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Boron deficiency inhibits root growth by controlling meristem activity under cytokinin regulation

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ABSTRACT

Significant advances have been made in the last years trying to identify regulatory pathways that control plant responses to boron (B) deficiency. Still, there is a lack of a deep understanding of how they act regulating growth and development under B limiting conditions. Here, we analyzed the impact of B deficit on cell division leading to root apical meristem (RAM) disorganization. Our results reveal that inhibition of cell proliferation under the regulatory control of cytokinins (CKs) is an early event contributing to root growth arrest under B deficiency. An early recovery of *QC46:GUS* expression after transferring B-deficient seedlings to control conditions revealed a role of B in the maintenance of QC identity whose loss under deficiency occurred at later stages of the stress. Additionally, the D-type cyclin *CYCD3* overexpressor and triple mutant *cycd3;1-3* were used to evaluate the effect on mitosis inhibition at the G1-S boundary. Overall, this study supports the hypothesis that meristem activity is inhibited by B deficiency at early stages of the stress as it does cell elongation. Likewise, distinct regulatory mechanisms seem to take place depending on the severity of the stress. The results presented here are key to better understand early signaling responses under B deficiency.

1. Introduction

Boron (B) is an essential micronutrient for vascular plants limiting crop productivity worldwide [1,2]. The tolerance of plants to B deficiency varies significantly depending on the plant species, genotype, and growing conditions [3] and relies, at least partially, on the capacity of B (as boric acid (H_3BO_3) or borate (BO_4^-)) to form complexes with polyhydroxyl compounds such as the apiose residues present in the rhamnogalacturonan II polymers of the cell wall [4,5] and on the abundance of these cell wall complexes which greatly varies among plant species [6]. Besides the demonstrated structural role of B as an assembly element in the cell wall, many biological functions of B in plants have been proposed including membrane stabilization [7–10] and the functioning of plant cell metabolism [11–14]. However, there is still a very limited knowledge of the signaling and regulatory mechanisms that act controlling B stress response in plants.

In plants, B deficiency symptoms include a drastic shortening of primary root growth and an altered root morphology accompanied by an increased number of root hairs (*hairy* phenotype), root swelling and the disorganization of the root apical meristem (RAM) [15]. The *Arabidopsis thaliana* RAM is the source of undifferentiated cells that leads and sustains root growth [16]. The maintenance of an organized RAM is necessary to ensure an appropriate root growth and development and depends on the fine tune regulation of the balance between cell division and differentiation rates which are controlled by hormone signaling and transcriptional networks that consequently act directing growth [17–21]. Despite the importance of RAM contributing to root growth providing newly dividing cells, previous studies performed in *Arabidopsis* have been exclusively associated the root growth inhibition observed under B deficiency with defects in cell elongation discarding a primary role of cell division in this response [22].

Phenotypical and molecular evidences suggests that ethylene is involved in the response of the primary root to short term B deprivation [23]. The phenotypic similarities found between 1-aminocyclopropane-1-carboxylic acid (ACC) treated plants and B deficient plants suggested that ethylene could be part of the B-deficiency response [24]. This hypothesis was later supported by the increased expression of ethylene GUS reporter lines (*EBS:GUS* and *ACS11:GUS*) under B deficiency

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together with the improved B deficiency tolerance shown by the *ethylene insensitive (ein2-1)* mutant or by *Arabidopsis* plants treated with ethylene antagonists [23]. Additionally, the point mutation located in the *CONSTITUTIVE TRIPLE RESPONSE 1 (CTR1)* gene, a negative regulator of ethylene response, was associated with a high B requirement to recover root growth [25].

Auxins are involved in B stress response regulation. In *Arabidopsis*, the use of the reporter line *DR5:GUS* showed a larger auxin accumulation under B deficiency [24]. Other authors have shown similar auxin responses [26]. Using the auxin sensor *DII-VENUS* [27], Li and coworkers found an increased auxin content in *Arabidopsis* B deficient meristems. This response was concomitant with the reduced accumulation of PIN1-GFP in B deficient plants and was later supported by the results of Li et al. that claimed defects in IAA synthesis and transport in citrus roots subjected to B deficiency [28]. Further studies indicated that the auxin transport inhibitor PEO-IAA rescued defects in cell elongation linking auxin and ethylene cascades under B deficiency [22].

Cytokinins (CKs), together with auxin and ethylene, may also act regulating B stress response in plants. Although their contribution has been little explored, the altered expression patterns of the *Arabidopsis CRE1/WOL/AHK4* and the *Citrus WOL* and *ARR12* (encoding proteins involved in CK signal transduction) under B deficiency suggest a role of this hormone in B stress that needs to be further explored [29,30].

To better understand the mechanisms mediating root growth inhibition under B deficiency and to identify signaling pathways mediating B stress response in plants, we have analyzed the effects of B deficiency on root cell division by characterizing the response of the *Arabidopsis pCYCB1:GUS* reporter line to a B gradient. The use of different reporter lines of quiescent center (QC) identity (*QC46:GUS*), CK signaling (*ARR5:GUS* and *TSCn:GFP*) and cell proliferation control (*pCCS52A2:GUS*) has allowed to stablish 1) that the loss of QC identity not preceds cell division inhibition under B deficiency and the identity is recovered early on time after B-deficient seedlings are transfer to control conditions, 2) that cell division and elongation are inhibited in a coordinated and simultaneous way contributing both to root growth arrest and 3) to detect rapid changes in CK-signaling pathways in response to B deficiency. Using the *Arabidopsis pCYCB1:GUS* line under a CK agonist and antagonist treatments and mutants in the CK signaling cascade (such as *ahk2/3* or *arr1arr10arr12*) further probed the CK regulatory mechanisms mediating cell division inhibition at the RAM in response to B deficiency. The effects of B deficiency on mitosis at the G1-S boundary through changes in *CYCD3* driven by CKs were analyzed using transgenic *Arabidopsis* plants overexpressing the D-type cyclin *CYCD3* and the *cycd3;1-3* triple mutant.

2. Materials and methods

2.1. Plant material

The *Arabidopsis thaliana* ecotype Columbia (Col-0) seedlings were used for root and meristem size measurements and qPCR analysis. Histochemical analysis were performed using different β-glucuronidase (GUS) reporter lines, including: *pCYCB1.1:Dbox-GUS* [31], *QC46:GUS* [32], *ARR5:GUS* [33] and *pCCS52A2:GUS* [34]. *TSCn:GFP* was used as a CK signaling reporter line [35].

Analysis of primary root growth and RAM was also performed in CK mutants including the double mutant *arr10-5/arr12-1*, the triple mutant *arr1-3/arr10-5/arr12-1*, *cre1-12/wol/AHK4* (obtained from the Nottingham Arabidopsis Stock Centre (NASC)), *ahk2-2/3-3* [36], the triple mutant *cycd3;1-3* [37] and also in the line constitutively expressing the D-type cyclin *CYCD3* under a CaMV 35S promoter (35S) (*CYCD3 OE*) [38].

2.2. Growth conditions

Seeds were surface-sterilized using EtOH 96% for 2 min, washed in distilled water and finally stratified at 4 °C for 24 h in the dark prior to each experiment. Stratified seeds were then transferred to a controlled growth chamber set at 22 °C with a 16 h: 8 h, light: dark photoperiod (with a light intensity of 120 μmol $m^{-2} s^{-1}$).

Seedlings were grown vertically on half-strength Murashige and Skoog (MS) medium [39] containing agarose (SERVA, Germany) 0.7% (w/v). In order to achieve B deficient conditions, B was first removed from the media using the B-binding specific resin Amberlite® IRA7-43 (Sigma) [40]. B, as H_3BO_3, was added into the media to a final concentration of 0.03 μM (low B conditions), 30 μM (B control conditions) or not added to reach severe B deficiency. In direct growth experiments, seedlings were grown in plates for 3 and 5 days after germination (3 DPG and 5 DPG, respectively). In transfer experiments, 5 days-old seedlings (5 DPG) were transferred to the appropriate media and measurements were taken 4 h post-transfer (HPT), 24 HPT and 48 HPT.

2.3. Root length and meristem size analysis

For primary root length measurements, plates were scanned and the resulting images were analyzed using the ImageJ software available online (https://imagej.nih.gov). Root length was measured from the root tip to the hypocotyl boundary.

Root meristem size was measured following the protocol described by Perilli & Sabatini, [41]. Images were taken with LAS v3.7 software (Leica DFC300 FX camera) using an Olympus BH2-RFCA optical microscope and analyzed using ImageJ software.

2.4. Plant hormone treatments

For the CK/CK inhibitor treatments the treatments applied were: 5 μM t-zeatin as the CK agonist or 10 μM S-4893 (phenylquinazoline compound, [42]) as a CK reception inhibitor. Chemicals were added to the media before solidification.

2.5. Detection of β-glucuronidase (GUS) activity

Histochemical analysis of β-glucuronidase (GUS) activity in reporter lines of *Arabidopsis* was performed as previously described [16]. Glycerol 80% 4 °C was used to stop the reaction and plants were stored under dark conditions at 4 °C till they were visualized.

GUS signal detection was performed by using an optical microscope Olympus BH2-RFCA. Images were obtained using LAS v3.7 software (Leica DFC300 FX camera) and analyzed using ImageJ software.

2.6. Lugol staining

The roots were firstly fixed in ethanol: acetic acid (3:1) and then submerged in 1% Lugol solution for 30 s. The roots were then washed in water and mounted onto microscopic slides using clearing buffer (chloral hydrate:glycerol:water in 8:3:1 ratio). Lugol stained seedlings were visualized using an optical microscope Olympus BX63.

2.7. RNA extraction and qRT-PCR

Root meristems of wild type *Arabidopsis* (2 mm from the RAM in pools of 30–60 seedlings) were collected and immediately frozen in liquid nitrogen at 3 and 5 days post-germination (DPG) to analyze the expression patterns of *arr1*, *cycb1.1* and *cycd3* genes. Total RNA was extracted using the RNeasy mini kit (Qiagen). RNA quality was determined using Nanodrop ND-1000. cDNA was synthesized from 0.5 μg total RNA using the QuantiTect Reverse Transcription kit (Qiagen). qRT-PCRs were performed with gene-specific primers (Supporting information Table S1) using SYBR Green Master mix (Roche) on a Techne Quantica apparatus. The $2^{-\Delta\Delta Ct}$ method [43] was used to normalize and calibrate transcript values relative to the endogenous *TIP41—like family protein* (At4G34270) gene.

2.8. Confocal microscopy

The reporter line *TSCn:GFP* was visualized and stack images (9.99 µm Z stepsize) were obtained in a Leica TCS SP5 confocal microscope using an Ar excitation laser (488 nm) with the emission maxima in the range 490–550 (520 max).

2.9. Statistical analysis

Each experiment was repeated, at least, 3 times. In each experiment, 3 plates containing 30–40 seedlings were used. Histochemical analysis were performed using an average of 20 seedlings (n = 15-35). The JMP® (ver.11.0) statistical package (SAS Institute) and the Free Software R (R-3.4.3; ScottKnott package [44]) were used for the statistical analyses. ANOVA was employed to test the effect of different treatments. LSMeans Tukey, ScottKnott or Student's *t* tests were used to compare means at a probability level of 5%. Levels of significance are represented by letters or asterisks in the graphs.

3. Results

3.1. B deficiency alters root growth modulating meristem activity

Primary root growth was monitored in *Arabidopsis* seedlings subjected to B deficiency at 3 and 5 days post-germination (DPG). Differences were found between severe deficiency (-B) and low B conditions (0.03 µM H_3BO_3) (Fig. 1). Under severe B deficiency, primary root growth was drastically inhibited at 3 DPG (approximately 80% inhibition compared to control conditions) and no additional primary root growth was detected 5 DPG (Fig. 1b). At low B conditions, primary root growth was reduced at 3 and 5 DPG in a time dependent manner (Fig. 1a and b).

