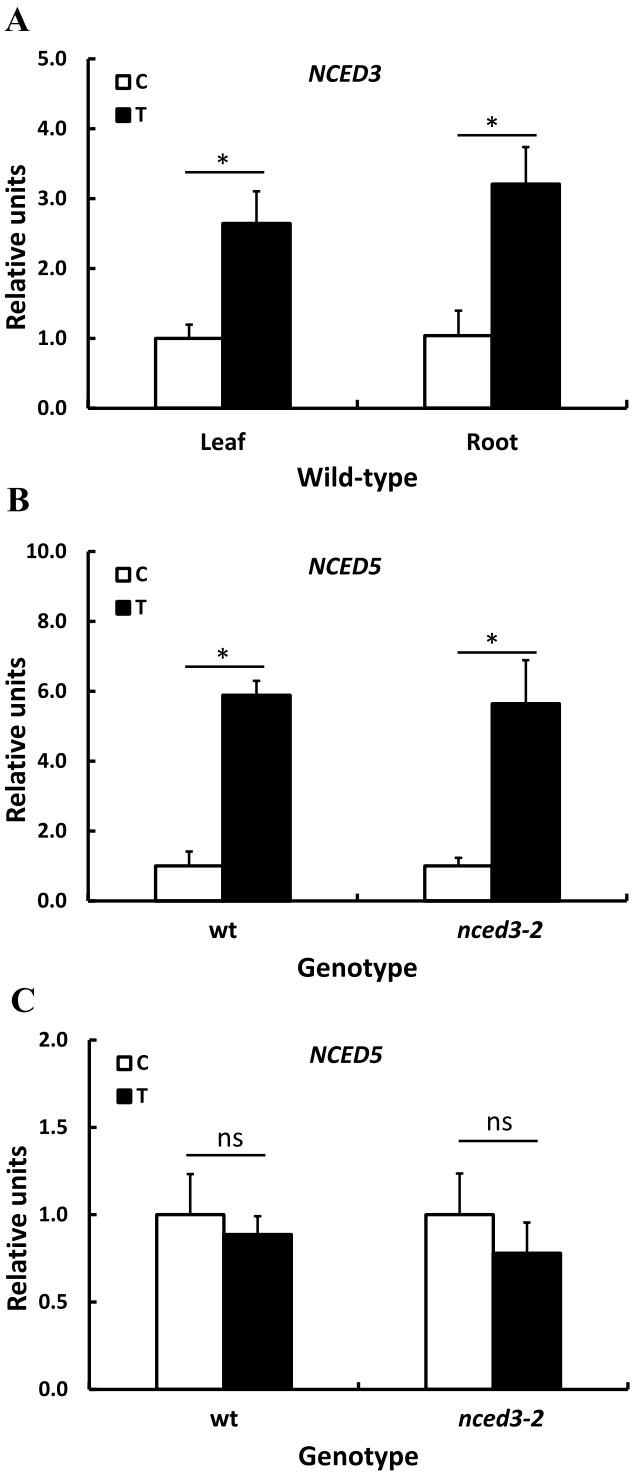

Fig. 4. Quantitative RT-PCR analysis of *NCED3* (A) and *NCED5* (B, C) transcript levels in Arabidopsis plants after treatment with 10 μM B (open squares) or 2 mM B (filled squares) for 7 d. (A) *NCED3* transcript levels in leaves and roots of wild-type plants. (B) *NCED5* transcript levels in leaves of wild-type and *nced3-2* plants. (C) *NCED5* transcript levels in roots of wild-type and *nced3-2* plants. Results are given as the mean ± SD from the analysis of five different plants for each genotype and treatment. Asterisks indicate statistically significant differences between treatments for each genotype according to Student's *t*-test ($P < 0.05$).

involved in the photosynthetic process, such as pigment levels, CO_2 assimilation, photosystem II efficiency, and electron transport rate (Princi et al., 2016; Landi et al., 2019). Accordingly, in the present work, the exposure of wild-type and *nced3-2* mutant plants to long-term B toxicity caused a significant decrease in photosynthetic rate (Fig. 2A). This fact cannot be attributed to a decrease in internal CO_2 concentration due to stomatal closure, since no significant effect of B toxicity on internal CO_2 concentration was observed in any of the two genotypes studied (Fig. 2B). In this regard, previous studies have described that the negative effect of B toxicity on photosynthesis could be the consequence of oxidative damage in thylakoid membranes owing to the overproduction of ROS (Cervilla et al., 2007; Ardic et al., 2009; Inal et al., 2009; Landi et al., 2013).

