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A molecular framework for the inhibition of *Arabidopsis* root growth in response to boron toxicity

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ABSTRACT

Boron is an essential micronutrient for plants and is taken up in the form of boric acid (BA). Despite this, a high BA concentration is toxic for the plants, inhibiting root growth and is thus a significant problem in semi-arid areas in the world. In this work, we report the molecular basis for the inhibition of root growth caused by boron. We show that application of BA reduces the size of root meristems, correlating with the inhibition of root growth. The decrease in meristem size is caused by a reduction of cell division. Mitotic cell number significantly decreases and the expression level of key core cell cycle regulators is modulated. The modulation of the cell cycle does not appear to act through cytokinin and auxin signalling. A global expression analysis reveals that boron toxicity induces the expression of genes related with abscisic acid (ABA) signalling, ABA response and cell wall modifications, and represses genes that code for water transporters. These results suggest that boron toxicity produces a reduction of water and BA uptake, triggering a hydric stress response that produces root growth inhibition.

Key-words: boric acid; environmental stress; phytohormones; plant nutrition.

INTRODUCTION

Boron is an essential microelement for plants and is extracted from the soil in the form of boric acid (BA). Plants regulate BA/borate homeostasis using uptake and efflux transporters (Takano, Miwa & Fujiwara 2008). The unusual nature of BA chemistry suggests that this micronutrient could have a wide variety of biological functions; however, its exact metabolic role is not completely understood (Hänsch and Mendel 2009). To date, the primordial function of boron is undoubtedly its structural role in the cell wall (Blevins & Lukaszewski 1998). More than 90% of the boron in plants is found in cell walls, forming borate ester cross-linked rhamnogalacturonan II (RG-II) dimers, essential for the structure and function of the extracellular matrix (O'Neill *et al.* 2001). Despite the great importance of boron for plants, only a narrow range of concentrations between deficiency and toxicity is considered optimal. Soils with insufficient or toxic levels of BA are widespread in agricultural areas throughout the world, limiting crop productivity. BA toxicity is more difficult to manage than BA deficiency, which can be avoided by fertilization. However, mismanaged fertilization with BA to avoid deficiency can result in toxicity problems. Boron toxicity is a significant problem in semi-arid, yet highly productive agricultural areas including South Australia, Turkey, Mediterranean countries, California and Chile. Toxic effects of boron in plants were well studied for decades and a number of physiological processes have been shown to be altered by an excess of boron. These include disruption of cell wall development; metabolic disruption by binding to the ribose moieties of ATP, NADH and NADPH; and inhibition of cell division and elongation (Stangoulis & Reid 2002; Reid *et al.* 2004). Although significant biochemical and physiological data have been obtained, the molecular mechanisms of boron toxicity remain unclear.

One of the main symptoms of boron toxicity is rapid inhibition of root growth (Nable 1988; Reid *et al.* 2004; Choi *et al.* 2007). Root growth depends on two basal developmental processes: cell division in the root apical meristem and elongation of cells that leave the root meristem (reviewed in Scheres, Benfey & Dolan 2002). Root cells first undergo repeated rounds of division in the root meristem and then subsequently experience rapid cell expansion in the elongation-differentiation zone. The rates of cell division and elongation-differentiation are integrated so that the size of the root meristem and the rate of root growth are coordinated.

Several hormonal pathways have been shown to be involved in the regulation of this balance, with auxin and cytokinin being the principal players (Moubayidin, Di Mambro & Sabatini 2009). Application of exogenous auxin increases the size of the root meristem (Dello Ioio *et al.* 2007) and mutations in the PIN auxin efflux facilitators produce a shorter root meristem compared with wild-type plants (Blilou *et al.* 2005). Cytokinin controls the rate of meristematic cell differentiation, thus contributing to the determination of the *Arabidopsis* root meristem size (Dello

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Ioio *et al*. 2007). In addition to auxin and cytokinin, other hormones have been recognized as modulating root development, such as gibberellins, ethylene and abscisic acid (ABA). It has recently been shown that gibberellins regulate *Arabidopsis* root growth by promoting cell proliferation (Achard *et al*. 2009; Ubeda-Tomás *et al*. 2009). In addition, ethylene regulates root growth by stimulating auxin biosynthesis and by modulating the transport machinery of this hormone (Růzicka *et al*. 2007). On the other hand, exogenous ABA application produces a reduction in root growth (Zeevaart & Creelman 1988) and promotes stem cell maintenance in *Arabidopsis* root meristems by both promoting quiescent centre (QC) quiescence and suppressing stem cell differentiation (Zhang *et al*. 2010).

Inhibition of root growth is not an exclusive effect of boron. Various abiotic stresses cause the same phenotype. In *Arabidopsis*, it has been previously reported that salt stress represses the cell cycle (Burssens *et al*. 2000; West, Inzé & Beemster 2004) and cell elongation (West *et al*. 2004), resulting in growth retardation of the primary root. Hormones not only exert intrinsic growth control but also mediate adaptation of plant development to transiently changing environmental conditions. ABA and ethylene synthesis are induced by salt stress (Achard *et al*. 2006; Huang *et al*. 2008) and salt-induced inhibition of root elongation seems to depend on ABA- and ethylene-mediated reduction in gibberellin levels and stabilization of DELLA proteins, as suggested by expression analysis and mutant studies (Achard *et al*. 2003, 2006). Furthermore, it was recently demonstrated that aluminium-induced inhibition of root elongation is also mediated by ethylene and auxin (Sun *et al*. 2010).

In this paper, we report the molecular basis for the inhibition of root growth caused by boron. We show that application of BA caused a decrease in meristem size because of a progressive decrease in the number of meristematic cells. We demonstrated that inhibition of root growth is a result of BA modulating cell division, probably mediated by changes in the expression of key cell cycle genes. Apparently, auxin and cytokinin are not involved in the suppression of root growth. Global gene expression analysis revealed that BA mainly triggers a water stress-related response. The participation of this response in the root growth inhibition caused by boron is discussed.

MATERIALS AND METHODS

Plant materials and growth conditions

Mutants and transgenic lines were derived from the Columbia (Col-0) ecotype. The transgenic lines *35S::LTI6b:GFP* (Kurup *et al*. 2005), *pCYCB1;1::CYCB1;1:GFP* (Ubeda-Tomás *et al*. 2009), *pCYCB1;1::CYCB1;1:GUS* (Colón-Carmona *et al*. 1999), *DR5rev::GFP* (Friml *et al*. 2003), *pPIN1::GFP* (Benkova *et al*. 2003), *pPIN3::GUS* (Friml *et al*. 2002), *pPIN7::GUS* (Friml *et al*. 2003), *35S::*miR393 (Navarro *et al*. 2006) were described previously. The reporter lines *pARR5::H2B:RFP and pIAA2::H2B:RFP* were developed in the Haseloff's lab (unpublished data), whereas 35S::*CKX4* was obtained from Miltos Tsiantis. In all experiments, seeds were surface sterilized and germinated on an agar-solidified nutrient medium in Petri dishes. The nutrient medium was based on half Murashige–Skoog salts (MS; Murashige & Skoog 1962) and the final pH was adjusted to 5,7. The seeds were vernalized at 4 °C for 2 d. Petri dishes were placed into a growth chamber (Percival Scientific, Inc., Perry, IA, USA), positioned vertically and kept under controlled environmental conditions at 22 °C and a 16/8 h day/night regime. After 5 d, seedlings were transferred to 1/2 MS plates containing BA (H_3BO_3, Merck®, Rahway, NJ, USA, concentrations as indicated). Methylboronic acid was supplied by Sigma-Aldrich® (cat n°165336, St Louis, MO, USA). For root length determinations, the lengths of roots (from root tip to hypocotyl base) were measured 5 d after transfer.

Fluorescent GFP assays

Confocal analysis was performed as described previously by Ubeda-Tomás *et al*. (2009) using a Leica SP5 microscope (Wetzlar, Germany)_ with objective 40× oil. Roots were stained with 10 mg mL^{-1} propidium iodide (Sigma) for 15 s, rinsed and mounted in water. Enhanced green fluorescent protein (EGFP) was excited with the 488 nm line of an argon laser and propidium iodide was excited with the 514 nm line. Fluorescence emission was collected between 505 and 530 nm for EGFP, and 606 and 635 nm for propidium iodide. The number of mitotic cells was quantified by manually counting the green fluorescent protein (GFP)-positive cells. A z-stack of images was taken for each root for analysis to avoid optical artefacts.

Root meristem size analysis

Root meristem size was analysed as described previously by Ubeda-Tomás *et al*. (2009). Roots were measured using the National Institutes of Health program ImageJ (Bethesda, MD, USA).

Histochemical GUS assays

GUS histochemical staining was performed as described previously by Aquea *et al*. (2010), followed by root clarification.

