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PLANT & CELL PHYSIOLOGY

Differential Roles of PIN1 and PIN2 in Root Meristem Maintenance Under Low-B Conditions in *Arabidopsis thaliana*

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(Received October 1, 2014; Accepted March 12, 2015)

Boron (B) is an essential element for plants; its deficiency causes rapid cessation of root elongation. In addition, B influences auxin accumulation in plants. To assess the importance of auxin transport in B-dependent root elongation, *Arabidopsis thaliana pin1–pin4* mutants were grown under low-B conditions. Among them, only the *pin2/eir1-1* mutant showed a significantly shorter root under low-B conditions than under control conditions. Moreover, the root meristem size of *pin2/eir1-1* was reduced under low-B conditions. Among the PIN-FORMED (PIN) family, PIN1 and PIN2 are important for root meristem growth/maintenance under normal conditions. To investigate the differential response of *pin1* and *pin2* mutants under low-B conditions, the effect of low-B on PIN1–green fluorescent protein (GFP) and PIN2–GFP accumulation and localization was examined. Low-B did not affect PIN2–GFP, while it reduced the accumulation of PIN1–GFP. Moreover, no signal from DII-VENUS, an auxin sensor, was detected under the low-B condition in the stele of wild-type root meristems. Taken together, these results indicate that under low-B conditions PIN1 is down-regulated and PIN2 plays an important role in root meristem maintenance.

Keywords: Boron deficiency • PIN1 • PIN2 • Root meristem.

Abbreviations: B, boron; Ca, calcium; CSLB5, cellulose synthase like 5; DII-VENUS, an auxin sensor fusing a fast maturing form of the yellow fluorescent protein to the DII domain of the repressor AUX/IAA protein IAA28; EXP, expansin; GFP, green fluorescent protein; Mn, manganese; PI, propidium iodide; PIN, PIN-FORMED; RG-II, rhamnogalacturonan-II; XTH, xyloglucan endotransglycosylase/hydrolase.

Introduction

Boron (B) is an essential microelement for higher plants, and B deficiency is an agricultural problem worldwide (Shorrocks and Bureau 1997). Typical B deficiency symptoms include inhibition of and/or defects in root elongation, leaf expansion, pollen tube growth and anther development (Gupta 1993, Rawson 1996). Plants require more B during reproductive growth than in the vegetative phase (Dell and Huang 1997). The symptoms of mild B deficiency include decreased seed production without any visible phenotype of B deficiency during the vegetative phase (Nyomora et al. 1997).

The only known physiological function of B is the stabilization of cell wall structures by forming the borate–rhamnogalacturonan-II (B–RG-II) complex. B cross-links two RG-II monomers to form a B–RG-II dimer by linking the *cis*-diol of apiosyl residues in RG-II (Kobayashi et al. 1996, O'Neil et al. 2004). A typical mutant that has less B–RG-II dimerization is the *Arabidopsis thaliana mur1* mutant. MUR1 encodes an isoform of GDP-D-mannose-4,6-dehydratase, and the GDP-L-fucose content in aerial components of *mur1-1* is <2% of that in the wild type (Reiter et al. 1993, Bonin et al. 1997). The loss of fucose causes structural changes in the lateral chain of pectic and hemicellulosic cell wall polysaccharides, which reduces the efficiency of pectic polysaccharide RG-II cross-linking through B. In the *mur1-1* mutant, a high concentration of B is required for both the cross-linking of RG-II and normal expansion of leaves, indicative of the importance of B–RG-II cross-linking in leaf expansion (O'Neill et al. 2001, O'Neill et al. 2004). The physiological function of B in the cell wall coincides with the hypothesis that B deficiency disrupts growing tissues by affecting cell elongation (Gupta 1993, Brown et al. 2002). Moreover, cell division is also affected by low-B treatments (Brown et al. 2002).

B also affects the expression of several cell-wall-modifying enzymes, such as expansins (EXPs) and xyloglucan endotransglycosylase/hydrolases (XTHs), which are down-regulated under low-B conditions, leading to the inhibition of cell elongation independently of B–RG-II cross-linking (Cosgrove 1999, Camacho-Cristóbal et al. 2008a). The activity and expression of EXPs and XTHs is regulated by the plant hormone auxin (Sánchez-Rodríguez et al. 2010). Camacho-Cristóbal et al. (2008b) suggested a possibility that B regulates the expression of these cell wall/membrane-related genes by modulating auxin.

Auxin is an important plant hormone for regulation of root development. Appropriate concentrations of auxin promote root elongation, while its overaccumulation inhibits elongation. Regulation of the auxin concentration gradient in the root is required for root elongation. Auxin concentration in the root is controlled by auxin biosynthesis and, more importantly, by its transport (Cheng et al. 2006, Tanaka et al. 2006, Cheng et al. 2007, Stepanova et al. 2008, Tao et al. 2008). Auxin is transported between cells via AUXIN1/LIKE AUX 1 influx facilitators, PIN-FORMED (PIN) efflux carriers and ATP-binding cassette-type transporters (Pickett et al. 1990, Murphy et al. 2002, Geisler et al. 2005, Cho et al. 2007, Vieten et al. 2007,

Plant Cell Physiol. 56(6): 1205–1214 (2015) doi:10.1093/pcp/pcv047, Advance Access publication on 25 March 2015, available online at www.pcp.oxfordjournals.org

Swarup et al. 2008). These transporters are responsible for regulation of auxin distribution.

PINs are the best characterized efflux auxin transporters. Among the eight PINs in *A. thaliana*, PIN1, PIN2, PIN3, PIN4 and PIN7 regulate auxin distribution in roots and are important for root elongation (Kleine-Vehn and Friml 2008, Krecek et al. 2009).