Root meristem size was measured as the root proliferating zone where division is the process responsible for root growth [41]. As previously described, under control conditions (30 µM H_3BO_3) it was observed that, although primary root growth predominantly ocurred at 5 DPG, the largest meristem growth increment occurred at 3 DPG (Fig. 1c), when division has the highest rate of influence in primary root growth [16]. At low B concentrations, root meristem growth was mainly inhibited at 3 DPG whereas under severe B deficiency, the length of the root meristem was reduced from 3 to 5 DPG (showing a strong reduction of more than 80% at 5 DPG) (Fig. 1c).

Transfer experiments were carried out to assess early responses associated with B deficiency in primary root growth (Fig. 2a). When 5-day-old seedlings were transferred from control conditions to severe B deficient media, growth inhibition was observed at 24 HPT. No recovery was observed in primary root length within the first 48 HPT when seedlings were transferred from severe B deficiency or low B to control conditions.

RAM length was measured to evaluate meristem maintenance in transfer experiments. Meristem length inhibition was observed as early as 4 HPT when seedlings were transferred from control to severe B deficiency (Fig. 2b). No recovery was observed when seedlings were transferred from severe B deficiency to control conditions (probably due to meristem exhaustion) but seedlings transferred from low B to control conditions were able to partially recover meristem growth at 24 HPT (Fig. 2b).

3.2. Cell division is an early target of B deficiency stress response

Mitotic activity was evaluated visualizing GUS activity using the reporter *Arabidopsis* line *pCYCB1.1:Dbox-GUS (pCYCB1:GUS)*. *Arabidopsis pCYCB1:GUS* seedlings showed a decrease of the mitotic activity under B deficiency at 3 and 5 DPG (Fig. 3). At 3 DPG the inhibition of the mitotic activity was similar under low and severe B deficiency conditions, however, at 5 DPG the mitotic activity almost disappeared under severe B deficiency (Fig. 3b).

Relative expression of *CYCB1.1* (At4G37490) was quantified by using qPCR analysis. A down-regulation of *CYCB1.1* under B deficiency (low B or severe B deficiency) was observed at 5 DPG while no changes at gene expression level were detected at 3 DPG (Fig. 3c).

In order to relate the early growth inhibition of root meristems and primary root growth with the maintenance of the mitotic activity under B deficiency, GUS activity analysis of *pCYCB1:GUS* was performed in transfer experiments that combined three B treatments (Fig. 4). While no recovery was observed in the mitotic activity of seedlings transferred from severe B deficiency to control conditions, a recovery in cell division occurred rapidly (4 HPT), when seedlings were transferred from low B conditions to control media (Fig. 4b). A steep decrease in mitotic activity was detected when seedlings were transferred from control to severe B deficiency at 4 HPT or to low B at 24 HPT (Fig. 4b and Table S2).

To temporally compare the defects observed on the mitotic activity and on root cell elongation associated with B deficiency under our experimental conditions, we determined the length of the first epidermal cell with visible root hair bulge (LEH) as a parameter to evaluate root elongation as previously described [45]. No differences were found in LEH between seedlings transferred to severe B deficiency and those transferred to control conditions at 4 HPT (Fig. S1). However, a significant inhibition of LEH was detected 24 HPT in seedlings transferred to severe B deficient conditions (Fig. S1), coinciding in time with cell division inhibition (Fig. 4b).

3.3. Early recovery of a Quiescent Center (QC) reporter line expression reveals a role of B in the maintenance of QC identity

The Quiescent Center (QC) of the RAM ensures post-embryonic root growth providing stem cells that then will divide and elongate/differentiate, leading to root growth [46,47]. The failure in root meristem maintenance observed under B deficiency could be related to a rapid cell differentiation of stem cell daughters or to the lack of QC activity. In order to test this hypothesis, the *Arabidopsis* QC reporter line *QC46:GUS* was used in direct growth (3 and 5 DPG) and transfer experiments using two different B conditions: severe B deficiency (-B) and control conditions (30 µM) (Fig. 5). Three and 5 days-old seedlings growing under severe B deficiency showed a reduced GUS expression of the quiescent center (QC) marker *QC46* (Fig. 5a).

Aiming to analyze short term effects of B on QC identity, transfer experiments were performed. QC signal was maintained 48 HPT after transferring the seedlings to a B deficient media. On the contrary, seedlings growing under B deficiency for 5 DPG and later transferred to control media, were able to rapidly recover QC signal as soon as 24 HPT (Fig. 5c).

To further determine if B can affect stem cell differentiation we analyzed Lugol-stained root tips and starch granules present in the columella cells. The results revealed that seedlings growing under B deficiency reduced the number of starch-containing elongated columella cells (three compared to four found under B control conditions) supporting that B deficiency influences in the maintenance of stem cell fate (Fig. 5b and d).

CCS52A2, an activator of the anaphase promoting complex/cyclosome (APC/C), plays an essential role controlling the identity and maintenance of the QC and stem cells, respectively, favoring low mitotic state in certain cell types of the root meristem [34]. To assess whether the reduction in meristem size could be caused by changes in QC activity we examine the effects of B nutrition on *CCS52A2* expression by GUS expression analysis. As shown in Fig. S2, a characteristic GUS signal was localized in the columella and QC cells of *Arabidopsis* roots growing under control conditions (Fig. S2a). On the contrary, when seedlings were growing under severe B deficiency, *pCCS52A2:GUS* expression appeared mislocalized along the root with a higher GUS signal localized in the vascular tissue of the root maturation

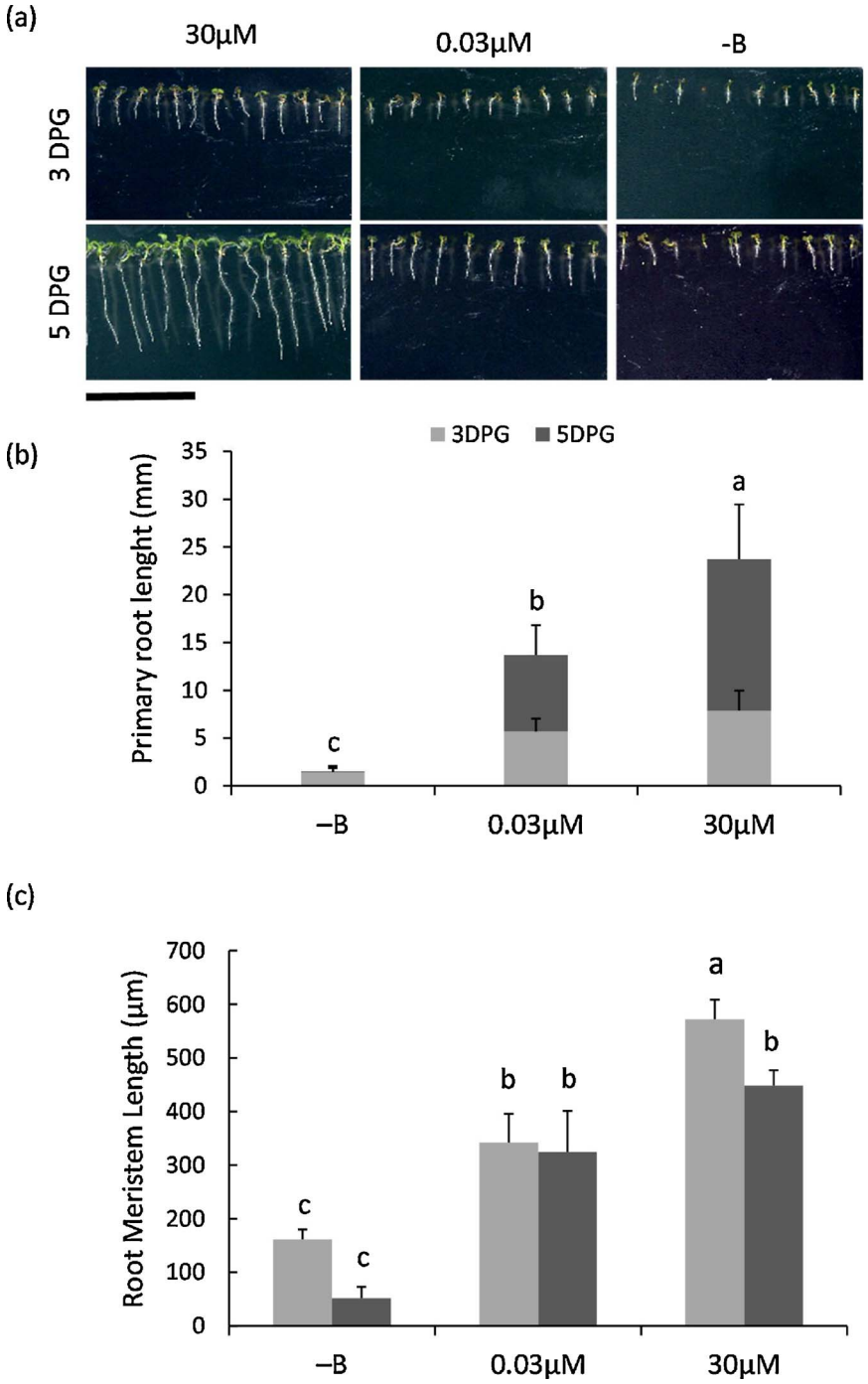

Fig. 1. *Arabidopsis thaliana* Col-0 seedling phenotype growing under B deficiency. (a) Phenotype of 3-day-old and 5-day-old wild-type Col-0 seedlings growing in a B gradient. From left to right: control conditions (30 µM), low B (0.03 µM) and absence of B (-B). (b) Primary root length (mm) and (c) root meristem size (µm) of *Arabidopsis* seedlings after 3 and 5 days post germination (DPG) growing in a B gradient (-B, 0.03 µM and 30 µM). Values shown in (b) and (c) represent the mean of 30 seedlings ± StdDev. Different letters indicate statistical differences at $P < 0.05$ (Tukey *t*-test). Scale bar = 2 cm.

zone and a decrease in the GUS intensity in the apical meristem indicating that the mitotic stage of QC and stem cells could be altered in response to B deprivation (Fig. S2b).

3.4. Cytokinin treatment led to the increase of root meristem size by promoting cell division under severe B deficiency

The role of CKs in the control of root meristem growth and cell division under B deficiency was first evaluated carrying out experiments applying hormone or hormone inhibitor treatments using 5-day-old *pCYCB1:GUS Arabidopsis* seedlings.

CKs might play relevant roles in the regulation of plant response to B deficiency [29]. *t*-Zeatin, used as CK treatment, caused a significant increment of the mitotic activity of *Arabidopsis* RAM transferred from control conditions to severe B deficiency (Fig. 6b). This effect that was concomitant with the increase of root meristem size (Fig. 6c). On the contrary, under this B condition, the application of S-4893 (as CK inhibitor) was not associated with changes in GUS activity but resulted still in the increase of root meristem size at 48 HPT (Fig. 6c).