Quantitative RT-PCR analyses

Total RNA was extracted with Trizol reagent TRIzol® Reagent (Sigma) from 5-day-old roots treated with 5 mM H_3BO_3 and controls. One μg of total RNA treated with DNAse I (RQ1, Promega, Madison, WI, USA) was reverse transcribed with random hexamer primers using StrataScript® reverse transcriptase (Statagene, La Jolla, CA, USA), according to the manufacturer's instructions. Real-time RT-PCR was performed using the Brilliant SYBR Green QPCR Master Reagent Kit (Stratagene) and the

Mx3000P detection system (Stratagene) as described in the manufacturer's manual. The *CLATHRIN* and At4g26410 (unknown function) genes were used as internal controls. The relative expression level of each gene in BA treatment was compared with control conditions and calculated as described previously by Matus, Aquea & Arce-Johnson (2008). Normalization was performed using the *CLATHRIN* cDNA level and averaged over three replicates. qRT-PCR analyses were performed with two biological repeats. The primers used are listed in Supporting Information Table S1.

Statistical analysis

The data were statistically analysed using the GraphPad Prism 5 Program (GraphPad Software, Inc., La Jolla, CA, USA). Student's *t*-test was used for the comparison of means, which were considered significantly different at $P < 0.05$.

Microarray hybridization

Three biological replicates for control and BA treatments were used for global gene expression analysis. RNA samples were quantified and analysed in terms of their quality using a Nanodrop Spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA), according to the manufacturer's instructions. RNA samples were further processed (GeneChip 3′ IVT Express Kit aRNA amplification, Affymetrix, Santa Clara, CA, USA) according to the manufacturer's directions. Single-stranded cDNA synthesis was performed with 0.5 μg RNA of each sample, using the oligo-dT-T7Promoter Primer and the Superscript II reverse transcriptase system (Invitrogen, Carlsbad, CA, USA). Subsequently, double-stranded cDNA was synthesized and used as template to generate biotinylated-targeted aRNA, following the manufacturer's specifications. Fifteen μg of the biotinylated aRNA was fragmented to between 35 and 200 bases in length and the fragmented aRNA (10 μg) was hybridized on a GeneChip® Arabidopsis ATH1 Genome Array using standard procedures (45 °C for 16 h). The arrays were washed and stained in a Fluidics Station 450 (Affymetrix).

Data processing and analysis

The chip is composed of approximately 22 500 *Arabidopsis thaliana* probe sets and was designed in collaboration with The Institute for Genome Research (TIGR). Data from the TIGR database (ATH1- 121501) are available from the NetAffxTM Analysis Center (http://www.affymetrix.com). Array scanning was carried out with the GeneChip® scanner 300 and image analysis was performed using the GeneChip® Operating Software. GeneChip® array data were first assessed using a set of standard quality control steps described in the Affymetrix manual 'GeneChip® Expression Analysis: Data Analysis Fundamentals'. Calls of all three spike-in controls BioC, BioD and Cre were present, and their intensity values increased from BioC to Cre as expected. Average background values ranged from 25 to 27. Digestion curves displaying trends in RNA degradation between the 5′ and 3′ end in each probe set were also inspected, and all proved very similar.

Arrays data were processed and normalized by robust multi-array average (RMA) (Irizarry *et al*. 2003) using the R package known as 'affy' (Gautier *et al*. 2004). Pearson rank coefficients were computed on the RMA expression values (log2-transformed) for each set of biological replicates. Pearson coefficients ranged between 0.97 and 0.99. Differentially expressed genes were identified using the RankProduct method (Breitling *et al*. 2004). Genes with a $P < 0.05$ were identified as differentially expressed genes and selected for further analysis. The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (Edgar, Domrachev & Lash 2002) and are accessible through GEO Series accession number GEO32659 (http://www.ncbi.nlm.nih.gov/geo/query/ acc.cgi?acc= GEO32659).

RESULTS

Boron decreases root meristem size and cell production rate

To evaluate the toxic effect of boron on root growth of seedlings, 5-day-old *Arabidopsis* were transferred to different BA concentrations and the lengths of roots were measured 5 d after transfer from the root tip to the base of the hypocotyl (Fig. 1a). As expected, an inhibition of root growth was observed. In this experiment, we determined that 5 mM BA is the minimum concentration that produces the maximum effect, stunting growth by ~50% (Fig. 1b). We also grew *Arabidopsis* seedlings with 5 mM of the BA analog methylboronic acid, NaCl and mannitol (Supporting Information Fig. S1). We observed that 5 mM BA drastically inhibits root growth in comparison with the other solutions and conclude that the phenotypes shown in Fig. 1a,b are genuinely associated with boron toxicity.

Root growth depends on the production of new cells, and their subsequent differentiation and elongation. Therefore, we investigated the cellular basis for the inhibition of root growth. To determine which process is affected by boron, we used the overexpression of *LTI6b:GFP*, a fusion protein that is localized at the cell plasma membrane, as a marker of cells. These transgenic lines were transferred to different concentrations of BA and their roots were observed in a confocal microscope. Root meristem size was expressed as the length of the meristematic zone and the number of cortex cells in a file extending from the QC to the first elongated cell exhibiting vacuolization (Dello Ioio *et al*. 2007). We found that BA repressed root meristem size (Fig. 1c–f) and that this reduction correlates with the BA concentration in the medium and the inhibition of root growth (Fig. 1a,b). At higher concentrations (7 mM BA), alterations in the pattern of cell division were also observed (inset Fig. 1c,f). To quantify this phenotype, we analysed the