ABA is a stress hormone that accumulates in response to both abiotic and biotic stresses leading to stress tolerance. For instance, ABA accumulation in leaves reduces transpiration rate by closing stomata, which limits the water loss of plants subjected to different stresses. ABA-mediated stomatal closure is a well-known process in which second messengers such as ROS/NO/Ca^{2+} play an important role (Bharath et al., 2021). Among the various enzymatic proteins involved in ABA biosynthetic pathways in plants, NINE-CIS-EPOXYCAROTENOID DIOXYGENASE 3 (NCED3) is known as an important enzyme for ABA accumulation during drought stress (Iuchi et al., 2001; Endo et al., 2008; Sato et al., 2018). Similarly, it has been described that NCED3 plays an important role in regulating ABA levels under short-term B toxicity conditions. Thus, B toxicity for 24 h increased the root *NCED3* transcripts, leaf ABA levels, and decreased transpiration rate and stomatal conductance in Arabidopsis wild-type plants (Macho-Rivero et al., 2017, 2018). This physiological response to short-term B toxicity, which could be a mechanism to restrict B accumulation in shoots (Macho-Rivero et al., 2017, 2018), failed in the ABA-deficient *nced3-2* mutant (Macho-Rivero et al., 2017). These results showed the significance of NCED3 in the regulation of rapid stomatal closure through the control of shoot ABA levels to cope with short-term B toxicity (Macho-Rivero et al., 2017). Present results also demonstrated the importance of NCED3 in the ABA-dependent stomatal closure under prolonged stress conditions; in fact, the *nced3-2* mutant had higher values of stomatal conductance (Fig. 2C) and lower values of leaf ABA content (Fig. 3C) than wild-type plants. However, unlike in the short-term stress response, B toxicity for 7 d led to a stomatal closure not only in wild-type but also in *nced3-2* Arabidopsis plants (Fig. 2C, E), which correlated with the observed increase in their leaf ABA contents (Fig. 3C). These results seem to indicate that, in addition to the increase in leaf ABA content driven by NCED3 (Fig. 4A), there must be other ABA biosynthetic genes involved in the ABA-dependent stomatal closure of Arabidopsis plants in response to prolonged B toxicity. Interestingly, an increase in the leaf transcript level of *NCED5* was observed in both wild-type and *nced3-2* mutant plants treated with B toxicity for 7 d (Fig. 4B), which suggests a possible role of *NCED5* in the control of leaf ABA level under prolonged B toxicity. This hypothesis would explain the increased leaf ABA contents (Fig. 3C) and the subsequent stomatal closure (Fig. 2C, E) observed in the *nced3-2* mutant under prolonged stress conditions. In agreement with this, it has been shown that NCED5 contributes, together with NCED3, to ABA production in response to water deficit (Frey et al., 2012).

While the observed increase in leaf ABA levels correlates with stomatal closure, it is important to mention that other factors than ABA may contribute to stomatal closure in response to prolonged B toxicity. In fact, besides ABA, several other compounds increase when plants are exposed to stress, which closes stomata and favors plant adaptive responses. For instance, several phytohormones, including methyl jasmonate and salicylic acid, can induce stomatal closure by elevating pH, ROS, NO, and/or Ca^{2+} leading to activation of anion channels, like ABA action (Bharath et al., 2021 and references cited therein). Similarly, aquaporins have also been described to be involved in stomatal regulation by directly controlling water and hydrogen peroxide transport