When seedlings were transferred to control or low B conditions combined with CK treatment, a reduction in the mitotic activity of *pCYCB1:GUS* reporter line was observed (Fig. 6b). In addition, this

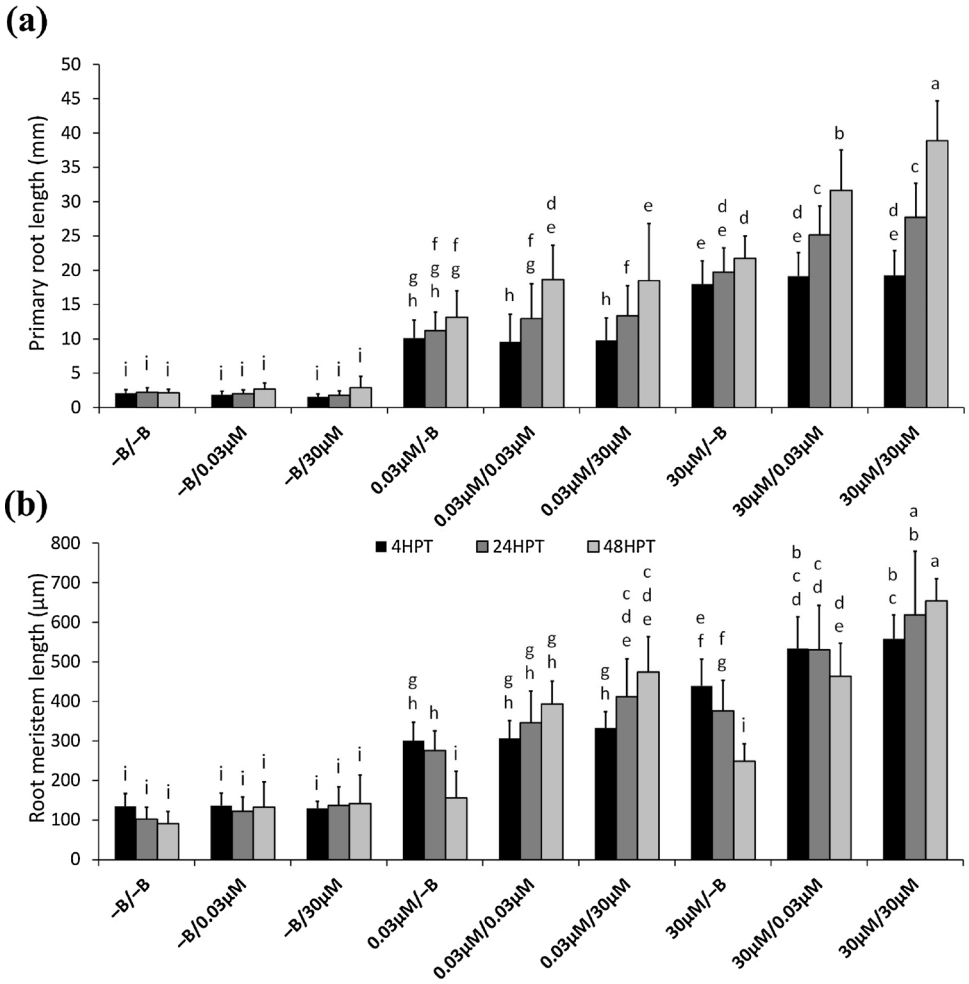

Fig. 2. Short-term effects of B deficiency on primary root growth and meristem size of *Arabidopsis thaliana* wild-type Col-0 seedlings. Transfer experiments using a B gradient (absence of B (-B), low B (0.03 µM) and control conditions (30 µM)) were performed to calculate (a) primary root length (mm) and (b) root apical meristem length (µm) using 5-day-old wild-type Col-0 (WT) seedlings after 4 h post transfer (HPT), 24HPT and 48HPT. Root/meristem length values show total length of seedlings at different time after transfer. Values represent the mean of 30 seedlings ± StdDev. Different letters indicate statistical differences at $P < 0.05$ (Tukey *t*-test).

inhibition was well correlated with a decrease in root meristem size (Fig. 6c). The CK inhibitor S-4893 resulted as well in the inhibition of *pCYCB1:GUS* activity, although no significant differences were observed in RAM length (Fig. 6c).

3.5. CK signaling is altered under B deficiency

The *Arabidopsis* reporter line *ARR5:GUS* was used in order to report changes in CK signaling in seedlings subjected to B deficiency (Fig. 7). An increased GUS signal appeared localized in the root vascular cylinder and root meristematic zone 5 DPG when seedlings were grown under B deficiency (low B or severe B deficiency) (Fig. 7a) with a higher intensity localized in the meristematic zone under severe B deficiency. Increments of GUS activity appear soon after transferring the seedlings from control conditions to B deficient media (at 4 HPT). The transfer to B deficiency led to changes in GUS activity that increased progressively and was localized extended along the root at 48 HPT in seedlings growing under severe B deficiency. Seedlings initially growing under B deficiency (low or severe B deficiency) were able to restore GUS signal to control conditions at 48 HPT, with an early reduced signal detected as soon as 4HPT. The *hairy* phenotype and the abnormal root meristem thickening that appear as morphological and cellular symptoms of B deficiency, accompanied GUS increments and disappeared when the GUS activity was reduced (Fig. 7b).

A complementary analysis was performed as well using the reporter line *TSCn:GFP* [35], which showed a very similar pattern to what was observed in *ARR5:GUS* seedlings (Fig. S3). When *TSCn:GFP* seedlings were transferred from control conditions to B deficiency, a rapid increase of GFP signal in the RAM was detected. Moreover, the root extended GFP signal observed in B deficient seedlings was diminished progressively along the root when seedlings were transferred from B deficient media to control conditions (Fig. S3).

To further analyze the implication of CKs in B deficiency stress response, meristem size and primary root length of mutants affected in CK perception and signaling were determined at 3 and 5 DPG when growing under a B gradient (Figs. 8 and S4). Increments in meristem size of seedlings growing under low B conditions were observed in meristems of all the mutants tested at 3 DPG (Fig. 8a). Under severe B deficiency, only the CK-signaling mutants *arr1/arr12* and *arr1/arr10/arr12* showed meristem size increments at 3 DPG. At 5 DPG, the mutants affected in CK perception (*wol* and *ahk2/3*) showed an increased meristem size compared to Wt under all the conditions tested. Under severe deficiency (at 5 DPG) the double mutant *arr1/arr12* presented longer meristems when compared to Wt meristems (Fig. 8b). Positive, negative or no correlation were found between meristem size and primary root length (Fig. S4).

3.6. Effect on mitosis inhibition at the G1-S boundary

The use of *Arabidopsis* plants overexpressing the D-type cyclin

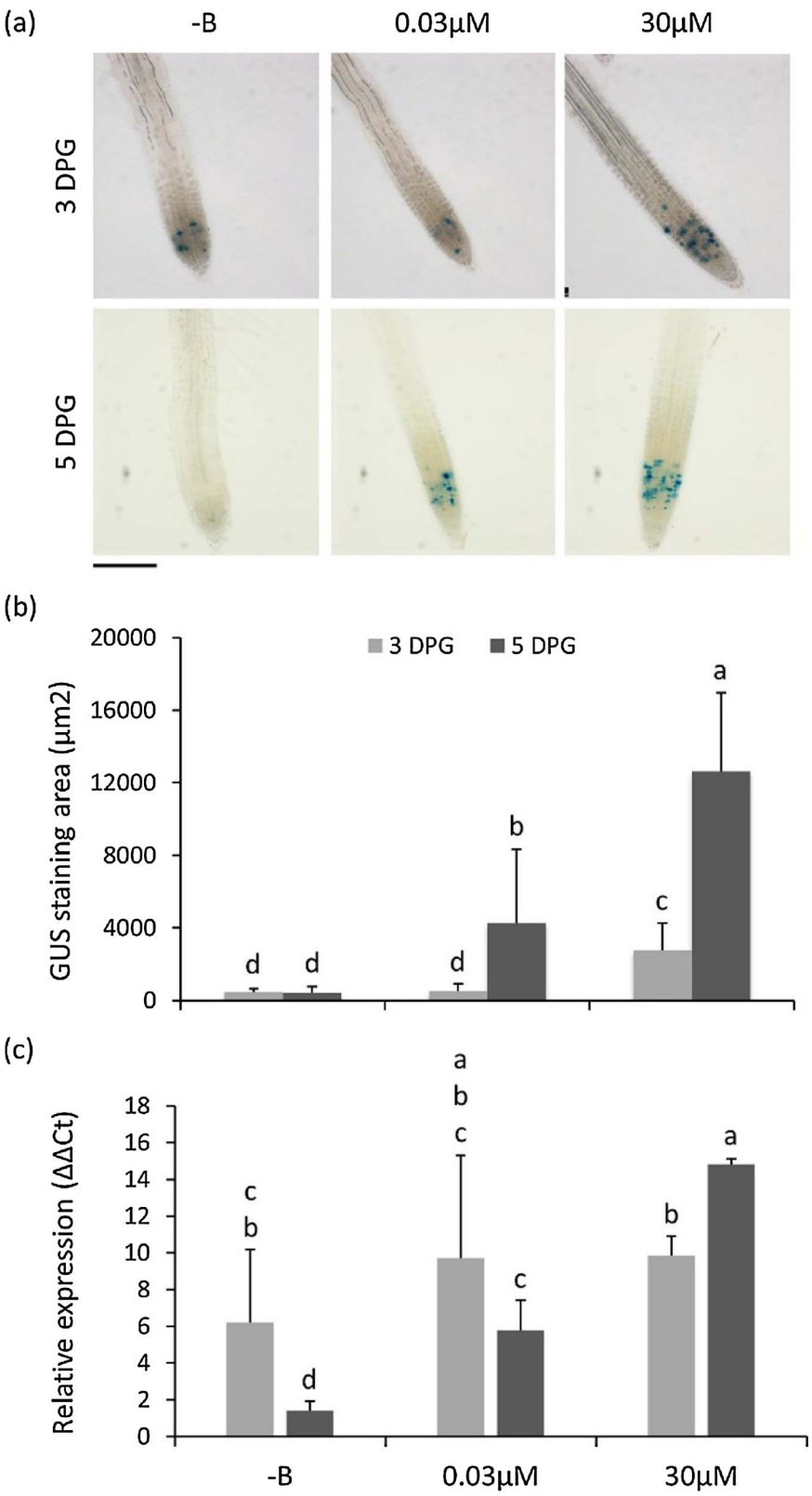

Fig. 3. Effects of B deficiency on the mitotic activity of *Arabidopsis thaliana* roots. Images show β-glucuronidase (GUS) activity of *pCYCB1:GUS* reporter *A. thaliana* line growing in a B gradient. Images shown are representative of at least 30 seedlings analysed from three different B conditions: absence of B (-B), low B (0.03 µM) and control conditions (30 µM), (a) 3 days post germination, 3 DPG, and 5 days post germination, 5 DPG. (b)Activity of the reporter gene was calculated as the blue stained area that appear at the proximal meristem zone at 3 DPG (light grey columns) and 5 DPG (dark grey columns). Values shown in (b) represent the mean of 30 seedlings ± StdDev. (c) Relative expression of *cycb1.1* (AT4G37490) in *A. thaliana* root meristems of 3- and 5-day-old seedlings growing under B deficiency (-B), low B (0.03 µM) and B control conditions (30 µM). RNA was extracted from root meristems collected from seedlings 3 (light grey columns) or 5 days (dark grey columns) post germination (DPG). Values represent the mean of 3 pools of at least 35 root meristems ± StdDev. Different letters indicate statistical differences at $P < 0.05$ (Tukey *t*-test). Scale bar = 200 µm.

CYCD3 and the *cycd3;1-3* triple mutant aimed to assess, on one hand, if B deficiency responses were associated with an effect on cell division at the G1-S transition and also to analyze if the changes observed in CK signaling were causing cell division inhibition through changes in *CYCD3*. As shown in Fig. 9, increments in meristem size appeared in the *cycd3* triple mutant when plants were subjected to severe B deficiency. Meristem size remained unchanged in plants overexpressing *CYCD3* (*CYCD3-OE*) under both control and severe B deficiency. While a decrease in primary root length of these lines compared to Wt was detected under control conditions, no changes were detected in the root length of plants subjected to severe B deficiency (Fig. S6).