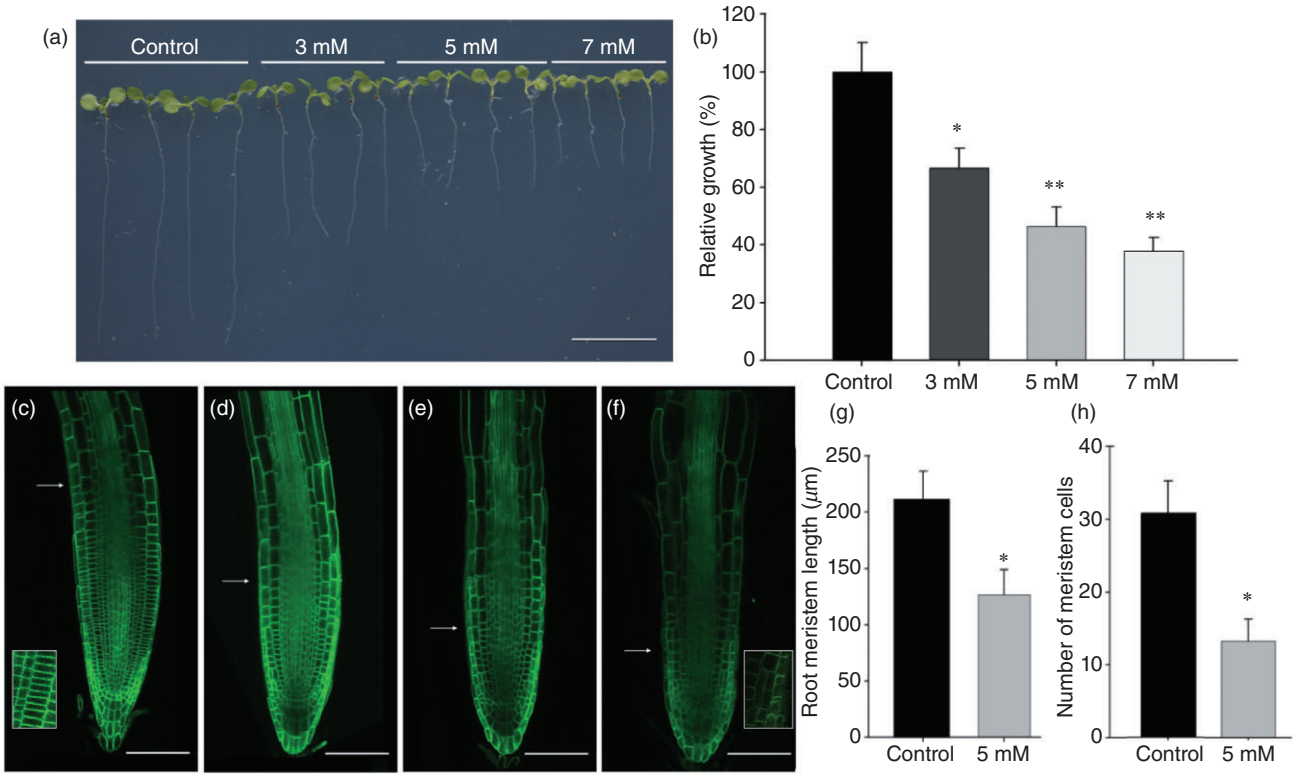


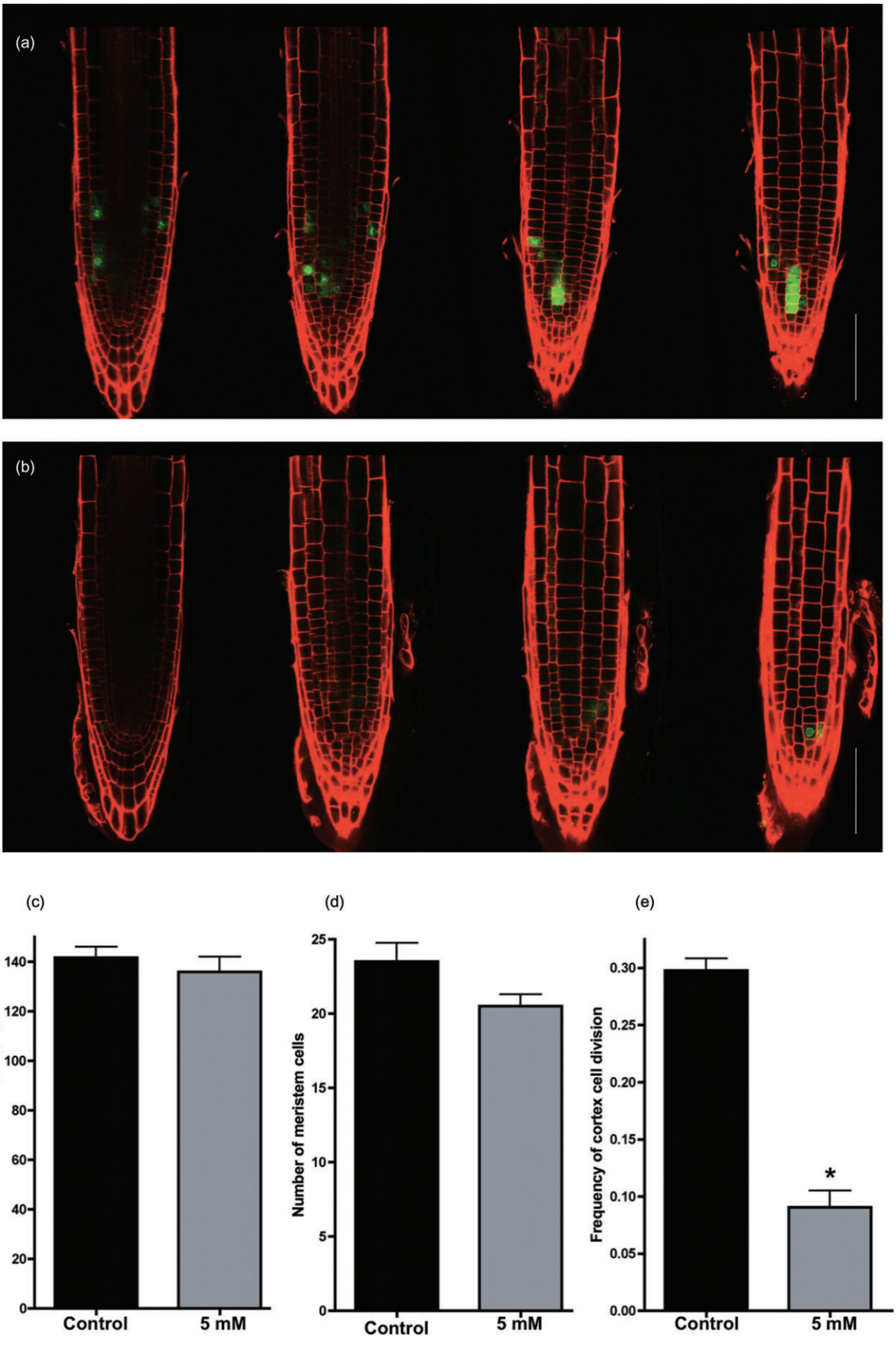
Figure 1. Boron inhibits root growth through regulation of meristem size. (a) *Arabidopsis* seedlings (5-day-old) were transferred to different concentrations of boric acid (BA) and the root growth was visualized 5 d later. (b) Quantification of root growth of (a) from the root tip to the hypocotyl base ($n = 50$). (c–f) 35S:Lti6b:GFP lines treated with different BA concentrations and visualized by confocal microscopy after 5 d. (c) Control (0.05 mM BA); (d) 3 mM BA; (e) 5 mM BA; (f) 7 mM BA. White arrows indicate the position of the transition zone. (g) Quantification of meristem length from the quiescent centre (QC) to the transition zone ($n = 20$). (h) Quantification of meristem cell numbers ($n = 20$). Asterisk indicates statistical significance. Scale bars represent: (a) 1 cm. (c–f) 60 μm. A detail of the pattern of cell division in control condition and 7 mM BA is showed in inset (C and F, respectively).

plants grown in 5 mM BA and found significant differences in the root meristem length (Fig. 1g) and in the number of meristem cells between control conditions and treatment (Fig. 1h). Untreated meristems reached their final size when a fixed number of approximately 30 cells were established in the meristem. In contrast, application of 5 mM BA reduced the number to 13 cells after 5 d of treatment (Fig. 1h). To demonstrate that inhibition of root growth was due to the reduction of the meristem size, we analysed the effect of BA effect in the short term. We observed no significant difference in the root length but there were reductions in meristem length and root meristem cell number at 24 h (Supporting Information Fig. S2). Moreover, root growth inhibition was also observed in the lateral roots (Supporting Information Fig. S3).

Boron represses mitotic activity in the root meristem

Reducing the number of cells in the root meristem by the application of higher BA concentrations suggests that cell proliferation was severely reduced. To test this, we monitored how changes in BA levels affect the expression of the mitotic marker *pCYCB1;1::CYCB1;1:GFP* (Fig. 2). *CYCB1;1* belongs to the cyclin protein family that regulates G2-to-M cell cycle progression and can be used as a marker of mitosis (Doerner *et al.* 1996). Fig. 2a shows confocal images of four radial optical sections of the root meristem of the same transgenic line *pCYCB1;1::CYCB1;1:GFP* in control (Fig. 2a) and BA treatment (Fig. 2b). Applications of BA significantly decreased the frequency of mitotic cells,

Figure 2. Analysis of the role of boron in cell division in the root meristematic region. (a–b) Confocal images of four radial optical sections of the root meristem of pCYCB1;1:CYCB1;1:GFP in (a) control conditions and (b) 5 mM boric acid (BA) at 12 h. The cells that express green fluorescent protein (GFP) are in mitotic division. (c) Quantification of meristem length from the QC to the transition zone. (d) Quantification of meristem cell numbers. (e) Quantification of cells in division within a region of active proliferation in the root meristem. Thirty cortex cells from the 2nd to the 20th position from the quiescent centre (QC) in two adjacent files of cortex cells were scored in batches of 15 roots for GFP expression. Propidium iodide was used as a red counterstain. Asterisk indicates statistical significance. Scale bars represent 50 μm.


(a)
(b)
(c)
(d)
(e)
Root meristem length (μm)
0
20
40
60
80
100
120
140
Control
5 mM
Number of meristem cells
0
5
10
15
20
25
Control
5 mM
Frequency of cortex cell division
0.00
0.05
0.10
0.15
0.20
0.25
0.30
*
Control
5 mM

observed as loss of fluorescence within a fixed number of cells capable of division (Fig. 2b). This assay was carried out after 12 h of BA treatment because at this time we did not observe any differences in the root meristem length or in the number of meristem cells but there were significant differences in the frequency of mitotic cells of the roots analysed (Fig. 2c–e). These results suggest that boron inhibits root growth by reducing the rate of cell division in the root meristem.