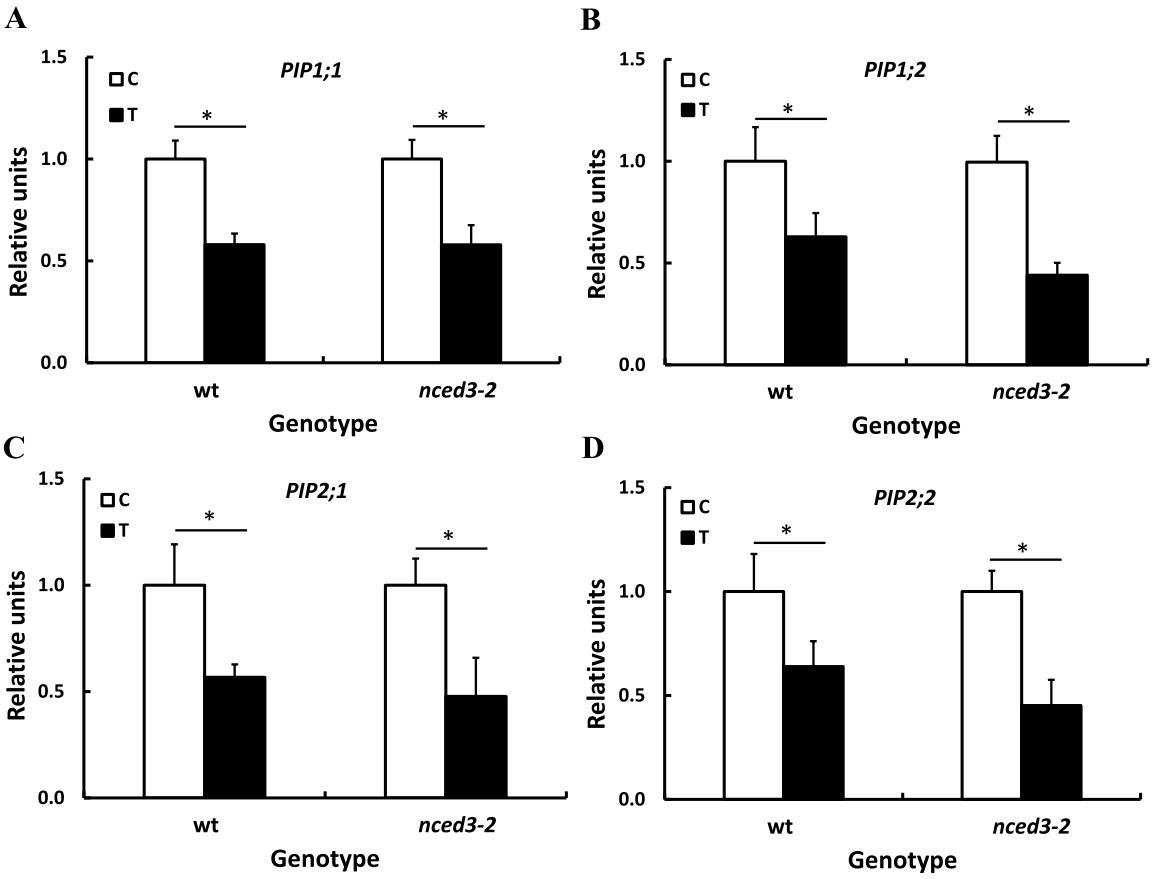

Fig. 5. Quantitative RT-PCR analysis of *PIP1;1* (A), *PIP1;2* (B), *PIP2;1* (C) and *PIP2;2* (D) transcript levels in wild-type and *nced3-2* Arabidopsis roots after treatment with 10 μM B (open squares) or 2 mM B (filled squares) for 7 d. Results are given as the mean ± SD from the analysis of five different plants for each genotype and treatment. Asterisks indicate statistically significant differences between treatments for each genotype according to Student's *t*-test ($P < 0.05$).

into guard cells and/or through their role in the transmission of hydraulic signal for stomatal closure (Scharwies and Dinneny, 2019 and references cited therein).