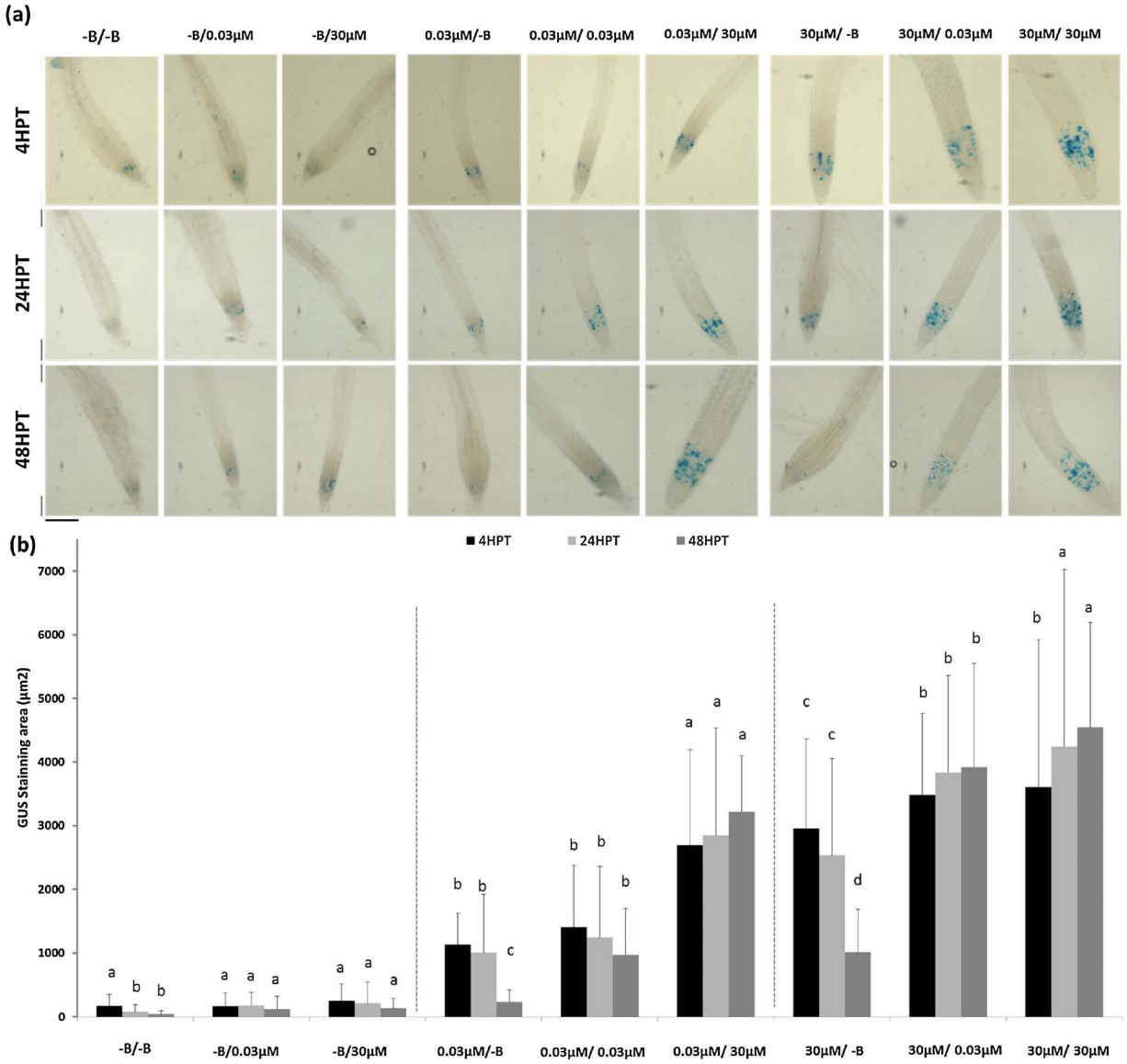

Fig. 4. Short-term effects of B deficiency on the mitotic activity of *Arabidopsis thaliana* roots. GUS staining patterns of *pCYCB1:GUS A. thaliana* 5-day-old seedlings in transfer experiments under a B gradient 4 h post transfer (HPT), 24HPT and 48HPT. Transfer experiments were performed combining three different B conditions: absence of B (-B), low B (0.03 µM) and control conditions (30 µM). Upper labels indicate the previous growing condition and the condition after transference (i.e. −B/30 µM from absence of B to control conditions). (a) Representative images of GUS staining area for each transfer condition. (b) GUS staining area quantification where values represent the mean of 15–20 seedlings ± StdDev. Statistics were performed per group (separated by dashed lines in the graph) considering the final media and the time post transfer. Different letters indicate statistical differences at $P < 0.05$ (ScottKnott test). Scale bar = 200 µm.

4. Discussion

B deficiency causes root growth inhibition accompanied by an altered root system architecture [24,28,29,48]. Still, the sensing and signaling pathways controlling plant response to B stress are not yet well characterized nor are fully understood. The growth arrest and altered development observed in *Arabidopsis* roots under B deprivation have been related to defects in cell elongation due to the role of B in cell wall assembly [4,22,23]. However, cell division inhibition may act parallel to elongation contributing to root growth arrest in *Arabidopsis* as has been claimed for different plant species [49–51].

4.1. Boron regulates cell proliferation in Arabidopsis contributing to root growth inhibition under deficiency

When analyzing B deficiency responses it is important to consider the exceptional B requirement in developing tissues and organogenesis processes, which could be related with the role of this micronutrient in the meristem maintenance [26,52,53]. RAM growth inhibition (measured as total meristem size reduction) under two different B deficient conditions (severe B deficiency and low B conditions) was evaluated. RAM growth impairment was detected earlier on time than primary root growth arrest under B scarcity, suggesting that the inhibition of root meristem growth (as a source of newly dividing cells) precedes and contributes to primary root growth inhibition under B starvation. These results would support recent findings that have shown that maximum B contents are located at the root tip (coinciding with the QC and cell division zone) while lower amounts of B are found in the elongated/differentiated zones of the root [53] and would fit as well with the notion that higher B contents are required for the growth of newly formed tissues. Alternatively, these findings might merely be a consequence of differences in the abundance of B binding ligands [6].

According to Perilli et al., the *Arabidopsis* RAM could be divided into four different parts: the stem cell niche, the proximal meristem, the

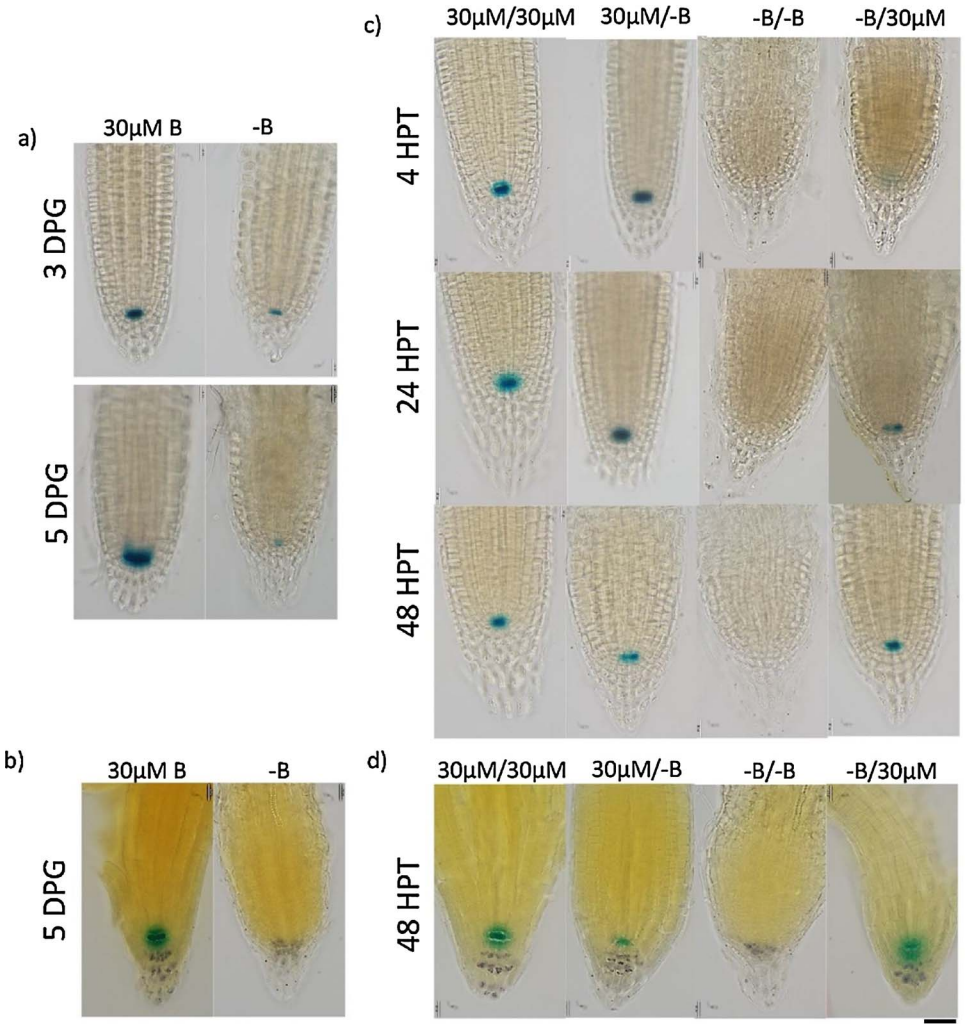

Fig. 5. Quiescent center (QC) identity under B deficiency. B deficiency effect on QC identity maintenance was evaluated based on the expression analysis of the QC-specific marker *QC46:GUS* in *A. thaliana* seedlings grown under control (30 µM) and B deficiency (-B) conditions at (a) 3 and 5 days post germination (3 DPG and 5 DPG) and (b) by analyzing the double labelling of QC and differentiated columella cells (Lugol staining). (c) *QC46:GUS* expression was also examined in transfer experiments using 5-day old seedlings at 4 h post transfer (HPT), 24 HPT and 48 HPT and (d) double QC and Lugol staining was used in seedlings 48HPT. Transfer experiments were performed combining two different B conditions: absence of B (-B) and control conditions (30 µM). Labels in the Figure are indicating previous condition and the condition after transfer (i.e. −B/30 µM from absence of B to control conditions). Representative images are shown among n = 15–20 for each condition. Scale bar = 40 µm.

transition zone and the elongation/differentiation zone. Root growth is supported by a balanced rate between cell division and cell elongation occurring at the proximal meristem and elongation/differentiation zones, respectively [16,54]. In order to determine how B interferes in these processes and to define the developmental basis of plant response (s) to B deficiency, cell proliferation was monitored. Our results showed that cell division inhibition occurs rapidly under B deficiency. Interestingly, despite an inhibition of cell division as early as 3 DPG, gene expression of *cycb1* transcript remained unchanged till 5 DPG, suggesting that posttranscriptional regulation may be the early target to control cell proliferation under B deficiency.

A reduction of cell division (accompanying the decrease in meristem size and primary root growth arrest) was especially noticeable when seedlings were transferred to severe B deficiency and appeared as soon as 4 HPT, preceding in time cell elongation inhibition observed under our experimental conditions at 24 HPT (Fig. 4 and Fig. S1). Supporting what was previously suggested by Abreu and coworkers [29], this result would confirm that B deprivation would prevent primary root growth by the simultaneous inhibition of division and elongation contrary to what has previously reported in *Arabidopsis* [22,24]. Even moe, based on our data, severe B defiency may lead to cell division inhibition before elongation is affected. Nevertheless, it is important to highlight that different experimental conditions may lead to differences in the response observed between this and previous works. While in the present study no B or low B at 0.03 µM H_3BO_3 concentration were used, previous studies applied higher H_3BO_3 concentrations (0.4 µM) to achieve deficiency [24].

4.2. B influences the maintenance and rapid recovery of QC identity

Quiescent center (QC) maintenance is needed to ensure a proper RAM structure and spatial organization to sustain root growth after germination [18,47]. A role of the QC as an environmental sensor controlling growth has been previously proposed. For example, phosphate starvation causes changes in QC activity that ends modulating cell division and differentiation [55]. To determine if B deficiency affects meristem activity by altering QC identity, we used the *QC46:GUS* reporter line growing under a B gradient in direct growth and transfer experiments. We observed that *QC46:GUS* signal diminished at 3 and 5 DPG under B deficiency. However, in transfer experiments from control to B deficient conditions did not reveal a shutdown of the signal 48 HPT indicating that QC loss was not an early response associated to B deficiency confirming that cell division inhibition in the meristematic zone preceded this event. This effect of QC maintenance could be related to the role of the QC sustaining growth, ensuring nonexhaustion of stem cells under stress conditions [56].