Boron affects the expression levels of the key cell cycle regulators and modulates the meristem root division

Plant cells have evolved a complex circuitry to regulate cell division, a process controlled by the activity of inducer and repressor proteins. To gain insight into the molecular basis of the regulatory mechanism of the repression of mitotic activity in the root meristem, we next determined the expression levels of key core cell cycle regulators. We evaluated the expression of the positive regulator genes that code for cyclin-dependent kinases (*CDKA1*, *CDKB1;1* and *CDKB2;1*), cyclins (*CYCA1;1, CYCA2, CYCB1;1* and *CYCD3;1*) and transcription factors (*E2Fa*, *E2Fb*), and the negative regulator genes that code for a transcription factor (*DEL1*), a kinase (*WEE1*), cyclin-dependent kinase inhibitors (*KRP1*, *KRP2*, *KRP4*, *SIM*, *SMR1*, *SMR2*, *SMR3, SMR4* and *SMR5*) and a retinoblastoma-related (RBR) protein. Roots from 5-day-old seedlings treated for 12 and 24 h with BA were used for qRT-PCR analysis. The expression of the positive regulators *CDKB1;1*, *CDKB2;1*, *CYCA1;1* and *CYCB1;1* was down-regulated at 12 h of BA treatment and then recovered at 24 h, except in the case of *CDKB2;1* (Fig. 3). In addition, the level of expression of negative regulators *KRP1*, *SMR3*, *SMR4* and *SMR5* was up-regulated and that of *DEL1*, *SIM* and *SMR1* was down-regulated at 12 h of BA treatment (Fig. 4). At this time, the expression of *WEE1*, *KRP2* and *KRP4* was not modified (Fig. 4). These expression patterns change after 24 h of BA treatment. The expression level of *DEL1*, *KRP1*, *SMR1* and *SMR5* returned to pretreatment levels; *WEE1*, *KRP2*, *SMR3* and *SMR4* were induced and *KRP4* and *SIM* were repressed (Fig. 4). There were no significant differences in the expression of *CDKA1*, *CYCA2*, *CYCD3;1*, *E2Fa*, *E2Fb*, *SMR2* and *RBR* (data not shown). These results suggest that after BA treatment, cell cycle progression is repressed and subsequently resumed after 24 h. This phenomenon has been described as cell cycle modulation and is a general mechanism of stress adaptation (West *et al.* 2004). To further evaluate if BA treatment modulates the root cell cycle, we studied the changes in mitotic activity using the transgenic line *pCYCB1;1::CYCB1;1:GUS* in function of time after transfer of the seedlings to the medium with 5 mM BA. We observed a significant difference in the number of dividing cells at 6 h of transfer, which is drastically reduced at 12 h before returning at 24 h to a level similar to baseline (Fig. 5). The reduction in the number of dividing cells is reversible (Supporting Information Fig. S4),

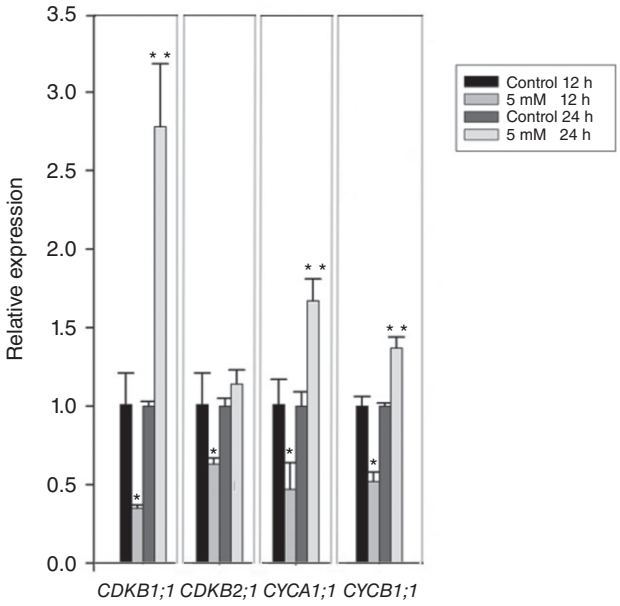


Figure 3. Expression of positive regulators of cell cycle. Relative levels of gene expression determined by quantitative RT-PCR in roots of 5-day-old wild-type Col-0 treated with 5 mM boric acid (BA). Data are means ± SE. Similar results were obtained in two independent experiments. Asterisk indicates statistical significance.

suggesting indeed that it experiences a mechanism of stress adaptation. These results suggest that high levels of boron inhibit root growth by modulating the cell cycle.

Boron toxicity does not appear to act through cytokinin and auxin signalling

Our results show that boron toxicity inhibits root growth. It is known that cytokinin and auxin are key regulators of cell division in the root. For this reason, we monitored the distribution and response of both hormones after 24 h of exposure to toxic concentrations of BA (Fig. 6). At this time, we observed differences in meristem sizes, the number of meristem cells and in cell division (Fig. 5 and Supporting Information Fig. S2). Using the reporter line *pARR5::H2B:RFP* (a cytokinin-inducible promoter), no visible differences in the pattern of *RFP* expression and distribution were observed (Fig. 6a–d). Moreover, the expression of ARR5 and ARR7 was unchanged in the presence of BA (Supporting Information Fig. S5), suggesting that cytokinin signalling is normal in the presence of BA. Using the reporter lines *DR5::GFP* and *pIAA2::H2B:RFP* (auxin-inducible promoters), we observed that GFP and red fluorescent protein (RFP) expression was unaffected in the presence of BA (Fig. 6e,h,f,i respectively), suggesting that auxin signalling is unchanged in the presence of BA. Furthermore, the pattern of expression of the auxin efflux protein *PIN1* (Fig. 6g,j and Supporting Information Fig. S5), AUX1 (Fig. S5), *PIN3* and *PIN7* (Supporting Information Fig. S6) was unchanged.

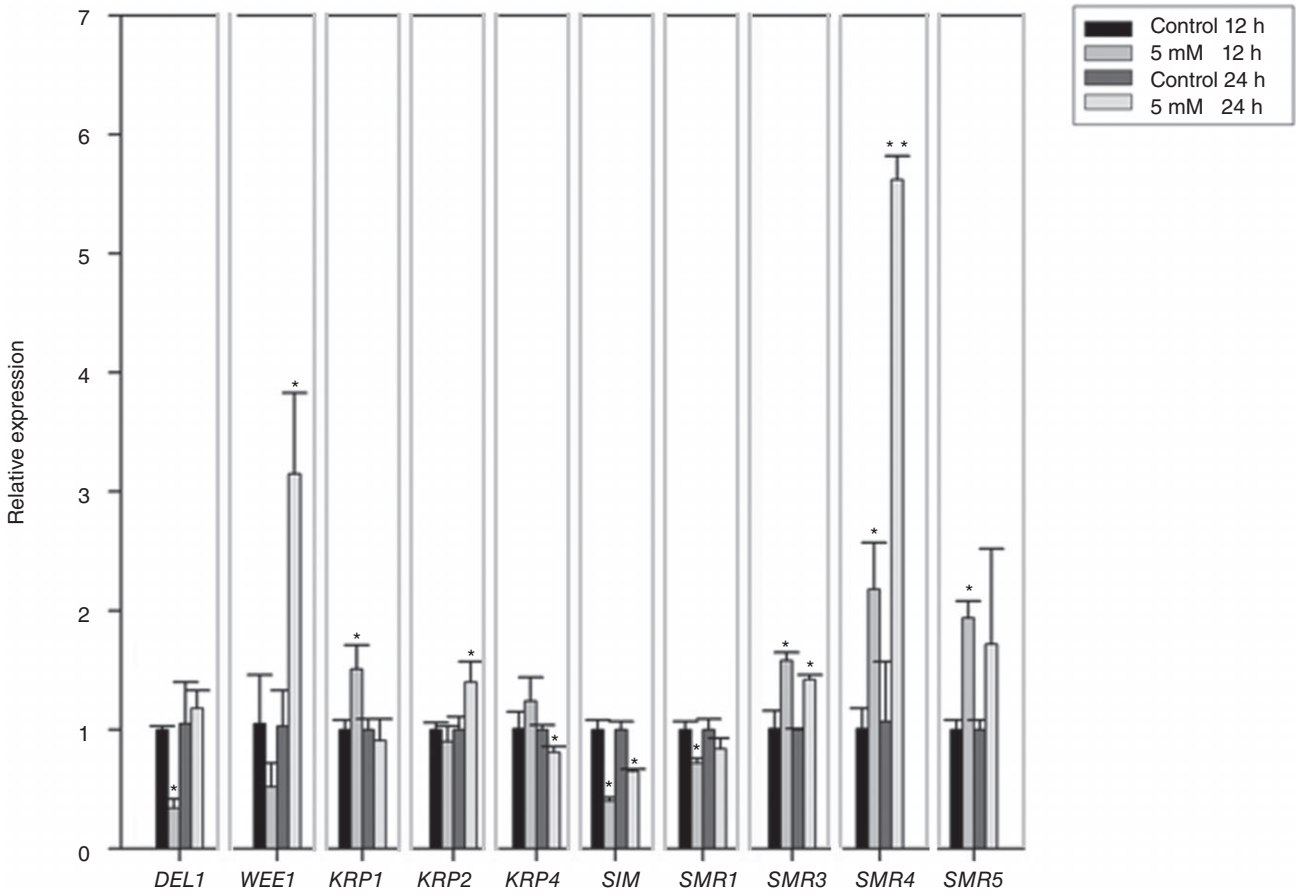


Figure 4. Expression of negative regulators of cell cycle. Relative levels of gene expression determined by quantitative RT-PCR in roots of 5-day-old wild-type Col-0 treated with 5 mM boric acid (BA). Data are means ± SE. Similar results were obtained in two independent experiments. Asterisk indicates statistical significance.