Each member of the PINs has different physiological roles and expression patterns. PIN1 is expressed in the vascular bundle of the root, with a basal subcellular localization on the plasma membrane. The *pin1* mutant usually produces no flowers, and shows a pin-like stem in the shoot and a slightly shorter root (Okada et al. 1991, Bennett et al. 1995, Blilou et al. 2005, Bandyopadhyay et al. 2007, Kleine-Vehn and Friml 2008, Krecek et al. 2009). PIN2 is expressed mainly in the epidermis and cortex in the root meristem, with an apical and lateral subcellular localization on the plasma membrane. The *pin2* mutant shows short roots and the loss of gravitropic response in roots (Müller et al. 1998, Blilou et al. 2005, Vieten et al. 2005, Kleine-Vehn and Friml 2008). PIN2 is reported to be the most important PIN for the regulation of meristem enlargement in the root. The *pin1pin2* double mutant and all triple and quadruple *pin* mutants that contain *pin2* exhibit a growth defect in the root meristem. Irrespective of the established importance of PIN2 in meristem maintenance, it is important to note that the meristem of single *pin2* mutants is not much smaller than that of the wild type. This is because in the absence of PIN2, the PIN1 protein is expressed in the PIN2 domain, the region of the root where PIN2 is expressed; this ectopically expressed PIN1 may compensate for PIN2 function in meristem elongation (Blilou et al. 2005, Vieten et al. 2005). The expression and function of PIN3 overlap with those of PIN7. They are expressed in the stele of the central and distal elongation zone and the columella in the roots, with an apolar subcellular localization on the plasma membrane. PIN3 and PIN7 also have partial functional redundancy with PIN1. Single *pin3* or *pin7* mutants usually show growth phenotypes similar to the wild type, while the *pin3/pin7* double mutant has a slightly shorter root elongation than the wild type and loss of gravitropic responses (Blilou et al. 2005, Kleine-Vehn and Friml, 2008, Kleine-Vehn et al. 2010). PIN4 is expressed in the quiescent center and its surrounding cells, showing functional redundancy with PIN1, PIN3 and PIN7. The *pin4* mutant has no visible phenotype under normal conditions (Friml et al. 2002, Blilou et al. 2005, Kleine-Vehn and Friml 2008).

A number of reports have observed a relationship between B and auxin. B is required for root development in response to auxin (Jarvis et al. 1983). Wang et al. (2006) reported that auxin export from the shoot apex is affected under low-B supply conditions in pea plants, and auxin oxidase activity is affected under B starvation conditions (Camacho-Cristóbal et al. 2008b). B deprivation significantly decreases polar IAA transport and reduces cytokinin levels in the shoot apex and roots of pea plants (Li et al. 2001). The endogenous IAA/cytokinin ratios change significantly in somatic carrot embryo cultures according to changes in the B concentration in the culture (Neumann et al. 2009). Low-B conditions also affect auxin accumulation in the roots of *A. thaliana* (Martín-Rejano et al. 2011).

Although previous reports have established a relationship between B and auxin, it remains unclear how B affects auxin distribution. One possible mechanism for B-mediated regulation of auxin accumulation is an effect on PIN transporters. In this report, we examined the relationship between B and PIN function. We found that PIN2 was essential for root meristem enlargement under low-B conditions and that PIN1 protein accumulation was down-regulated by B starvation.

Results

The *pin2* mutant is overly sensitive to low-B conditions

To examine whether auxin efflux PIN transporters are required for B starvation tolerance, the *A. thaliana pin1–pin4* mutants were cultured in solid medium with 0.1 and 30 μM B, referred to hereafter as low- and normal-B conditions, respectively. Under both conditions, the root lengths of *pin1* (SALK_047613) and *pin3-4* were similar to those of the wild type (**Fig. 1A**). Under the control conditions, root lengths of *pin2/eir1-1* were shorter than those of the wild type, while *pin4-3* roots were slightly longer than those of the wild type (**Fig. 1A**). Under low-B conditions, *pin2/eir1-1* roots were shorter than those of the wild type, which was not observed for *pin4-3* mutants (**Fig. 1A**). We then compared the relative root elongation of *pin2/eir1-1* between the control and low-B conditions. *pin2/eir1-1* also exhibited a shorter relative root length under low-B conditions (**Fig. 1A**). The root length of *pin2/eir1-1* relative to that of the wild type was about 79% under normal-B conditions, while low-B treatment reduced the relative root length to about 49% (**Fig. 1A**), suggesting that *pin2/eir1-1* is overly sensitive to low-B conditions.

To determine the sensitivity of the *pin2/eir1-1* mutant to deficiencies in other nutrients, root elongation was measured under −manganese (Mn) and low-calcium (Ca) conditions. The root length of the *pin2/eir1-1* mutant was shorter than that of the wild type under −Mn or low-Ca treatment (**Fig. 1B**, **C**), but the root length of *pin2/eir1-1* relative to that of the wild type was not affected by the −Mn or low-Ca treatment (**Fig. 1B**, **C**), suggesting that among the three nutrients tested, *pin2/eir1-1* is sensitive only to low-B.

Although the root elongation of *pin2/eir1-1* was much reduced under low-B conditions, shoot growth was not apparently affected (**Fig. 2A**). This is consistent with PIN2 expression only in the root (Vieten et al. 2005, Kleine-Vehn and Friml, 2008). The root elongation under 0.1 μM B was very low and it is possible that the root elongation of the *pin2/eir1-1* mutant may be strongly affected under conditions that strongly reduce root elongation, rather than reacting to the reduced B condition. We examined root elongation of *pin2/eir1-1* under 0.3 μM B, a mild- to low-B condition (**Fig. 2B**). The root length of the wild type increased 137% from 0.1 to 0.3 μM B conditions after growing for 1 week, while the root length of *pin2/eir1-1* increased only 17% from 0.1 to 0.3 μM B conditions (**Fig. 2B**).