On one hand, QC fate depends on the local increment of auxins mediated by PIN1 efflux transporters [32]. On the other, B deficiency causes reduced PIN1 protein accumulation resulting in an altered auxin transport and distribution [28,26]. The effect on QC identity observed under B deficiency might then be a consequence of the disturbance of the auxin homestasis. Also, *CCS52A2* has been implicated in QC maintenance by inactivating mitosis. The addition of the polar auxin transport inhibitor 1-N-naphthylphthalamic acid (NPA) or the auxin agonists 2,4-dichlorophenoxyacetic acid (2,4-D) led to an accumulation

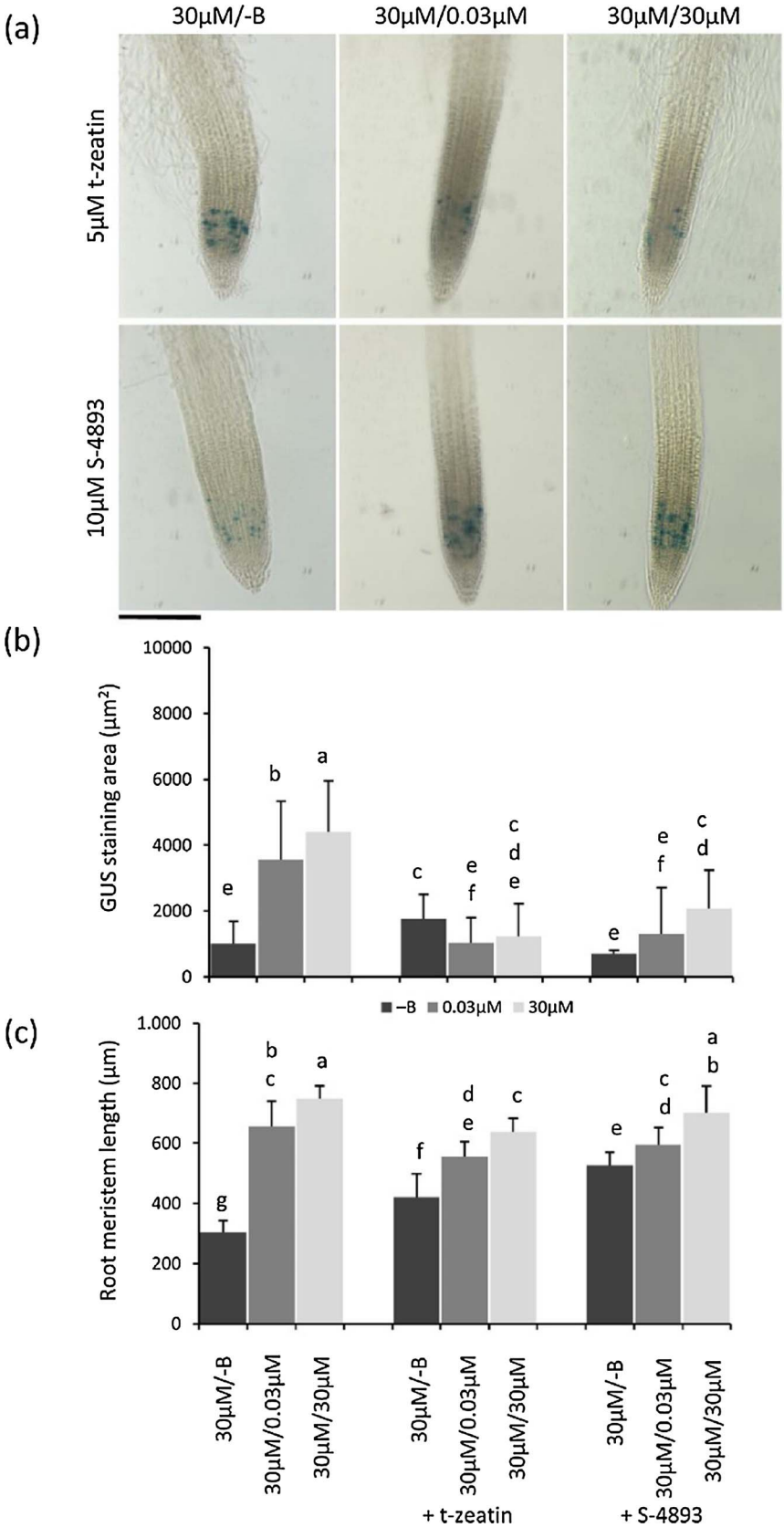

Fig. 6. Cytokinins, cell division and root meristem length under B deficiency. *pCYCB1:GUS* relative activity in root meristems and root meristem length of *Arabidopsis thaliana* cytokinin/cytokinin (CK) inhibitor-treated seedlings in transfer experiments. Transfer experiments were performed using 5-day-old seedlings growing under B control conditions (30 µM B). Seedlings were transferred to three different B conditions (control (30 µM), low B (0.03 µM) and severe B deficiency (-B)) combining three different hormone treatments (no treatment (used as control), CK treatment (5 µM t-zeatin) and a CK inhibitor treatment (10 µM S-4893)). Image pictures and measurements were taken at 48 HPT. (a) Representative images of transfer experiments (48HPT) to CK/CK inhibitor treatment; (b) Blue stained area as the *pCYCB1:GUS* relative activity and (c) root meristem lengths of Arabidopsis seedlings growing under different hormone and B treatments. Values represent the mean of 10–15 seedlings ± StdDev. Different letters indicate statistical differences at $P < 0.05$ (Tukey *t*-test). Scale bar = 200 µm.

of auxin signal spreading *pCCS52A2:GUS* signal along the meristem [34] similarly to the expression patterns observed under severe B deficiency (Fig. S2). Altogether, our findings would support the notion that B deficiency could result in a non-rapid QC identity loss through the down-regulation of *pCCS52A2* mediated by auxin accumulation.

Interestingly, a fast recovery of QC signal was achieved just 24 HPT. QC cells are infrequently divided and act regulating the differentiation of neighboring cells by complex signaling cascades driven by hormones

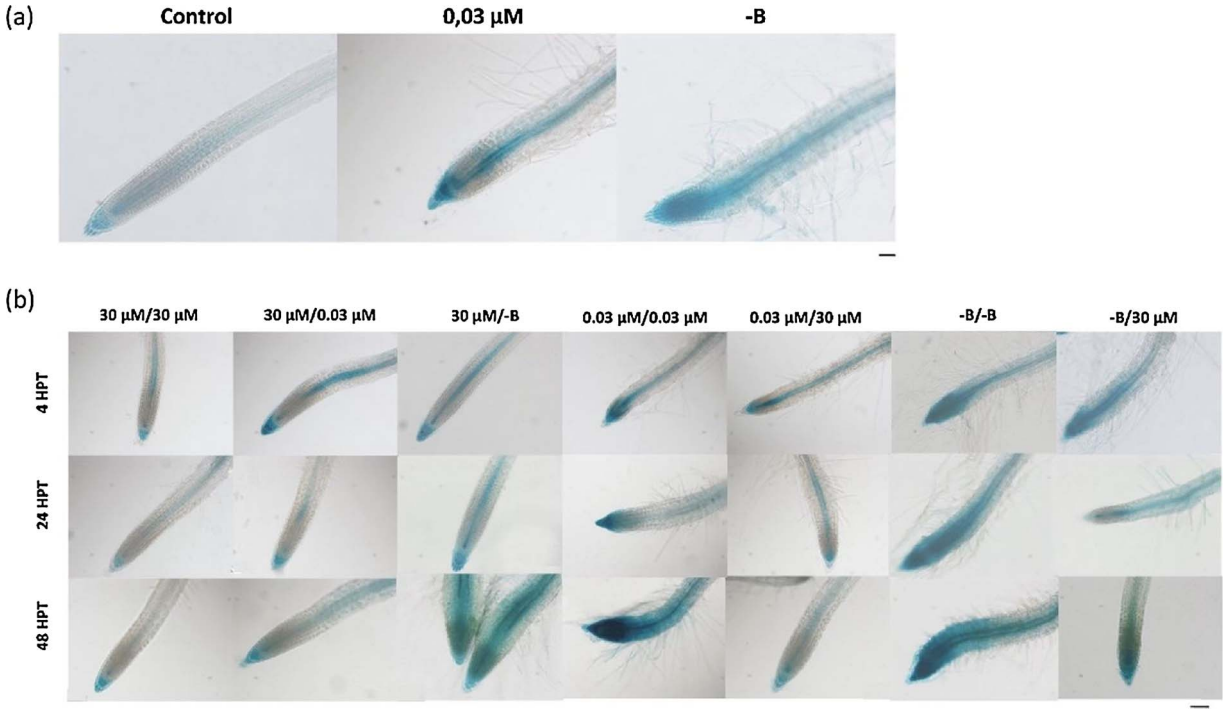

Fig. 7. Cytokinin signaling in response to B deficiency. GUS activity of *ARR5:GUS* root meristems in direct growth (a) and transfer experiments (b). (a) Direct growth assays were performed using 5-day-old seedlings growing under different boron concentrations: severe boron deficiency, −B; low boron, 0.03 µM, and control conditions, 30 µM. (b) In transfer experiments, GUS activity was determined 4, 24 and 48 h post-transfer (HPT) using 5-day-old *ARR5:GUS* seedlings. Different B conditions were used in transfer experiments as indicated by the top labels that show the condition before transfer/the condition after transfer. Scale bar in (a) = 50 µm. Scale bar in (b) = 100 µm.

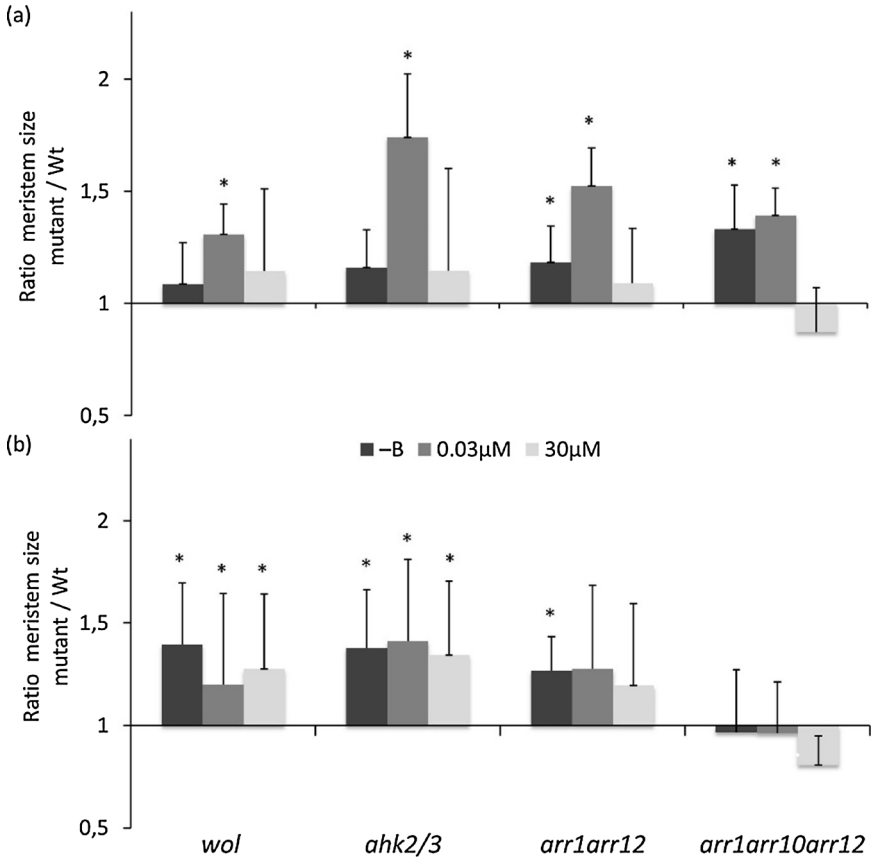

Fig. 8. Meristem size of CK-related mutants under B deficiency. The ratios of mutant meristems (µm) compared to Wt were calculated under boron deficiency (-B), low boron (0.03 µM) and control boron conditions (30 µM) at (a) 3 days-after-germination (3 DPG) and (b) 5 DPG to report changes associated with cytokinin response. CK-related mutants included mutants affected in CK perception (*wol*, *ahk2/3*) and signaling (*arr1arr12* and *arr1arr10arr12*). Columns represent the Mean of the ratios calculated from at least 15 seedlings ± StdDev (n = 15–25). Asterisks indicate statistical differences at $P < 0.05$ (Student *t*-test) comparing the meristem size of each mutant with the meristem size of Wt Col.0.