To further study the involvement of cytokinin and auxin in boron-induced root growth inhibition, we applied BA to plants that overexpress *CYTOKININ OXIDASE 4 (CKX4)*, which catalyzes the degradation of cytokinin; and miR393, a microRNA that targets the auxin receptors TIR1, AFB1, AFB2 and AFB3. Plants that overexpress *CKX4* were sensitive to BA toxicity in a similar manner as wild-type plants (Supporting Information Fig. S7). Similarly, plants expressing miR393 had the same phenotype as control plants (Supporting Information Fig. S7). These results suggest that auxin and cytokinin do not participate in the inhibition of root growth caused by BA.

Boron toxicity produces gene expression changes associated to water-stress related response

To further investigate the molecular mechanisms underlying the inhibition of root growth by toxic boron treatments, we analysed the transcript profiles in roots by microarray analysis (Affymetrix ATH1 Genome Array). We compared the transcripts obtained at 12 h of BA treatment. We found 211 genes down-regulated and 240 genes up-regulated by more than twofold ($Log_2 > 1, P < 0.05$) in roots treated with BA compared with those under control conditions. The key core cell cycle genes previously identified by the quantitative RT-PCR analysis, as described previously, were not identified as being significantly differentially regulated in the affymetrix analysis. To get a global overview of these differentially expressed genes, we first investigated which Gene Ontology categories were represented. The main biological processes among the up-regulated and down-regulated genes were 'response to stress' and 'response to abiotic or biotic stimulus', respectively (Supporting Information Fig. S8). Interestingly, the 'transport' category appears only in down-regulated genes (Supporting Information Fig. S8). The main molecular functions among the down-regulated genes were 'transporter activity' and 'transferase activity' (Supporting Information Fig. S9). Descriptions of selected up-regulated and down-regulated genes are shown in Tables 1 and 2, respectively. The up-regulated genes are mainly involved in ABA signalling (phosphatase 2C, transcription factors, kinase), ABA response (LEA proteins, COR genes) or in cell wall modifications (suberin, lignin and cutin biosynthesis genes). The down-regulated genes are mainly involved in glucosinolate biosynthesis,

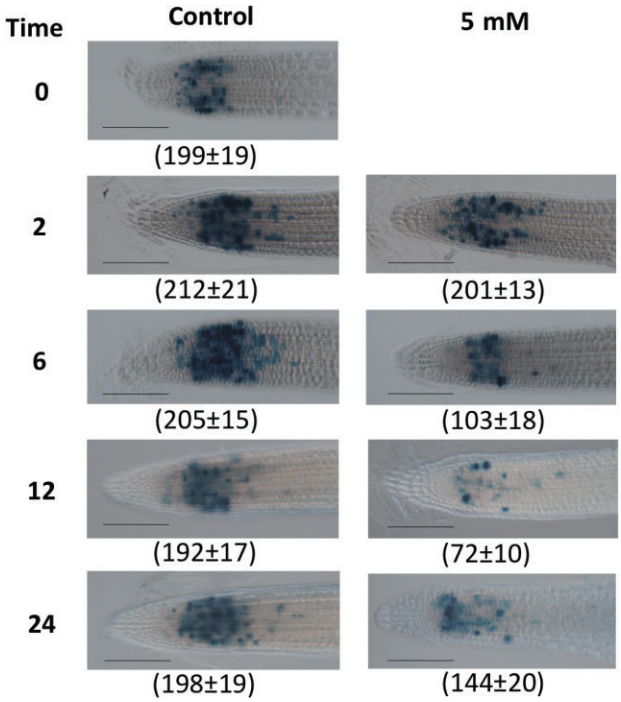


Figure 5. Temporal analysis of cell division in the root meristematic region. pCYCB1;1:CYCB1;1:GUS staining in root meristems of 5-day-old seedlings treated with 5 mM boric acid (BA) for the times (h) indicated in the figure. Numbers indicate average length ($n = 10$, ± SE) of the β-glucuronidase-stained region in the longitudinal axis of the root meristem. Scale bars represent 120 μm.

water transport or aquaporins (NIP, TIP and PIP) and genes that code for nutrient transporter proteins (sulfate, nitrate, nickel, ammonium, sucrose and boron). The global gene expression changes indicate that boron mainly triggers a molecular response associated with a water-stress related response.

Quantitative real-time RT-PCR was used to confirm the results of the microarray studies for the selected genes. We selected the ABA-responsive gene At3g02480, the transcription factors ATHB7 and MYB41, the ABA signal transducer ABI1, and the water channels NIP1;1 and TIP2;1 (Fig. 7). All of the genes tested were confirmed to be either induced or repressed in BA treatment compared with the control conditions. As expected, the magnitude of changes calculated from quantitative real-time RT-PCR data was greater than from array data.

DISCUSSION

Plants have developed several strategies for taking up and utilizing nutrients from the soil for normal growth. However, when nutrients are present in excess, their toxic effects can be severe in higher plants and are considered an abiotic stress for growth. In this work, we report a molecular framework of how *Arabidopsis* respond to the toxic effect of boron, an essential plant micronutrient. When BA concentrations are increased in the growth medium, we observed cellular alterations in the root meristem, leading to the inhibition of root growth (Fig. 1). Several reports have shown that the main effect of excess nutrients and abiotic stress conditions is observed in root growth. For example, zinc is essential for plants as a cofactor of a large number of enzymes and proteins. However, excess zinc causes serious growth defects such as chlorosis and root growth inhibition (Marschner 1995). A stunted root system is also a significant symptom of excess levels of ammonium (Britto & Kronzucker 2002), copper (Lequeux *et al*. 2010), sodium (Flowers, Hajibagheri & Yeo 1991) and chloride (White & Broadley 2001). Some processes, such as changes in cell division and hormonal homeostasis, have been postulated to be involved in this response (Jiang, Liu & Liu 2001; López-Bucio, Cruz-Ramírez & Herrera-Estrella 2003; Potters *et al*. 2006, 2009). In the case of boron toxicity, the cellular alterations in root meristems are related to a reduction of mitotic activity (Fig. 2) and modifications of the expression patterns of key core cell cycle genes (Figs 3 & 4). In *Arabidopsis*, it has been previously reported that salt stress represses the cell cycle (Burssens *et al*. 2000; West *et al*. 2004), resulting in growth retardation of the primary root. This phenomenon has been named as cell cycle modulation and is important for stress adaptation. In the case of boron toxicity, we observed the same phenotype as salt stress, suggesting that toxic concentrations of NaCl and BA could act in the same way. It has been proposed that this adaptation involves two phases: firstly, a rapid transient inhibition of the cell cycle that results in fewer cells remaining in the meristem, and secondly, when the meristem reaches the appropriate size for the given conditions, the cell cycle duration returns to its pre-stress state (West *et al*. 2004). Interestingly, there is evidence that boron is also involved in cell growth and proliferation in animals (Park *et al*. 2005) and BA has a chemo-preventive effect against prostate cancer, inhibiting cell proliferation in humans (Gallardo-Williams *et al*. 2004).

Notably, a quantitative RT-PCR analysis showed that expression of the negative cell cycle regulators *WEE1* and *SMR4* increases significantly after 24 h of BA treatment (Fig. 4). *WEE1* codes for a kinase protein and is transcriptionally activated upon the cessation of DNA replication or DNA damage, inhibiting plant growth by arresting dividing cells in the G2-phase of the cell cycle (De Schutter *et al*. 2007). Moreover, it has been reported that expression of the SIM gene family responds to diverse biotic and abiotic stress treatments and it was suggested that these proteins decouple the cell cycle during unfavourable environmental conditions (Peres *et al*. 2007). Our results suggest that boron treatment produces genotoxic damage to root cells, thus triggering a molecular response that modifies the cell cycle and inhibits root growth. Recently, it has been suggested that boron toxicity mechanism involves DNA double-strand breaks and possibly replication blocks triggered by a genotoxic stress caused by BA (Sakamoto *et al*. 2011).