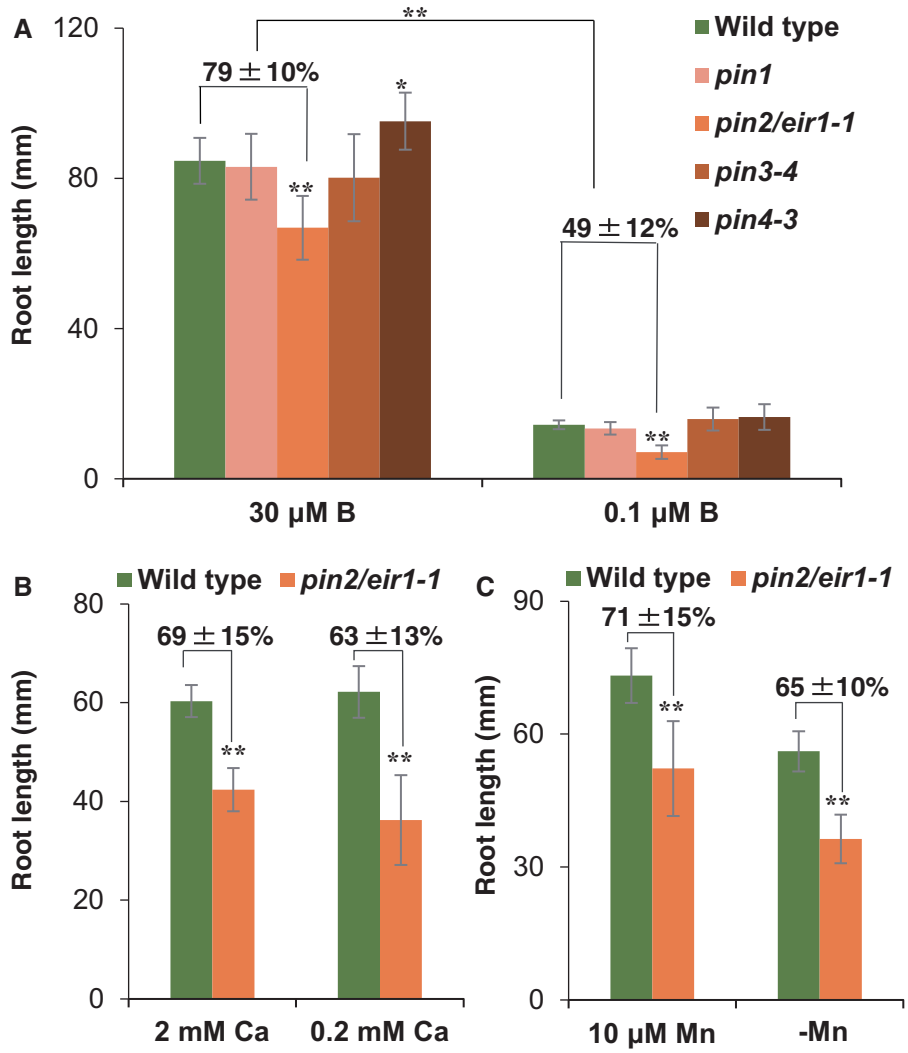

Fig. 1 Root elongation of the *pin1*, *pin2*, *pin3* and *pin4* mutants. (A) Root length of the *pin1–pin4* mutants and the wild type. *pin1* used in the experiment is the homozygous line of the SALK_047613 line. The seedlings were grown under 30 and 0.1 μM B conditions. The value on the line indicates the root length of *pin2* relative to that of the wild type under the same B conditions. (B) Root length of the wild type and *pin2/eir1-1* under 2 and 0.2 mM Ca conditions. The value on the line indicates the root length of *pin2* relative to that of the wild type under the same Ca conditions. (C) Root length of the wild type and *pin2/eir1-1* under 10 μM Mn and −Mn conditions. −Mn indicates that no Mn was added to the culture medium. The value on the line indicates the root length of *pin2* relative to that of the wild type under the same Mn conditions. The plants were grown on the indicated media for 10 d. Data were expressed as means ± SD ($n = 10$). Asterisks on the graphs indicate significant differences between the wild type and *pin* mutant under the same B condition. The asterisks on the line indicate significant differences of the relative root length of *pin2/eir1-1* between normal- and low-B conditions. Student's *t*-test: $*P < 0.05$, $**P < 0.01$. Wild type indicates Col-0.

This means that the root elongation of the *pin2/eir1-1* mutant was much more sensitive to the mild- to low-B conditions than that of the wild type. This result indicates that the differential response of *pin2/eir1-1* is due to low-B treatments, but not due to the severity of the stress

The *pin2* mutant shows a strong defect in root meristem enlargement under low-B conditions

PIN2 is important for the control of root meristem size (Blilou et al. 2005), and the root elongation of the *pin2/eir1-1* mutant was found to be highly sensitive to low-B conditions (**Figs. 1A**, **2**). We examined the meristem size of the wild type and *pin2/eir1-1* mutants under normal- and low-B conditions. Under normal-B conditions, the length of the *pin2/eir1-1* root meristems was about 65% that of the wild type, and their width was about 82% that of the wild type (**Fig. 3A**, **B**; **Table 1**). Under the low-B conditions, the root meristem length of *pin2/eir1-1* was approximately 28% that of the wild type, and their width was approximately 47% that of the wild type (**Fig. 3C**, **D**; **Table 1**). These observations confirmed that the low-B condition resulted in a greater reduction in the meristem size of *pin2/eir1-1* than of the wild type.