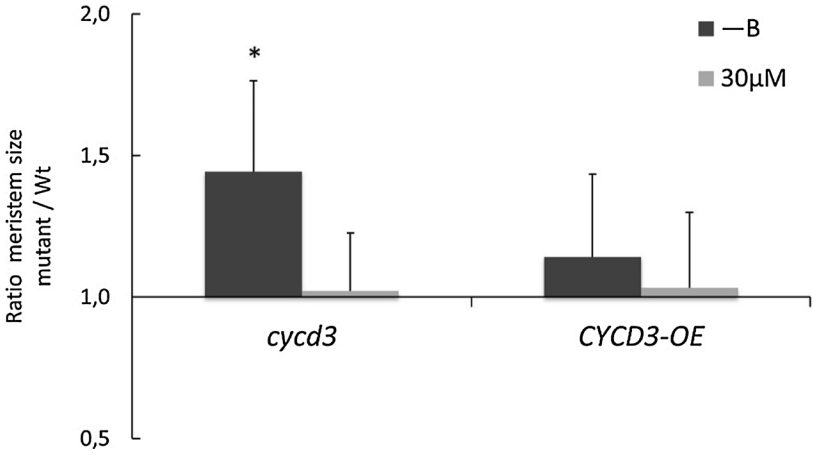

Fig. 9. Meristem size of CYCD3 mutant and overexpressor line under B deficiency. Ratios of meristem size comparing primary root meristem length of *cycd3* mutant and CYCD3 overexpressor line (OE) with Wt are presented. Meristem size was measured in both *cycd3;1-3* mutant and CYCD3-overexpressor seedlings under severe boron deficiency (-B) and control boron conditions (30 μM) at 5 DPG. Columns represent the Mean ± StdDev of the ratios of meristem size in mutant or overexpressor compared to the meristem size of Wt, of at least, 15 seedlings (n = 15–25). Asterisks indicate statistical differences at $P < 0.05$ (Student *t*-test) between Wt Col.0 *A. thaliana* and mutants or overexpressor for each condition.

or specific transcription factors [57,58]. The rapid QC restoration after B supply would ensure long-term growth after the stress episode as a prerequisite to reconstitute the pool of dividing and differentiating cells. Besides, QC recovery coincided in time with the CK signal recovery (to control conditions) observed in this study by using different CK-signaling reporter lines. Taken into consideration the function of CKs in controlling division of QC, it is suggested that this hormone is playing important roles regulating QC identity under B deficiency [59]. Nonetheless, further studies are still needed to determine the factors and implications of this fast QC recovery and the specific role of hormones regulating the QC rescue.

4.3. Changes in cytokinin homeostasis are associated with root meristem growth inhibition under B deficiency

Likewise, the maintenance of the meristematic activity of primary roots in *Arabidopsis* responds to a fine-tune regulation driven by different factors including hormones, ROS and a transcriptional regulation [16,19,60–62]. All these growth regulatory factors influence or are potentially involved in the control of plant growth and development when plants are subjected to B deficiency [22,23,26,29,63] and they are expected to act controlling the sensing, transport and mobilization of the micronutrient in the plant.

Cytokinins (CKs) influence on the control of cell division under B deficiency response regulation was assessed by using t-zeatin and the CK inhibitor S-4893 in transfer experiments of 5-day-old *pCYCB1:GUS* seedlings together with the use of the CK-signaling reporter lines *ARR5:GUS* (Fig. 7) and *TSCn:GFP* (Fig. S3). CKs are key hormones regulating meristem size establishment and the meristem maintenance during post-embryonic development [64]. Among their functions, CKs are hormones able to regulate the transition from proliferation stage to differentiation cooperating with auxins to induce endocycling events in the root transition zone [19,60]. While under our experimental conditions, control seedlings showed a characteristic response previously described under *t*-zeatin treatments showing a reduced meristem sized positively correlated with a decrease in the mitotic activity [60], the addition of *t*-zeatin to seedlings transferred to severe B deficiency led to the increase of root meristem size by promoting cell division. This result is in agreement with previous works that revealed a role of CKs in the induction of auxin synthesis in young and developing roots promoting cell proliferation [65]. Besides, the addition of CKs to B deficient roots could influence auxin redistribution leading to increments in division in a concentration dependent manner [66,67]. Significant increments of *ARR5:GUS* and *TSCn:GFP* signals were identified as early events that occur after transferring the seedlings to B deficiency (just 4 HPT). Under severe B deficiency a negative feedback driven by the type-A of ARRs (such as ARR5) may trigger CK-deficit associated effects on cell division at the RAM that are restored after CK application [68]. Furthermore, an increased root meristem size without changes in division were found after the CK inhibitor treatment under severe B deficiency suggesting that different mechanisms other than division are involved in root meristem growth inhibition (i.e. endoreduplication, [69]). Therefore, changes in CK distribution along the root under severe B deficiency might act deregulating (inhibiting) ploidity-dependent elongation as well [70,71] and the increment in the meristem size observed under severe B deficiency with the inhibitor treatment could be related with a delay in elongation/differentiation rates at the RAM [60,72].

The analysis of meristem and root growth in mutants affected in CK perception or signaling were used in this study to further determine the function(s) of this hormone in B deficiency stress response. Changes in the meristem size under B deficiency (severe deficiency or low B conditions) in CK perception (*wol* and *ahk2/3*) and signaling (*arr1/arr12* and *arr1/arr10/arr12*) mutants would support the idea of a role of CKs regulating cell division inhibition under B deficiency. Mutants affected in CK perception lead to increments in the meristem size under all conditions analyzed probably because of a general effect on the delay of elongation/differentiation rates that would favour the augment of meristem size [60]. Instead, the triple mutant *arr1/arr10/arr12* at 3 DPG showed a specific increment of the meristem only under B deficiency when compared to Wt (revealing positive effects of CKs signaling suppression under severe B deficiency) but no differences were observed at 5 DPG. The mode of action of the type-A ARRs should be considered here [68]. If they act negatively regulating CK response in Wt seedlings grown under B deficiency, the CK signaling pathway would be suppressed and no differences could be observed when compared with the triple mutant. Interestingly, the CK response and signaling repression may be playing important roles coping with the stress as previously observed in plants subjected to drought [72]. Also, differences were detected between the double (*arr1arr12*) and triple mutant (*arr1ar10arr12*) in the root meristem growth (Fig. 8). The absence of *arr1* and *arr12* but not *arr10* has somehow a positive effect on the meristematic growth under severe B deficiency. This result might point to specific roles of the type-B ARRs in B stress response what can be related to their regulatory role of auxin and ethylene signaling controlling cell proliferation and elongation at the RAM [67].

Interestingly, the changes observed in CK-signaling under B deficiency (Figs. 7 and S3) were rapidly restored after transferring the seedlings to control conditions, what was accompanied by the disappearance of the characteristic *hairy* phenotype of B deficient roots and by a root swelling decrease. The *hairy* phenotype has been shown to be associated with increments in ethylene and B deficiency [23,25]. Our results might suggest that CKs act together with ethylene leading to the increment of root hairs that characteristically appear under B deprivation and it is in agreement with recent published works that point to a role of CK (together with auxin and ethylene) in root hair formation

[73]. Besides, the effect of B deficiency on root thickening could be also related to CK increments and changes in the expression of *CYCD3* [74–76].

The *Arabidopsis* CYCD3 D-type cyclins are involved in CK response (s) and have been implicated in endocycling delay by prolonging the mitotic stage of cells [37,77]. The increased RAM size observed under severe B deficiency in the loss-of-function triple mutant *cycd3* and the unchanged RAM size in the *CYCD3 OE* together with the increased CK signaling along the root detected under severe B deficiency suggest that B deprivation induces changes in *CYCD3* expression through the alteration in CK signaling that results in a reduced RAM and primary root growth by inhibiting both endocycling and division [37]. The mutation of the three *CYCD3* cyclins would impair CK responses ameliorating the defects found at the RAM under B deficiency. Due to the important roles that play other types of D-cyclin regulating cell proliferation in roots [78], further analyses should be performed to evaluate the role of B deficiency response in cell cycle inhibition through the regulatory action of CKs.

Altogether our results supports the notion that a coordinated and simultaneous inhibition of cell division and elongation takes place under B deficiency conditions under the regulatory control of CKs and downstream pathways resulting in an altered root growth and development. Nonetheless, the fact that several authors were able to propagate cell cultures under B deficiency suggests that the effect on cell division is not primary [10,79,80]. We propose here that cell division inhibition under B deficiency is more likely a consequence of the alteration in the signaling pathways that act balancing cell division and differentiation/elongation at the RAM resulting in growth arrest. Besides, although the transfer experiments carried out in this work aimed to evaluate short-time responses to B deficiency (hours), still major changes may occur within minutes in response to B deprivation. It is yet to be elucidated then the sequence of primary and secondary events that take place when B availability is limited. A deeper analysis of how plants initially sense B availability and cell wall stress, changing hormone homeostasis and triggering different signaling pathways regulating the root developmental program is key to advance in the understanding of how plants cope with B deficiency stress.

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Author contributions

M.R., I.A.S and L.B. conceived the original screening and research plans; M.R., L.B. and I.B.M. supervised the experiments; L.P.V and M.R. performed most of the experiments; P.A. provided technical assistance to L.P.V.; M.R., L.P.V., I.A.S and M.P.G-G designed the experiments and analyzed the data; M.R. and L.B. conceived the project and wrote the article with contributions of all the authors.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.plantsci.2018.02.005.