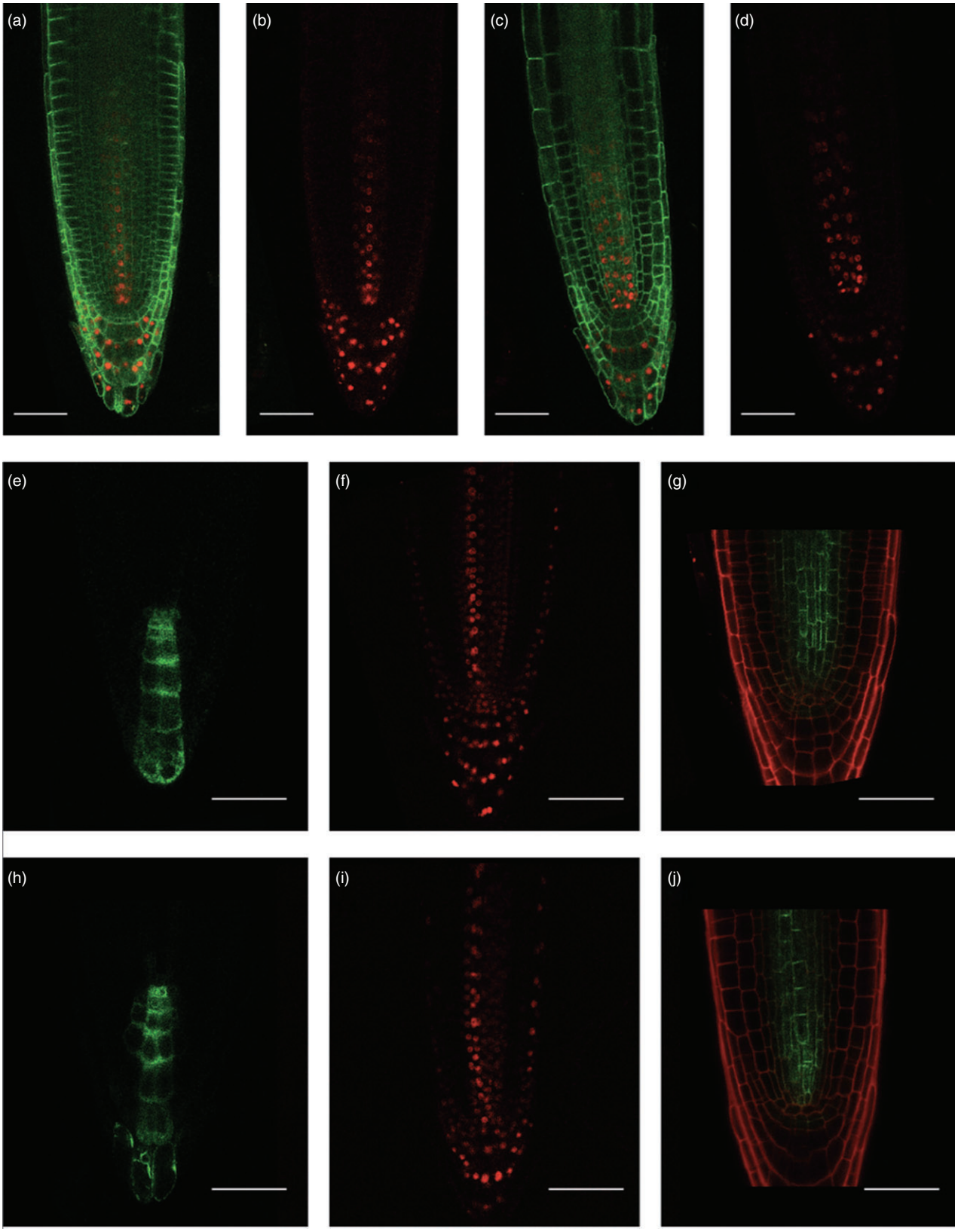


Figure 6. Analysis of cytokinin and auxin response in presence of boron. Analysis of *pARR5:H2B:RFP* in control conditions (a–b) and 5 mM boric acid (BA) (c–d) after 24 h of treatment. Analysis of *DR5:GFP* in control conditions (e) and 5 mM BA (h). Analysis of *pIAA2:H2B:RFP* in control conditions (f) and 5 mM BA (i). Analysis of *pPIN1:GFP* in control conditions (g) and 5 mM BA (j) ($n = 10$). Scale bars represent 60 μm.

Table 1. Up-regulated genes in roots treated with boron

ID Affymetrix	Locus	Name	Description	Fold	*P* value
ABA signalling and response					
258498_at	AT3G02480	–	Abscisic acid (ABA)-responsive protein-related	5.99	0
250648_at	AT5G06760	LEA4-5	Late embryogenesis abundant (LEA) proteins	4.97	0
247723_at	AT5G59220	HAI1	Putative protein phosphatase 2C	4.08	0
262128_at	AT1G52690	–	LEA proteins	3.79	0
266327_at	AT2G46680	ATHB7	Transcription factor that contains a homeodomain	3.43	0.0010
251272_at	AT3G61890	ATHB12	Homeodomain leucine zipper class I (HD-Zip I) protein	3.37	0.0009
253408_at	AT4G32950	–	Putative protein phosphatase 2C	3.08	0.0006
260357_at	AT1G69260	AFP1	ABI five binding protein	3.02	0.0005
266462_at	AT2G47770	TSPO	Membrane-bound protein	2.97	0.0005
246097_at	AT5G20270	HHP1	Heptahelical transmembrane protein	2.52	0.0017
254215_at	AT4G23700	CHX17	Member of Putative Na^+/H^+ antiporter family	2.50	0.0016
253851_at	AT4G28110	MYB41	Member of the R2R3 factor gene family	2.30	0.0043
246481_s_at	AT5G15960	KIN1/KIN2	Cold and ABA-inducible protein	2.30	0.0026
264436_at	AT1G10370	ERD9	Early-responsive to dehydration	2.26	0.0034
248337_at	AT5G52310	RD29A/COR78	Cold regulated gene	2.08	0.0041
256576_at	AT3G28210	SAP12	Putative zinc finger protein (PMZ)	1.98	0.0056
247957_at	AT5G57050	ABI2	Protein phosphatase 2C	1.83	0.0099
253264_at	AT4G33950	OST1	Calcium-independent ABA-activated protein kinase	1.74	0.0112
258347_at	AT3G17520	–	LEA proteins	1.74	0.0129
254562_at	AT4G19230	CYP707A1	Protein with ABA 8′-hydroxylase activity	1.73	0.0145
246908_at	AT5G25610	RD22	Responsive to dehydration mediated by ABA	1.61	0.0162
258310_at	AT3G26744	ICE1	MYC-like bHLH transcriptional activator	1.56	0.0168
253994_at	AT4G26080	ABI1	Involved in ABA signal transduction	1.56	0.0192
267372_at	AT2G26290	ARSK1	Root-specific kinase 1	1.46	0.0441
253453_at	AT4G31860	–	Putative protein phosphatase 2C	1.43	0.0278
247095_at	AT5G66400	RAB18	Dehydrin protein family	1.42	0.0385
259922_at	AT1G72770	HAB1	Protein phosphatase 2C	1.41	0.0285
266544_at	AT2G35300	LEA4-2	LEA proteins	1.33	0.0406
Cell wall modification					
252209_at	AT3G50400	–	GDSL-motif lipase/hydrolase family protein	3.42	0.001
251428_at	AT3G60140	DIN2	Protein similar to beta-glucosidase	3.22	0.0008
251229_at	AT3G62740	BGLU7	Beta glucosidase 7	2.87	0.0008
250674_at	AT5G07130	LAC13	Member of laccase family of genes	2.60	0.0013
250770_at	AT5G05390	LAC12	Member of laccase family of genes	2.48	0.0017
259975_at	AT1G76470	–	Cinnamoyl-CoA reductase	2.42	0.0015
249881_at	AT5G23190	CYP86B1	Cytochrome P450	2.37	0.0026
249289_at	AT5G41040	MEE6.11	Feruloyl-CoA transferase	2.37	0.0026
259149_at	AT3G10340	PAL4	Phenylalanine ammonia-lyase	2.29	0.0026
264318_at	AT1G04220	KCS2	Member of the 3-ketoacyl-CoA synthase family	2.15	0.0042
252639_at	AT3G44550	FAR5	Alcohol-forming fatty acyl-CoA reductases	2.13	0.0042
254543_at	AT4G19810	–	Glycosyl hydrolase family 18 protein	2.07	0.0046
248100_at	AT5G55180	–	Glycosyl hydrolase family 17 protein	2.06	0.0046
256779_at	AT3G13784	ATCWINV5	Arabidopsis thaliana cell wall invertase 5	1.98	0.0051
259282_at	AT3G11430	ATGPAT5	Glycerol-3-phosphate acyltransferase	1.96	0.0078
249123_at	AT5G43760	KCS20	Member of the 3-ketoacyl-CoA synthase family	1.84	0.0083
261899_at	AT1G80820	CCR2	Cinnamoyl CoA reductase	1.77	0.013
252638_at	AT3G44540	FAR4	Alcohol-forming fatty acyl-CoA reductases	1.71	0.016
262414_at	AT1G49430	LACS2	Long chain acyl-CoA synthetase	1.68	0.019
263825_at	AT2G40370	LAC5	Member of laccase family of genes	1.60	0.019
256186_at	AT1G51680	4CL1	4-coumarate-CoA ligase	1.47	0.026
264433_at	AT1G61810	BGLU45	Beta glucosidase 45	1.41	0.029

Expression changes are presented as log2.