Boron concentration of the *pin2* mutant is unaffected

Because *pin2/eir1-1* mutants exhibit defects in meristem enlargement and roots of reduced size, they may have been

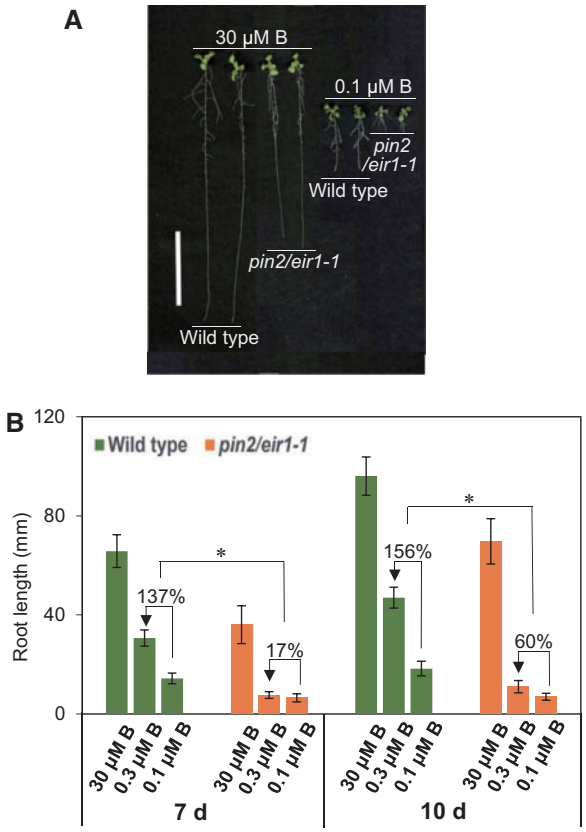

Fig. 2 Growth of the *pin2* mutant under low-B conditions. (A) Phenotype of the wild type and *pin2/eir1-1* under 30 and 0.1 μM B conditions. The plants were grown on the indicated media for 10 d. (B) Root length of the wild type and *pin2/eir1-1* under 30, 0.3 and 0.1 μM B conditions. Data above the arrow indicate the increased root length from 0.1 μM to 0.3 μM B. The plants were grown on the indicated medium for 1 week or 10 d, respectively. Data were expressed as means ± SD ($n = 10$). Asterisks indicate significant differences between the wild type and *pin2/eir1-1* mutant under the same B condition. Student's *t*-test: *$P < 0.05$. Scale bar = 3 cm. Wild type indicates Col-0.

unable to obtain sufficient B from the medium. We determined the B concentration in the roots and shoots of the wild type and *pin2/eir1-1* under normal and low-B conditions; no strong significant difference was observed (**Supplementary Fig. S1**), suggesting that *pin2/eir1-1* could absorb and transport B normally. Therefore, the defect in meristem enlargement of *pin2/eir1-1* was not caused by abnormal B absorption or distribution in the root.

PIN1–GFP accumulation and auxin distribution are altered under low-B conditions

PIN1 has partial functional redundancy with PIN2 (Vieten et al. 2005). Under normal conditions, in the *pin2* mutant, PIN1 can be expressed in the cortex of the root meristem, and partially complement the function of PIN2. Therefore, under normal conditions, the root meristem size of the *pin2* mutant is just a little smaller than that of the wild type, and the root meristem size of the *pin1pin2* double mutant is greatly reduced, compared with that of the wild type (Blilou et al. 2005, Vieten et al. 2005). In this study, the root meristem size of *pin2/eir1-1* was significantly much smaller than that of the wild type under low-B conditions (**Fig. 3**; **Table 1**). These results suggest that PIN1 cannot complement the function of PIN2 under low-B conditions. Thus it is possible that low B affects PIN1 protein accumulation or function. To examine this, we evaluated the expression of PIN1–green fluorescent protein (GFP) signals under different B conditions. The expression of PIN1–GFP signals was reduced under low-B conditions (**Fig. 4**), indicating that low B down-regulates the abundance of PIN1–GFP.

To confirm that the down-regulation of PIN1–GFP accumulation is caused specifically by low-B conditions, the expression of PIN1–GFP was also observed under −Mn and low-Ca conditions (**Supplementary Fig. S2**). Although the detailed expression pattern of PIN1–GFP could be different, the abundance of the GFP signal was not decreased, indicating that −Mn and low-Ca conditions did not affect the abundance of PIN1, suggesting a low-B-specific effect among the elements examined.

In our imaging conditions, part of the PIN1–GFP signals appeared not to be localized on the basal sides of the plasma membrane under low-Ca conditions (**Supplementary Fig. S2C**). This is because the structures of the cells in the root stele under low-Ca conditions were changed, compared with the control conditions. It was difficult to focus on the same cell layers in all the root stele cells in one image under low-Ca conditions. PIN–GFP has been observed with the focus on different cell layers under low-Ca. Also, PIN1–GFP signals on the basal sides of the plasma membrane were found in most cells in the root stele. Therefore, the polar localization of PIN1–GFP proteins under low-Ca (**Supplementary Fig. S2C**) was similar to that under normal conditions.

We also examined auxin distribution using the auxin-specific DII-VENUS marker (Brunoud et al. 2012) (**Fig. 5**). Under normal-B (**Fig. 5A**, **B**) but not low-B (**Fig. 5C**, **D**) conditions, a VENUS signal was detected in the stele of the meristem in the wild type. These data indicate that the low-B treatment increases auxin accumulation in the stele of the root meristem.

Low-B conditions have no effect on PIN2–GFP expression and localization

The expression of PIN2–GFP proteins was also observed (**Fig. 6**). Strong PIN2–GFP signals were detected from the cortical and epidermal cells under both normal-B (**Fig. 6A**) and low-B (**Fig. 6B**) conditions. The relative fluorescence intensities of PIN2–GFP on the plasma membrane were measured in the cortical and epidermal cells, and they were similar between normal-B and low-B conditions (**Fig. 6G**). These results indicate that the accumulation of PIN2–GFP is not changed by low-B.