Despite the clear decrease in the rate of water loss by transpiration caused by prolonged B toxicity (Fig. 2D and 3A), this stress did not cause significant changes in the LWC of wild-type plants (Fig. 3B). These results suggest the occurrence of an adjustment of plant water relations to maintain leaf water balance under conditions of B toxicity. In addition to transpiration rate, PIP aquaporins have been identified as a key component for the maintenance of water balance of plants due to their involvement in the water uptake by roots and its transport within plant tissues (Maurel et al., 2008, 2015; Chaumont and Tyerman, 2014). Interestingly, previous studies have shown a relationship between B nutrition and water transport involving aquaporins; for instance, foliar fertilization with B to young B-deficient almond trees causes an increase in the expression of PIPs (mainly PIP2.2), which could improve water movement (Rios et al., 2021). On the other hand, it has been shown that B toxicity reduces the transcript levels of several *PIP* genes (Aquea et al., 2012; Macho-Rivero et al., 2018) and water transport from root to shoot in Arabidopsis (Macho-Rivero et al., 2018). In the present work, a decrease in the expression of several root *PIP* genes was observed after 7 d of B toxicity in wild-type plants (Fig. 5), which could result in a repressed uptake and/or distribution of water through plant tissues. Therefore, the results of this work, together with those previously published, indicate that prolonged B toxicity causes not only a decrease in water loss by transpiration (Fig. 2D and 3A), but also in water uptake and/or transport through plant tissues via PIP aquaporins (Macho-Rivero et al., 2018). In addition, as already discussed, the decrease in the expression of aquaporins under B toxicity may also contribute to stomatal closure and, consequently, to a decrease in the transpiration rate. The coordinated action of both mechanisms (transpiration rate and water transport via PIP aquaporins) would allow wild-type plants to maintain their leaf water balance and restrict B accumulation in leaves under conditions of B toxicity (Macho-Rivero et al., 2017, 2018). Interestingly, the decrease in the expression of root *PIP* genes by B toxicity was also observed in the ABA-deficient *nced3-2* mutant (Fig. 5); however, this mutant showed a clear decrease in LWC (Fig. 3B) and an increase in leaf B content (Fig. 6A) when compared to those of wild-type plants under prolonged B toxicity. This fact could be explained by the higher stomatal conductance and water loss rate by transpiration of the mutant in comparison to wild-type plants (Fig. 2C, D, and 3A), which suggests that NCED3 plays an important role in the regulation of LWC through the control of leaf ABA levels.

Plants can regulate the expression of nutrient transporters for maintaining homeostasis in response to nutrient availability (Aibara and Miwa, 2014). For instance, it has long been known that B toxicity tolerance in plants is related to the ability to restrict B accumulation in plant tissues (Reid, 2007b; Sutton et al., 2007), which can be achieved by regulating the expression of several B transporters. Thus, gene expression of NIP5; 1 [an influx-type B channel for efficient B uptake in Arabidopsis (Takano et al., 2006)] and translation of the BOR1 mRNA [an efflux-type B transporter for xylem loading (Takano et al., 2002)] have been described to decrease in Arabidopsis roots when treated with toxic concentrations of B (Takano et al., 2010; Tanaka et al., 2011; Aibara et al., 2018). In the present work, the root transcript levels of *NIP5;1* and *BOR1* genes decreased under prolonged B toxicity in wild-type and *nced3-2* mutant plants (Fig. 7A and B), which would contribute to a decreased accumulation of B in plant tissues under this