Plant hormones, mainly auxin and cytokinin, control most of the characteristics of the root system, including principal root growth and formation of lateral roots and root hairs (Moubayidin *et al*. 2009). Furthermore, there are several reports that associate the biosynthesis, transport and sensitivity of auxin with the modifications of root growth caused by abiotic stress (Wang, Li & Li 2009; Sun *et al*. 2010), including boron deficit (Martín-Rejano *et al*. 2011). In our work, we observed that BA toxicity does not alter the distribution of both hormones in the root (Fig. 6). Additionally, plants that overexpress miR393 and *CKX4* are just as sensitive to boron toxicity as wild-type *Arabidopsis* plants

Table 2. Down-regulated genes in roots treated with boron

ID Affymetrix	Locus	Name	Description	Fold	*P* value
Glucosinolate biosynthesis					
249867_at	AT5G23020	MAM3	Methylthioalkymalate synthase-like	–5.95	0
257021_at	AT3G19710	BCAT4	Branched-chain amino acid aminotransferase	–5.26	0
251524_at	AT3G58990	PMI SSU3	Isopropylmalate isomerase 1	–4.40	0
249866_at	AT5G23010	MAM1	Methylthioalkylmalate synthase	–4.12	0
254687_at	AT4G13770	CYP83A1	Cytochrome p450	–3.61	0
264052_at	AT2G22330	CYP79B3	Cytochrome p450	–3.58	0
252827_at	AT4G39950	CYP79B2	Cytochrome p450	–3.21	0
254862_at	AT4G12030	BAT5	Bile acid transporter	–2.48	0.0004
266395_at	AT2G43100	PMI SSU2	Isopropylmalate isomerase 2	–2.66	0
252870_at	AT4G39940	APK2	Adenosine-5′-phosphosulfate-kinase	–2.29	0.0002
263714_at	AT2G20610	SUR1	C-S lyase	–2.19	0.0005
253534_at	AT4G31500	CYP83B1	Cytochrome p450	–2.16	0.0005
267153_at	AT2G30860	ATGSTF09	Glutathione transferase	–1.95	0.002
263706_s_at	AT5G14200	IPMDH1	Methylthioalkylmalate dehydrogenase	–1.89	0.004
258851_at	AT3G03190	ATGSTF11	Glutathione transferase	–1.87	0.005
255934_at	AT1G12740	CYP87A2	Cytochrome p450	–1.77	0.006
255773_at	AT1G18590	SOT17	Desulfoglucosinolate sulfotransferase	–1.68	0.017
260745_at	AT1G78370	ATGSTU20	Glutathione transferase	–1.56	0.018
260387_at	AT1G74100	SOT16	Desulfoglucosinolate sulfotransferase	–1.43	0.028
263477_at	AT2G31790	UGT74C1	UDP-glycosyltransferase activity	–1.34	0.038
260385_at	AT1G74090	SOT18	Desulfoglucosinolate sulfotransferase	–1.27	0.040
264873_at	AT1G24100	UGT74B1	UDP-glycosyltransferase activity	–1.22	0.049
Transporter proteins					
262133_at	AT1G78000	SULTR1;2	Sulfate transporter	–2.79	0
254606_at	AT4G19030	NIP1;1	Aquaporin and arsenite transport	–2.61	0
264734_at	AT1G62280	SLAH1	Homologue to SLAC1 (ion homeostasis)	–2.36	0.0003
258054_at	AT3G16240	TIP2;1	Water channel and ammonium transporter	–2.12	0.001
260693_at	AT1G32450	NRT1.5	Transmembrane nitrate transporter	–2.09	0.0012
258629_at	AT3G02850	SKOR	Member of Shaker family K^+ ion channel	–2.02	0.0022
246238_at	AT4G36670	–	Mannitol transporter	–1.84	0.0050
250952_at	AT5G03570	FPN2	Nickel transport protein	–1.72	0.0075
262883_at	AT1G64780	AMT1;2	Ammonium transporter protein	–1.60	0.014
257162_s_at	AT3G24300/AT3G24290	AMT1;3/AMT1;5	Ammonium transporter protein	–1.56	0.016
261895_at	AT1G80830	NRAMP1	Putative protein involved in iron homeostasis	–1.56	0.013
249765_at	AT5G24030	SLAH3	Homologue to SLAC1 (ion homeostasis)	–1.48	0.018
247440_at	AT5G62680	–	Proton-dependent oligopeptide transporter	–1.47	0.021
257939_at	AT3G19930	STP4	Sucrose hydrogen symporter	–1.45	0.021
262134_at	AT1G77990	SULTR2;2	Sulfate transporter	–1.41	0.024
252537_at	AT3G45710	–	Proton-dependent oligopeptide transporter	–1.35	0.034
247586_at	AT5G60660	PIP2;4	Plasma membrane intrinsic protein	–1.31	0.034
262813_at	AT1G11670	–	MATE efflux family protein	–1.29	0.042
245399_at	AT4G17340	TIP2;2	Tonoplast intrinsic protein	–1.29	0.047
254239_at	AT4G23400	PIP1;5	Plasma membrane intrinsic protein	–1.29	0.034
263319_at	AT2G47160	BOR1	Boron transporter	–1.28	0.048

Expression changes are presented as log2.

(Supporting Information Fig. S7), suggesting that both hormones do not participate in the inhibition of root growth caused by BA. It has been proposed that the reactive oxygen species (ROS) pathway may play a key role in response to local cues and controls the transition from proliferation to differentiation in the root, independently of auxin and cytokinin signalling (Tsukagoshi, Busch & Benfey 2010). Indeed, there is evidence that boron toxicity alters the antioxidant machinery and produces oxidative stress damage (Karabal, Yucel & Oktem 2003; Ardic *et al*. 2009), suggesting that ROS could be an important signal during boron toxicity.

Although a few boron-regulated genes have been identified previously (Kasajima & Fujiwara 2007), our study provides the first global expression profile, to our knowledge, of the toxic effect of this micronutrient in *Arabidopsis roots*. Transcriptome analysis revealed that boron toxicity had impacts on the genes involved in metabolism, transport and stress responses. The majority of the genes up-regulated by boron treatment is not specific to toxicity of this micronutrient and is also induced by many other stresses, such as exposure to salt, drought and/or osmotic shock (Kilian *et al*. 2007). This suggests that boron toxicity triggers a common molecular response to most abiotic stresses.