To confirm the subcellular localization of PIN2–GFP, it was observed in the cortical and epidermal cells (**Fig. 6C**, **D**) and in the epidermis (**Fig. 6E**, **F**). In the cortical and epidermal cells, the subcellular localization of PIN2–GFP was similar between normal-B (**Fig. 6C**) and low-B (**Fig. 6D**) conditions. In the epidermis, PIN2–GFP signals were detected from the apical and lateral sides on the plasma membrane under both

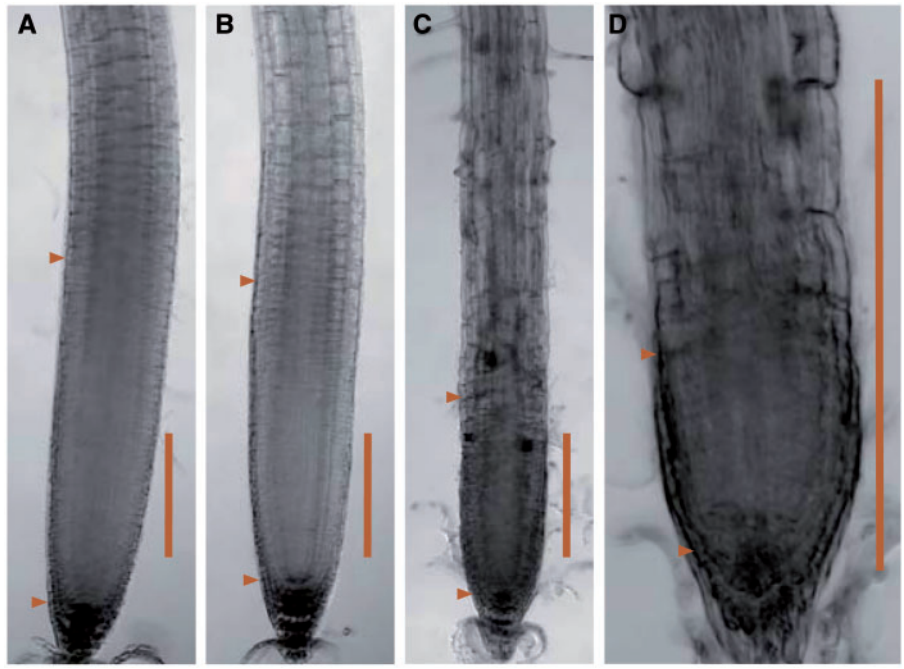

Fig. 3 Morphology of root tips under different B conditions. Root tips under 30 μM B (A, B) and 0.1 μM B (C, D). (A) and (C) show the wild-type. (B) and (D) show *pin2/eir1-1*.The arrowheads indicate the meristem border. The plants were grown on the indicated media for 10 d. The meristem length and width data are shown in **Table 1**. Scale bar = 200 μm. Wild type indicates Col-0.

Table 1 The size of the meristem in the roots of the wild type and *pin2/eir1-1*

B concentration in the medium	Length (μm)		Width (μm)	
	Wild type	*pin2/eir1-1*	Wild type	*pin2/eir1-1*
30 μM	648 ± 120	419 ± 69[a]	180 ± 15	148 ± 19[a]
0.1 μM	403 ± 67[b]	112 ± 46[a,b]	199 ± 21[b]	94 ± 23[a,b]

The seedlings were grown for 10 d under 30 and 0.1 μM B conditions. Data are expressed as means ± SD ($n = 20$).
Superscipt letters indicate a significant difference between the wild-type and *pin2/eir1-1*, [a]under the same B conditions and [b]between the 30 and 0.1 μM B treatments, by Student's *t*-test: $P < 0.05$. Wild type indicates Col-0.

normal- (**Fig. 6E**) and low-B (**Fig. 6F**) conditions, indicating that the subcellular localization of PIN2–GFP was also similar in the epidermis.

Taking all these results together, the accumulation and localization of PIN2–GFP under low-B conditions were similar to those under normal-B conditions (**Fig. 6**), revealing that PIN2 is unaffected by low-B treatments.

Discussion

In the present study, we demonstrated that PIN2 is essential for the maintenance of root elongation under low-B conditions (**Figs. 1–3**). We also demonstrated that the accumulation of PIN1–GFP, but not PIN2–GFP, decreased under low-B conditions (**Figs. 4**, **6**). With the down-regulation of PIN1 protein accumulation under low-B conditions, PIN1 cannot complement the PIN2 function, and the relative meristem length of *pin2/eir1-1* was approximately 37% less in the low-B condition compared with the normal-B condition (**Table 1**). This is similar to the approximately 39% reduced meristem length of the *pin1pin2* double mutant compared with the *pin2* single mutant under normal conditions (Vieten et al. 2005). These findings suggest that PIN1 and PIN2 play different roles in the low-B response. Thus, we discuss two issues: the possible mechanism underlying PIN1 protein down-regulation under low B; and the implications of the differential PIN responses to low-B conditions.

Possible mechanisms of down-regulation low-B-dependent PIN1 accumulation

PIN1 proteins undergo constitutive endocytic recycling between the plasma membrane and the endosomes (Geldner et al. 2001). It is possible that specific steps in this recycling are affected by B conditions. The GNOM-dependent polar recycling pathway controls both PIN1 and PIN2 proteins. Through this pathway, both PIN1 and PIN2 are transported to the vacuole for degradation (Kleine-Vehn and Friml 2008). Thus the GNOM pathway may be involved in the B-dependent down-regulation of PIN1 accumulation. However, based on the differential response of PIN1–GFP and PIN2–GFP (**Figs. 4**, **6**), the GNOM pathway is probably not responsible for the differential regulation of PIN1 accumulation under low-B conditions.