References

[1] R.D. Graham, Micronutrient deficiencies in crops and their global significance, Micronutrient Deficiencies in Global Crop Production, Springer, Netherlands, Dordrecht, 2008, pp. 41–61, http://dx.doi.org/10.1007/978-1-4020-6860-7_2.
[2] M. Sillanpää, Micronutrients and the nutrient status of soils: a global study, In: FAO Soils Bull. CN – S592.6. T7 S538 1982, 1982, p. 444.
[3] V.M. Shorrocks, The occurrence and correction of boron deficiency, Plant Soil 193 (1997) 121–148, http://dx.doi.org/10.1023/A:1004216126069.
[4] M.A. O'Neill, T. Ishii, P. Albersheim, A.G. Darvill, Rhamnogalacturonan II: structure and function of a borate cross-linked cell wall pectic polysaccharide, Annu. Rev. Plant Biol. 55 (2004) 109–139, http://dx.doi.org/10.1146/annurev.arplant.55.031903.141750.
[5] M.A. O'Neill, S. Eberhard, P. Albersheim, A.G. Darvill, Requirement of borate cross-linking of cell wall rhamnogalacturonan II for arabidopsis growth, Science (80-.) 294 (2001).
[6] H. Hu, P.H. Brown, J.M. Labavitch, Species variability in boron requirement is correlated with cell wall pectin, J. Exp. Bot. 47 (1996) 227–232 http://citeseerx.ist.psu.edu/viewdoc/download?doi=10.1.1.1010.2080&rep=rep1&type=pdf . (Accessed 6 November 2017).
[7] L. Bolaños, K. Lukaszewski, I. Bonilla, D. Blevins, Why boron? Plant Physiol. Biochem. PPB/Société Fr. Physiol. Végétale. 42 (2004) 907–912, http://dx.doi.org/10.1016/j.plaphy.2004.11.002.
[8] M.A. Wimmer, G. Lochnit, E. Bassil, K.H. Mühling, H.E. Goldbach, Membrane-associated, boron-interacting proteins isolated by boronate affinity chromatography, Plant Cell Physiol. 50 (2009) 1292–1304, http://dx.doi.org/10.1093/pcp/pcp073.
[9] N. Ferrol, A. Belver, M. Roldan, M.P. Rodriguez-Rosales, J.P. Donaire, Effects of boron on proton transport and membrane properties of sunflower (Helianthus annuus L.) cell microsomes, Plant Physiol. 103 (1993) 763–769 http://www.ncbi.nlm.nih.gov/pubmed/12231978 . (Accessed 1 October 2016).
[10] A. Voxeur, S.C. Fry, Glycosylinositol phosphorylceramides from Rosa cell cultures are boron-bridged in the plasma membrane and form complexes with rhamnogalacturonan II, Plant J. 79 (2014) 139–149, http://dx.doi.org/10.1111/tpj.12547.
[11] D.G. Blevins, K.M. Lukaszewski, Boron in plant structure and function, Annu. Rev. Plant Physiol. Plant Mol. Biol. 49 (1998) 481–500, http://dx.doi.org/10.1146/annurev.arplant.49.1.481.
[12] H.E. Goldbach, M.A. Wimmer, Boron in plants and animals: is there a role beyond cell-wall structure? J. Plant Nutr. Soil Sci. 170 (2007) 39–48, http://dx.doi.org/10.1002/jpln.200625161.
[13] J.J. Camacho-Cristóbal, J. Rexach, A. González-Fontes, Boron in plants: deficiency and toxicity, J. Integr. Plant Biol. 50 (2008) 1247–1255, http://dx.doi.org/10.1111/j.1744-7909.2008.00742.x.
[14] P.H. Brown, N. Bellaloui, M.A. Wimmer, E.S. Bassil, J. Ruiz, H. Hu, H. Pfeffer, F. Dannel, V. Römheld, Boron in plant biology, Plant Biol. 4 (2002) 205–223, http://dx.doi.org/10.1055/s-2002-25740.
[15] B. Dell, L. Huang, Physiological response of plants to low boron, Plant Soil 193 (1997) 103–120, http://dx.doi.org/10.1023/A:1004264009230.
[16] S. Perilli, R. Di Mambro, S. Sabatini, Growth and development of the root apical meristem, Curr. Opin. Plant Biol. 15 (2012) 17–23, http://dx.doi.org/10.1016/j.pbi.2011.10.006.
[17] L. Moubayidin, S. Perilli, R. Dello Ioio, R. Di Mambro, P. Costantino, S. Sabatini, The rate of cell differentiation controls the arabidopsis root meristem growth phase, Curr. Biol. 20 (2010) 1138–1143, http://dx.doi.org/10.1016/j.cub.2010.05.035.
[18] L. Moubayidin, R. Di Mambro, R. Sozzani, E. Pacifici, E. Salvi, I. Terpstra, D. Bao, A. van Dijken, R. Dello Ioio, S. Perilli, K. Ljung, P.N. Benfey, R. Heidstra, P. Costantino, S. Sabatini, Spatial coordination between stem cell activity and cell differentiation in the root meristem, Dev. Cell 26 (2013) 405–415, http://dx.doi.org/10.1016/j.devcel.2013.06.025.
[19] H. Takatsuka, M. Umeda, Hormonal control of cell division and elongation along differentiation trajectories in roots, J. Exp. Bot. 65 (2014) 2633–2643, http://dx.doi.org/10.1093/jxb/ert485.
[20] J.K.H. Jung, S. McCouch, Getting to the roots of it: genetic and hormonal control of root architecture, Front. Plant Sci. 4 (2013) 186, http://dx.doi.org/10.3389/fpls.2013.00186.
[21] M.-P. González-García, J. Vilarrasa-Blasi, M. Zhiponova, F. Divol, S. Mora-García, E. Russinova, A.I. Caño-Delgado, Brassinosteroids control meristem size by promoting cell cycle progression in Arabidopsis roots, Development 138 (2011) http://dev.biologists.org/content/138/5/849 . (Accessed 4 August 2017).
[22] J.J. Camacho-Cristobal, E.M. Martin-Rejano, M.B. Herrera-Rodriguez, M.T. Navarro-Gochicoa, J. Rexach, A. Gonzalez-Fontes, Boron deficiency inhibits root cell elongation via an ethylene/auxin/ROS-dependent pathway in Arabidopsis seedlings, J. Exp. Bot. 66 (2015) 3831–3840, http://dx.doi.org/10.1093/jxb/erv186.
[23] A. González-Fontes, M.B. Herrera-Rodríguez, E.M. Martín-Rejano, M.T. Navarro-Gochicoa, J. Rexach, J.J. Camacho-Cristóbal, Root responses to boron deficiency