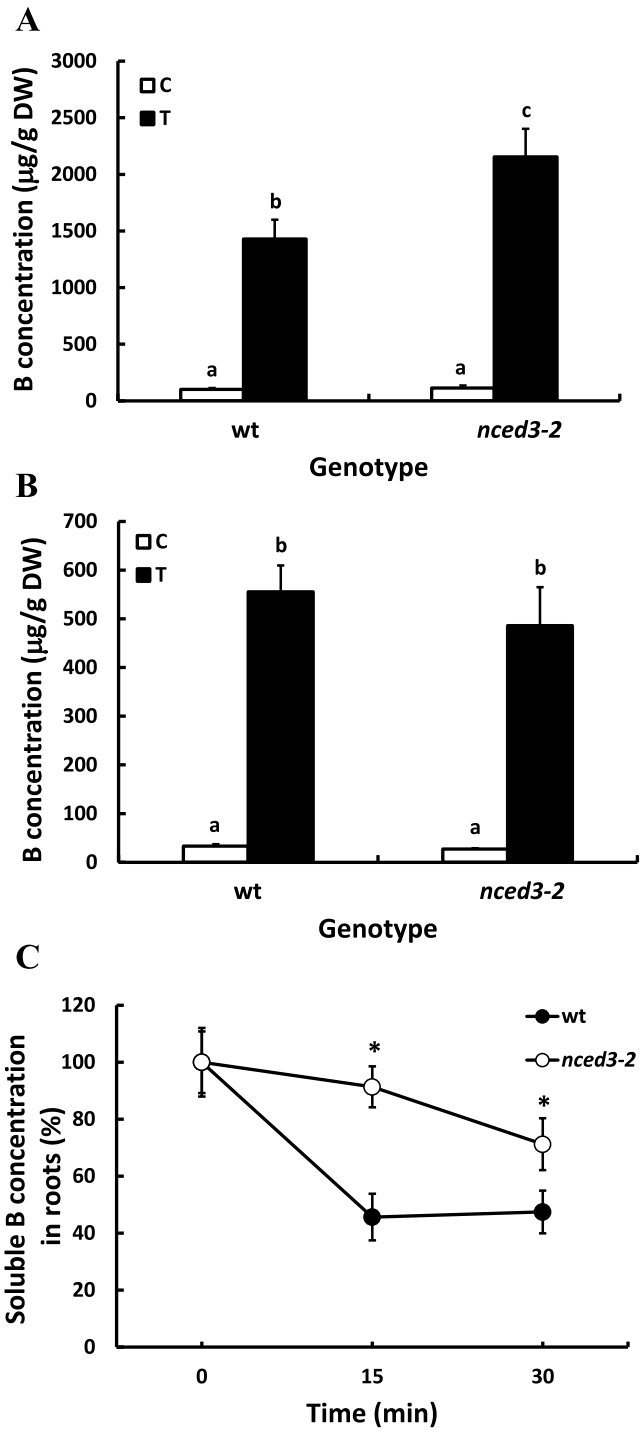

Fig. 6. Total B concentration in shoots (A) and roots (B) of wild-type and *nced3-2* Arabidopsis plants after treatment with 10 µM B (open squares) or 2 mM B (filled squares) for 7 d. Results are given as the mean ± SD from the analysis of five different plants for each genotype and treatment. Different letters indicate statistically significant differences between treatments according to ANOVA with Tukey's HSD test ($P < 0.05$). (C) Relative soluble B concentration in roots of wild-type (filled circles) and *nced3-2* (open circles) plants during short-term efflux of B after treatment with 2 mM B for 7 d. Results are given as the mean ± SD from the analysis of five different plants for each genotype and treatment. Absolute values of soluble B concentration at time zero were 12.9 ± 1.6 and 11.7 ± 1.3 µg/g FW in wild-type and *nced3-2*, respectively. The asterisks represent the significant difference in efflux of B between genotypes at the indicated times according to Student's *t*-test ($P < 0.05$).