A possible mechanism of low B regulation of PIN1 accumulation is the modulation of cytokinin levels in roots. Exogenous cytokinin treatments decrease the root meristem length by decreasing the cell numbers in the meristem zone of *A. thaliana* (Dello Ioio et al. 2007). Exogenous cytokinin treatments also decrease the accumulation of PIN1–GFP, but not that of

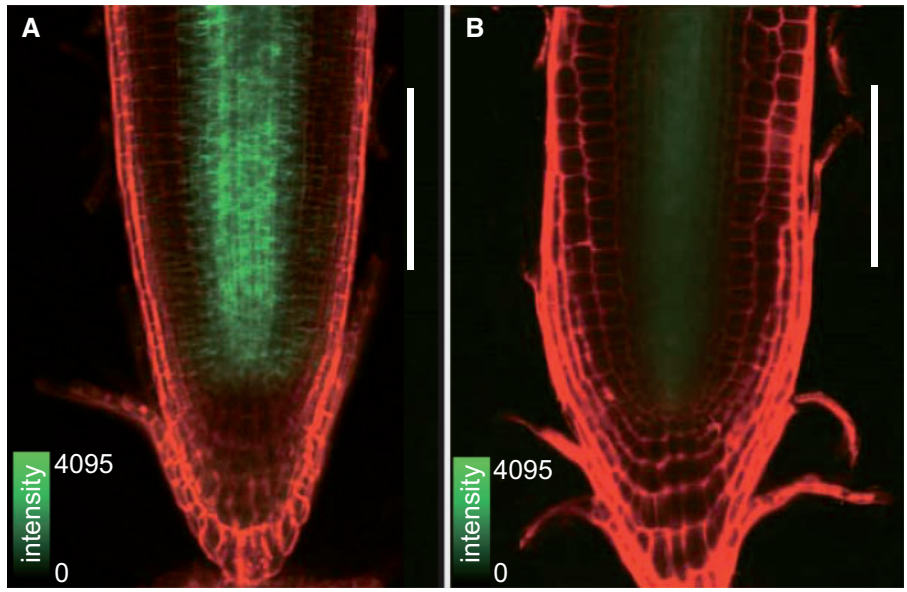

Fig. 4 Expression pattern of PIN1–GFP. Fluorescence images of PIN1–GFP under 30 μM (A) and 0.1 μM (B) B conditions. Col-0 with the pPIN1::PIN1–GFP marker was used. GFP fluorescence intensity is green coded. The cell wall was stained with PI (red). The plants were grown on the indicated media for 1 week. A total of 10 seedlings for 30 μM B and 20 seedlings for 0.1 μM were imaged for each treatment; representative photographs are shown. Scale bar = 100 μm.

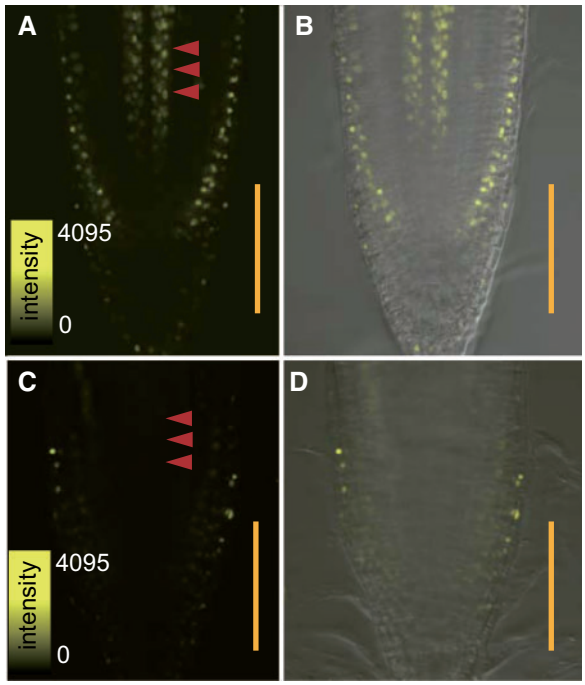

Fig. 5 Auxin distribution under different B conditions. DII-VENUS fluorescence images of the roots of Col-0 with the DII-VENUS marker under 30 μM (A, B) and 0.1 μM (C, D) B conditions. (B, D) Merged image of the VENUS and visible light signals under 30 μM (B) and 0.1 μM (D) B conditions. The red arrows indicate the VENUS signal in the central part of the meristem. VENUS fluorescence intensity is yellow coded. The plants were grown on the indicated media for 1 week. A total of 10 seedlings was imaged for each treatment; representative photographs are shown. Scale bar = 100 μm.

PIN2–GFP, PIN3–GFP or PIN7–GFP, in the roots of *A. thaliana* (Marhavý et al. 2011, Marhavý et al. 2014). In our study, we demonstrated that low-B treatment decreases the root meristem size in both the wild type and *pin2/eir1-1* (**Table 1**). We also observed that low-B treatments down-regulate PIN1–GFP, but not PIN2–GFP accumulation (**Figs. 4**, **6**). The effects of cytokinin and low-B treatment on the root meristem size and on the accumulation of PIN1 and PIN2 were similar, suggesting that B deprivation increases the cytokinin levels in roots. It is also possible that the two treatments act independently to regulate the meristem size and PIN1 and PIN2 in a similar manner. To our knowledge, no report of cytokinin levels under low-B conditions in *A. thaliana* is available. It has been shown that B deprivation decreases the cytokinin level in the shoots of pea (Li et al. 2001), which is seemingly contradictory to our assumption. Růžička et al. (2009) reported that the cytokinin receptor AHK4 is required for the regulation of the accumulation of PIN1 by cytokinin. Abreu et al. (2014) reported that the expression of the *AHK4* gene is down-regulated in the root of *A. thaliana* under B deprivation treatment. Without the AHK4 function, cytokinin cannot regulate the accumulation of PIN1 protein. It seems that low B regulates PIN1 accumulation through a cytokinin-independent pathway.