mediated by ethylene, Front. Plant Sci. 6 (2016) 1103, http://dx.doi.org/10.3389/fpls.2015.01103.
[24] E.M. Martín-Rejano, J.J. Camacho-Cristóbal, J. Herrera-Rodríguez, M.T. Navarro-Gochicoa, A. González-Fontes, Auxin and ethylene are involved in the responses of root system architecture to low boron supply in Arabidopsis seedlings, Physiol. Plant 142 (2011) 170–178, http://dx.doi.org/10.1111/j.1399-3054.2011.01459.x.
[25] R. Tabata, T. Kamiya, S. Shigenobu, K. Yamaguchi, M. Yamada, M. Hasebe, T. Fujiwara, S. Sawa, Identification of an EMS-induced causal mutation in a gene required for boron-mediated root development by low-coverage genome re-sequencing in Arabidopsis, Plant Signal. Behav. 8 (2013) e22534, http://dx.doi.org/10.4161/psb.22534.
[26] K. Li, T. Kamiya, T. Fujiwara, Differential roles of PIN1 and PIN2 in root meristem maintenance under low-B conditions in arabidopsis thaliana, Plant Cell Physiol. 56 (2015) 1205–1214, http://dx.doi.org/10.1093/pcp/pcv047.
[27] G. Brunoud, D.M. Wells, M. Oliva, A. Larrieu, V. Mirabet, A.H. Burrow, T. Beeckman, S. Kepinski, J. Traas, M.J. Bennett, T. Vernoux, A novel sensor to map auxin response and distribution at high spatio-temporal resolution, Nature 482 (2012) 103–106, http://dx.doi.org/10.1038/nature10791.
[28] Q. Li, Y. Liu, Z. Pan, S. Xie, S. Peng, Boron deficiency alters root growth and development and interacts with auxin metabolism by influencing the expression of auxin synthesis and transport genes, Biotechnol. Biotechnol. Equip. 30 (2016) 661–668, http://dx.doi.org/10.1080/13102818.2016.1166985.
[29] I. Abreu, L. Poza, I. Bonilla, L. Bolaños, Boron deficiency results in early repression of a cytokinin receptor gene and abnormal cell differentiation in the apical root meristem of Arabidopsis thaliana, Plant Physiol. Biochem. 77 (2014) 117–121, http://dx.doi.org/10.1016/j.plaphy.2014.02.008.
[30] C.-Q. Yang, Y.-Z. Liu, J.-C. An, S. Li, L.-F. Jin, G.-F. Zhou, Q.-J. Wei, H.-Q. Yan, N.-N. Wang, L.-N. Fu, X. Liu, X.-M. Hu, T.-S. Yan, S.-A. Peng, Digital gene expression analysis of corky split vein caused by boron deficiency in newhall navel orange (Citrus sinensis osbeck) for selecting differentially expressed genes related to vascular hypertrophy, PLoS One 8 (2013) e65737, http://dx.doi.org/10.1371/journal.pone.0065737.
[31] A. Colón-Carmona, R. You, T. Haimovitch-Gal, P. Doerner, Technical advance: spatio-temporal analysis of mitotic activity with a labile cyclin-GUS fusion protein, Plant J. 20 (1999) 503–508 http://www.ncbi.nlm.nih.gov/pubmed/10607302 . (Accessed 17 October 2016).
[32] S. Sabatini, D. Beis, H. Wolkenfelt, J. Murfett, T. Guilfoyle, J. Malamy, P. Benfey, O. Leyser, N. Bechtold, P. Weisbeek, B. Scheres, An auxin-dependent distal organizer of pattern and polarity in the arabidopsis root, Cell 99 (1999) 463–472, http://dx.doi.org/10.1016/S0092-8674(00)81535-4.
[33] I.B. D'Agostino, J. Deruere, J.J. Kieber, Characterization of the response of the arabidopsis response regulator gene family to cytokinin, Plant Physiol. 124 (2000) 1706–1717.
[34] M. Vanstraelen, M. Baloban, O. Da Ines, A. Cultrone, T. Lammens, V. Boudolf, S.C. Brown, L. De Veylder, P. Mergaert, E. Kondorosi, APC/C-CCS52A complexes control meristem maintenance in the Arabidopsis root, Proc. Natl. Acad. Sci. U. S. A. 106 (2009) 11806–11811, http://dx.doi.org/10.1073/pnas.0901193106.
[35] E. Zürcher, D. Tavor-Deslex, D. Lituiev, K. Enkerli, P.T. Tarr, B. Müller, A robust and sensitive synthetic sensor to monitor the transcriptional output of the cytokinin signaling network in planta, Plant Physiol. 161 (2013) 1066–1075, http://dx.doi.org/10.1104/pp.112.211763.
[36] M. Higuchi, M.S. Pischke, A.P. Mähönen, K. Miyawaki, Y. Hashimoto, M. Seki, M. Kobayashi, K. Shinozaki, T. Kato, S. Tabata, Y. Helariutta, M.R. Sussman, T. Kakimoto, In planta functions of the Arabidopsis cytokinin receptor family, Proc. Natl. Acad. Sci. U. S. A. 101 (2004) 8821–8826, http://dx.doi.org/10.1073/pnas.0402887101.
[37] W. Dewitte, S. Scofield, A.A. Alcasabas, S.C. Maughan, M. Menges, N. Braun, C. Collins, J. Nieuwland, E. Prinsen, V. Sundaresan, J.A.H. Murray, Arabidopsis CYCD3 D-type cyclins link cell proliferation and endocycles and are rate-limiting for cytokinin responses, Proc. Natl. Acad. Sci. U. S. A. 104 (2007) 14537–14542, http://dx.doi.org/10.1073/pnas.0704166104.
[38] C. Riou-Khamlichi, R. Huntley, A. Jacqmard, J.A. Murray, Cytokinin activation of Arabidopsis cell division through a D-type cyclin, Science 283 (1999) 1541–1544 http://www.ncbi.nlm.nih.gov/pubmed/10066178 . (Accessed 26 October 2016).
[39] T. Murashige, F. Skoog, A revised medium for rapid growth and bio assays with tobacco tissue cultures, Physiol. Plant 15 (1962) 473–497, http://dx.doi.org/10.1111/j.1399-3054.1962.tb08052.x.
[40] A. Asad, R.W. Bell, B. Dell, L. Huang, Development of a boron buffered solution culture system for controlled studies of plant boron nutrition, Plant Soil 188 (1997) 21–32, http://dx.doi.org/10.1023/A:1004291225723.
[41] S. Perilli, S. Sabatini, Analysis of root meristem size development, Methods Mol. Biol. (2010) 177–187, http://dx.doi.org/10.1007/978-1-60761-765-5_12.
[42] Y. Arata, A. Nagasawa-Iida, H. Uneme, H. Nakajima, T. Kakimoto, R. Sato, The phenylquinazoline compound S-4893 is a non-competitive cytokinin antagonist that targets arabidopsis cytokinin receptor CRE1 and promotes root growth in arabidopsis and rice, Plant Cell Physiol. 51 (2010) 2047–2059, http://dx.doi.org/10.1093/pcp/pcq163.
[43] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method, Methods 25 (2001) 402–408, http://dx.doi.org/10.1006/meth.2001.1262.
[44] E. Jelihovschi, J.C. Faria, I.B. Allaman, ScottKnott. A package for performing the Scott-Knott clustering algorithm in R, TEMA (São Carlos) 15 (2014) 3, http://dx.doi.org/10.5540/tema.2014.015.01.0003.
[45] J. Le, F. Vandenbussche, D. Van Der Straeten, J.P. Verbelen, In the early response of Arabidopsis roots to ethylene, cell elongation is up- and down-regulated and uncoupled from differentiation, Plant Physiol. 125 (2001) 519–522 http://www.ncbi.nlm.nih.gov/pubmed/11161008 . (Accessed 21 October 2016).
[46] J. Sebastian, K.H. Ryu, J. Zhou, D. Tarkowská, P. Tarkowski, Y.-H. Cho, S.-D. Yoo, E.-S. Kim, J.-Y. Lee, PHABULOSA controls the quiescent center-independent root meristem activities in Arabidopsis thaliana, PLoS Genet. 11 (2015) e1004973, http://dx.doi.org/10.1371/journal.pgen.1004973.
[47] K. Nakajima, P.N. Benfey, Signaling in and out: control of cell division and differentiation in the shoot and root, Plant Cell 14 (Suppl) (2002) S265–S276 http://www.ncbi.nlm.nih.gov/pubmed/12045282 . (Accessed 4 August 2017).
[48] B.D. Gruber, R.F.H. Giehl, S. Friedel, N. von Wirén, Plasticity of the Arabidopsis root system under nutrient deficiencies, Plant Physiol. 163 (2013) 161–179, http://dx.doi.org/10.1104/pp.113.218453.
[49] H.M. Moore, A.M. Hirsch, Effects of boron deficiency on mitosis and incorporation of tritiated thymidine into nuclei of sunflower root tips, Am. J. Bot. 70 (1983) 165, http://dx.doi.org/10.2307/2443260.
[50] R.W. Krueger, C.J. Lovatt, L.S. Albert, Metabolic requirement of Cucurbita pepo for Boron', Plant Physiol. 83 (1987) 254–258.
[51] C.J. Lovatt, Evolution of xylem resulted in a requirement for boron in the apical meristems of vascular plants, New Phytol. 99 (1985) 509–522, http://dx.doi.org/10.1111/j.1469-8137.1985.tb03679.x.
[52] M. Reguera, A. Espí, L. Bolaños, I. Bonilla, M. Redondo-Nieto, Endoreduplication before cell differentiation fails in boron-deficient legume nodules. Is boron involved in signalling during cell cycle regulation? New Phytol. 183 (2009) 8–12, http://dx.doi.org/10.1111/j.1469-8137.2009.02869.x.
[53] A. Shimotohno, N. Sotta, T. Sato, M. De Ruvo, A.F.M. Marée, V.A. Grieneisen, T. Fujiwara, Mathematical modeling and experimental validation of the spatial distribution of boron in the root of Arabidopsis thaliana identify high boron accumulation in the tip and predict a distinct root tip uptake function, Plant Cell Physiol. 56 (2015) 620–630, http://dx.doi.org/10.1093/pcp/pcv016.
[54] J.-P. Verbelen, T. De Cnodder, J. Le, K. Vissenberg, F. Baluska, The root apex of Arabidopsis thaliana consists of four distinct zones of growth activities: meristematic zone, transition, fast elongation zone and growth terminating zone, Plant Signal. Behav. 1 (2006) 296–304 http://www.ncbi.nlm.nih.gov/pubmed/19517000 . (Accessed 20 October 2016).
[55] L. Sánchez-Calderón, J. López-Bucio, A. Chacón-López, A. Cruz-Ramírez, F. Nieto-Jacobo, J.G. Dubrovsky, L. Herrera-Estrella, Phosphate starvation induces a determinate developmental program in the roots of Arabidopsis thaliana, Plant Cell Physiol. 46 (2005) 174–184, http://dx.doi.org/10.1093/pcp/pci011.
[56] R.C. Drisch, Y. Stahl, Function and regulation of transcription factors involved in root apical meristem and stem cell maintenance, Front. Plant Sci. 6 (2015) 505, http://dx.doi.org/10.3389/fpls.2015.00505.
[57] T. Nawy, J.-Y. Lee, J. Colinas, J.Y. Wang, S.C. Thongrod, J.E. Malamy, K. Birnbaum, P.N. Benfey, Transcriptional profile of the Arabidopsis root quiescent center, Plant Cell 17 (2005) 1908–1925, http://dx.doi.org/10.1105/tpc.105.031724.
[58] B. Scheres, C. van den Berg, V. Willemsen, G. Hendriks, P. Weisbeek, Short-range control of cell differentiation in the Arabidopsis root meristem, Nature 390 (1997) 287–289, http://dx.doi.org/10.1038/36856.
[59] W. Zhang, R. Swarup, M. Bennett, G.E. Schaller, J.J. Kieber, Cytokinin induces cell division in the quiescent center of the Arabidopsis root apical meristem, Curr. Biol. 23 (2013) 1979–1989, http://dx.doi.org/10.1016/j.cub.2013.08.008.
[60] R. Dello Ioio, F.S. Linhares, E. Scacchi, E. Casamitjana-Martinez, R. Heidstra, P. Costantino, S. Sabatini, Cytokinins determine Arabidopsis root-meristem size by controlling cell differentiation, Curr. Biol. 17 (2007) 678–682, http://dx.doi.org/10.1016/j.cub.2007.02.047.
[61] L. Yang, J. Zhang, J. He, Y. Qin, D. Hua, Y. Duan, Z. Chen, Z. Gong, ABA-mediated ROS in mitochondria regulate root meristem activity by controlling PLETHORA expression in arabidopsis, PLoS Genet. 10 (2014) e1004791, http://dx.doi.org/10.1371/journal.pgen.1004791.
[62] H. Tsukagoshi, W. Busch, P.N. Benfey, Transcriptional regulation of ROS controls transition from proliferation to differentiation in the root, Cell 143 (2010) 606–616, http://dx.doi.org/10.1016/j.cell.2010.10.020.
[63] Y. Oiwa, K. Kitayama, M. Kobayashi, T. Matoh, Boron deprivation immediately causes cell death in growing roots of Arabidopsis thaliana (L.) Heynh, Soil Sci. Plant Nutr. 59 (2013) 621–627, http://dx.doi.org/10.1080/00380768.2013.813382.
[64] A. Skylar, X. Wu, Regulation of meristem size by cytokinin signaling, J. Integr. Plant Biol. 53 (2011) 446–454, http://dx.doi.org/10.1111/j.1744-7909.2011.01045.x.
[65] B. Jones, S.A. Gunnerås, S.V. Petersson, P. Tarkowski, N. Graham, S. May, K. Dolezal, G. Sandberg, K. Ljung, Cytokinin regulation of auxin synthesis in Arabidopsis involves a homeostatic feedback loop regulated via auxin and cytokinin signal transduction, Plant Cell 22 (2010) 2956–2969, http://dx.doi.org/10.1105/tpc.110.074856.
[66] K. Ruzicka, M. Simásková, J. Duclercq, J. Petrásek, E. Zazímalová, S. Simon, J. Friml, M.C.E. Van Montagu, E. Benková, Cytokinin regulates root meristem activity via modulation of the polar auxin transport, Proc. Natl. Acad. Sci. U. S. A. 106 (2009) 4284–4299, http://dx.doi.org/10.1073/pnas.0900060106.
[67] I.H. Street, D.E. Mathews, M.V. Yamburkenko, A. Sorooshzadeh, R.T. John, R. Swarup, M.J. Bennett, J.J. Kieber, G.E. Schaller, Cytokinin acts through the auxin influx carrier AUX1 to regulate cell elongation in the root, Development 143 (2016) 3982–3993, http://dx.doi.org/10.1242/dev.132035.
[68] J.P.C. To, J. Deruère, B.B. Maxwell, V.F. Morris, C.E. Hutchison, F.J. Ferreira, G.E. Schaller, J.J. Kieber, Cytokinin regulates type-A Arabidopsis Response Regulator activity and protein stability via two-component phosphorelay, Plant Cell 19 (2007) 3901–3914, http://dx.doi.org/10.1105/tpc.107.052662.
[69] K. Hayashi, J. Hasegawa, S. Matsunaga, J. Engelhardt, S.W. Hell, The boundary of the meristematic and elongation zones in roots: endoreduplication precedes rapid cell expansion, Sci. Rep. 3 (2013) 2723, http://dx.doi.org/10.1038/srep02723.
[70] R. Dello Ioio, F.S. Linhares, S. Sabatini, Emerging role of cytokinin as a regulator of

cellular differentiation, Curr. Opin. Plant Biol. 11 (2008) 23–27, http://dx.doi.org/10.1016/j.pbi.2007.10.006.
[71] P. Valente, W. Tao, J.-P. Verbelen, Auxins and cytokinins control DNA endoreduplication and deduplication in single cells of tobacco, Plant Sci. 134 (1998) 207–215, http://dx.doi.org/10.1016/S0168-9452(98)00062-4.
[72] G.T. Beemster, T.I. Baskin, Stunted plant 1 mediates effects of cytokinin, but not of auxin, on cell division and expansion in the root of Arabidopsis, Plant Physiol. 124 (2000) 1718–1727, http://dx.doi.org/10.1104/PP.124.4.1718.
[73] S. Zhang, L. Huang, A. Yan, Y. Liu, B. Liu, C. Yu, A. Zhang, J. Schiefelbein, Y. Gan, Multiple phytohormones promote root hair elongation by regulating a similar set of genes in the root epidermis in Arabidopsis, J. Exp. Bot. 67 (2016) 6363–6372, http://dx.doi.org/10.1093/jxb/erw400.
[74] M. Matsumoto-Kitano, T. Kusumoto, P. Tarkowski, K. Kinoshita-Tsujimura, K. Václavíková, K. Miyawaki, T. Kakimoto, Cytokinins are central regulators of cambial activity, Proc. Natl. Acad. Sci. U. S. A. 105 (2008) 20027–20031, http://dx.doi.org/10.1073/pnas.0805619105.
[75] R. Spicer, A. Groover, Evolution of development of vascular cambia and secondary growth, New Phytol. 186 (2010) 577–592, http://dx.doi.org/10.1111/j.1469-8137.2010.03236.x.
[76] R.S. Randall, S. Miyashima, T. Blomster, J. Zhang, A. Elo, A. Karlberg, J. Immanen, K. Nieminen, J.-Y. Lee, T. Kakimoto, K. Blajecka, C.W. Melnyk, A. Alcasabas, C. Forzani, M. Matsumoto-Kitano, A.P. Mähönen, R. Bhalerao, W. Dewitte, Y. Helariutta, J.A.H. Murray, AINTEGUMENTA and the D-type cyclin CYCD3;1 regulate root secondary growth and respond to cytokinins, Biol. Open 4 (2015) 1229–1236, http://dx.doi.org/10.1242/bio.013128.
[77] W. Dewitte, C. Riou-Khamlichi, S. Scofield, J.M.S. Healy, A. Jacqmard, N.J. Kilby, J.A.H. Murray, Altered cell cycle distribution, hyperplasia, and inhibited differentiation in Arabidopsis caused by the D-type cyclin CYCD3, Plant Cell 15 (2003) 79–92, http://dx.doi.org/10.1105/TPC.004838.
[78] N.H. Masubelele, W. Dewitte, M. Menges, S. Maughan, C. Collins, R. Huntley, J. Nieuwland, S. Scofield, J.A.H. Murray, D-type cyclins activate division in the root apex to promote seed germination in Arabidopsis, Proc. Natl. Acad. Sci. U. S. A. 102 (2005) 15694–15699, http://dx.doi.org/10.1073/pnas.0507581102.
[79] A. Fleischer, C. Titel, R. Ehwald, The boron requirement and cell wall properties of growing and stationary suspension-cultured Chenopodium album L. cells, Plant Physiol. 117 (1998) 1401–1410, http://dx.doi.org/10.1104/pp.117.4.1401.
[80] D. Chormova, D.J. Messenger, S.C. Fry, Boron bridging of rhamnogalacturonan-II, monitored by gel electrophoresis, occurs during polysaccharide synthesis and secretion but not post-secretion, Plant J. 77 (2014) 534–546, http://dx.doi.org/10.1111/tpj.12403.