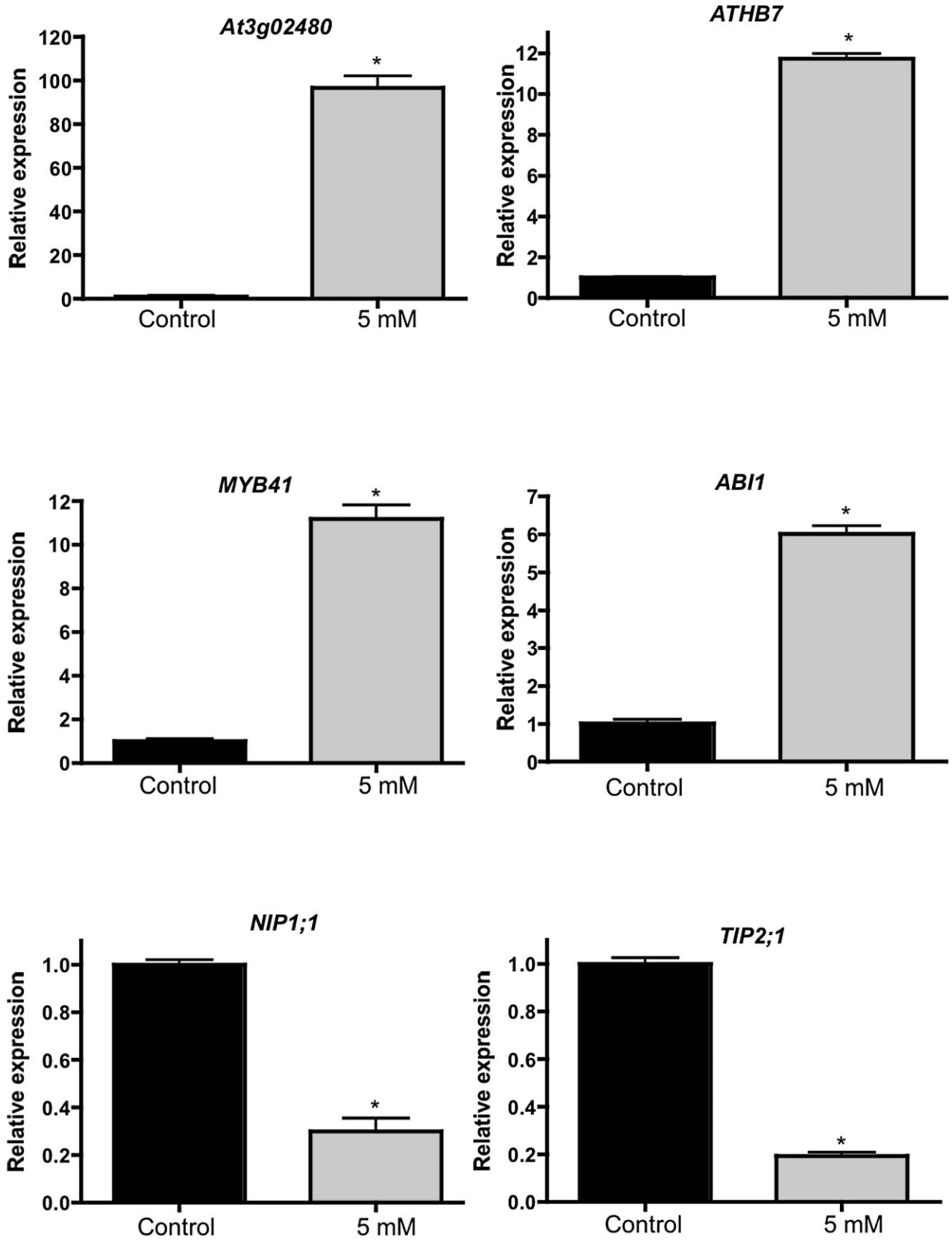


Figure 7. Validation of selected genes by qRT-PCR. Relative levels of gene expression determined by quantitative RT-PCR in roots of 5-day-old wild-type Col-0 treated with 5 mM boric acid (BA) for 12 h. Data are means ± SE. Similar results were obtained in two independent experiments. Asterisk indicates statistical significance.

A detailed analysis of most genes that are significantly induced reveals that ABA signalling and ABA responses are the main molecular changes that occur in roots after treatment (Table 1), indicating that this hormone is involved in the response of *Arabidopsis* to boron toxicity. ABA plays a key role in plant adaptation to adverse environmental conditions including drought, osmotic and salt stress (Hirayama & Shinozaki 2010). Several studies have shown that ABA accumulation is a key factor in controlling downstream responses essential for adaptation to environmental stress (Hirayama & Shinozaki 2010). These results suggest again that the response of *Arabidopsis* to boron toxicity is similar to the plants' response to other abiotic stresses. Another group of genes that are induced in a significant manner are those involved in cell wall modifications (Table 1). These genes participate in the biosynthesis of lignin, cutin and suberin. It has been reported that suspension tobacco cells treated with excess BA have an increase in the content of lignin and suberin in their cell walls (Ghanati, Morita & Yokota 2002). Moreover, an extensive suberization of cells was observed in root tips of soybean seedlings exposed to 5 mM BA (Ghanati, Morita & Yokota 2005). These cell wall modifications have important roles in the stress response because they alter the fluxes of gases, solutes, water and nutrients (Pollard *et al*. 2008). In the *Arabidopsis* mutant *esd1* that is characterized by increased root suberin, the shoot concentration of boron was significantly reduced by approximately 25–40% compared with wild-type plants (Baxter *et al*. 2009). This decrease in the content of endogenous boron could be associated with a reduction of water uptake by the roots, because initially BA is taken up from the soil in a passive form and by aquaporins (Takano *et al*. 2008). Interestingly, the expression of genes that code for aquaporins is significantly repressed by toxic levels of boron (Table 2). Therefore, there is a possible relationship between the deposition of suberin, the down-regulation of aquaporin genes, and the reduction of water and boron uptake. These results suggest that once a plant senses toxic concentrations of boron, a molecular response to reduce water absorption as a mechanism that inhibits the incorporation of boron is elicited. This response causes plant dehydration mediated by ABA. Probably, the inhibition of root meristem cell division observed previously is associated with the abiotic stress response triggered by boron and finally root growth is stalled, leading to plant death. Further experiments are necessary to prove this model and elucidate whether this mechanism is specific to boron or is common to nutritional stress conditions.

In addition to aquaporins, several genes that codify nutrient transporters are repressed (Table 2), suggesting that the plants attempt to avoid nutrient uptake, including boron, given that the borate transporter *BOR1* is repressed as well.

The observation that glucosinolate-biosynthetic genes are the most repressed is interesting (Table 2). Glucosinolates are secondary metabolites well known for their role in plant resistance to insects and pathogens in the brassicales order and are derived from amino acids (Sønderby, Geu-Flores & Halkier 2010). The repression of 22 biosynthetic genes suggests that *Arabidopsis* respond to boron toxicity by limiting several glucosinolate synthesis pathways. As a consequence, the unused amino acids could be used for protein synthesis or to increase the concentration of intracellular solutes to prevent water loss.

The observation that boron toxicity produces the modulation of root cell division and modifies the expression pattern of genes associated to ABA, cell wall modifications and water transport, indicates that there is a tight correlation between inhibition of root growth and water-stress related responses.

An interesting challenge in plant biotechnology is to produce crops that are tolerant of excess boron. Such a challenge is currently being met by manipulating boron transport (Miwa *et al*. 2007; Sutton *et al*. 2007; Pang *et al*. 2010; Schnurbusch *et al*. 2010). However, we conclude that boron toxicity triggers a water-stress response associated with root growth inhibition, and suggest that the use of plants tolerant to drought or salt stress may represent a novel approach for improving the boron tolerance of crops.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Phenotypic analysis of boron and other abiotic stresses on root growth inhibition. *Arabidopsis* seedlings (5-day-old) were transferred to different solutions and root growth was visualized 14 d after transfer. (a) Control conditions (0.05 mM BA), (b) 5 mM BA, (c) 5 mM methylboronic acid, (d) 5 mM NaCl, (e) 5 mM mannitol. Scale bars represent: 1.5 cm

Figure S2. Evaluation of the root meristem in presence of boron for 24 h. *35S::Lti6b:GFP* lines were treated with 5 mM BA and visualized by confocal microscopy 24 h after transfer. (a) Control conditions (0.05 mM BA), (b) 5 mM BA. White arrowheads indicate the position of the transition zone. (c) Quantification of root growth from the root tip to the hypocotyl base. (d) Quantification of meristem length from the quiescent centre to the transition zone. Asterisk indicates statistical significance. Scale bars represent 60 μm.

Figure S3. Analysis of the effect of boron on lateral root growth. Lateral root formation was induced by cutting the root tip and transferring to (a) control conditions and (b) 5 mM BA. Lateral root growth was recorded after 5 d. Scale bars represent 1 cm.

Figure S4. Recovery of root meristem cell division after boron treatment. *pCYCB1;1::CYCB1;1:GUS* staining in root meristems of 5-day-old seedlings treated with (a) control conditions, (b) 5 mM BA for 24 h and (c) 5 mM BA

for 24 h followed by 24 h in control conditions. Numbers indicate average length ($n = 10$, ±SE) of the β-glucuronidase-stained region in the longitudinal axe of the root meristem. Scale bars represent 90 μm.

Figure S5. Expression of auxin and cytokinin responsive genes by quantitative RT-PCR. Relative levels of gene expression determined by quantitative RT-PCR in roots of 5-day-old wild-type Col-0 treated with 5 mM BA for 12 h. Data are means ± SE. Similar results were obtained in two independent experiments. Black bar, control; grey bar, 5 mM BA.

Figure S6. Evaluation of PIN expression in boron treatment. Analysis of *pPIN3::GUS* in (a) control conditions and (b) 5 mM BA. Analysis of *pPIN7::GUS* in (c) control conditions and (d) 5 mM BA. GUS activity was recorded after 24 h of BA treatment. Scale bars represent 40 μm.

Figure S7. Phenotypic analysis of 35S::miR393 and 35S::CKX4 in presence of boron. (a) Col-0, (b) *35S::CKX4*, (c) *35S::miR393*. Seedling phenotypes were recorded 5 d after transfer to control conditions (left side) and 5 mM BA (right side). The % relative growth ± SE is indicated in parentheses ($n = 10$). Scale bars represent 0.8 cm.

Figure S8. Biological processes affected in roots treated with boron. Data obtained from Arabidopsis Gene Ontology (http://www.arabidopsis.org/ tools/bulk/go/index.jsp) using the Affymetrix results. Up-regulated genes are shown in red and down-regulated genes are shown in green.

Figure S9. Molecular functions affected in roots treated with boron. Data obtained from Arabidopsis Gene Ontology (http://www.arabidopsis.org/ tools/bulk/go/index.jsp) using the Affymetrix results. Up-regulated genes are shown in red and down-regulated genes are shown in green.

Table S1. Primers used for qRT-PCR expression analysis.

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