Another possible mechanism of B-regulated PIN1 accumulation is alteration of the cell wall properties. B is essential for the cross-linking of RG-II to form the B–RG-II dimer (Kobayashi et al. 1996); B–RG-II is required for linkage of the pectic polysaccharide network to stabilize the pectic polysaccharide–cellulose network in the cell wall (Wilson et al. 2000, Somerville et al. 2004). In addition, the expression of genes encoding several cell-wall-modifying enzymes is down-regulated by low-B treatments (Camacho-Crisobal et al. 2008a).

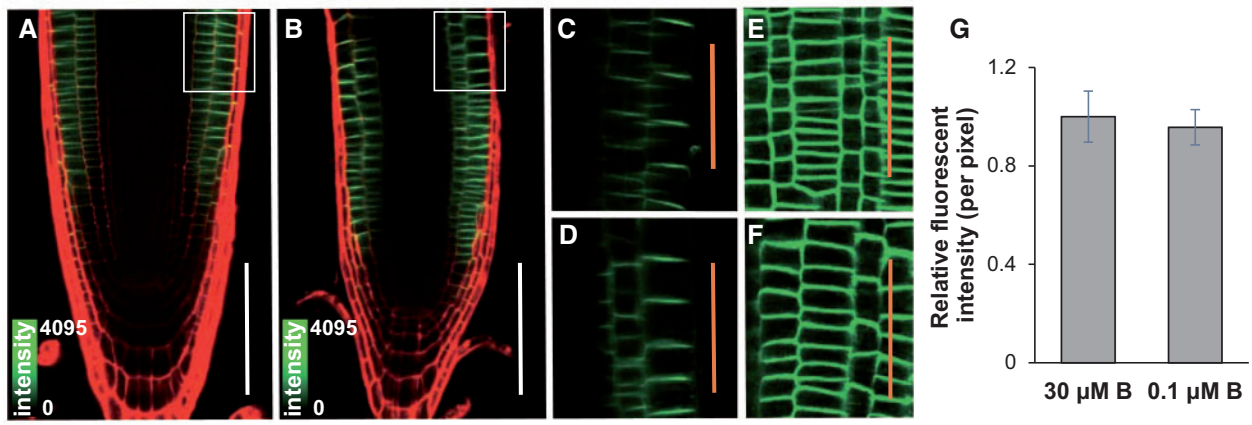

Fig. 6 The expression and localization of PIN2–GFP under different B conditions. The fluorescence of PIN2–GFP protein was imaged with the pPIN2::PIN2–GFP marker under 30 μM (A, C, E) and 0.1 μM B (B, D, F) conditions. (A–D) The PIN2–GFP localized in the cortical and epidermal cells. (C) and (D) are enlarged images from the boxed area in (A) and (B), respectively. (E, F) PIN2–GFP localization in the meristem zone of the epidermis. (G) The relative fluorescent intensities of PIN2–GFP on the plasma membrane under normal-B (E) and low-B (F) conditions. GFP fluorescence intensity is green coded (A–F). The cell wall was stained with PI (red) (A, B). The plants were grown on the indicated medium for 1 week. A total of 10 seedlings were imaged for each treatment, and representative photos are shown. White scale bar = 100 μm; orange scale bar = 50 μm.

Among the genes examined, *CSLB5* was down-regulated. *CSLB5* encodes a protein similar to cellulose synthases and is involved in cellulose biosynthetic processes (Roberts and Bushoven 2007). These findings suggest that low-B treatment may reduce cellulose biosynthesis through down-regulation of *CSLB5*. A reduction in cellulose biosynthesis would lead to changes in cell wall properties. Feraru et al. (2011) reported that a reduction in the cellulose-based extracellular matrix leads to PIN1 polarity. Taken all these findings together, low-B treatment reduces B–RG-II cross-linking and CSLB5 activity. It is possible that these responses reduce the content of cellulose, resulting in the change in polar localization of PIN1. The polar localization change of PIN1 may disturb its endocytic recycling and cause a reduction in its accumulation. In this research, the polar localization of PIN1 was not observed. This will be demonstrated in our future work to test the hypothesis of cellulose-based PIN1 polarity change.

The role of PIN1 and PIN2 in root elongation under low-B conditions

Our study demonstrated that the responses and roles of PIN1 and PIN2 differ under low-B conditions. We discuss the possible functions of PIN1 and PIN2 in response to low-B in *A. thaliana*.

Plant growth is suppressed under low-B conditions. Such growth suppression enables plants to avoid unnecessary use of resources for root elongation until B becomes available. Plants probably detect B conditions in the surrounding environment and regulate their growth accordingly. In this sense, it is reasonable to assume that root elongation is regulated in a manner dependent on the availability of nutrients. To suppress root growth, regulation of auxin levels could be used and, for such regulation, down-regulation of PIN1 and PIN2 accumulation can be a useful strategy.

In this study, PIN1 accumulation was down-regulated under low-B conditions (**Fig. 4**). The low PIN1 abundance could repress basal auxin transport to the root tip and may enhance the auxin content in the root stele. Abnormal auxin overaccumulation in the stele of the root meristem was observed under low-B conditions (**Fig. 5**), which may be caused by the reduced PIN1 abundance. A reduced PIN1 abundance enhances auxin accumulation (Yuan et al. 2013), and auxin overaccumulation inhibits meristem enlargement (Friml 2003, Friml et al. 2003, Overoorde et al. 2010). *Arabidopsis thaliana* may thus regulate root meristem enlargement via PIN1 in a low-B-dependent manner.