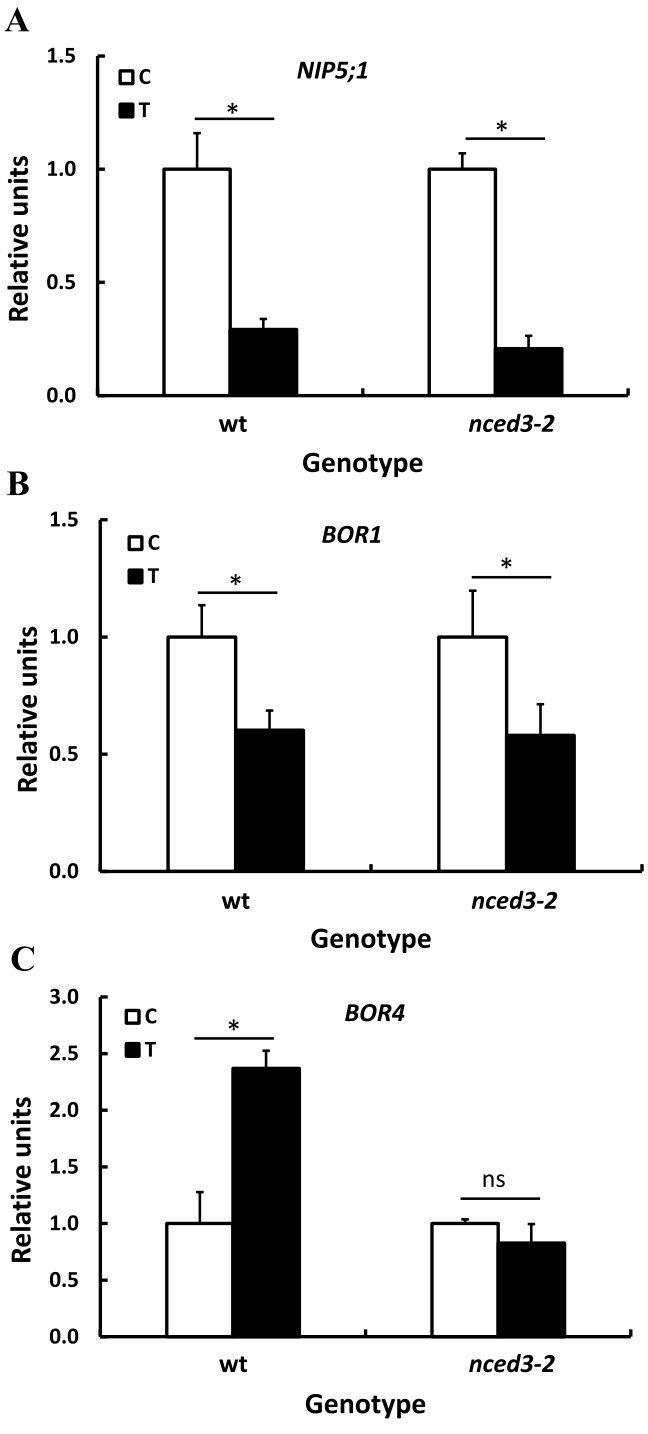

Fig. 7. Quantitative RT-PCR analysis of *NIP5;1* (A), *BOR1* (B), and *BOR4* (C) transcript levels in wild-type and *nced3-2* Arabidopsis roots after treatment with 10 µM B (open squares) or 2 mM B (filled squares) for 7 d. Results are given as the mean ± SD from the analysis of five different plants for each genotype and treatment. Asterisks indicate statistically significant differences between treatments for each genotype according to Student's *t*-test ($P < 0.05$).

stress condition. However, B efflux from the root to the outside under B toxicity conditions has been proposed to be the main mechanism to prevent excess B accumulation in roots (Reid, 2007a, 2007b). In this regard, different efflux-type B transporters involved in tolerance to B toxicity have been identified, such as BOR4 in Arabidopsis (Miwa et al., 2007, 2014) and *Citrus macrophylla* (Martínez-Cuenca et al., 2015), BOR2 in barley and wheat (Reid, 2007a, 2007b; Sutton et al., 2007), and

RTE3 and Zm00001d030297 in maize (Mamani-Huarcaya et al., 2022). Consequently, it was reported that Arabidopsis transgenic plants over-expressing the B efflux transporter BOR4 had lower root and leaf B contents than those of wild-type plants grown in B toxicity (Miwa et al., 2007). Interestingly, an increase in the root transcript level of the *BOR4* gene was observed in Arabidopsis wild-type plants under prolonged B toxicity (Fig. 7C), which was concomitant with a slight increase in root ABA content (Fig. 3C). This fact was not observed in the ABA-deficient *nced3-2* mutant (Fig. 7C), which correlated with its lower B efflux rate (Fig. 6C) and root ABA content (Fig. 3D), and higher leaf B content (Fig. 6A). These results would indicate that endogenous root ABA levels are involved in the induction of root *BOR4* expression and B efflux rate under long-term B toxicity.

Finally, although this study relies on the use of a single mutant line (*nced3-2* mutant) to investigate the role of ABA in the response to long-term B toxicity, it is important to note the following two facts. First, the strong correlation between *NCED3* gene expression and ABA levels is widely contrasted in the literature, as already highlighted in the Introduction. Second, the contrasted phenotype of the *nced3-2* mutant (lower ABA content and higher stomatal conductance than wild-type plants) reasonably explains the response of this mutant to prolonged B toxicity.

5. Conclusions

In conclusion, this study provides the following evidence: (i) Regulation of stomatal closure is important for the adjustment of plant water relations to sustain LWC under conditions of prolonged B toxicity. In fact, unlike wild-type plants, the ABA-deficient *nced3-2* mutant was unable to maintain the LWC under B excess condition due to its higher stomatal conductance, stomatal aperture index, and transpiration rate. (ii) Long-term B toxicity induced stomatal closure in both wild-type and *nced3-2* plants, which correlated with an increase in leaf ABA levels. These results would indicate that, in addition to *AtNCED3*, there are other ABA biosynthetic genes involved in the ABA-dependent stomatal closure of Arabidopsis plants in response to prolonged B toxicity. Our results would suggest a possible role of *AtNCED5*. (iii) The ABA-deficient *nced3-2* mutant failed to induce root *BOR4* gene expression and had lower B efflux rate than wild-type under prolonged B toxicity; whether this is directly related to ABA remains to be shown.

Contributions

J.J.C-C. and M.B.H–R. planned and designed the research. J.J.C-C. carried out the analysis and interpretation of data. P.M-M. performed the experiments, with a substantial contribution from the remaining authors. J.J.C-C. wrote the first draft of the manuscript. All authors critically revised the manuscript with significant contributions. All authors approved the final version of the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2023.107965.

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