In contrast to PIN1, PIN2 is essential for gravitropism and phototropism of root apices (Chen et al. 1999, Sukumar et al. 2009). PIN2-dependent polar auxin flow in the meristem cortex is required to maintain the gravitropic response (Rahman et al. 2010). Root elongation is suppressed under low-B conditions, but gravitropism needs to be maintained. This may explain why PIN2 accumulation is not regulated by low-B conditions, in contrast to PIN1. Root elongation is crucial for tolerance to low-nutrient conditions. The present findings of the differential roles of PINs in root elongation under low-B condition may allow us to regulate the root elongation through modulating PIN1 under low-B condition.

Materials and Methods

Plant materials

Arabidopsis thaliana wild-type Col-0 was obtained from our laboratory stock. *pin3-4* (Friml et al. 2003) and *pin4-3* (Friml et al. 2002) were kindly provided by Professor Klaus Palme, University of Freiburg. Transgenic plants expressing the *pPIN2::PIN2-GFP* marker (Xu et al. 2005) were kindly provided by Dr. Jiří Friml, VIB. Mutant lines of *pin1* (SALK_047613) were provided by the Arabidopsis Biological Resource Center. Mutant lines of *pin2/eir1-1* (Roman et al. 1995),

pPIN1::PIN1-GFP (Friml et al. 2003) and *DII-VENUS* (Brunoud et al. 2012) were obtained from The European Arabidopsis Stock Center.

As described (Yuan et al. 2013), the *pin1* (SALK_047613) homozygous line did not produce seeds. The root length of the homozygous mutant was measured using seeds from the heterozygous parents. *pin1* homozygous lines were selected based on the pin-formed phenotype.

Plant culture

Seeds were sown on MGRL solid medium (Fujiwara et al. 1992) containing 1% sucrose and 1% Gellan Gum (Wako) with various B concentrations. The B concentration was adjusted by adding boric acid solution without changing the pH. For −Mn and low-Ca treatments, seeds were grown in MGRL solid medium containing 1% ultrapure sucrose (Sigma) and 1% agar (Nacalai Tesque). The Ca concentration was adjusted by adding $Ca(NO_3)_2$ and $CaCl_2$, and the Mn concentration was adjusted by adding $MnSO_4$. The seeds were vernalized at 4°C for 48 h and then grown at 22°C under a 16 h light/8 h dark cycle.

Observation of root meristem size

Following culture of the wild type and *pin2/eir1-1* under 0.1 and 30 μM B for 10 d, 2 cm of the root tips were cut and observed with laser scanning confocal microscopy, as described below. To measure the meristem size, propidium iodide (PI) (Molecular Probes) was used to stain the cell wall. The root meristem length was measured from the quiescent center to the cortical cells which are rapidly elongated as described (Blilou et al. 2005). The meristem width was measured in the middle of the meristem zone.

Propidium iodide and fluorescence imaging

For PI staining, 2 cm of root tips was cut, treated with 10 μg ml^{-1} PI (Molecular Probes) for 10 min and then loaded on glass slides. PI fluorescence was observed using a FV-1200 confocal laser scanning microscope (Olympus) with 559 nm excitation and 575–675 nm emission. For fluorescent proteins, wild-type Col-0 harboring the *pPIN1::PIN1-GFP*, *pPIN2::PIN2-GFP* and *DII-VENUS* markers were grown under different B conditions for 1 week. Next, 1.5 cm of the root tips was cut and imaged as above. Excitation was at 488 nm and GFP fluorescence was detected with a 505–540 nm band-pass filter. For the VENUS fluorescence, 515 nm excitation and 535–565 nm emission filters were used. For confirmation of PIN1–GFP expression, the strength of the 488 nm laser was 10%, with Hv as 579 V, gain as 1.5× and offset as 5%. To demonstrate the relative signal intensity of PIN2–GFP, the photographs were analyzed by ImageJ software (W.S. Rasband, National Institutes of Health). Since PIN2 is a membrane protein, the intensity should be compared on the basis of membrane areas. In the optical section used to demonstrate PIN2–GFP, the membrane area was represented by the length of the membrane. We compared the mean signal intensities of the membrane for PIN2–GFP. To measure the membrane areas, we first defined them in a standard image. We set the areas in the central part inside the cells as the background and then we took pixels with six times higher signal intensities than the background as the pixels representing membranes. The total number of the membrane pixels and the total signal intensities from the membrane pixels were calculated. Then the mean intensities (per pixel) on the membrane were calculated.

Determination of B concentration

Ten-day-old roots and shoots were used. The samples were washed with ultrapure water and then dried at 60°C for 2 d. After measuring the dry weight, samples were digested with 13 N HNO_3 and suspended with 0.08 N HNO_3 containing 1 p.p.b. In. B concentrations were determined using ICP-MS (SPQ-9700) (Seiko Instrument Inc.), as described in Nozawa et al. (2006).

Supplementary data

Supplementary data are available at PCP online.

Funding

This work was supported in part by grants from the Japan Society for the Promotion of Science (JSPS) [Grant-in-Aid for Scientific Research No. 25221202) to T.F.].

Disclosures

The authors have no conflicts of interest to declare.

Acknowledgments

We thank Professor Klaus Palme and Professor Jiří Friml for sharing their published material, Dr. Baohai Li, Ms. Sae Kageshima and Ms. Emiko Yokota for preparing −Mn and low-Ca solid medium, Zengyu Liu for technical assistance, and Dr. Baohai Li for discussions.

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